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SHOX gene defects and selected dysmorphic signs in patients of idiopathic short stature and Léri–Weill dyschondrosteosis

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ABSTRACT

The aim of the study was to analyze frequency of *SHOX* gene defects and selected dysmorphic signs in patients of both idiopathic short stature (ISS) and Léri–Weill dyschondrosteosis (LWD), all derived from the Czech population.

Overall, 98 subjects were analyzed in the study. Inclusion criteria were the presence of short stature (−2.0 SD), in combination with at least one of the selected dysmorphic signs for the ISS + group; and the presence of Madelung deformity, without positive karyotyping for the LWD + group. Each proband was analyzed by use of P018 MLPA kit, which covers *SHOX* and its regulatory sequences. Additionally, mutational analysis was done of the coding portions of the *SHOX*.

Both extent and breakpoint localizations in the deletions/duplications found were quite variable. Some PAR1 rearrangements were detected, without obvious phenotypic association. In the ISS + group, MLPA analysis detected four PAR1 deletions associated with a *SHOX* gene defect, PAR1 duplication with an ambiguous effect, and two *SHOX* mutations (13.7%). In the LWD + group, MLPA analysis detected nine deletions in PAR1 region, with a deleterious effect on *SHOX*, first reported case of isolated *SHOX* enhancer duplication, and *SHOX* mutation (68.8%). In both ISS + and LWD + groups were positivity associated with a disproportionately short stature; in the ISS + group, in combination with muscular hypertrophy.

It seems that small PAR1 rearrangements might be quite frequent in the population. Our study suggests disproportionateness, especially in combination with muscular hypertrophy, as relevant indicators of ISS to be the effect of *SHOX* defect.

1. Introduction

The *SHOX* (Short Stature Homeobox-Containing Gene; Gene ID: 6473) is located in the main pseudoautosomal region (PAR1) and is grouped among the so-called homeobox genes, which are distinguished by a homeodomain. Like all genes in the pseudoautosomal region, it escapes X inactivation and shows a "pseudo-autosomal" inheritance pattern (Rao et al., 1997). Mutations or deletions/duplications affecting the production of the *SHOX* are associated with: Turner syndrome; Léri–Weill dyschondrosteosis (LWD; MIM ID: 127300); its homozygote form, Langer mesomelic dysplasia (MIM ID: 249700); as well as with idiopathic short stature (ISS; MIM ID: 300582) (Chen et al., 2009; Ellison et al., 1996; Sabherwal et al., 2007). In these syndromes, *SHOX* defects are implicated in inaccurate bone development and longitudinal body growth.

The clinical symptoms of the *SHOX* deficiency are highly variable, and the phenotype can be markedly different, even among affected

Abbreviations: A, adenine; c, cDNA level; C, cytosine; CEN, centromeric; CNE, conserved non-coding DNA element; DMSO, dimethylsulfoxide; fhGH, recombinant human growth hormone; G, guanine; ISS, idiopathic short stature; Kb, kilobase(s) or 1000 bp; L, leucine; LWD, Léri–Weill dyschondrosteosis; Mb, megabase(s) or 1000 000 bp; MLPA, multiplex ligand-dependent probe amplification; N, asparagine; OAR, otp, aristaless, and rax domain; p, protein level; P, proband; PAR1, main pseudoautosomal region; R, arginine; S, serine; *SHOX*, short stature homeobox-containing gene; SD, standard deviation; T, thymine; TEL, telomeric.

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members of the same family (Grigelioniene et al., 2001). The symptoms are usually more pronounced in women, which is the effect of estrogen (Fukami et al., 2004; Ogata et al., 2001).

The variability of the *SHOX* pathology contains both deletions/duplications (mainly cryptic) and point mutations (Chen et al., 2009; Sabherwal et al., 2007). Size of deletions does not correlate with the intensity of the clinical phenotype (Schiller et al., 2000). Deletions can include both the *SHOX* and its regulating regions (Benito-Sanz et al., 2005; Fukami et al., 2006). Partial *SHOX* duplications appeared to have a more deleterious effect on skeletal dysplasia and duplication height gain than complete *SHOX* duplications (Benito-Sanz et al., 2011b). However, the effect of duplications and the associated phenotype is highly variable (Hirschfeldova et al., 2011; Thomas et al., 2009). One exon which is mostly affected by causal point mutations is exon 3 (Marchini et al., 2007), coding the greater part of the homeodomain. The management of short stature in ISS and LWD patients, caused by SHOX-related haploinsufficiency includes treatment with recombinant human growth hormone (fhGH), in order to improve the final adult height (Blum et al., 2007). Early diagnosis is thus highly advantageous.

The aim of the study was to analyze the frequency of *SHOX* defects, as well as selected dysmorphic signs in patients of both ISS and LWD (all derived from the Czech population, and in order to improve its diagnosis within the Czech Republic).

2. Materials and methods

2.1. Subjects

The study's participants were screened for deletions or mutations in the *SHOX*, and its known regulatory sequences. Overall, 98 subjects were analyzed in the study. To follow the indication criteria for extended *SHOX* gene analysis in our laboratory practice the ISS+ and LWD+ study samples were designed. The ISS+ study sample consists of 51 unrelated probands and 11 relatives; the LWD+ study sample included 16 probands and 20 relatives. Study subjects were recruited from the Department of Medical Genetics of the 1st Faculty of Medicine and the General Teaching Hospital, Charles University in Prague, in close cooperation with the Department of Medical Genetics of the University Hospital in Brno as well as the Department of Paediatrics of the University Hospital in Olomouc. All of the patients that were introduced into the study (or their legal representatives) signed an informed consent form for the taking of blood, DNA analysis, plus an agreement to submit to the study. If required, an informed consent form for blood taking and DNA analysis was signed with the proband's relative, as well.

The probands' selection criteria for the ISS+ study sample were the presence of short stature (−2.0 SD), in combination with at least one of following dysmorphic signs: disproportionate stature, cubitus valgus, short forearm, bowing of forearm, muscular hypertrophy, or dislocation of ulna (at elbow). The recommended dysmorphic signs were obtained from the study by Rappold et al. (2007) that provides quantitative clinical guidelines for testing of the *SHOX* gene.

No GH deficiency or resistance, as well as no known growth-influencing medications, were detected in the selected probands. For the LWD+ study sample, the probands' inclusion criteria were the presence of at least unilateral Madelung deformity, but without a positive cytogenetic finding during standard cytogenetic karyotyping.

2.2. DNA analysis

The EDTA blood was stored at 4 °C, and then processed within 48 h after venesection. The genomic DNA was isolated from the peripheral blood using QIAGEN spin columns on a QIAcube (QIAGEN, GmbH, Germany). Detection of the PAR1 rearrangements was carried out by use of the multiplex ligand-dependent probe amplification (MLPA) molecular genetic method (MRC-Holland, the Netherlands) (Schouten et al., 2002).

Each proband was analyzed by use of a MLPA kit P018, which covers the *SHOX*, its regulatory sequences, and the adjacent X-specific region. The MLPA reaction was run with 50–150 ng of DNA, according to the manufacturer's instructions. The MLPA analysis was carried out by fragmentation analysis, conducted on an ABI PRISM 310 Genetic Analyser (Applied Biosystems, CA, USA). At first, using the raw data, a visual examination of the peak patterns was performed for each sample, as well as between each sample; additionally, negative controls were also run. The peak areas were normalized, according to the instructions of the manufacturer. In order to obtain the final results, the normalized probe ratios of each sample were divided by the normalized probe ratios of the negative control(s) run. A personally constructed Microsoft Excel table was used for the entry of all of these calculations.

Additionally, DNA sequencing was done of the coding portions of the *SHOX* exons 2, 3, 4, 5, 6a, and 6b. We used primers by Huber et al. (2001), with minor modifications to amplify exons 2 through 5. The following primers were used to amplify exons 6a and 6b: 5′ taggggagaagaggcacgtt 3′ as a forward, and 5′tcctcaggcctcttgcag 3′ as a reversed primer for 6a amplification; and 5′ttcaccgtgttagccaggaa 3′ as a forward, and 5′ggatcacctgaggtcaggagtt 3′ as a reversed primer for 6b amplification. The 25 μl reaction mixture contained: 50–120 ng of genomic DNA, 1.5 mM $MgCl_2$, 0.2 μM of each primer, 0.1 mM of each dNTP, and 0.5 unit of *Taq* DNA Polymerase (recombinant) in the manufacturer's provided $(NH_4)_2SO_4$ buffer (Fermentas, Vilnius, Lithuania). The amplification conditions were: 95 °C for 2 min as a denaturation step, followed by 40 cycles of 1 min at 94 °C, 30 s at 68 °C (exon 3, exons 4–5); or 61 °C (exon 6a and 6b), and 30 s at 72 °C, ending with 3 min at 70 °C. Exon 2 was amplified at adjusted conditions of 95 °C for 2 min as a denaturation step, followed by 10 cycles of 1 min at 94 °C, 45 s at 72 °C, followed by 30 cycles of 1 min at 94 °C, 45 s at 69 °C, ending with 3 min at 70 °C. The sequencing reaction was performed according to the manufacturer's instructions (we added DMSO), using the BigDye® Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA), and run on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

3. Results

All probands were screened for copy number variation in the PAR1 region, including the *SHOX*, and its regulatory sequences, using the MLPA kit P018. The largest deletion is in the range of around ~6 Mb (P61); the smallest deletion has less than 0.5 Mb (P24, P25). Duplications in the PAR1 region range of ~130 kb (P7), ~500 kb (P10), and 200–600 kb (P67). Both the extent and telomeric (TEL) or centromeric (CEN) breakpoint localization in the deletions/duplications found were quite variable (Fig. 1). The *SHOX* mutation analysis involved the coding portions of the *SHOX* exons 2, 3, 4, 5, 6a, and 6b.

3.1. The ISS + group

The ISS+ probands included 31 women and 20 men, ranging in age from 5 to 29. The primary inclusive criteria were growth retardation, diagnosed as a height below −2.0 SD. In 64% of the patients, the short stature was familial. One proband was adoptive. The listing and frequencies of selected dysmorphic signs can be found in Table 1.

The MLPA analysis detected four PAR1 deletions associated with the *SHOX* defect (7.8%); three *SHOX* deletions, and one enhancer CNE9 deletion (CNE; conserved non-coding DNA element). The extent of the deletion is outlined in Fig. 1. In a boy proband (P3), the familial short stature (−2.0 SD) was associated with muscular