Prevalence of *SHOX* haploinsufficiency among short statured children

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BACKGROUND: The aim of this clinical study was to determine the prevalence of *SHOX* haploinsufficiency in a population of short stature patients and describe their anthropometric measurements.

METHODS: 574 short statured patients were evaluated in a single center (1992–2015). *SHOX* copy number was detected by quantitative polymerase chain reaction (qPCR) in 574 subjects, followed by multiplex ligation-dependent probe amplification (MLPA) and DNA sequencing in subjects with *SHOX* haploinsufficiency. We evaluated anthropometric measurements at birth, and at first examination. Skeletal abnormalities were recorded for patients with *SHOX* haploinsufficiency.

RESULTS: Thirty-two patients were excluded due to Turner syndrome ($n = 28$), *SRY*-positive 46,XX male karyotype ($n = 1$), or lacked clinical follow-up information ($n = 3$). The prevalence of *SHOX* haploinsufficiency was 9 out of 542 (1.7%). The nine children had decreased height −2.85 (0.6) SD scores (SDS) (mean (SD)) and weight −2.15 (1.36) SDS, $P < 0.001$ and $P = 0.001$, respectively. The sitting height/height ratio was increased, $P = 0.04$. Madelung deformity was diagnosed in three patients. Mean height was −2.9 (0.4) SDS at baseline and increased by 0.25 (0.2) SDS, $P = 0.046$, after 1 y of growth hormone (GH) treatment.

CONCLUSION: The prevalence of *SHOX* haploinsufficiency was 1.7%. The clinical findings indicating *SHOX* haploinsufficiency among the nine children were disproportionate short stature and forearm anomalies.

Short stature is defined by a height below −2 SD scores (SDS) from an age- and gender-related population mean. The etiologies of short stature are numerous including endocrine disease such as growth hormone deficiency and various chronic diseases (1).

Longitudinal growth is regulated by the growth hormone/insulin-like growth factor-I axis (GH-IGF-I axis). The free biological active form of IGF-I is inducing the chondrocytes of the epiphyseal plates in the tubular bones to proliferate (2). In addition, FGF receptors (3) and the short stature homeobox-containing *(SHOX)* gene are regulating linear growth of the skeleton (4).

In the absence of any known causes of short stature, the patient is diagnosed with idiopathic short stature, which is an exclusion diagnosis defined by a height below −2 SDS without any causative disorders (1). However, it has been estimated that 1.1–16.9% of the idiopathic short stature patients have a haploinsufficiency of the *SHOX* gene (4–16).

The *SHOX* gene resides in the pseudoautosomal region 1 (PAR1) of both sex chromosomes; it escapes X inactivation (17) and the gene is expressed by growth plate chondrocytes (18,19). *SHOX* haploinsufficiency disorders include Leri-Weill dyschondrosteosis (20), Langer mesomelic dysplasia (21) and is involved in short stature and skeletal abnormalities of Turner syndrome (22). It is important to identify a patient with *SHOX* haploinsufficiency in order for the patient to benefit from possible GH therapy, and to convey the necessary genetic counseling. The aim of this study was (i) to evaluate the prevalence of *SHOX* haploinsufficiency in a population of Danish short stature patients and (ii) to describe clinical and biochemical characteristics of the patients.

METHODS

Study Population

This study was a retrospective clinical study of 574 patients with growth retardation refereed to our tertiary pediatric endocrine department during the period of May 1992 to April 2015. The patients were selected due to the following inclusion criteria: (i) A diagnosis of short stature (ICD10 DE34.3) (ii) *SHOX* gene evaluation by qPCR. (iii) Available record files with necessary follow-up information. Data on anthropometric measurements and hormone analyses were collected from the patients' files.

Clinical Examination

Height and sitting height were measured on a wall-mounted stadiometer to the nearest 0.1 cm by trained staff. Weight was measured using electronic scales to the nearest 0.1 kg and BMI was calculated (weight in kilogram/height in meters^2). SDS were derived for sitting height, birth weight, birth length, height, weight, and BMI using normal reference populations (23–25). Target height (SDS) was calculated as the mean of the maternal and paternal height (SDS). Data on pubertal development (Tanner stage (26,27)), age at menarche, and phenotypic characteristics (Madelung deformities, sitting height, brachymetacarpy, hyperconvex nails, cubitus valgus, scoliosis, short neck, micrognathia, high arched palate, muscular hypertrophy, increased papillae mammae gap, thoracic hypertrophy, naevi, and short lower leg) were obtained from the patient record files if available.

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Table 1. Clinical characteristics of nine children with *SHOX* gene haploinsufficiency.

ID no.	1	2	3	4	5	6	7	8	9
Gender (M = male, F = female)	M	M	M	F	F	F	F	F	F
Birth weight (g)	3,430	3,800	2,380	2,450	3,650	3,800	3,500	3,400	1,700
Birth length (cm)	50	53	48	47	53	–[a]	–	52	–
Gestational age (weeks)	40	42	40	40	40	40	40	40	–
SGA (yes = 1, no = 0)	0	0	1	1	0	0	0	0	–
Maternal height (cm)	169.4	163.5	149.0	160.0	156.7	–	158.0	172.0	–
Maternal height (SDS)[b]	-0.02	-0.96	-3.25	-1.51	-2.03	–	-1.82	0.39	–
Paternal height (cm)	184.0	180.2	175.4	172.0	153.0	–	173.1	182.0	–
Paternal height (SDS)	0.55	-0.04	-0.9	-1.31	-4.25	–	-1.14	0.24	–
Target Height (SDS)	0.27	-0.50	-2.1	-1.41	-3.14	–	-1.48	0.32	–
Evaluation									
Age at referral for short stature and/or dyschondrosteosis (years)	5.03	14.10	0.49	5.86	13.86	6.12	12.22	8.53	10.93
Height at referral (cm)	98.5	150.5	64.0	103.0	138.5	103.7	139.2	118.8	123.0
Weight at referral (kg)	17.0	40.0	6.7	15.4	31.8	15.7	41.5	24.1	19.3
BMI at referral (kg/m^2)	17.5	17.7	16.3	14.5	16.6	14.6	21.4	17.3	12.8
Sitting height/height ratio[c]	0.57	0.53	–	0.58	0.57	–	0.54	0.57	0.52
Increased SH/H ratio (yes = 1, no=0)	1	0	–	1	1	–	0	1	0
Madelung deformity (yes=1, no=0)	0	1	0	0	1	–	–	1	–
Parent with Madelung deformity (M = mater, P = pater, no = 0)	–	–	M	–	–	–	–	-	–
Scoliosis (yes = 1, no = 0)	0	0	0	1	0	0	0	0	–
Leri-Weill dyschondrosteosis (yes = 1, no = 0)	–	1	–	–	1	–	–	1	–
Bone age—Chronological age[d]	-0.88	-2.41	-1.22	-0.30	-2.41	-0.97	1.24	-0.50	-1.89
Prepubertal (yes = 1, no = 0)	1	0	1	0	0	1	0	1	1
Menarche (yes = 1, no = 0)	0	0	0	1	1	0	1	0	0
Age at menarche (years)				13.47	14.55		11.35		
GH treatment (yes = 1, no = 0)	1	0	0	1	1	1	1	1	0
Age at GH treatment start up (years)	5.25			7.53	14.38	7.73	12.54	8.73	
***SHOX* haploinsufficiency**	Del. PAR1[e] and 2 MB region	Del.[f] *SHOX* gene and regulatory regions	c.46I T>C, p.L154L mutation of *SHOX* gene	Del. PAR1 and 113 kb regulatory region	Del. *SHOX* gene	Del. *SHOX* gene	Del. *SHOX* gene	Del. PAR1	Del. *SHOX* gene
Inherited mutation	–	Paternal	Maternal	–	Paternal	–	–	–	–
First deletion of MLPA probe (coordinates in hg19)[g]	227417	227417		227417	227417	–	–	500427	–
Last deletion of MLPA probe (coordinates in hg19)	3549335	949779		1431352	634287	–	–	1712162	–
Size of deletion (kb)	3321.918	722.362		1203.935	406.87	–	–	1211.735	–

[a]Not available. [b]SD score. [c]First available sit height. [d]First available bone age with bone health index (SDS). [e]Deletion of pseudoautosomal region 1. [f]Deletion. [g]Human genome reference no. 19.
MLPA, multiplex ligation-dependent probe amplification.

X-ray Investigations

Bone age and bone health index (BHI) was determined using an automated evaluation system (BoneXpert, Visiana, Denmark) (28). BHI is a size-corrected measure of the cortical thickness of the three middle metacarpals and is used to assess the bone mass of the patient. The calculation of the BHI (SDS) was based upon a large normative dataset of individual sex- and bone age-dependent SDS (29).

Karyotyping

Karyotyping was performed on lymphocytes isolated from peripheral blood using routine G-banding and counting of at least 10 metaphases, three of which were fully analyzed.

SHOX Copy Number Analysis by qPCR

The copy numbers of the *SHOX* gene were analyzed by qPCR with a forward and reverse primer targeting exon 1. Forward primer:

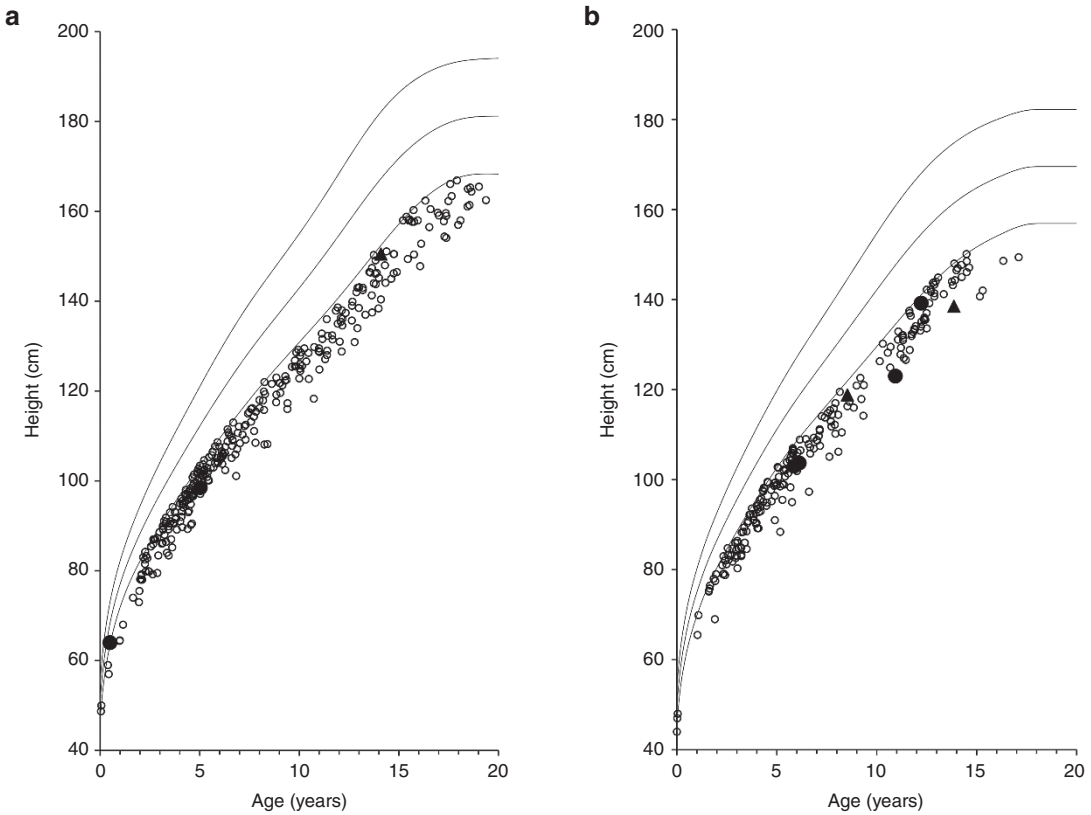

Figure 1. Longitudinal growth in short statured children with *SHOX* haploinsufficiency compared to short statured children with two copies of *SHOX*. (**a**) Male. (**b**) Female. Longitudinal growth in short statured children with two SHOX copies (open circles, $n = 533$), short statured children with SHOX gene haploinsufficiency (black circles, $n = 6$) and children with Leri-Weill dyschondrosteosis, short stature and SHOX gene haploinsufficiency (black triangles, $n = 3$) compared to a Danish normal reference population (25). Black lines correspond to −2 SD, mean and +2 SD.

5′-CTC-CTA-CCC-GCC-TGT-CCA-3′. Reverse primer: 5′-TCC-GCG-CGT-CTC-TTT-CTA-CT-3′. The *GAPDH* gene, which is located on an autosome (chromosome 12), was used as the internal control, using forward primer: 5′-CTC-CCC-ACA-CAC-ATG-CAC-TTA-3′, and the reverse primer: 5′-TTG-CCA-AGT-TGC-CTG-TCC-TT-3′. The qPCR was performed on a Mx3000P platform from Stratagene (Cedar Creek, TX) as previously described (30).

In cases where the gene copy number assessed by qPCR was incompatible with the patient's karyotype or if the phenotype clearly indicated *SHOX* haploinsufficiency, the patient was further screened for deletions or mutations in other exons of the *SHOX* gene by multiplex ligation-dependent probe amplification (MLPA) and *SHOX* gene sequencing.

MLPA

SHOX deletions were detected by using the MRC-Holland MLPA kit (Salsa P018-E and F1; MRC-Holland, Netherlands) according to the instructions of the manufacturer. The P018-G1 *SHOX* probe-mix contains 48 MLPA probes. Seven of the probes were specific for each exon of the *SHOX* gene. In total 26 probes targeted sequences located in the *SHOX* gene and in regions corresponding to the regulatory areas of *SHOX* transcription. The probe-mix also included 13 probes located outside of the PAR regions and 9 autosomal probes. Data analysis was performed using either the GeneMarker v.2.0 (Softgenetics, Stage College, PA) or Coffalyser software v. 9.4 (software by MRC-Holland, Netherlands) (31).

DNA Sequencing

The coding and intron flanking sequences of *SHOX* (NM_000451.3 and NM_006883.2) were PCR amplified and the products were subjected to Sanger sequencing by a standard approach on an ABI3130XL sequencer. Primers and PCR conditions are available upon request.

Hormone Assays

All blood samples were analyzed at Department of Growth and Reproduction, Rigshospitalet, Copenhagen, Denmark. Serum IGF-I concentrations were determined by three different assays during the 23-y study period. Until 2008 IGF-I concentrations were determined by radioimmunoassay serum as previously described (32). Limit of detection for IGF-I was 20 ng/ml and the coefficients of variation for the inter- and intra-assay were 15 and 6%, respectively. IGF-I (SDS) was calculated from our reference data (32). From 2008 to 2013, serum IGF-I concentrations were determined by using a solid-phase enzyme-labeled chemiluminescent immunometric assay (Immulite 2000, Diagnostic Products Corporation, L.A., CA) (33). Limit of detection was 20 ng/ml and the inter- and intra-assay coefficients of variations were 5.9 and 2.0%, respectively. IGF-I (SDS) was calculated from our reference data (34). From 2013, IGF-I concentrations were measured by an iSYS assay on IDS-iSYS Multidiscipline Automated Analyser (Immunodiagnosticsystems, UK). Limit of detection for IGF-I was 8.8 µg/l with an inter-assay coefficients of variation of 4.7–7.2%.

Statistical Analysis

The frequency of the *SHOX* haploinsufficiency is presented in percentage. We performed descriptive statistics on all patients. Variables are shown as standard deviation scores. Statistical analyses on SDS were done by using a One-Sample *T*-test. A P value < 0.05 was considered significant. For the evaluation of height (SDS) and IGF-I (SDS) prior to GH therapy and after 1 y of treatment, we performed a Wilcoxon Signed Ranks test. We used nonparametric testing by Mann-Whitney *U*-test when comparing the clinical characteristics of the patients with *SHOX* haploinsufficiency to the patients with a normal copy number of the *SHOX* gene. All statistical analyses were performed by using IBM SPSS Statistics for Windows, version 22.0 Armonk, NY: IBM Corp.

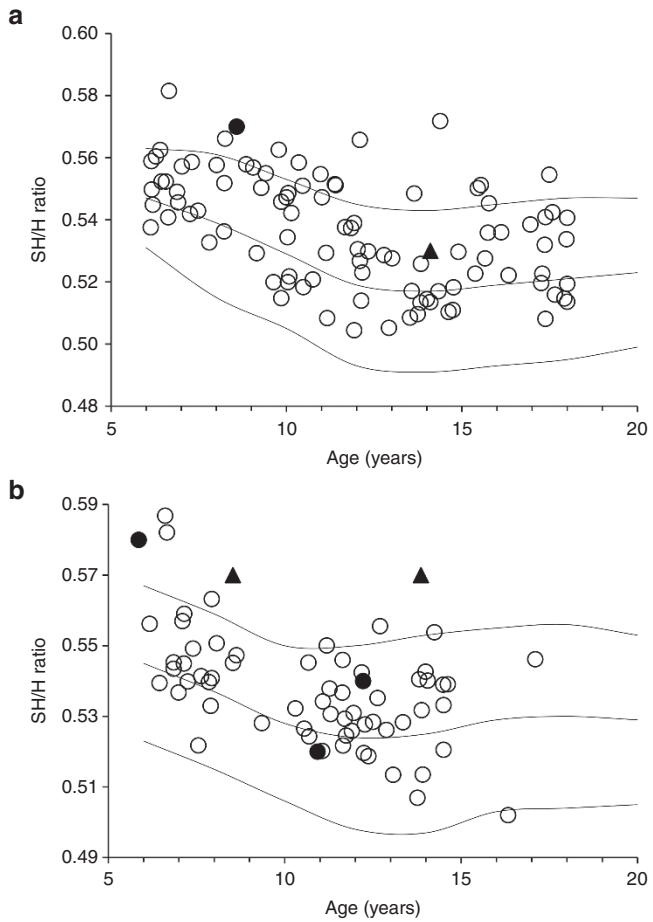

Figure 2. Sitting height/height ratio in short statured children with *SHOX* haploinsufficiency compared to short statured children with two copies of *SHOX*. (**a**) Male. (**b**) Female. Sitting height/height ratio in short statured children with two *SHOX* copies (open circles, $n = 157$), short statured children with *SHOX* gene haploinsufficiency (black circles, $n = 4$) and children with Leri-Weill dyschondrosteosis, short stature and *SHOX* gene haploinsufficiency (black triangles, $n = 3$) compared to a Danish normal reference population (23). Black lines correspond to −2 SD, mean and +2 SD.

Ethical Considerations

All blood samples and genetic analyses were taken as part of the clinical follow-up of the patients. The project was approved by Danish Data Protection Agency permission for biobank: RH-2016–177, I-Suite no: 04732.

RESULTS

In the cohort of 574 patients, 41 patients had one copy number of the *SHOX* gene. Thirty-two of these had either Turner syndrome ($n = 28$), a *SRY*-positive 46,XX male karyotype ($n = 1$), or lacked clinical follow-up information ($n = 3$).

Nine children (three boys) out of 542 patients (318 boys) had one copy number of the *SHOX* gene, and 533 patients had 2 *SHOX* gene copy numbers, resulting in a prevalence of 1.7%. The age of the children with *SHOX* haploinsufficiency at referral ranged between 0.5 and 14 y. Two girls (ID no. 6 and 9) were adopted from China and India, respectively.

SHOX gene point mutations or deletions are shown for each child ($n = 9$) in **Table 1**. Three children (two boys) had known affected relatives.

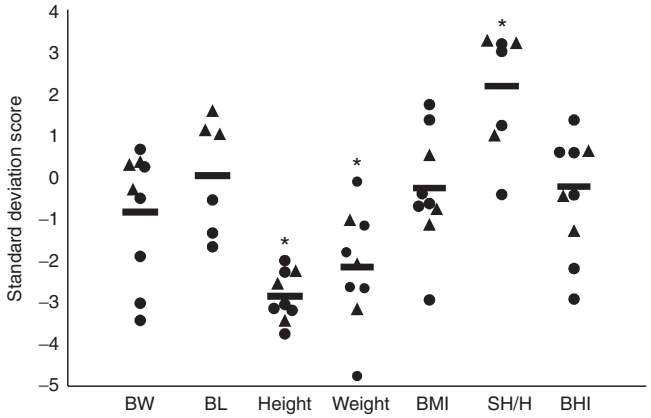

Figure 3. Anthropometric measurements at first presentation for patients with *SHOX* gene haploinsufficiency (black circles, $n = 6$) and patients with Leri-Weill dyschondrosteosis and *SHOX* gene haploinsufficiency (black triangles, $n = 3$). Black stars represent the anthropometric measurements that differed significantly. Black lines represent mean values for each variable. BHI, bone health index; BW, birth weight; BL, birth length; SH/H, sitting height/height ratio. All data are expressed as SD scores.

Anthropometric Measurements

Height, weight, BMI, and pubertal stage at referral for each child with *SHOX* haploinsufficiency are shown in **Table 1**. Height at referral and first available sitting height/height ratio are illustrated in **Figures 1** and **2** comparing the nine children with *SHOX* haploinsufficiency to the 533 patients with two *SHOX* copies. The sitting height/height ratio was above +2 SDS in 4 children (1 boy) with *SHOX* haploinsufficiency. Anthropometric measurements at birth and at time of first examination for the children with *SHOX* haploinsufficiency are presented as standard deviation scores in **Figure 3**. The children with *SHOX* haploinsufficiency had significantly decreased height −2.85 (0.6) SDS (mean (SD)) and weight −2.15 (1.36) SDS, $P < 0.001$ and $P = 0.001$, respectively. The sitting height/height ratio was significantly increased 2.17 (1.28) SDS, $P = 0.04$, whereas BMI did not differ. We found a decreased BHI in five children (two boys) but this did not reach statistical significance.

Comparison of anthropometric measurements between the children with *SHOX* haploinsufficiency and patients with two *SHOX* copies are shown in **Table 2**. Height (SDS) and the SH/H ratio were significantly different between the two groups, $P = 0.002$ and $P = 0.048$, respectively.

X-ray Investigations

Madelung deformities were diagnosed in three children (one boy). One girl presented with Madelung deformity and bowing of corpus radii. One girl (ID no. 4) had a wrist deformity of the left wrist, but the deformity was not compatible with Madelung deformity. One girl with *SHOX* haploinsufficiency presented with scoliosis (**Table 1**).

The bone age showed a mean delay of −1.04 (1.1) years (mean, (SD)) compared to chronological age (**Table 1**).

Table 2. Short stature patients with and without *SHOX* gene haploinsufficiency

	Patients with one copy of *SHOX*	Patients with two copies of *SHOX*	*P* value[a]
***N*[b] = 542 (male = 318)**	9	533	NS[c]
Gestational age (weeks)	40 (40.0 - 40.5)[d]	38 (35.0 - 40.0)	NS
Birth weight (gram)	3,415 (2,425 - 3,688)	2,870 (2,268 - 3,261)	NS
Birth length (cm)	51.0 (47.8 - 53.0)	49.0 (45.0 - 51.0)	NS
Maternal height (SDS)[e]	-1.51 (-2.03 to -0.02)	-1.35 (-2.02 to -0.67)	NS
Paternal height (SDS)	-0.90 (-1.31 - 0.24)	-0.96 (-1.74 to -0.22)	NS
Height (SDS)	-2.54 (-3.14 to -2.24)	-2.77 (-3.17 to -2.44)	P = 0.002
SH/H ratio[f]	0.57 (0.53 - 0.57)	0.54 (0.52 - 0.55)	P = 0.048

[a]Nonparametric testing by Mann-Whitney *U*-test. [b]Number of patients. [c]Not significant. [d]Data are presented as median (25th-75th). [e]SD scores. [f]Sitting height/height ratio.

Growth Hormone Therapy

Six children (one boy) of the nine patients with *SHOX* haploinsufficiency were treated with GH. One girl (ID no. 4) was treated with combined GH and gonadotropin-releasing hormone (GnRH) analog therapy. Changes in GH dose, height (SDS), and IGF-I (SDS) before and during the first year of GH treatment are shown in **Figure 4**. Mean height (SDS) was −2.9 (0.4) SDS (mean (SD)) at baseline, and −2.67 (0.3) SDS, P = 0.046 after 1 y of treatment. Thus, average height gain was 0.25 (0.2) SDS after 12 mo of GH therapy.

IGF-I (SDS) did increase from the low normal range to the high normal range during the first year of treatment in all patients, P = 0.028. Mean dose of GH was 28.8 (13.7) (μg/kg/day) (**Figure 4**).

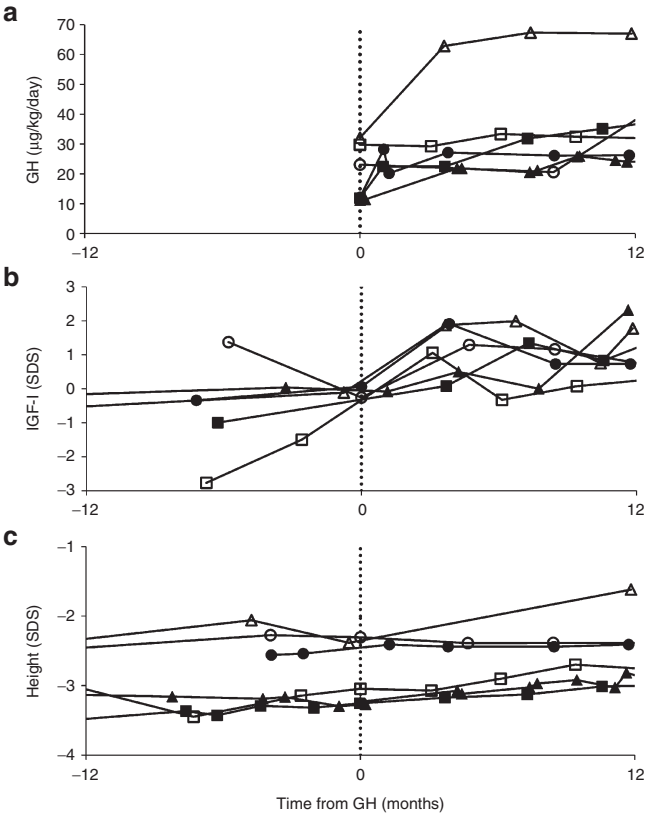

Figure 4. Changes in GH dose, height (SDS) and IGF-I (SDS) before and during the first year of GH therapy. (**a**) Changes in GH dose (μg/kg/day) during the first 12 mo of GH therapy. (**b**) Changes in IGF-I (SDS) before and during the first 12 mo of GH therapy. (**c**) Changes in height (SDS) before and during the first 12 mo of GH therapy. Data are shown for six patients with *SHOX* gene haploinsufficiency. Black dashed lines represent the start of the GH therapy. Open square = ID no. 1. Open triangle = ID no. 4. Black square = ID no. 5. Black triangle = ID no. 6. Open circle = ID no. 7. Black circle = ID no. 8.

DISCUSSION

In this large single-center study of 574 short statured patients, we found a prevalence of *SHOX* haploinsufficiency by qPCR of 1.7%. Children with *SHOX* haploinsufficiency showed moderate height gain following GH therapy.

Former studies have shown a prevalence of point mutations or deletions of the *SHOX* gene between 1.1–16.9% among patients with idiopatic short stature (4–16). Our clinical findings suggest that patients with forearm anomalies and short stature with an increased sitting height/height ratio are the most likely to have *SHOX* haploinsufficiency.

In our study population, four children were known with Madelung deformity or wrist deformity prior to *SHOX* gene analysis. Another study has shown that the prevalence of Madelung deformity is increasing substantially when radiographic evidence is included (35). It is likely that the frequency of Madelung deformity would be even higher if all patients in our study population were evaluated radiologically. Thus, we do not know if some of the 533 patients with two *SHOX* copies may have any undiagnosed skeletal lesions.

A phenotypic scoring system has been proposed by Rappold *et al.* as a way to select patients for *SHOX* screening, which included a variety of clinical characteristics (7). Our findings of short stature, low weight, and an increased sitting height/height ratio as a critical phenotypic characteristic are consistent with this scoring system. By contrast, BMI did not differ for the nine children with *SHOX* haploinsufficiency.

The sitting height/height ratio was significantly increased among the children with *SHOX* haploinsufficiency compared to patients with two *SHOX* copies. This confirms that an asymmetrical short stature is more frequently seen in our patients with *SHOX* haploinsufficiency.

Three children (two boys) included in this study had known affected relatives. A careful family history according to height (SDS), body proportions, and dysmorphic characteristics of relatives is necessary when selecting patients for *SHOX* screening, since *SHOX* is inherited in a pseudoautosomal dominant manner. Former studies have shown that it is not uncommon for patients with *SHOX* haploinsufficiency to have an affected parent (7,13).

GH therapy of children with *SHOX* haploinsufficiency resulted in increasing serum IGF-I levels during the first

year of GH treatment. The children experienced a moderate height gain of 0.25 (0.2) SDS, which is less compared to previous studies of *SHOX*-deficient patients receiving GH therapy (11,35,36). This could be due to the use of lower GH doses in our study of 29 μg/kg/d compared to a previously randomized, controlled study that was using GH doses of 50 μg/kg/d (36).

In our study, one girl (ID no. 4) initiated GnRH analog therapy 1 y after her GH treatment was started due to precocious puberty. She was furthermore a part of the North European Small for Gestational Age (NESGAS) study and on this behalf she received a fixed high GH dose. She reached a height above −1 SDS with 3 y of combined treatment. Other case studies have shown an improvement of the height gain in *SHOX*-deficient patients treated with combined GH and GnRH analog therapy (37,38), but the long-term outcome of this combined treatment remains still to be elucidated in a large cohort of patients.

In addition, it is important to remember that the *SHOX* gene is expressed in the chondrocytes of the growth plates in the tubular bones (18,19). It has been estimated that *SHOX* haploinsufficiency is causing a more rapid fusion of the growth plates resulting in a downward shift of the growth velocity by the time the patients reach puberty (39). This could be an explanation of why some *SHOX*-deficient patients do not benefit from GH therapy. Furthermore, patients with deletions or mutations delimited to the *SHOX* gene's enhancer regions might show a greater respond to GH therapy (40). For this reason, it may be advantageous to start GH therapy as early as possible and carefully examine which *SHOX*-deficient patients that are responding appropriate to the GH therapy. Whether the *SHOX*-deficient patients would benefit even more with a combined treatment of GH and GnRH analog therapy is still unclear.

In conclusion, we demonstrated *SHOX* haploinsufficiency in nine children corresponding to a prevalence of 1.7% among 542 patients with short stature. Due to the importance of early diagnosis and treatment, screening for *SHOX* haploinsufficiency among disproportionate short statured children must be considered.

STATEMENT OF FINANCIAL SUPPORT

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