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ORIGINAL ARTICLE

Detection of *SHOX* gene aberrations in routine diagnostic practice and evaluation of phenotype scoring form effectiveness

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Heterozygous aberrations of *SHOX* gene have been reported to be responsible for Léri–Weill dyschondrosteosis (LWD) and small portion of idiopathic short stature. The study was established to assess effectiveness of using phenotype 'scoring form' in patients indicated for *SHOX* gene defect analysis. The submitted study is based on a retrospective group of 352 unrelated patients enrolled as a part of the routine diagnostic practice and analyzed for aberrations affecting the *SHOX* gene. All participants were scanned for deletion/duplication within the main pseudoautosomal region (PAR1) using the multiplex ligation-dependent probe amplification (MLPA) method. The phenotype 'scoring form' is used in our laboratory practice to preselect patients for subsequent mutation analysis of *SHOX* gene-coding sequences. The overall detection rate was 11.1% but there was a significant increase in frequency of *SHOX* gene defect positive with increasing achieved score ($P<0.0001$). The most frequent aberration was a causal deletion within PAR1. In three probands, MLPA analysis indicated a more complex rearrangement. Madelung deformity or co-occurrence of disproportionate short stature, short forearm and muscular hypertrophy had represented the most potent markers to determine the likelihood of *SHOX* gene defect detection. We conclude that appliance of phenotype 'scoring form' had saved excessive sample analysis and enabled effective routine diagnostic testing.
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INTRODUCTION

Heterozygous aberration of *SHOX* gene (Short stature HOmeoboX; ID 6473) or deletion/duplication of one of its numerous enhancers have been reported to be responsible for Léri–Weill dyschondrosteosis (LWD; MIM 127300) and small portion of idiopathic short stature (MIM 300582).[1,2] There is a phenotype variability within afflicted family members ranging from small stature (frequently disproportionate) to a fully expressed LWD phenotype. It is partly due to estrogen effect, still it means a thin line between both of these phenotypes. Homozygous defect of *SHOX* gene is responsible for a rare Langer mesomelic dysplasia (MIM 249700) characterized with more severe phenotype. Haploinsufficiency of *SHOX* in LWD patients is associated with variable degrees of growth impairment with or without a spectrum of skeletal abnormalities.

SHOX gene is situated within the main pseudoautosomal region (PAR1; 2.7 Mb) on the p arm of sex chromosomes and its protein product acts as an important transcription factor during limb development.[3] Besides the limb bud, there is a significant expression of the *SHOX* gene within the first and second pharyngeal arches mesenchyma.[4] Spatio-temporal fine-tuning of *SHOX* gene expression is regulated by multiple mechanisms.[5–8]

Our study summarizes experiences concerning genetic analysis of patients suspected of LWD collected in the course of routine laboratory practice. The 'scoring form' of clinical criteria evaluating likelihood of *SHOX* gene defect established on the original Rappold's scoring[9] is an integral part of medical indication for the relevant molecular analysis in our laboratory routine. Our study is a retrospective type of study where no other criteria except for medical indication (for *SHOX* gene analysis) made by local medical specialists (endocrinologists and clinical geneticists as well as pediatricians) were established. The 'scoring form' is assumed to help these specialists decide if the *SHOX* gene aberration is under consideration. Even though not all forms are filled out correctly and completely, we have collected a representative cohort of patients with sufficient clinical data tested for PAR1 deletion/duplication and *SHOX* gene-coding sequences mutation. Numerous publications have proofed strong association of *SHOX* gene haploinsufficiency with LWD phenotype; however, strictly selected project participants are often indicated by well-trained specialist. Our not so strictly selected cohort of patients was indicated by numerous local medical specialists and generally represents individuals with more variable phenotype. The main purpose of the study was to evaluate the effectiveness of using 'scoring form' in patients indicated for *SHOX* gene defect analysis and to establish whether selected phenotypic markers remain valid even in such a cohort of patients.

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MATERIALS AND METHODS

Subjects

Our study is based on a retrospective group of patients from the database of the Institute of Biology and Medical Genetics, General University Hospital and The First Faculty of Medicine of Charles University in Prague (IBMG) in close cooperation with other medical genetic, endocrinology and pediatric departments. Participants were enrolled as part of the routine diagnostic practice. Indication for molecular analysis of *SHOX* gene defects is possible only on the basis of prior medical specialist examination (mostly endocrinologist or clinical geneticist) in the Czech Republic. We highly recommend the completed phenotype 'scoring form' (Table 1) enclosed with such routine indication as well as information regarding height and mean height s.d. score of proband and height of both parents if possible. All patients introduced to the study (or their legal representatives) signed an informed consent form for blood withdrawal and DNA analysis.

Table 1 The phenotype 'scoring form' for evaluation of LWD represents a modified version of the original Rappold's scoring[9]

Score item	*Criterion*	*Score points*
Arm span/height ratio	<0.965	2
Sitting height/height ratio	>0.555	2
Madelung deformity (in proband or first-degree relative)	Presence	6
Cubitus valgus	Presence	2
Short forearm	Presence	3
Bowing of forearm	Presence	3
Appearance of muscular hypertrophy	Presence	3
Dislocation of ulna (at elbow short forearm)	Presence	5

Abbreviation: LWD, Léri–Weill dyschondrosteosis.

Methods

All participants (352 unrelated probands) were screened for PAR1 deletion/duplication using the Multiplex Ligation-Dependent Probe Amplification (MLPA) Kit P018-G1 (or its older version B; D1; E1; F1) SHOX probemix (MRC-Holland, Amsterdam, The Netherlands).[10]

The MLPA reaction was run with 50–150 ng of DNA, according to the manufacturer's instructions with subsequent fragmentation analysis conducted on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Both visual and computational evaluation was performed for each sample against the run negative control. The peak areas were normalized according to the instructions of the manufacturer. A personally constructed Microsoft Excel (Microsoft, Redmond, WA, USA) table was used for the entry of all of these calculations. Probands suspected of X chromosome mosaicism were analyzed by the use of Devyser Resolution XY v2 Kit (Devyser AB, Hägersten, Sweden). Only those patients who reached the score of at least 4 in the 'scoring form' (168 patients) were further assigned for mutation analysis of the coding portion of *SHOX* gene (exons 2–6a/6b) employing the direct Sanger sequencing method. Sequencing reaction was prepared according to the manufacturer's instructions with the use of BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and ran on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Primers and amplification conditions are indicated elsewhere.[11] Statistical comparisons were performed using Fisher exact tests for categorical variables at a two-sided *P*-value (STATISTICA Ver 9.1) (StatSoft, Inc., Dell Software Inc., Aliso Viejo, CA, USA, 2010).

RESULTS

The overall detection rate based on 352 unrelated probands was 11.1%. The most frequent was deletion encompassing the *SHOX* gene (46.2%), followed by *SHOX* gene sequence mutation (17.9%) and exclusive deletion of downstream enhancer elements (12.8%). An overview of detected *SHOX* gene sequence mutations and polymorphisms is summarized in Table 2. Besides, duplication of

Table 2 Sequence variants detected in *SHOX* gene, their localization, known or assumed functional impact and population frequency (1000 Genomes project)

Localization	*Reference—SHOX database[a]; dbSNP (rs); HGMD (CM)*	*Variant*	*Functional impact*	*Status*	*Minor allele frequency*
Exon 2	SHOX_01270	c.-10delG	Ambiguous (change in transcription efficiency?)	Mutation	—
	SHOX_00068; rs142306835	c.63C>T; p.Gly21Gly	Non-pathogenic	Polymorphism	0.006
	None	c.124G>T; p.Gly42*	Premature translation termination	Mutation	—
Intron 2	SHOX_00071; rs2239402	c.277+17G>T	Non-pathogenic	Polymorphism	0.041
Exon 3	SHOX_00230; CM119853	c.361C>T; p.Arg121Cys	Defect of homeobox domain (no dimerization and no DNA binding)	Mutation	—
Exon 4	SHOX_00038; CM014910	c.518G>A; p.Arg173His	Defect of homeobox domain (no nuclear localization and no dimerization)	Mutation	—
	SHOX_00034; CM042777	c.502C>T; p.Arg168Trp	Defect of homeobox domain (no dimerization and no DNA binding)	Mutation	—
Intron 4	SHOX_00190	c.544+10G>A	Ambiguous (effect on splicing?)	Mutation	—
Exon 6b	SHOX_00135; rs28474801	c.657A>G; p.Pro219Pro	Non-pathogenic	Polymorphism	0.261
	SHOX_00136	c.676T>C; *226Argext*22	Protein extension of 22 amino acids	Mutation	—

Abbreviation: SHOX, Short stature HOmeoboX.
[a]Rappold's *SHOX* research group, University of Heidelberg, Germany (http://grenada.lumc.nl/LOVD2/MR/home.php?select_db=SHOX).

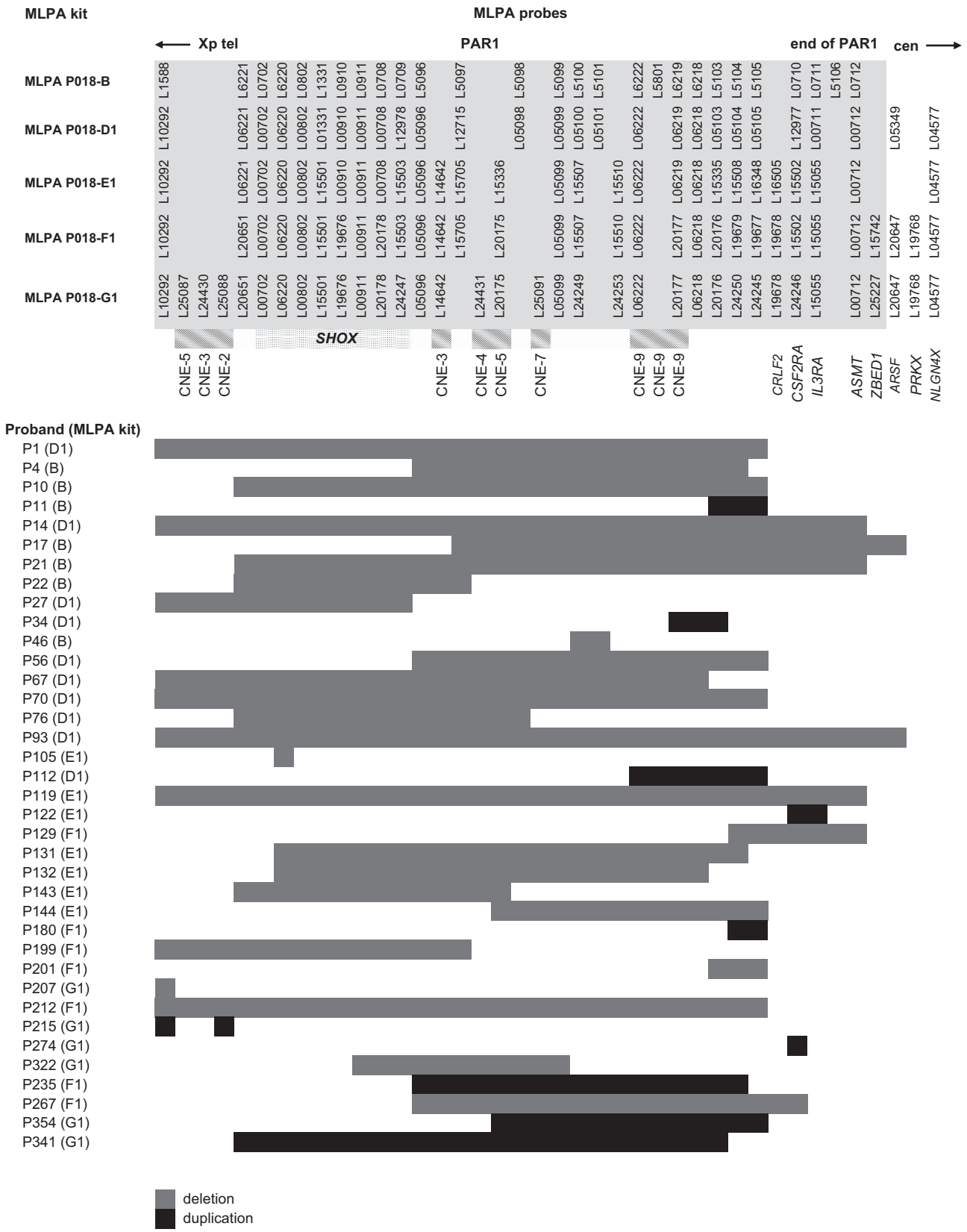

Figure 1 Scheme indicating breakpoints of microdeletions and microduplications within PAR1 evaluated on the basis of SALSA MLPA P018 probemixes (various versions) in our cohort of probands (P). Placing of *SHOX* gene as well as upstream and downstream evolutionarily conserved non-coding elements (CNEs) acting as *SHOX* enhancers are depicted.

downstream enhancer elements (10.3%), single *SHOX* gene duplication and single duplication of upstream enhancer element (CNE-2) have occurred. In two female patients, there was a 68–75% reduction of all probes focusing on PAR1 and PAR2 as well as on X-specific regions. This reduction is slightly higher than expected for complete deletion of X chromosome. Although both female probands display disproportionate short stature (height −2.5 s.d.) combined with short forearm and muscular hypertrophy, they are fertile and lack other features typical for Turner syndome. We thus hypothesized a mosaicism with partial cell line lacking X chromosome. We used the Devyser Resolution XY Kit to support our hypothesis. There was a clear imbalance of peak areas of heterozygous markers and the portion of XO cell line was estimated to be approximately 60% in both cases.

In one male proband, the MLPA analysis detected duplication of all PAR1 probes as well as deletion of PAR2 probe and absence of both Yq control probes. The suggested rearrangement was the isochromosome Yp (iYp).

The extent of detected PAR1 deletions/duplications was highly variable and there was not a significant recombination hotspot within this region (Figure 1).

Several non-pathogenic rearrangements were observed. There was a recurrent duplication of *ASMT* gene probe (MLPA Kit P018-G1, probe L00712) with frequency of 2.3% and common small PAR1 ~4.9 kb deletion (MLPA Kit P018-D1, probe L05101) some 200 kb downstream of *SHOX* gene. Moreover, four unique deletions and four duplications of various extent and localization within the PAR1 region were detected with no obvious association with the considered phenotype.

The following data were obtained from the subgroup of 247 unrelated individuals with sufficiently completed 'scoring form'. In 168 of them was the score of ⩾4. Detection rate of *SHOX* gene defects in the group with score <4 points (low-scoring group) was 3.8%, which significantly differs ($P=0.0013$) from the detection rate of 18.5% in the group of individuals with score of ⩾4 (high-scoring group). Such difference could not be caused by the absence of *SHOX* gene mutation analysis in the low-scoring group as such mutations are quite rare and represent only a small fraction (22.6%) of *SHOX* gene defects detected in the high-scoring group. Patients with score <4 points covered 32.0% of all the assessed indications. The PAR1 aberrations in the low-scoring group comprised of deletion/duplication of downstream regulatory sequences and of a carrier with the iYp rearrangement. For further evaluation, the subgroup of 247 individuals was divided into following categories according to the achieved score: 0 points, 1–3 points, 4–10 points, 11–15 points, and ⩾16 points. There was no significant difference in proportion of men and women in each category as well as in average height s.d. (~2.56) and median height s.d. (~2.59). The first category (0 points) represents patients indicated primarily on the presence of isolated short stature (frequently with family history). Detection rate for individual categories is indicated in Figure 2.

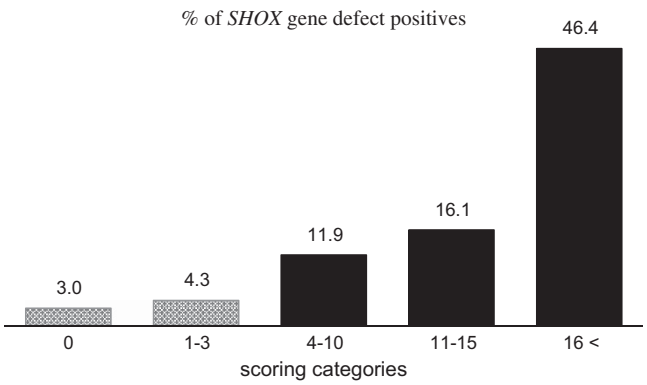

Figure 2 Detection rate in individual categories according to the achieved score (points).

It is obvious that the 'scoring form' was helpful in selecting patients with higher probability of *SHOX* defect. The pronounced increase of positive patients with last category (⩾16 points) is due to the presence of Madelung deformity either in the proband or in a first-degree relative. Madelung deformity still represents the most potent marker for evaluation of probability of *SHOX* gene defect detection that is evident from the frequency of selected clinical features among patients with and without detected *SHOX* locus aberration listed in Table 3. Besides Madelung deformity, a co-occurrence of disproportionate short stature, short forearm and appearance of muscular hypertrophy is present in 59.3% of positive patients compared with 14.3% of *SHOX* gene defect negative.

DISCUSSION

Recombination rate in PAR1 is consistent with one obligatory crossover per male meiosis having a crossover rate 17-fold greater than the genome-wide average.[12] High resolution sperm-typing measured a single recombination hotspot within the *SHOX* gene.[13] All of it results in a high frequency of rearrangements of both pathogenic and non-pathogenic within the PAR1 region. Duplication of *ASMT* gene is mainly discussed as a risk factor for autism spectrum disorders; to our knowledge, none of these patients had a history of this condition.[14] Frequency of a common small ~4.9 kb PAR1 rearrangement was determined to be 13.1% in the Czech population.[15] There was not a significant difference between its population frequency and frequency in patients with idiopathic short stature. There is a quite high variability of SHOX defect detection rate among different studies of patients with short stature;[2,11,16,17] it is partly because of different study inclusion criteria; however, population-specific dependencies could exist. The inclusion criteria for our study were very flexible and thus the lower overall incidence of SHOX defects in our sample (11.1%) is consistent.

Only a small portion of patients with diagnosed short stature have aberration of *SHOX* gene. It is highly desirable to narrow down the

Table 3 Frequency of monitored clinical features among patients with and without detected aberration of *SHOX* gene

Clinical feature	*SHOX defect positives*	*SHOX defect negatives*	P-*value*
Disproportionate short stature	82.1%	58.1%	0.0215
Arm span/height ratio <0.965	69.6%	48.2%	0.0741
Sitting height/height ratio >0.555	73.9%	44.8%	0.0130
Madelung deformity			
In proband	60.0%	9.1%	0.0001
In proband or first-degree relative	70.0%	17.8%	0.0001
Cubitus valgus	44.8%	37.9%	0.8387
Short forearm	80.0%	34.8%	0.0001
Bowing of forearm	41.4%	16.7%	0.0046
Appearance of muscular hypertrophy	75.9%	46.2%	0.0029
Dislocation of ulna	0.0%	2.1%	1.0000

Abbreviation: SHOX, Short stature HOmeoboX.

number of investigated patients by using the appropriate phenotype scoring system. We had introduced such 'scoring form' in our laboratory practice, based on the original Rappold's scoring system, to preselect patients intended for advanced *SHOX* gene mutation analysis. Our 'scoring form' was purposely modified, including high score for Madelung deformity (6 points), what reflects its high informative potential and no body mass index criteria inclusion. High body mass index in SHOX defect positives is mostly due to disproportionate short stature and muscular hypertrophy; both of these symptoms are already included in our 'scoring form', and moreover, significance of body mass index would fade with higher obese children in our population (special report from the The National Institute of Public Health of the Czech Republic: The change in body proportions, prevalence of overweight and obesity; www.szu.cz).

There was an obvious increase in frequency of *SHOX* gene defect positives with increasing achieved score. Madelung deformity or co-occurrence of disproportionate short stature, short forearm and muscular hypertrophy had represented the most potent markers to determine likelihood of *SHOX* gene defect detection.

We can conclude that phenotype 'scoring form' appliance had saved excessive sample analysis and enabled effective routine diagnostic testing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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