The growth response to growth hormone treatment is greater in patients with SHOX enhancer deletions compared to SHOX defects.

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Abstract

Objective

Short stature caused by point mutations or deletions of the short stature homeobox (SHOX) gene (SHOX haploinsufficiency, SHI) is a registered indication for growth hormone (GH) treatment. Patients with a SHOX enhancer deletion (SED) have a similar phenotype, but their response to GH is unknown. It is uncertain if duplications of SHOX or its enhancer (SDUP) cause short stature. This study aimed to describe the clinical characteristics and growth response to GH treatment in patients with aberrations of SHOX and its enhancers.

Design

In this retrospective multi-center study (2002 to March 2014) clinical information was available from 130 patients (72 SHI, 44 SED, 14 SDUP) of whom 52 patients were treated with GH. We evaluated height, sitting height, arm span, dysmorphic features and indicators of the growth response to GH (delta height SDS, height velocity and Index of Responsiveness).

Results

Patients with SEDs showed similar height SDS to patients with SHI (-2.3 and -2.6, respectively, p=0.2), but they were less disproportionate (sitting height/height ratio SDS 2.0 vs. 3.1 (p<0.01), and extremities-trunk ratio 2.57 vs. 2.43 (p=0.03)). The first year growth response to GH treatment was significantly greater in prepubertal patients with SEDs than SHI. None of the patients with an SDUP was disproportionate and SDUP cosegregated poorly with short stature; their growth response to GH treatment (n=3) was similar to the other groups.

Conclusions

Patients with SEDs are equally short, but less disproportionate than patients with SHI, and show a greater response to GH.
Introduction

The short stature homeobox \( (SHOX) \) gene encodes a homeodomain protein that acts as a transcriptional activator \(^1\). It is strongly expressed in the developing limb and is thought to contribute to longitudinal growth by regulating proliferation and differentiation of the chondrocytes in the growth plate \(^1\). The gene is located on the short arm of the X- and Y-chromosomes in the pseudoautosomal region 1 (PAR1) and escapes X-inactivation, which leads to a pseudoautosomal inheritance pattern \(^2,^4\). A homozygous deletion or point mutation of \( SHOX \) results in an extreme phenotype of osteodysplasia called Langer Syndrome. The classical presentation of \( SHOX \) haploinsufficiency (SHI), due to heterozygous \( SHOX \) defects, is Léri-Weill Dyschondrosteosis (LWD), characterized by the typical clinical triad of short stature, mesomelia and Madelung deformity. However, \( SHOX \) defects can also be found in short children without dysmorphic features and normal body proportions, who had initially been considered as having idiopathic short stature.

Recent studies have shown that deletions in the downstream enhancer region of \( SHOX \), and (less frequently) in the upstream enhancer region (SEDs), can result in a clinical picture similar to that of SHI \(^5,^10\). In about two thirds of cases LWD is caused by intragenic point mutations or deletions of the complete coding sequence of \( SHOX \), and in one third of cases by deletions in the enhancer sequences in the 3′- or 5′-flanking region of \( SHOX \), leaving the gene itself intact \(^11\).

A duplication of \( SHOX \) would be expected to be associated with tall stature. The relatively tall height of individuals with Klinefelter syndrome and XXX syndrome is indeed thought to be caused by increased \( SHOX \) expression. Surprisingly, duplications of \( SHOX \) and/or its enhancers (SDUPs) have also been found in short children \(^12,^14\), although only in few of them additional clinical features characteristic for \( SHOX \) dysfunction were noted, and parents carrying the duplication were usually of normal stature. Therefore, the pathogenicity of SDUPs is still uncertain \(^12,^15\).
Growth hormone (GH) treatment is registered for children with short stature caused by SHI, currently defined as a deletion or point mutation in SHOX itself, based on studies showing a significant increase in height SDS during the first two years of treatment \(^{16,17}\). The effect of GH treatment, however, had not yet been evaluated for patients with an SED or SDUP \(^{17}\).

The principal aim of this study is to investigate whether the effect of GH treatment in children with an SED is comparable to that in children with SHI. Because of the phenotypic similarity between the two groups \(^2,10\), we hypothesized that the effect of GH treatment in children with a deletion of the down- or upstream enhancer region of SHOX would be similar to the effect in children with a SHOX point mutation or deletion. In addition, the clinical characteristics of patients with point mutations or deletions of the SHOX gene, deletions of the SHOX enhancer regions, or duplications of SHOX or its enhancer were investigated.

**Subjects and Methods**

**Study design:**

In this retrospective study patients diagnosed with SHI, SEDs and SDUPs in the Laboratories for Diagnostic Genome Analysis of the Departments of Clinical Genetics of the Leiden University Medical Center (LUMC, Leiden), VU University Medical Center (VUMC, Amsterdam), Erasmus Medical Center (EMC, Rotterdam) and University Medical Center Groningen (UMCG, Groningen) in the Netherlands from 2002 onwards were included.

Deletions and duplications of SHOX and its enhancer regions were diagnosed with Multiplex Ligation Probe Amplification (MLPA) with the P018 kit from MRC-Holland (Amsterdam, The Netherlands) using standard procedures. Most deletions and duplications were characterized with the P018-D1 MLPA kit but other versions of the kit had been used as well. A graphical picture of the position of the various probes is shown in Supplementary Figure 1. If a deletion of the SHOX gene was detected, Sanger sequencing was not performed. If MLPA analysis revealed no deletions in the SHOX gene or
if it revealed a deletion of the \textit{SHOX} enhancer region, Sanger sequence analysis of the complete coding region of \textit{SHOX}, including intron-exon boundaries, was performed \cite{18}. The \textit{SHOXa} isoform was sequenced using the Ensembl transcript ‘ENST00000381575’ (NM_000451.3).

In general, patients with short stature were referred for DNA analysis by pediatric endocrinologists or clinical geneticists. After approval from the Medical Ethical Committee of the Leiden University Medical Center patients diagnosed with an aberration of \textit{SHOX} or its enhancer region were asked to participate in this study. Written informed consent was obtained from the participating subjects or from the subjects’ parents.

\textit{Participants}

Until March 2014 390 patients were diagnosed with defects of \textit{SHOX} or its enhancer regions (figure 1). Patients living in other countries than the Netherlands (n=23) and patients in whom the \textit{SHOX} variants were considered non-pathogenic (n=27) were not asked to participate in the study. Eight patients were not included because the treating physicians were reluctant to ask the patients and their parents for permission and another eight patients were diagnosed with other disorders also contributing to short stature: Multiple Epiphyseal Dysplasia (n=2), Smith Magenis Syndrome (n=1), \textit{IGFI} mutation (n=1), Albright’s Hereditary Osteodystrophy (n=1), an additional deletion of chromosome 6 (n=1), bio-inactive GH (n=1) and an isodicentric Y-chromosome (n=1). The affected parents of these patients were excluded as well (n=4).

The remaining 320 patients were asked to participate in this study. At closure of the database, we had received 157 informed consent forms and collected clinical information from 130 patients (88 probands, from 74 families). Fifty-six patients from 47 families were treated with a variety of commercially available recombinant GH products.

\textit{Clinical assessment}
Clinical data from the referring hospital and the Dutch National Registry for GH Treatment in children located at the Dutch Growth Research Foundation (“Stichting Kind en Groei”, Rotterdam, The Netherlands) were collected from all patients who provided informed consent. These included: birth length, birth weight, head circumference at birth, height, weight, sitting height (SH), arm span, forearm length, dysmorphic signs (Madelung deformity, cubitus valgus, bowing of the radius and ulna or muscular hypertrophy), serum IGF-I and IGFBP-3 and skeletal age. Information about linear growth, serum IGF-I, IGFBP-3 and skeletal age, was collected every 12 ± 3 months up to 4 years for patients treated with GH. From the parents, height, body proportions and SHOX test results were obtained.

Patient analysis

Patients were divided into three groups: group 1 consisted of patients with SHI; group 2 of patients with a SED; and group 3 of patients with an SDUP. Subgroups of each group were treated with GH (Fig. 1). For all patients we analyzed the available clinical data and compared the clinical data of probands to those of their parents.

To assess the effect of GH therapy in patients with a deletion in the SHOX enhancer region and patients with a SHOX defect we used four outcome measures for the first 4 years of treatment. The first three of these, the difference in height SDS (ΔHtSDS), height velocity (cm/year) and height velocity as SDS for age (based on the Swiss longitudinal growth study 19), are parameters of the growth response. The fourth measure is the “Index of Responsiveness (IoR)”, based on prediction models for growth during the first 4 years on GH treatment in girls with Turner Syndrome 20. We chose this approach because the growth response to GH treatment is reported as being similar for patients with SHOX defects and patients with Turner Syndrome 21. Because previous studies have shown that prepubertal growth is largely independent of sex, we applied the prediction models for Turner Syndrome in males and females 22. For each patient the predicted growth velocity (based on
clinical predictors of the growth response) as described by Ranke et al.\textsuperscript{20, 23, 24} was subtracted from the observed height velocity and transformed into a Studentized Residual. A Studentized Residual above 0 is an indicator of a relatively high responsiveness to GH treatment, adjusted for potential confounders. Strictly speaking, the prediction formulas for Turner syndrome are not applicable for children who undergo spontaneous puberty. However, since prepubertal children only represented a relatively small part of the total number of patients treated with GH, we also applied the formulas to all patients, irrespective of their pubertal status, and compared the results between groups 1 and 2.

Secondary outcome measures included the increase in bone age/calendar age ratio during GH therapy ($\Delta$BA/$\Delta$CA ratio), the increase in serum IGF-I Standard Deviation Score (SDS) during GH treatment ($\Delta$IGF-I) and the effect of GH on body proportions (the change in sitting height/height SDS, $\Delta$SH/H SDS). Skeletal age, serum IGF-I and SH/H SDS closest to start of GH were compared to the last measured BA, serum IGF-I and SH/H SDS. All analyses were carried out separately for patients who remained prepubertal during the observation period, and for all patients. Results for prepubertal children were compared to published data on the growth response to GH treatment in patients with SHI. In this study by Blum et al, the percentage of $SHOX$ deletions and mutations was similar to our study, as was the GH dose\textsuperscript{21}.

**Statistical Analysis**

Height SDS and SH/H SDS were calculated based on Dutch nation-wide references\textsuperscript{25, 26}. Height velocity SDS was calculated based on the Swiss longitudinal study\textsuperscript{19}. Midparental height adjusted for gender, assortative mating and parent-offspring correlations, adapted to the most recent Dutch population reference\textsuperscript{26}, was expressed as SDS (conditional target height SDS)\textsuperscript{27}. Body Mass Index (BMI) SDS was calculated based on the 1980 Dutch nation-wide growth study\textsuperscript{28}. Birth weight and birth length for gestational age were transformed to SDS values using the standards of Niklasson et al\textsuperscript{29}. IGF-I and IGFBP-3 data were collected as measured by local laboratories. Since the IGF-I assays
in the Netherlands have been harmonized and titrated on the original assay used for preparing reference data. Levels were expressed as SDS. Bone age of patients was analyzed as reported by the treating physicians. The extremities-trunk ratio, which compares extremities length to trunk length, was calculated as follows: (calculated subischial length + arm span) / sitting height.

To calculate the predicted height velocity, according to the prediction model for Turner syndrome, height and weight were converted to SD scores using the height standards of Prader et al and the weight standards of Freeman et al, and mid-parental height was calculated as (father’s height SDS + mother’s height SDS) / 1.61.

Data were expressed as means and SD and compared between groups with the Student’s t-test for continuous variables with a Gaussian distribution and chi-squared test for categorical variables. If continuous variables were not normally distributed the Mann-Whitney U test was used. Significance was considered at the 5% level (p<0.05).
Results

Of the 88 probands (from 74 families), 55 (from 42 families) inherited the mutation from one of the parents, 26 from their father (47%) and 29 from their mother (53%). Seven patients had a de novo defect, and for the remaining cases (n=26) no information was available.

Mutations of SHOX and its enhancer

Of the 88 probands, 54 were diagnosed with a point mutation (n=11, from 10 families) or deletion (n=43, from 35 families) of SHOX (SHI, group 1). Growth data were similar, except for a slight but statistically significant difference in height (-3.0 for SHOX point mutations, -2.5 for SHOX deletions, p=0.04). There was no statistically significant correlation between height SDS of the index cases and that of the affected or unaffected parents. In order to increase statistical power, we clustered both types of SHI in one group.

There were 26 patients (from 22 families) with a deletion of the SHOX downstream or upstream enhancer region (SED, group 2). Table 1 shows the clinical characteristics at first visit of groups 1 and 2. Children from group 2 were less disproportionate than children from group 1 (SH/H SDS 3.1 vs. 3.0, p<0.01 and Extremities-Trunk ratio 2.43 vs. 2.57, p=0.03). Madelung deformity was present in 23 out of 74 individuals (31%), 17 in group 1, 6 in group 2 (p=0.11). Information on other dysmorphic features, such as cubitus valgus, bowing of the forearm and muscular hypertrophy were often not reported and could therefore not be compared between groups.

Height SDS of patients with a de novo mutation was compared to the conditional Target Height SDS. In case of a familial mutation, height SDS was compared to height SDS of the affected and the unaffected parent (Table 1). As expected, in both groups the proband’s height SDS was closer to height SDS of the parent carrying the SHOX aberration than to the other parent’s height SDS. Affected parents in group 2 were significantly less short than affected parents in group 1 (-1.9 vs -2.4, p=0.03).
Duplications of SHOX and its enhancer

The clinical features of the 8 patients (from 7 families) with duplications of SHOX or its enhancer (SDUP, group 3) are shown in Table 2. In none of them SH/H SDS was above +2 SDS, and only one parent carrying the duplication was short. The duplicated SHOX probes of these patients are shown in Supplementary Fig. 1.

Growth response to GH

From the 56 probands who were treated with biosynthetic GH, we obtained clinical information during GH treatment about 37 patients from group 1 (66%), 12 patients from group 2 (22%) and 3 patients from group 3 (5%) (Fig. 1). Table 3 shows the baseline data and growth response for patients from groups 1 and 2 who remained prepubertal. For comparing HV and IoR between groups 1 and 2 we only included patients of whom HV before start of GH was available. Except for a small, but statistically significant, difference in HV before start of GH treatment (5.0 for group 1, 6.1 for group 2, p=0.02), there were no significant differences between the two groups at start of GH. In the first year all three parameters of growth response, delta height SDS, HV and HV SDS were significantly greater in group 2. The difference between the two groups for the IoR was just above the level of statistical significance (Table 3). A graphical representation of these data is shown in Fig. 2 and Fig. 3, showing height SDS and HV in response to GH treatment for four cohorts of children who remained prepubertal during GH treatment for 1-4 years, in comparison to data reported by Blum et al. 16. In both groups the growth response was greatest in the first year of GH treatment and declined thereafter.

For all patients from groups 1 and 2, irrespective of pubertal status during treatment, numerical data are shown in Supplementary Table 1, and graphical representations in Supplementary Figures 2 and 3. All parameters of the growth response and responsiveness in the first year of GH treatment were significantly greater for group 2 compared with group 1.
Only three patients from group 3 (SDUP), all prepubertal, were treated with GH, and their response is shown in Table 2. In the first year of GH treatment these patients gained on average 0.8 SDS in height and achieved a HV of 9.6 cm in one year, which is similar to patients from group 1 and 2. Because of the uncertainty about the pathogenicity of these duplications, and the small number of patients, no further statistical analysis was carried out.

**Effect of GH on serum IGF-I, skeletal maturation and body proportions**

Serum IGF-I SDS (mean ±SD) increased similarly in groups 1 and 2 (2.4±1.0, n=29 and 2.4±0.9, n=9 respectively). Baseline median IGF-I was -0.4 SDS (range -3.3 to 0.9 SDS) and on treatment median (SD) serum IGF-I was 1.9 (range 0 to 2.9 SDS), after a median treatment duration of 2.7 (0.2 to 5) years. The ratio between bone age and chronological age increased by 0.1 in both groups over a median duration of 2.7 years (range 1 to 5 years after start GH treatment), and body proportions did not significantly change during treatment (ΔSH/H ratio SDS of 0.4 and -0.1 in groups 1 and 2, respectively). There were no significant differences between the two groups.

**Discussion**

Children with a deletion of the *SHOX* enhancer region are equally short, but less disproportionate than patients with a *SHOX* deletion or point mutation and have a similar frequency of Madelung deformity. Their parents carrying the variant are less short and disproportionate than parents carrying a *SHOX* deletion or point mutation. For children who remained prepubertal during GH treatment, the growth response was slightly, but statistically significantly, greater in patients with SEDs, even though the serum IGF-I response was similar for both groups. The growth response of patients with SHI was similar to that reported previously.¹⁶

The results of genetic testing in our patients, showing deletions of *SHOX* and its enhancer in 35 and 22 out of 74 families (47 and 30%, respectively), far more than *SHOX* point mutations (10/74 families, 14%), demonstrates the efficiency of our stepwise diagnostic approach, consisting of an
MLPA for the detection of copy number variants followed by Sanger sequencing. Deletions of *SHOX*
or its enhancer region were found in around 80% of patients in other reports as well \(^{34,35}\).

Our findings confirm those of previous reports \(^{5,6,10}\) that patients with SEDs show a similar degree of short stature compared to patients with SHI. We also confirm the remarkable heterogeneity of statural growth in carriers of *SHOX* defects both in probands and affected parents, as well as in carriers of SEDs. Similarly to body stature, also for body proportions the variability was wide for both groups. Patients with SEDs were on average less disproportionate than patients with SHI if the SH/H ratio and the extremities/trunk ratio were taken as criteria, but the arm span/height ratio was not different. Consistent with an earlier report \(^{35}\), patients with SEDs also had a slightly lower BMI than patients with SHI, although mean BMI SDS in both groups was close to average for the population. These observations suggest that the predictive value of a BMI above the mean as part of the clinical score developed by Rappold et al. \(^{36}\) may have been overestimated and that normal body proportions should not be considered a contraindication for *SHOX* testing.

While duplications of *SHOX* have been reported to be associated with normal to tall stature \(^{37-39}\), there are also reports on a possible association with short stature \(^{12-14}\). In one of these reports it was hypothesized that patients with smaller duplications may be more severely affected than patients with larger duplications \(^{13}\). In another report \(^{12}\) it was speculated that patients with duplications which included the *SHOX* enhancer could impair gene regulation by interfering with the three dimensional chromatin spatial organization, resulting in impaired contact between the enhancer and promoter \(^{40}\). The phenotype of these patients is quite variable \(^{13,39}\), and the clinical characteristics of patients with duplications of only the *SHOX* downstream enhancer region have not been described so far. Based on these reports we tried to collect as much as possible clinical information about the children and their parents with *SHOX* duplications (n=5) and duplications of the enhancer region (n=9) (Table 2). Height SDS in our patients ranged from -3.5 to -2.2, and SH/H SDS from 0.7 to 1.9. Height SDS of parents carrying the duplication varied from -2.5 SDS to +1.2 SDS and in unaffected parents from -
3.5 to -0.5. In some patients a causal association between the \textit{SHOX} duplication and the child’s short stature appeared unlikely, for example in the child born severely SGA with normal body proportions and a normally statured parent carrying a small duplication (Table 2, case 8). Even though the absence of body disproportion might be partially explained by the young age of the majority of these children, the observation that body disproportion is absent in all children, and the absence of clinical features in their parents, cast doubt on the pathogenicity of these genetic variants.

This is the first study assessing the effect of GH treatment in patients with SEDs in comparison with SHI. The growth response in patients with SHI was similar to previously reported data \textsuperscript{16}, but the conventional outcome measures of the first year growth response (height velocity, height velocity SDS and change in height SDS) showed a greater effect in prepubertal patients with SEDs. The GH dose between the two groups was similar, and the recommended dose for SHOX haploinsufficiency as reported by Blum et al \textsuperscript{16}, seems efficacious for patients with SHOX defects as well as enhancer deletions. Since the growth response in patients with SHI has been reported as similar to that of girls with Turner syndrome, we used the prediction models for Turner syndrome \textsuperscript{20} to calculate the Index of Responsiveness (IoR). The IoR in group 2 was slightly higher than the IoR in group 1, but this difference was not significantly different in prepubertal children. The IoR was, however, significantly different if all patients were included, regardless of their pubertal status. The reason why children with SEDs seem to respond slightly greater to GH treatment remains unclear. We speculate that if GH, via downstream GH-dependent transcription factors, promotes expression of \textit{SHOX}, the presence of two intact (functional) copies of \textit{SHOX} in SED may cause a higher responsiveness to GH. Another possible explanation for the higher responsiveness to GH could be that SHOX deficiency is less severe in the presence of enhancer deletions. This hypothesis would explain the lower degree of skeletal disproportion and Madelung deformity observed, but not, the same degree of short stature.
This study has several limitations. First, there may be an ascertainment bias towards patients with typical clinical features of LWS, because screening for \textit{SHOX} deficiency is not performed routinely in all cases of short stature. Second, due to the retrospective character of the study, not all variables were available and we had to depend on data as reported by multiple physicians. Third, we were only able to investigate 40\% of potentially eligible cases. However, it seems unlikely that this has generated sampling bias. Fourth, similarly to previous studies, the effect of GH treatment could not be compared with untreated controls. However, our data are well in line with previous reports \cite{16,17}, and there is little doubt that GH is effective for this indication. Finally, the number of patients with \textit{SHOX} or \textit{SHOX} enhancer duplications was too small to draw any firm conclusion on the pathogenicity of these duplications, and on their growth response to GH treatment.

In conclusion, we show that children with SEDs are less disproportionate than children with SHI, and that in both groups height SDS and body proportions vary widely. Children with a SED show a slightly greater growth response to GH treatment to children with SHI. The clinical significance of SDUPs remains uncertain.
Declaration of interest and funding

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children born small for gestational age and short children with normal birth size (idiopathic

15. Wit JM, van Duyvenvoorde HA, van Klinken JB, Caliebe J, Bosch CA, Lui JC, Gijssbers AC,
Bakker E, Breuning MH, Oostdijk W, Losekoot M, Baron J, Binder G, Ranke MB &
Ruivenkamp CA. Copy number variants in short children born small for gestational age. Horm

hormone is effective in treatment of short stature associated with short stature homeobox-
containing gene deficiency: Two-year results of a randomized, controlled, multicenter trial. J
Clin Endocrinol Metab 2007 92 219-228.

17. Massart F, Bizi M, Baggiani A & Miccoli M. Height outcome of the recombinant human
growth hormone treatment in patients with SHOX gene haploinsufficiency: a meta-analysis.
Pharmacogenomics 2013 14 607-612.

18. Bunyan DJ, Baker KR, Harvey JF & Thomas NS. Diagnostic screening identifies a wide range of
mutations involving the SHOX gene, including a common 47.5 kb deletion 160 kb

19. Prader A, Largo RH, Molinari L & Issler C. Physical growth of Swiss children from birth to 20
years of age. First Zurich longitudinal study of growth and development. Helv Paediatr Acta
Suppl 1989 52 1-125.

Roelants M. Accurate long-term prediction of height during the first four years of growth
hormone treatment in prepubertal children with growth hormone deficiency or Turner

G & Cutler GB, Jr. GH treatment to final height produces similar height gains in patients with
SHOX deficiency and Turner syndrome: results of a multicenter trial. J Clin Endocrinol Metab
2013 98 E1383-1392.

22. Ranke MB & Lindberg A. Observed and predicted growth responses in prepubertal children
with growth disorders: guidance of growth hormone treatment by empirical variables. J Clin
Endocrinol Metab 2010 95 1229-1237.

Prediction of long-term response to recombinant human growth hormone in Turner
syndrome: development and validation of mathematical models. KIGS International Board.


25. Fredriks AM, van Buuren S, van Heel WJ, Dijkman-Neerinx RH, Verloove-Vanhorick SP & Wit
JM. Nationwide age references for sitting height, leg length, and sitting height/height ratio,
and their diagnostic value for disproportionate growth disorders. Arch Dis Child 2005 90 807-
812.

S. The world’s tallest nation has stopped growing taller: the height of Dutch children from

27. van Dommelen P, Schonbeck Y & van Buuren S. A simple calculation of the target height.
Arch Dis Child 2012 97 182.

28. Cole TJ & Roede MJ. Centiles of body mass index for Dutch children aged 0-20 years in 1980-

Swedish reference standards for weight, length and head circumference at birth for given


Legends to the figures

Figure 1. Flow chart of study design and patient distribution.

Figure 2. Mean height SDS in children of groups 1 and 2 who remained prepubertal during GH treatment in four cohorts (followed for 1, 2, 3 and 4 years). The dotted lines with circles represent group 1, the lines with squares group 2 and the dashed lines with triangles represent data as reported by Blum et al. One year of GH treatment (Fig. 2A): group 1 (n=25), group 2 (n=8). Two years of GH treatment (Fig. 2B): group 1 (n=17), group 2 (n=6). Three years of GH treatment (Fig. 2C): group 1 (n=10), group 2 (n=5). Four years of GH treatment (Fig. 2D): group 1 (n=8), group 2 (n=3).

Figure 3. Mean height velocities in children of groups 1 and 2 who remained prepubertal during GH treatment in four cohorts (followed for 1, 2, 3 and 4 years). The dotted lines with circles represent group 1, the lines with squares group 2 and the dashed lines with triangles represent data as reported by Blum et al. One year of GH treatment (Fig. 3A): group 1 (n=20), group 2 (n=8). Two years of GH treatment (Fig. 3B): group 1 (n=13), group 2 (n=6). Three years of GH treatment (Fig. 3C) group 1 (n=9), group 2 (n=5). Four years of GH treatment (Fig. 3D): group 1 (n=8), group 2 (n=3).

Statistically significant differences are indicated with an asterisk.

Supplementary Figure 1. Schematic representation of the SHOX gene and its 5’ and 3’ flanking regions, showing the probes of the P018-D1 MLPA kit (MRC Holland). The box indicates the probe locations of the SHOX gene and the size of the duplications is indicated by the numbered bars, representing cases 1-8 (Table 2)

Supplementary Figure 2. Mean height SDS in all children of groups 1 and 2 (prepubertal and pubertal) during GH treatment in four cohorts (followed for 1, 2, 3 and 4 years). The dotted lines with circles represent group 1 and the lines with squares group 2. One year of GH treatment (Suppl. Fig. 2A): group 1 (n=32), group 2 (n=12). Two years of GH treatment (Suppl. Fig. 2B): group 1 (n=28), group 2 (n=8). Three years of GH treatment (Suppl. Fig. 2C): group 1 (n=21), group 2 (n=7). Four years of GH treatment (Suppl. Fig. 2D): group 1 (n=17), group 2 (n=4). Statistically significant differences are indicated with an asterisk.
**Supplementary Figure 3.** Mean height velocities in all children of groups 1 and 2 (prepubertal and pubertal) during GH treatment in four cohorts (followed for 1, 2, 3 and 4 years). The dotted lines with circles represent group 1 and the lines with squares group 2. One year of GH treatment (Suppl. Fig. 3A): group 1 (n=23), group 2 (n=10). Two years of GH treatment (Suppl. Fig. 3B): group 1 (n=21), group 2 (n=7). Three years of GH treatment (Suppl. Fig. 3C): group 1 (n=17), group 2 (n=6). Four years of GH treatment (Suppl. Fig. 3D): group 1 (n=15), group 2 (n=4). Statistically significant differences are indicated with an asterisk.
Figure 1.

SHOX cases
390 cases (233 families)

Excluded:
62 patients (33 families):
- patients from foreign countries n = 23
- patients with aberrations considered non-pathogenic n = 27
- other diagnoses influencing growth n = 12

Potential participants
328 cases (200 families)

Excluded:
- physicians reluctant to request parental consent n = 8

Informed consents
157 cases (92 families)

Excluded:
- index patients > 18 years old n = 11
- grandparents n = 3
- no clinical information n = 10
- other disorders influencing growth n = 3

Patient analysis
130 cases (74 families)

Genetic subgroups

- SHOX mutations
  17 cases (10 families)

- SHOX deletions
  55 cases (35 families)

- SHOX downstream enhancer deletions
  40 cases (20 families)

- SHOX upstream enhancer deletions
  4 cases (2 families)

- SHOX downstream enhancer duplications
  9 cases (4 families)

- SHOX duplications
  5 cases (3 families)

- SHOX mutations
  7 cases (6 families)

- SHOX deletions
  30 cases (25 families)

- SHOX downstream enhancer deletions
  11 cases (9 families)

- SHOX upstream enhancer deletions
  1 case (1 family)

- SHOX downstream enhancer duplications
  3 cases (2 families)

- SHOX duplications
  0 cases

GH treatment
Table 1. Clinical characteristics at first visit in patients with *SHOX* mutations and deletions and *SHOX* enhancer deletions (mean (SD)).

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th><em>SHOX</em> mutations or deletions</th>
<th><em>SHOX</em> upstream and downstream enhancer deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Range</td>
<td>N</td>
</tr>
<tr>
<td>Age at first visit (yrs)</td>
<td>80</td>
<td>8.35 (3.6) [1.2;16.2]</td>
<td>54</td>
</tr>
<tr>
<td>Male/Female</td>
<td>34/46</td>
<td></td>
<td>23/31</td>
</tr>
<tr>
<td>Birth weight SDS</td>
<td>66</td>
<td>-0.4 (1.3) [-3.3;3.2]</td>
<td>43</td>
</tr>
<tr>
<td>Birth length SDS</td>
<td>37</td>
<td>-1.1 (1.2) [-4.3;1.5]</td>
<td>23</td>
</tr>
<tr>
<td>Height SDS</td>
<td>80</td>
<td>-2.5 (0.8) [-4.4;0.3]</td>
<td>54</td>
</tr>
<tr>
<td>Conditional Target Height SDS</td>
<td>74</td>
<td>-1.0 (0.6) [-2.9;0.7]</td>
<td>50</td>
</tr>
<tr>
<td>Sitting Height/Height ratio SDS</td>
<td>72</td>
<td>2.8 (1.3) [-0.1;5.5]</td>
<td>50</td>
</tr>
<tr>
<td>Armspan/Height ratio</td>
<td>33</td>
<td>0.95 (0.03) [0.87;1.01]</td>
<td>21</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>76</td>
<td>0.4 (1.0) [-2.0;2.7]</td>
<td>51</td>
</tr>
<tr>
<td>Extremities-trunk ratio&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32</td>
<td>2.48 (0.2) [2.06;2.80]</td>
<td>21</td>
</tr>
</tbody>
</table>

**Comparison to parents’ phenotype**

| Height SDS affected parent | 49 | -2.2 (0.9) [-4.8;0.3] | 28 | -2.4 (0.9) | 21 | -1.9 (0.9) | 0.032 |
| SH/H ratio affected parent | 23 | 2.7 (1.7) [0.5;6.1] | 9 | 3.3 (1.4) | 14 | 2.3 (1.8) | 0.168 |
| Ht SDS - TH SDS (de novo mutations) | 7 | -1.9 (0.7) [-3.2;1.1] | 6 | -1.9 (0.7) | 1 | -2.1 | |
| Ht SDS - Ht SDS affected parent | 46 | -0.3 (1.1) [-2.2;2.6] | 25 | -0.2 (0.9) | 21 | -0.5 (1.3) | 0.419 |
| Ht SDS - Ht SDS unaffected parent | 43 | 1.7 (0.9) [-3.5;0.2] | 24 | 1.7 (0.9) | 19 | 1.6 (1.0) | 0.579 |

<sup>a</sup>Binders’ extremities-trunk ratio compares the extremities length to the trunk length and is calculated as follows: (calculated subischial length + arm span) / sitting height. The normal values are dependent on height. A lower score indicates more severe disproportionate stature.

<sup>b</sup>p-value from t-test comparing patients with *SHOX* mutations and deletions to patients with *SHOX* upstream and downstream enhancer deletions.
Table 2. Clinical characteristics of patients with SHOX or SHOX enhancer duplications at first visit (n=8).

<table>
<thead>
<tr>
<th></th>
<th>1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>6&lt;sup&gt;b,c&lt;/sup&gt;</th>
<th>7&lt;sup&gt;c&lt;/sup&gt;</th>
<th>8</th>
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</thead>
<tbody>
<tr>
<td>Age at first visit (yrs)</td>
<td>5.6</td>
<td>3.3</td>
<td>11.9</td>
<td>4.5</td>
<td>9.2</td>
<td>5.5</td>
<td>3.8</td>
<td>3.2</td>
</tr>
<tr>
<td>Male(M)/Female(F)</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>F</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>Birth weight SDS</td>
<td>-</td>
<td>-</td>
<td>0.22</td>
<td>-1.7</td>
<td>-0.5</td>
<td>-0.5</td>
<td>-1.0</td>
<td>-3.6</td>
</tr>
<tr>
<td>Birth length SDS</td>
<td>-</td>
<td>-</td>
<td>0.9</td>
<td>-1.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-4.8</td>
</tr>
<tr>
<td>Height SDS</td>
<td>-2.4</td>
<td>-3.5</td>
<td>-2.4</td>
<td>-2.4</td>
<td>-2.2</td>
<td>-2.7</td>
<td>-3.4</td>
<td>-2.9</td>
</tr>
<tr>
<td>Conditional target height SDS</td>
<td>-</td>
<td>-0.9</td>
<td>-0.8</td>
<td>-1.7</td>
<td>-1.1</td>
<td>-1.1</td>
<td>-1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Sitting Height/Height ratio SDS</td>
<td>1.5</td>
<td>1.8</td>
<td>-</td>
<td>0.7</td>
<td>1.3</td>
<td>1.9</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Armspan/Height ratio</td>
<td>-</td>
<td>-</td>
<td>0.94</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>0.6</td>
<td>0.2</td>
<td>-1.2</td>
<td>-1.9</td>
<td>-1.5</td>
<td>-1.1</td>
<td>-1.3</td>
<td>0</td>
</tr>
<tr>
<td>IGF-I SDS</td>
<td>0.8</td>
<td>-</td>
<td>-2.7</td>
<td>-</td>
<td>-1.4</td>
<td>-2.1</td>
<td>-0.3</td>
<td>-</td>
</tr>
<tr>
<td>IGFBP-3 SDS</td>
<td>0.6</td>
<td>-</td>
<td>-0.1</td>
<td>-</td>
<td>-2.7</td>
<td>-4.3</td>
<td>-3.5</td>
<td>-</td>
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**Parents’ phenotype**

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<tbody>
<tr>
<td>Carrier parent Ht SDS</td>
<td>-</td>
<td>1.0</td>
<td>-0.4</td>
<td>-1.5</td>
<td>-1.4</td>
<td>-1.4</td>
<td>-2.5</td>
</tr>
<tr>
<td>SH/H SDS</td>
<td>-</td>
<td></td>
<td></td>
<td>3.1</td>
<td>-</td>
<td>-</td>
<td>1.4</td>
</tr>
<tr>
<td>Unaffected parent Ht SDS</td>
<td>-</td>
<td>-3.5</td>
<td>-1.5</td>
<td>-3.0</td>
<td>-1.6</td>
<td>-1.6</td>
<td>-1.0</td>
</tr>
<tr>
<td>SH/H SDS</td>
<td>-</td>
<td>3.2</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
</tr>
</tbody>
</table>

**Growth response & responsiveness**

<p>| | | | | | | | |</p>
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<tr>
<td>First year of GH</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>∆ Ht SDS</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.0</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>HV (cm/year)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.5</td>
<td>8.7</td>
<td>9.6</td>
</tr>
<tr>
<td>IoR</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.9</td>
<td>0.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The size of the duplications are shown in Supplementary Figure 1.

<sup>a</sup>adopted from Taiwan, therefore clinical information concerning birth and height and proportions of the parents is missing.

<sup>b</sup>patients treated with GH

<sup>c</sup>patient 5 and 6 are siblings
Table 3. Clinical characteristics at start of GH and GH response in prepubertal patients with SHOX mutations and deletions and SHOX enhancer deletions, mean (SD).

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>SHOX mutations and deletions</th>
<th>SHOX upstream and downstream enhancer deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Range</td>
<td>N</td>
</tr>
<tr>
<td><strong>At start of GH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>33</td>
<td>8.07 (2.3) [4.0;12.1]</td>
<td>25</td>
</tr>
<tr>
<td>Male/Female</td>
<td>13/20</td>
<td></td>
<td>8/17</td>
</tr>
<tr>
<td>Height SDS</td>
<td>33</td>
<td>-2.8 (0.7) [-4.3;-1.8]</td>
<td>25</td>
</tr>
<tr>
<td>HV year before GH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cm/year)</td>
<td>28</td>
<td>5.4 (1.1) [3.1;7.2]</td>
<td>20</td>
</tr>
<tr>
<td>HV SDS before GH</td>
<td>28</td>
<td>-0.5 (1.0) [-2.8;1.2]</td>
<td>20</td>
</tr>
<tr>
<td>BMI SDS</td>
<td>33</td>
<td>0.6 (0.9) [-1.4;2.7]</td>
<td>25</td>
</tr>
<tr>
<td>IGF-I SDS</td>
<td>29</td>
<td>-1.0 (1.1) [-3.3;0.7]</td>
<td>22</td>
</tr>
<tr>
<td>IGFBP-3 SDS</td>
<td>9</td>
<td>0.1 (0.8) [-1.6;1.1]</td>
<td>5</td>
</tr>
<tr>
<td>BA / CA ratio</td>
<td>21</td>
<td>0.9 (0.1) [0.5;1.1]</td>
<td>15</td>
</tr>
<tr>
<td>GH dose (mg/m2·day)</td>
<td>33</td>
<td>1.24 (0.2) [0.6;2.0]</td>
<td>25</td>
</tr>
<tr>
<td>GH dose (ug/kg·day)</td>
<td>33</td>
<td>48.0 (9.4) [28.6;77.2]</td>
<td>25</td>
</tr>
<tr>
<td><strong>After one year of GH therapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HV (cm/year)</td>
<td>28</td>
<td>9.2 (1.3) [7.0;12.8]</td>
<td>20</td>
</tr>
<tr>
<td>HV SDS</td>
<td>28</td>
<td>3.5 (1.9) [0.2;8.6]</td>
<td>20</td>
</tr>
<tr>
<td>∆ Ht SDS</td>
<td>33</td>
<td>0.6 (0.2) [0.2;1.2]</td>
<td>25</td>
</tr>
<tr>
<td>IoR</td>
<td>28</td>
<td>0.9 (0.9) [-0.8;3.2]</td>
<td>20</td>
</tr>
<tr>
<td><strong>After two years of GH therapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HV (cm/year)</td>
<td>19</td>
<td>7.9 (1.9) [4.4;14.1]</td>
<td>13</td>
</tr>
<tr>
<td>HV SDS</td>
<td>19</td>
<td>2.5 (2.1) [-1.2;9.4]</td>
<td>13</td>
</tr>
<tr>
<td>∆ Ht SDS</td>
<td>23</td>
<td>0.3 (0.2) [-0.3;0.6]</td>
<td>17</td>
</tr>
<tr>
<td>IoR</td>
<td>19</td>
<td>0.9 (1.6) [-1.8;6.2]</td>
<td>13</td>
</tr>
<tr>
<td><strong>After three years of GH therapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HV (cm/year)</td>
<td>14</td>
<td>6.9 (0.9) [5.3;8.2]</td>
<td>9</td>
</tr>
<tr>
<td>HV SDS</td>
<td>14</td>
<td>1.6 (1.1) [0.1;3.6]</td>
<td>9</td>
</tr>
<tr>
<td>∆ Ht SDS</td>
<td>15</td>
<td>0.3 (0.2) [0.0;0.5]</td>
<td>10</td>
</tr>
<tr>
<td>IoR</td>
<td>14</td>
<td>0.6 (0.9) [-1.3;1.6]</td>
<td>9</td>
</tr>
<tr>
<td><strong>After four years of GH therapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HV (cm/year)</td>
<td>11</td>
<td>6.2 (0.9) [4.7;7.7]</td>
<td>8</td>
</tr>
<tr>
<td>HV SDS</td>
<td>11</td>
<td>0.8 (0.8) [-0.2;2.8]</td>
<td>8</td>
</tr>
<tr>
<td>∆ Ht SDS</td>
<td>11</td>
<td>0.2 (0.2) [-0.1;0.6]</td>
<td>8</td>
</tr>
<tr>
<td>IoR</td>
<td>11</td>
<td>0.5 (0.9) [-0.9;1.8]</td>
<td>8</td>
</tr>
</tbody>
</table>
*p-value from t-test comparing patients with SHOX mutations and deletions to patients with SHOX upstream and downstream enhancer deletions.