1

Genotypes and phenotypes in children with short stature: clinical indicators of *SHOX* haploinsufficiency

Gudrun Rappold[1*], Werner F. Blum[2,3], Elena P. Shavrikova[4], Brenda J. Crowe[5], Ralph Roeth[1], Charmian A. Quigley[5], Judith L. Ross[6], Beate Niesler[1]

[1]Department of Molecular Human Genetics, University of Heidelberg, Im Neuenheimer Feld 366, D-69120 Heidelberg, Germany
[2]Lilly Research Laboratories, Eli Lilly and Company, Bad Homburg, Germany
[3]University Children's Hospital, Giessen, Germany
[4]Pharma Support Inc., St. Petersburg, Russia
[5]Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, USA
[6]Department of Pediatrics, Thomas Jefferson University, Philadelphia, USA

To whom correspondence should be addressed:

Gudrun A. Rappold, PhD
Institute of Human Genetics
University of Heidelberg
Im Neuenheimer Feld 366
69120 Heidelberg, Germany
Phone: +49 6221 56-5059, -5066, -5068
Fax: +49 6221 56-5155
gudrun.rappold@med.uni-heidelberg.de

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ABSTRACT

Introduction: Short stature affects approximately 2% of children, representing one of the more frequent disorders for which clinical attention is sought during childhood. Despite assumed genetic heterogeneity, mutations or deletions of the <u>S</u>hort <u>S</u>tature <u>H</u>omeob<u>ox</u>-containing gene (*SHOX*) are found quite frequently in subjects with short stature. Haploinsufficiency of the *SHOX* gene causes short stature with highly variable clinical severity, ranging from isolated short stature without dysmorphic features to Léri-Weill syndrome and, with no functional copy of the *SHOX* gene, Langer syndrome.

Methods and Results: To characterize the clinical and molecular spectrum of *SHOX* deficiency in childhood we assessed the association between genotype and phenotype in a large cohort of short-statured children from 14 countries. Screening of 1608 unrelated individuals with sporadic or familial short stature revealed *SHOX* mutations or deletions in 68 individuals (4.2%): complete deletions in 48 (70.6%), partial deletions in 4 (5.9%) and point mutations in 16 subjects (23.5%). While mean height standard deviation score (SDS) was not different between short-stature subjects with or without identified *SHOX* gene defects (-2.6 versus -2.6), detailed examination revealed that certain bone deformities and dysmorphic signs, such as short forearm and lower leg, cubitus valgus, Madelung deformity, high-arched palate and muscular hypertrophy, differed significantly between subjects with or without *SHOX* gene defects (p<0.001).

Discussion: Phenotypic data were also compared for 33 children with Turner syndrome in whom haploinsufficiency of *SHOX* is thought to be responsible for the height deficit. A phenotype scoring system was developed that may assist in identifying the most appropriate subjects for *SHOX* testing. This study offers a detailed genotype-phenotype analysis in a large cohort of children with short stature and provides quantitative clinical guidelines for testing of the *SHOX* gene.

Short stature is a developmental, multifactorial condition with a strong genetic component. Although several genes have been reported to be associated with longitudinal growth, they account only for a small proportion of cases of short stature [1],[2]. One such gene is the Short-Stature Homeobox-containing gene (*SHOX*), which encodes a homeodomain transcription factor[3] and is believed to be responsible for the height deficit associated with Turner syndrome (TS)[4-6]. Mutations in the *SHOX* gene are one of the more frequent genetic causes of short stature in individuals with isolated or familial short stature[7-11].

Significant evidence points to an important role for SHOX as a mediator of linear growth. First, the gene is expressed in the developing skeleton during fetal life[6] and is specifically expressed in bone marrow fibroblasts and proliferating and hypertrophic chondrocytes[3-5, 12, 13], second, deficiency of SHOX at the growth plate is associated with marked disorganization of chondrocyte proliferation[14]; and third, there is a dose-dependent association between the number of active copies of the SHOX gene and height, such that *SHOX* haploinsufficiency is associated with short stature, whereas *SHOX* overdose as seen in sex chromosome polyploidy is associated with tall stature[15].

More than 50 different mutations in the *SHOX* gene associated with short stature in subjects previously described as "idiopathic" or in subjects with Léri-Weill syndrome (LWS) or Langer syndrome have been reported and assembled in the *SHOX* gene mutation database (www.shox.uni-hd.de)[16]. This database has been established to provide clinicians and scientists access to a central source of information about *SHOX* mutations. Indeed, the phenotype can vary from extreme dwarfism, mesomelia (short forearms and short lower legs) and limb deformity as seen in Langer syndrome (two defective or absent *SHOX* alleles) to disproportionate short stature and mesomelia as seen in LWS and Turner syndrome (one absent *SHOX* allele), to apparently isolated short stature with no other obvious clinical signs, often referred to as "idiopathic" short stature[5, 17, 18]. The typical mesomelia and Madelung deformity (clinical finding of dinner fork-like deformity of the wrist) of LWS are more prevalent in females, but often do not become apparent before puberty[10, 19, 20]. The clinical phenotype represents a continuum from very severe disproportionate short stature to milder short stature with or without a number of other clinical and radiological abnormalities, making the distinction between LWS and non-LWS rather arbitrary. Even within a single family with an identical genetic defect in all affected members, the phenotype can vary considerably[21]. This phenotypic variability is seen in many other genetic disorders, such that the classic boundaries between syndromic and non-syndromic forms are blurred, although identical mutations are present[22]. Thus, there is no reliable basis for strictly distinguishing syndromic and non-syndromic phenotypes caused by the same mutation genotypes.

The aim of this study was to determine the phenotypical and molecular spectrum of SHOX deficiency in a large group of children with short stature and to develop a scoring system to aid in future selection of the most appropriate patients for diagnostic *SHOX* testing.

SUBJECTS AND METHODS

The study was conducted in accordance with the principles of the Declaration of Helsinki/ Good Clinical Practice and was approved by the ethics committees of the participating institutions. Investigators obtained written informed consent from the subjects' parent(s) or legal guardian(s) before conducting study-related procedures.

Subjects
We studied 1,608 pre-pubertal children with short stature from 14 countries: Belgium (n=96), Canada (96), Croatia (74), Czech Republic (171), France (138), Germany (200), Hungary

(55), India (216), Netherlands (6), Poland (93), Russia (23), Spain (181), Turkey (114), USA (145). The subjects' ethnic origins were: Caucasian 1348 (83.8%), Western Asian 227 (14.1%), Eastern Asian 9 (0.6%), Hispanic 10 (0.6%), African American 7 (0.4%) and Other 7 (0.4%). Subjects were invited to participate in genetic screening for this study if they fulfilled the following entry criteria: chronological age $\geq$ 3 years and pre-pubertal (males: genital stage Tanner 1 and testes $\leq$ 2mL; females: breast stage Tanner 1); height < 3rd percentile of the local reference range or height < 10th percentile with height velocity < 25th percentile; bone age < 10 years for boys and < 8 years for girls or < 9 years for patients with TS; no GH deficiency or GH resistance; no chronic disease; and no known growth-influencing medications.

Study subjects were screened for deletions or mutations in the *SHOX* gene to determine their eligibility for participation in a growth hormone intervention study [23]. ..This cohort of subjects with short stature does not overlap with subjects from a previous study that investigated the prevalence of SHOX deficiency in children with idiopathic short stature (ISS)[7]. For comparison, a group of subjects with TS (n=33) was included. The ethnic backgrounds of these subjects were: 24 Caucasian (72.7%), 7 Western Asian (21.2%) and 2 Eastern Asian (6.1%).

Clinical assessments

Height was measured with calibrated stadiometers and a number of anthropometric measures were assessed using standardized procedures and equipment to quantify possible disproportion: sitting height, arm span, forearm and upper arm length, lower leg length and the circumferences of forearm, upper arm, lower leg and upper leg [24], [25]. Investigators were trained on these anthropometric techniques at study initiation. In addition, investigators were requested to report the presence or absence of a number of defined dysmorphic signs often observed in subjects with LWS that were listed on the case report forms.

DNA analyses

DNA preparation and PCR

Genomic DNA was prepared from 0.5 ml peripheral blood using the PUREGENE DNA Isolation kit (Gentra Systems, Minneapolis, MN, USA). PCR were performed as previously described by Rappold et al, 2002 [7].

PCR-based microsatellite analysis

To look for evidence of a *SHOX* gene deletion, two microsatellites in the vicinity of the *SHOX* locus (CA-SHOX, CAII) were PCR-amplified and analyzed on polyacrylamide gels for homo- or heterozygosity for the size of the microsatellite. Genomic DNA (100 ng) was amplified with 20 pmol of each of the primers DXYS201 (SHOX 5CA forward/reverse: CAT GTC ATA TAT ATA TGT GAT CC/ GAC ACA GAA ATC CTT CAT AAA). PCR was performed using 2 U of Goldstar DNA Polymerase (Eurogentec, Cologne, Germany) in the buffer provided with the enzyme, 10 pmol dNTP (MBI) and 75 mmol MgCl$_2$ in a Peltier Thermal Cycler (PTC-200) starting with denaturation (95°C, 5 min) followed by 35 cycles of amplification (95°C 30 s, 55°C 30 s and 72°C 45 s) and a final elongation step (72°C, 5 min). The PCR products were separated on polyacrylamide gels (10%) and analyzed after silver staining.

FISH analysis

Biotinylated cosmid DNA was hybridized to metaphase chromosomes from stimulated lymphocytes of 247 subjects under conditions previously described[26]. FISH was performed using the *SHOX*-containing cosmid 34F5 (LLN0YCO3´M´34F5)[5].

Mutation analysis

Mutation screening was performed by denaturing high performance liquid chromatography (DHPLC), using a WAVE DNA-Fragment Analysis System (Transgenomic Inc., Cheshire,

UK). Mutation detection relies on differential binding to the column of homo- and heteroduplexes, which are generated by denaturing PCR products at 95°C for five minutes and cooling them slowly (-2°C per cycle, 44 cycles) to allow renaturing. Analysis was confined to the coding regions of the *SHOX* gene (exons 2 – 6). Primer sequences and PCR conditions were: exon 2 forward/reverse: CGC GGG GAG ACG CGC GCA TCC/GGC GCC GAA CCC CAG GAG GAG GGC; 63°C/65°C/66°C; exon 3 forward/reverse: GCC ACG TTG CGC AAA ACC TC/CCC GAG GAC CAG GCG ATG; 64°C/65°C/67°C; exons 4 and 5 were amplified together: exon 4 forward: GGG AGG CTG GGC TGG GTT C; 62°C/64°C/65°C; exon 5 reverse: GGA AGG GAG CAG CAG GTC C; 62°C/64°C/65°C; exon 6a forward/reverse: GTC CCC ATC CTG CGC CCT CAC CC/GCG CGG AGC CCG GGA GTC CG; 66°C/68°C/67°C).

Sequencing

Sequencing was performed on a MegaBACE sequencer (Amersham Bioscience, Piscataway, NJ, USA) using the DYEnamic™ ET terminator Cycle Sequencing Kit following the manufacturer's protocol. Sequencing reactions were performed on both DNA strands. Sequences were analyzed using the Clustal program (German Cancer Research Center, Biocomputing Facility HUSAR, Heidelberg, Germany).

Single Nucleotide Polymorphism (SNP)-Based Screening

While the majority of DNA samples were analyzed according to the methods described above, 275 subjects had DNA analyses for *SHOX* deletions and mutations performed using a commercially available assay (**SHOX***DNAdx*^SM, Esoterix Inc, Calabasas Hills, CA, USA)[27].

Statistical analyses

Because the majority of subjects originated from European countries, standard deviation scores (SDS) for height and the various anthropometric parameters were calculated using central European references[24]. The exception was lower leg length, for which the reference range of Ross et al.[25] was used. Values were adjusted for the actual chronologic age by linear interpolation. Body mass index (BMI, kg/m^{2}) was converted to SDS using the method of Cole[28]. Between-group comparisons were performed using one-way ANOVA for continuous variables and Fisher exact tests for categorical variables at a two-sided significance level of 0.05.

For the development of a quantitative scoring system to assist in identifying patients for *SHOX* gene testing, logistic regression models were developed with confirmed SHOX deficiency (yes/no) as the dependent variable and various explanatory variables, including clinical diagnosis of LWS in the subject or relative(s), dysmorphic signs and anthropometric measures. The continuous variables were converted to categorical variables by applying defined cutoffs. For each group (SHOX and non-SHOX) the median for the particular variable was determined, then the mean of the two medians was calculated and used as the cut-off. Variables were first tested one at a time and included in the models only if there was a significant mean difference between the SHOX deficient and non-SHOX deficient groups. Next, the stepwise selection procedure was used to develop the models and the models were tested for lack of fit by applying Hosmer-Lemeshow statistic. Scoring systems were developed that included as score items only those variables that contributed significantly to the logistic models. The odds ratios generated by the logistic models were then rounded to the nearest integer and used as the score weights. Various score systems were applied to the study population and sensitivity, specificity, accuracy, and positive predictive values were calculated for different score point cutoffs.

RESULTS

A large cohort of 1608 children with short stature was screened for deletions and intragenic mutations of the *SHOX* gene. The physician-designated clinical diagnosis (LWS vs. ISS) was collected at study entry. Fifty-five subjects (3.4%) were given the diagnosis of LWS; of the remaining 1553 subjects 1534 were designated as having ISS while in 19 subjects no diagnosis or diagnoses other than LWS or ISS were provided. *SHOX* defects were identified in 68 of 1608 screened subjects (4.2%), comprising 32/55 (58%) with LWS and 34/1534 (2.2%) with ISS, while 2 subjects were not classified. The analysis for *SHOX* defects followed a step-wise process. To screen for a possible gene deletion, the first analysis determined the presence or absence of heterozygosity for a CA-repeat marker 14 kb (*DXYS201)* and CAII 34 kb upstream of the *SHOX* gene. When only a single allele size of these 2 markers was detected, implying homo- or hemizygosity, the next step was a FISH analysis using the *SHOX*-containing cosmid LLN0YCO3´M´34F5 to verify a putative deletion. Forty-eight subjects (70.6%) had complete *SHOX* deletions and 4/68 (5.9%) had partial (small intragenic) deletions as indicated by a reduced FISH signal on one of the sex chromosomes. Intragenic mutations were detected in exons 2 - 6 of the coding sequence in 16/68 (23.5%) subjects: 10 subjects had missense mutations, 5 had nonsense mutations and 1 had a small duplication (Table 1). Seven of the 16 mutations have not been reported previously. *In vitro* functional analyses of four missense mutations demonstrated reduced DNA binding (R168W), reduced dimerization (L132V) or impaired nuclear translocation (A170P, R173H)[29], [30].

Demographic and baseline clinical characteristics of the children with or without *SHOX* gene defects are shown in Table 2. Although more males than females were enrolled in this study (952 [60%] male *vs.* 656 [40%] female), the sex distribution of confirmed SHOX defects was reversed, with 54% of molecular defects being in females and 46% in males. As expected, children with SHOX deficiency had a significantly higher percentage of first-degree relatives with LWS or Langer syndrome than children without SHOX deficiency: fathers: 9.7% vs. 0.5% (*P*<0.001), mothers: 26.6% vs. 1.6% (*P*<0.001), siblings: 26.0% vs. 2.0% (*P*<0.001). This finding is reflected by the decreased target height, which supports the concept of pseudoautosomal dominant inheritance of SHOX deficiency. SHOX testing was also performed in either one (n=8) or both (n=17) parents of 25 subjects with SHOX deficiency. In 14 of 17 (82%) families in which both parents were tested, either the father or the mother had a *SHOX* defect; in no family did both parents have a *SHOX* defect. A *SHOX* mutation was found in ten of 18 tested fathers (56%) and 8 of 24 tested mothers (33%). In all 25 cases in which DNA was also available for family members the mutations co-segregated with the phenotype of LWS or short stature.

Investigators were instructed to examine the subjects at screening for the presence of a specified list of dysmorphic signs (Table 3). A significant number of subjects with a pre-study diagnosis of ISS who were subsequently confirmed to have a *SHOX* defect had some dysmorphic signs typical of LWS. Only a few signs such as micrognathia, high-arched palate, short forearm and Madelung deformity were significantly more frequent in subjects with a baseline clinical diagnosis of LWS (Table 3). For subjects with a clinical diagnosis of LWS there was no statistically significant difference in the frequency of dysmorphic signs between subjects who were subsequently confirmed to have a *SHOX* defect (n=32) and those without an intragenic mutation of the *SHOX* gene (n=23; data not shown). For subjects with a clinical diagnosis of non-syndromic short stature ("ISS") a number of clinical signs were more prevalent in those with a molecularly confirmed *SHOX* defect (n=34) than in those without a *SHOX* defect (n=1500; Table 4). Therefore, these physical signs (shortening or bowing of the forearm, dislocation of the ulna at the elbow, bowing of the tibia and appearance of muscular

hypertrophy) appear to represent clinical indicators for SHOX deficiency in short children. In addition, subjects with proven *SHOX* defects had significantly greater mean BMI SDS than those without.

Because *SHOX* haploinsufficiency is also believed to underlie the short stature and skeletal anomalies of TS, frequencies of dysmorphic signs were compared between subjects with TS (n=33) and those with (n=68) or without (n=1540) confirmed SHOX deficiency (Table 5). While the majority of dysmorphic signs were not different in frequency between the SHOX-deficient and TS groups, there were a few notable exceptions. High-arched palate and cubitus valgus were substantially more common in TS, whereas short forearm or short lower leg, Madelung deformity and muscular hypertrophy were markedly more prevalent in the SHOX-deficient group.

To obtain an objective assessment of body proportions a number of anthropometric measurements were performed. Values were adjusted for standing height by calculating the ratio (%) of the anthropometric measure divided by height (Table 6). Subjects with SHOX deficiency had a significantly greater sitting height and forearm, upper arm and upper leg circumference adjusted for standing height. In contrast, height-adjusted arm span, forearm length and lower leg length were significantly shorter in subjects with SHOX deficiency compared to those without. These findings underline the differences in frequency of dysmorphic signs between the groups with and without SHOX deficiency and suggest mesomelic disproportion and appearance of muscular hypertrophy as the most indicative features of SHOX deficiency. There were no significant differences in dysmorphic signs or anthropometric measures between subjects with a missense mutation within (n=6) or outside (n=5) the homeobox (data not shown).

Although the above analyses elucidate the spectrum of phenotypic findings in children with various forms of SHOX deficiency, the variability of the phenotype makes it difficult to decide whether or not SHOX testing is warranted for an individual child. Therefore, to provide evidence-based scoring systems for identifying the most appropriate subjects for *SHOX* gene testing on clinical grounds, various logistic regression models were developed with SHOX deficiency (yes/no) as the dependent variable. Combinations of variables, including diagnosis (LWS in subject or relatives), dysmorphic signs and anthropometric variables that were significantly different between subjects with or without SHOX deficiency, were included in the models as explanatory variables, and odds ratios were calculated. The diagnosis of LWS or the presence of Madelung deformity in the subject or in a family member resulted in very high odds ratios in the models. Therefore, to focus on the clinically more challenging subjects with non-syndromic short stature ("ISS"), these strong indicators of SHOX deficiency were excluded from the models. The scoring system presented in Table 7 is based on the final logistic regression model, using rounded odds ratios as weighted score points. There was no evidence of lack of fit of the underlying logistic model (lack of fit *P*=0.81). The suggested scoring system includes three anthropometric variables (arm span/height, sitting height/height and BMI) and five dysmorphic signs. This scoring system, in which a maximum of 24 score points can be attained, was applied to the study population to evaluate the performance of the score. The dependence on various score point cutoffs of sensitivity, specificity, accuracy and positive predictive value is shown in Figure 1. At a cutoff of >4 points, sensitivity was 71% and the positive predictive value was 11%; at >7 points the sensitivity dropped to 61% while the positive predictive value increased to 19%.

DISCUSSION

This multi-center, multi-national study was performed to define a comprehensive clinical and molecular spectrum for *SHOX* haploinsufficiency. Whereas the involvement of the *SHOX* gene in short stature is well established, there is little prospective quantitative data addressing the more subtle clinical features and genotype-phenotype relationships in subjects with *SHOX* deficiency. We therefore evaluated the growth and skeletal features of a large cohort of children with short stature and performed a detailed analysis of their anthropometric measures and dysmorphic features, comparing the findings in subjects with proven SHOX defects to those in subjects without demonstrable molecular SHOX defects. We also compared the clinical features in children with isolated SHOX deficiency to those seen in another model of SHOX haploinsufficiency - children with TS; within the cohort with isolated SHOX deficiency (i.e. excluding subjects with TS), we compared the findings of subjects with the classical LWS phenotype with those subjects with more subtle phenotype, diagnosed prior to study entry as ISS. Finally, we defined diagnostic criteria that will allow clinicians to identify patients most likely to have a *SHOX* gene abnormality and to target SHOX testing to these patients.

SHOX mutations were identified in 4.2% of 1608 children studied. However, this cohort was enriched for subjects with LWS, in whom the frequency of *SHOX* defects is particularly high; when these subjects were excluded and only the subjects with non-syndromic (or idiopathic) short stature were included the frequency of SHOX deficiency was 2.2%. This frequency is similar to the rates reported in previous smaller studies in independent samples of children with ISS using similar genetic and molecular methodologies[7, 10]; however, it is lower than frequencies reported in other studies[8, 9, 11]. These variable rates of detection of *SHOX* defects may in part relate to methodological differences or to the different population studied[11, 31, 32]. We also did not screen for putative enhancer mutations ("downstream deletions") nor single exon deletions. It is therefore likely that intragenic mutations due to splice defects in the introns, regulatory mutations or single exon deletions have been missed.

Short stature is a more frequent reason for referral to a pediatric endocrine clinic for boys than for girls. Therefore, it was not surprising that 60% of our study subjects were boys. However, it is interesting to note that SHOX gene defects were confirmed in a greater proportion of tested girls than boys (54% vs. 46%). This finding is consistent with the greater reported prevalence of LWS in females[33], hypothesized to result from an effect of estrogen at the SHOX-deficient growth plate[20]. If true, this effect may have led to an ascertainment bias toward females with clinically significant skeletal anomalies.

Consistent with previous reports, the majority (52/68; 77%) of mutations detected were complete or partial gene deletions[7, 10, 11, 21]. The relatively high frequency of deletions has been attributed to the structural composition of the pseudoautosomal region, which may promote recombination, thereby predisposing to deletion events[34, 35]. Notably, the majority of deletions have been transmitted through the paternal allele suggesting that the high recombination rate during male meiosis is accountable for this phenomenon[36].

Although the majority of the *SHOX* gene defects in this study were large scale deletions, there were nevertheless a number of novel intragenic mutations that may elucidate some aspects of SHOX structure or function. The spectrum of intragenic mutations in the *SHOX* gene in this and other studies is highly heterogeneous. Including the seven novel mutations reported here, more than 60 different mutations have been identified, distributed throughout the coding regions of the gene (www.shox.uni-hd.de). The majority of intragenic mutations are missense mutations, and all missense mutations detected in this study affect amino acids conserved between human, chicken and fish, highlighting the functional importance of these affected residues. None of these missense mutations was detected in unaffected parents tested

in this study or in 100 unaffected control individuals screened outside this study. While the fact that the missense mutations segregate with the phenotype provides evidence of the functional role of the mutations, definitive proof requires *in vitro* demonstration of disturbed activity of the encoded transcription factor. Functional consequences have been demonstrated for the mutants R168W, L132V, A170P and R173H with respect to DNA binding, dimerization or nuclear translocation of the SHOX protein[29, 30]. However, the effect of Q112P, K116E, insH229-L232 and X226R on SHOX activity is unknown.

Although it might have been expected that children with molecular *SHOX* gene defects would have had more severe short stature than those with normal *SHOX* genes, we found no difference in the degree of short stature between those with or without an identifiable *SHOX* gene defect; average height was -2.6 SDS in both groups. In fact, the most striking anthropometric difference between SHOX-deficient and non-SHOX deficient subjects was the 1.0 SDS greater BMI in the SHOX-deficient group (p<0.001). This finding is interesting in light of the fact that subjects with a working diagnosis of ISS (who comprised half of the SHOX-deficient group in our study) typically have BMI values in the low to low-normal range, perhaps due to poor eating behavior[37]. However, rather than reflecting obesity, the above average BMI for age and sex in SHOX-deficient children likely reflects segmental disproportion with rather normal trunk size but short legs. Moreover, the relative increase in limb circumferences (discussed below) suggests increased muscle mass or muscular hypertrophy, which may translate to a greater body mass compared with the significantly reduced height of affected individuals, leading in turn to an increase in BMI.

Apart from the increased BMI, the most reliable clinical indicators of *SHOX* deficiency were disproportionate growth, with shortening of the limbs compared with the trunk and bowing of the forearm or lower leg. The limb shortening was evidenced by reductions in the ratios of arm span and forearm length to height and an increase in sitting height/height ratio in the SHOX-deficient vs. SHOX-sufficient group; furthermore, arm span and forearm length vs. height were all reduced in the SHOX-deficient group compared with reference values for age. These findings are consistent with other reports[10, 38]. All limb circumferences (upper arm, forearm, upper leg and lower leg) were relatively increased compared with height in SHOX deficient vs. non-SHOX deficient subjects, and were increased relative to reference values for all measures except upper leg circumference/height. These findings of increased limb circumferences were manifest clinically as the appearance of muscular hypertrophy, which was significantly more common in the SHOX-deficient subjects, as previously reported[21]. While it is possible that the apparent muscular hypertrophy may result from unfavorable lever proportions of the limbs, it may also represent a genuine developmental phenomenon due to *SHOX* haploinsufficiency. Notably, these findings were present in a significant number of subjects with the "ISS" phenotype as well as those with LWS, indicating that these diagnoses represent a continuum, rather than discrete entities.

The clinical features of SHOX deficiency beyond the effects on long bone length, appear to represent changes in the curvature or angularity of certain bones. As examples, the following features were present in a significantly greater proportion of subjects with SHOX deficiency than those without: forearm and tibial bowing, cubitus valgus and genu valgum, Madelung deformity and ulnar dislocation at the elbow. In addition, it should be mentioned that lower leg and forearm X rays can be very helpful to detect deformities, which are clinically not obvious. Furthermore, as reported previously, a number of these features were also seen with increased frequency in the TS group, including high-arched palate, forearm bowing, cubitus valgus, ulnar dislocation, and a variety of other dysmorphic signs. While the phenotypic differences between SHOX-deficient subjects with LWS vs. "ISS" were subtle, there were a number of features that were significantly more common in children with LWS than in those with the baseline clinical diagnosis of ISS, including high-arched palate, Madelung deformity, micrognathia and shortening and/or bowing of the forearm. These

alterations in the patterns of bone growth likely reflect the disorganization of the growth plate that has been reported in association with SHOX deficiency[14]. Despite some differences between the diagnostic groups within this study in terms of frequency of the various clinical findings, it has become clear that there is no single phenotype of SHOX deficiency, but rather that molecular defects of the *SHOX* gene may manifest a widely varying spectrum of clinical severity, most likely reflecting other generic influences on the expression of the genetic disorder.

The presence of LWS or Madelung deformity in an individual subject or in a first degree relative in this study was an overwhelming indicator for the presence of SHOX deficiency. Therefore, all children with these clinical diagnoses should undergo formal *SHOX* gene molecular analysis. However, the same is not true for the milder forms of SHOX deficiency detected in this study. In subjects with non-syndromic forms of short stature the key clinical features of SHOX deficiency, evidenced by significant differences between the SHOX-deficient and non-SHOX-deficient groups, were increased BMI, short limbs relative to overall height (e.g. short forearm: 21% vs. 7%; p=0.006), evidence of muscular hypertrophy (27% vs. 9%; p=0.003), and increased bowing or angularity of various bones, particularly those of the lower leg (tibial bowing: 15% vs. 3%; p=0.002) and forearm (12% vs. 3%; p=0.027).

Although this study undertook a thorough assessment of the phenotype of children with SHOX deficiency, we were unable to determine any clear correlation between the specific *SHOX* gene defect and the clinical features. For example, no consistent differences could be discerned between subjects with complete *SHOX* gene deletions and those with missense mutations resulting in conservative amino acid substitutions. Furthermore, although *SHOX* haploinsufficiency is present in both LWS and TS and might be expected to result in similar skeletal features, the findings of high-arched palate and cubitus valgus were significantly more frequent in children with TS, whereas children with isolated SHOX deficiency had a significantly greater frequency of Madelung deformity, short forearm, short lower leg and appearance of muscular hypertrophy. The basis for these phenotypic differences is speculative: one suggested explanation is that estrogen action at the growth plate in girls with isolated SHOX deficiency (i.e. normal gonadal function) may exacerbate the effects of *SHOX* haploinsufficiency[20]; alternatively, it may be hypothesized that haploinsufficiency of additional X-chromosomal genes in girls with TS may mitigate the effects of *SHOX* deficiency to some degree. In addition to the absence of a genotype-phenotype relationship for *SHOX* gene defects, there also appear to be other defects that manifest an indistinguishable clinical phenotype, evidenced by the fact that a significant minority of subjects with typical dysmorphic signs of LWS had no detectable SHOX gene defect in this and other studies[8, 9, 11]. The absence of a clear molecular basis for the LWS phenotype in a significant proportion of subjects suggests that a similar phenotype can be caused either by intragenic mutations that may escape detection due to their location in regulatory regions of the gene, or to defects at a different, but perhaps related, genetic locus.

To provide a quantitative approach to determining the most appropriate candidates for *SHOX* genetic testing, we integrated the clinical and anthropometric findings reported here into a pragmatic scoring system that optimizes the selection of patients who are less clinically obvious than those with LWS and Madelung deformity. The score combines three anthropometric variables (arm span/height ratio, sitting height/height ratio and BMI), with five clinical variables (cubitus valgus, short forearm, bowing of forearm, muscular hypertrophy and dislocation of the ulna at the elbow), each of which represents at least 2 points in the score system (Table 7). The system was then examined for sensitivity, specificity, positive predictive value and accuracy using various total score values. On the basis of these analyses, testing for SHOX deficiency is recommended for subjects with a score greater than 4 or 7 out of a total possible score of 24. The decision on whether to choose the increased threshold score needs to take into account the decrease in sensitivity, which means

more SHOX-deficient subjects will be missed, versus the increase in positive predictive value, which means more subjects will be tested positive for a *SHOX* defect.

In summary, *SHOX* defects represent a relatively frequent cause of short stature, particularly in children with evidence - which may sometimes be quite subtle – of shortening and/or bowing of the forearms and lower legs. The presence of some of the typical physical findings of LWS in children with the initial clinical diagnosis of ISS indicates that a very careful physical examination, including measurement of body proportions, must be performed before making the diagnosis of ISS. In addition, X ray analysis of the lower leg and forearm can be very important when distinguishing between ISS and LWS. Presence of any combination of the following physical findings: reduced arm span/height ratio, increased sitting height/height ratio, above average BMI, Madelung deformity, cubitus valgus, short or bowed forearm, dislocation of the ulna at the elbow, or the appearance of muscular hypertrophy, should prompt the clinician to obtain a molecular analysis of the *SHOX* gene.

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Table 1. Point mutations in the *SHOX* gene; the shaded area indicates the homeobox

Exon	Amino acid change	Nucleotide change	Mutation type	Diagnosis	New mutation/ Previously reported
2	E61X	c.181G>T	Nonsense	LWS	New
3	Q112P	c.335A>C	Missense	ISS	New
3	K116E	c.346A>G	Missense	ISS	New
3	L132V	c.394C>G	Missense	LWS	Grigelioniene et al. 2000[39] Schneider et al., 2005[29]
3	Y141D	c.421T>G	Missense	ISS	Schneider et al., 2005[29]
3	E148X	c.442G>T	Nonsense	LWS	New
4	W164X	c.492G>A	Nonsense	ISS	New
4	R168W	c.502C>T	Missense	ISS	Schneider et al., 2005[29]
4	R168L	c.503G>T	Missense	ISS	New
4	A170P	c.508G>C	Missense	ISS	Schneider et al., 2005[29] Sabherwal et al., 2004[30]
4	R173H	c.518G>A	Missense	LWS	Schneider et al., 2005[29] Binder et al. 2003[10]
5	R195X	c.583C>T	Nonsense	LWS	Rao et al 1997[5] Ross et al., 2005[38]
5	R195X	c.583C>T	Nonsense	LWS	Rao et al 1997[5] Ross et al., 2005[38]
6a	insH229-L232	c.685-696insCAC CCGCACCTG	Duplication	ISS	New
6b	X226R	c.676T>C	Missense	ISS	Ross et al., 2005[38]
6b	X226R	c.676T>C	Missense	LWS	Ross et al., 2005[38]

ISS: non-syndromic short stature, LWS: Léri-Weill syndrome

Table 2. Demographic and baseline data of patients with (SHOX) and without (Non-SHOX) a deletion or mutation in the *SHOX* gene and patients with Turner syndrome.

Variable	Non-SHOX	SHOX	TS	*P*-value Non-SHOX vs. SHOX	*P*-value TS vs. SHOX
N	1540	68	33		
Males/females (%)	60/40	46/54	0/100	0.023	
LWS (%)	1.5	48.5		<0.001	
Age (years)	7.7 ±2.4	7.4 ±2.5	7.2 ±2.0	0.311	0.767
Height SDS	-3.4 ±1.0	-3.4 ±1.1	-3.8 ±0.8	0.997	0.104
BMI SDS	-0.6 ±1.4	+0.4 ±1.1	-0.3 ±1.2	<0.001	0.023
TH SDS	-1.3 ±1.0	-1.5 ±1.3	-0.6 ±1.3	0.205	<0.001
Height SDS – TH SDS	-2.1 ±1.2	-1.9 ±1.1	-3.1 ±1.0	0.156	<0.001

LWS: Léri-Weill syndrome; TS: Turner syndrome; SDS: standard deviation score; BMI: body mass index; TH: target height.

Continuous variables are shown as mean ± SD. *P*-values of categorical variables were calculated by Fisher's exact test and of continuous variables by one-way ANOVA (two-sided *P*-values).

Table 3. Dysmorphic signs [n (%)] in patients with confirmed SHOX deficiency. Comparison between patients with non-syndromic short stature (ISS) and patients with Léri-Weill syndrome (LWS). Note: The assignment to ISS or LWS was not obtained in 2 patients.

Dysmorphic sign	ISS N=34	LWS N=32	P
Micrognathia	3 (8.8)	13 (40.6)	0.004
High-arched palate	9 (26.5)	25 (78.1)	<0.001
Short forearm	7 (20.6)	17 (53.1)	0.010
Bowing of forearm	4 (11.8)	11 (34.4)	0.040
Cubitus valgus	5 (14.7)	11 (34.4)	0.086
Short hands	7 (20.6)	5 (15.6)	0.752
Madelung deformity with pain	0	2 (6.3)	0.231
Madelung deformity without pain	2 (5.9)	12 (37.5)	0.002
Dislocation of ulna at elbow	3 (8.8)	1 (3.1)	0.614
Short lower leg	5 (14.7)	12 (37.5)	0.049
Bowing of tibia	5 (14.7)	10 (31.3)	0.145
Genu valgum	2 (5.9)	6 (18.8)	0.143
Short feet	6 (17.6)	4 (12.5)	0.734
Scoliosis	2 (5.9)	1 (3.1)	0.999
Muscular hypertrophy	9 (26.5)	14 (43.8)	0.197
Other dysmorphic signs	5 (14.7)	2 (6.3)	0.428

P-values were calculated using Fisher's exact test.

Table 4. Dysmorphic signs [n (%)] in patients with non-syndromic short stature (ISS) excluding patients with Léri-Weill syndrome, with unknown diagnosis or with a diagnosis other than ISS. Comparison between patients without confirmed SHOX deficiency (Non-SHOX) versus patients with confirmed SHOX deficiency (SHOX).

Dysmorphic sign	Non-SHOX N=1500	SHOX N=34	P
Micrognathia	208 (13.9)	3 (8.8)	0.613
High-arched palate	438 (29.2)	9 (26.5)	0.850
Short forearm	97 (6.5)	7 (20.6)	0.006
Bowing of forearm	49 (3.3)	4 (11.8)	0.027
Cubitus valgus	116 (7.7)	5 (14.7)	0.183
Short hands	287 (19.1)	7 (20.6)	0.836
Madelung deformity with pain	4 (0.3)	0	0.999
Madelung deformity without pain	22 (1.5)	2 (5.9)	0.097
Dislocation of ulna at elbow	6 (0.4)	3 (8.8)	<0.001
Short lower leg	90 (6.0)	5 (14.7)	0.055
Bowing of tibia	39 (2.6)	5 (14.7)	0.002
Genu valgum	64 (4.3)	2 (5.9)	0.655
Short feet	187 (12.5)	6 (17.6)	0.427
Scoliosis	40 (2.7)	2 (5.9)	0.238
Muscular hypertrophy	132 (8.8)	9 (26.5)	0.003
Other dysmorphic signs	235 (15.7)	5 (14.7)	0.999

P-values were calculated using Fisher's exact test.

Table 5. Dysmorphic signs [n (%)] in patients without SHOX deficiency (Non-SHOX), with SHOX deficiency (SHOX) and with Turner syndrome (TS); both the Non-SHOX and SHOX groups include patients with Léri-Weill syndrome.

Dysmorphic sign	Non-SHOX N=1540	SHOX N=68	TS N=33	*P* Non-SHOX vs. SHOX	*P* SHOX vs. TS	*P* Non-SHOX vs. TS
Micrognathia	221 (14.4)	16 (23.5)	3 (9.1)	0.052	0.106	0.613
High-arched palate	457 (29.7)	34 (50.0)	24 (72.7)	<0.001	0.034	<0.001
Short forearm	108 (7.0)	25 (36.8)	2 (6.1)	<0.001	<0.001	0.999
Bowing of forearm	61 (4.0)	15 (22.1)	3 (9.1)	<0.001	0.165	0.148
Cubitus valgus	129 (8.4)	16 (23.5)	20 (60.6)	<0.001	<0.001	<0.001
Short hands	304 (19.7)	13 (19.1)	7 (21.2)	0.999	0.796	0.826
Madelung deformity with pain	5 (0.3)	2 (2.9)	0	0.032	0.999	0.999
Madelung deformity without pain	30 (1.9)	14 (20.6)	1 (3.0)	<0.001	0.019	0.485
Dislocation of ulna at elbow	8 (0.5)	5 (7.4)	0	<0.001	0.170	0.999
Short lower leg	100 (6.5)	17 (25.0)	2 (6.1)	<0.001	0.029	0.999
Bowing of tibia	50 (3.2)	15 (22.1)	2 (6.1)	<0.001	0.050	0.298
Genu valgum	69 (4.5)	8 (11.8)	3 (9.1)	0.014	0.999	0.189
Short feet	198 (12.9)	11 (16.2)	2 (6.1)	0.459	0.212	0.423
Scoliosis	43 (2.8)	3 (4.4)	3 (9.1)	0.441	0.389	0.069
Muscular hypertrophy	142 (9.2)	24 (35.3)	1(3.0)	<0.001	<0.001	0.357
Other dysmorphic signs	251 (16.3)	8 (11.8)	19 (57.6)	0.400	<0.001	<0.001

P-values were calculated using Fisher's exact test.

Table 6. Anthropometric measures in patients with (SHOX) and without (Non-SHOX) SHOX deficiency. Measures were related to standing height to evaluate disproportions; anthropometric measures as percentage of height were compared to the corresponding means of normal 8 year-old children (Flügel et al.[24]) and were converted to differences in cm to give an impression of the magnitude of deviation.

Variable	Percent of height (%)			Deviation from normal (cm)	
	Non-SHOX N=1447	SHOX N=65	*P*	Non-SHOX	SHOX
Height SDS	-3.3 ±0.9	-3.4 ±1.1	0.673		
Sitting height SDS	-3.1 ±1.1	-2.4 ±1.4	<0.001		
Sitting height/ Height	54.5 ±3.0	56.5 ±3.0	<0.001	+1.2	+3.9
Arm span/ Height	97.5 ±3.4	95.2 ±3.3	<0.001	-2.8	-5.8
Forearm length/ Height	14.9 ±1.2	14.3 ±1.8	<0.001	+0.4	-0.4
Upper arm length/ Height	17.8 ±1.5	18.0 ±1.8	0.287	-0.5	-0.3
Lower leg length/ Height	21.9 ±1.7	21.1 ±2.3	<0.001		
Forearm circumference/ Height	14.9 ±1.4	15.7 ±1.6	<0.001	+0.3	+1.3
Upper arm circumference/ Height	15.5 ±1.7	16.6 ±1.8	<0.001	+0.1	+1.6
Lower leg circumference/ Height	21.9 ±1.7	21.1 ±2.3	<0.001	+2.5	+1.5
Upper leg circumference/ Height	29.5 ±3.2	31.8 ±3.8	<0.001	-3.1	-0.1

Two-sided *P* values were calculated using ANOVA; SDS: standard deviation score.

Table 7. Scoring system for identifying subjects that qualify for SHOX testing based on clinical criteria

Score item	Criterion	Score points
Arm span/height ratio	<96.5%	2
Sitting height/height ratio	>55.5%	2
Body mass index	>50th percentile	4
Cubitus valgus	Yes	2
Short forearm	Yes	3
Bowing of forearm	Yes	3
Appearance of muscular hypertrophy	Yes	3
Dislocation of ulna (at elbow)	Yes	5
Total		24

Legend to Figure

Figure 1. Dependence of sensitivity, specificity, accuracy and positive predictive value on cutoff score point; the dotted lines represent cutoffs of >4 and >7 score points, respectively.