HAL
open science

Enhancer mutations of the SHOX gene as a frequent cause of short stature - the essential role of a 250 kb downstream regulatory domain

Jianjun Chen, Gabriele Wildhardt, Zilin Zhong, Ralph Roeth, Birgit Weiss, Daniela Steinberger, Jochen Decker, Werner F Blum, Gudrun A Rappold

To cite this version:

Jianjun Chen, Gabriele Wildhardt, Zilin Zhong, Ralph Roeth, Birgit Weiss, et al.. Enhancer mutations of the SHOX gene as a frequent cause of short stature - the essential role of a 250 kb downstream regulatory domain. Journal of Medical Genetics, 2009, 46 (12), pp.834. 10.1136/jmg.2009.067785 . hal-00552699

HAL Id: hal-00552699

https://hal.science/hal-00552699v1

Submitted on 6 Jan 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Enhancer deletions of the *SHOX* gene as a frequent cause of short stature - the essential role of a 250 kb downstream regulatory domain

Jianjun Chen[1], Gabriele Wildhardt[2], Zilin Zhong[1], Ralph Röth[1], Birgit Weiss[1], Daniela Steinberger[2], Jochen Decker[2], Werner F. Blum[3], Gudrun Rappold[1,*]

1) Department of Molecular Human Genetics, University of Heidelberg, 69120 Heidelberg, Germany; 2) Bioscientia Institute of Medical Diagnostics, 55218 Ingelheim, Germany; 3) Lilly Research Laboratories, Eli Lilly and Company, Bad Homburg, Germany

[*]**To whom correspondence should be addressed:**

Prof. Dr. Gudrun A. Rappold
Institute of Human Genetics
Im Neuenheimer Feld 366
69120 Heidelberg, Germany
Tel: 49-6221-565059
Fax: 49-6221-568884
gudrun.rappold@med.uni-heidelberg.de

Abstract

BACKGROUND: Mutations and deletions of the homeobox transcription factor gene *SHOX* are known to cause short stature. We have analyzed *SHOX* enhancer regions in a large cohort of short stature patients to study the importance of regulatory regions in developmentally relevant genes like *SHOX*. METHODS: We tested for the presence of copy number variations in the pseudoautosomal region of the sex chromosomes in 735 individuals with idiopathic short stature and compared the results to 58 cases with Leri-Weill syndrome and 100 normal height controls, using FISH, SNP, microsatellites and MLPA analysis. RESULTS: A total of 31/735 (4.2%) microdeletions were identified in the pseudoautosomal region in patients with idiopathic short stature; eight of these microdeletions (8/31; 26%) involved only enhancer sequences residing a considerable distance away from the gene. In 58 Leri-Weill syndrome patients, a total of 29 microdeletions were identified; almost half of these (13/29; 45%) involve enhancer sequences and leave the *SHOX* gene intact. These deletions were absent in 100 control persons. CONCLUSION: We conclude that enhancer deletions in the *SHOX* gene region are a relatively frequent cause of growth failure in patients with idiopathic short stature and Leri-Weill syndrome. Our data highlight the growing recognition that regulatory sequences are of crucial importance in the genome when diagnosing and understanding the etiology of disease.

Introduction

Short stature is a developmental, multifactorial condition with a strong genetic component. Three percent of the population are defined as being short. Short stature can have many different causes, a multitude of which are genetic. Several large scale genome-wide association studies have recently determined variants that affect susceptibility to disease and identified a total of 54 validated SNP variants with an influence on height. [1-3] However, in total these large scale studies only pinpointed candidate genes of relatively minor effect within each locus, explaining only a small proportion of the phenotypic variance in the normal population, thus accounting together for little more than 5% of our height (0.4 cm per "increasing" allele).

Although mutations in several genes have been reported that cause pronounced short or tall stature with a drastic effect on height (10 to 30 cm per "mutant" allele), this normally accounts for only a very small proportion of patients with short stature. So we are left with the fact that for the vast majority of short individuals with a height below -2 standard deviations scores (SDS), no causing defects are known, clinically termed as "idiopathic" short stature. [4]

One of the more prevalent causes of short stature was shown to derive from a defect in a developmental gene on the X chromosome, *SHOX* (for: short stature homeobox gene; MIM 312865). Defects in the coding region of *SHOX* have been demonstrated in individuals with different short stature syndromes including Leri-Weill, Langer and Turner syndrome (MIM 127300), [5-8] but mutations are also found in patients with idiopathic short stature (ISS) (MIM 300582). [9]

Regulatory elements residing either 5´ or 3´of a gene can be sites of mutations in genetic disease and interfere with the normal expression of a gene. Some of these elements - defined as enhancers or repressors - can lie a considerable distance away from the coding portion of a gene. [10] Enhancer elements can act up- and downstream of a gene and function only in certain cell types. In concert with transcription factors

that bind to these enhancer sequences, they play a role in achieving the appropriate level of gene expression. Defects in enhancer sequences have as yet only rarely been identified, and like promoter mutations, are considered to play only a relatively minor role in disease.

Comparative genomic analysis has previously identified evolutionarily conserved non-coding DNA elements, termed CNE, several hundred kilobases downstream of the *SHOX* gene in patients with Leri-Weill syndrome.[11-14] Three of these elements have subsequently been shown to act as enhancers in the chicken limb bud and five elements as enhancers in the neural tube.[15] To establish the frequency and significance of long-range regulatory elements also in individuals with idiopathic short stature (ISS), we have analyzed the DNA of 735 patients with ISS and compared these results to 58 patients with a clinical manifestation of Leri-Weill syndrome (LWS) and 100 controls.

Material and Methods

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

Study Subjects

We studied 740 unrelated pre-pubertal children with ISS from 14 countries: Belgium (n = 61), Canada (n = 39), Croatia (n = 34), Czech Republic (n = 87), France (n = 60), Germany (n = 144), Hungary (n = 29), India (n = 7), Netherlands (n = 2), Poland (n = 49), Russia (n = 6), Spain (n = 107), Turkey (n = 61), and USA (n = 54). Inclusion criteria were: chronological age ≥ 3 years and pre-pubertal (males: genital stage Tanner 1 and testes ≤ 2 ml; females: breast stage Tanner 1); height ≤ 3rd percentile of the local reference range or height ≤ 10th percentile with height velocity ≤ 25th percentile; bone age ≤ 10 years for boys and ≤ 8 years for girls; no GH deficiency or GH resistance; no chronic disease; and no known growth-influencing medications.

Our study also included 67 patients with LWS from 8 different European countries: Germany (n = 37), Austria (n = 1), Croatia (n = 4), France (n = 10), Italy (n = 2), Netherlands (n = 8), Spain (n = 4), and Sweden (n = 1). The control cohort consisted of 100 individuals of European origin with normal stature. We used a combination of DNA sequencing, microsatellite analysis, fluorescence *in situ* hybridization (FISH), and multiplex ligand-dependent probe amplification (MLPA), [16] to search for copy number variations in the proximity of *SHOX* in the pseudoautosomal region (PAR1). Details are given in the Supplementary Appendix.

Results

The main focus of this study was to find out about the frequency of enhancer deletions in a large cohort of patients with ISS and compare the results to patients with LWS and control individuals. Patients that presented (point) mutations within the *SHOX* gene were excluded from the deletion analysis (5 patients with ISS, 9 patients with LWS), leaving altogether 735 patients with ISS, 58 patients with LWS and 100 controls for the enhancer deletion analysis.

To screen for enhancer deletions downstream of the *SHOX* gene, we carried out multiplex ligand-dependent probe amplification (MLPA) on 201 of the 735 ISS patients. These 201 patients were selected as they showed homozygosity with at least one of the microsatellite markers (*DXYS10085*, *DXYS10087*, and *DXYS10096*), in addition to the limb-specific enhancer CNE9, all residing at a distance of 150 to 250 kb from the *SHOX* gene (Figure 1).

We have also screened the 735 ISS patients for homozygosity of two markers in direct vicinity of the *SHOX* gene (DXYS201 and CAII) [14], to pinpoint *SHOX* containing deletions. In all individuals where all 2 markers were homozygous, FISH analysis was carried out and in 18 individuals, a deletion of the *SHOX* gene was demonstrated by FISH. In these 18 individuals, MLPA was also carried out additionally as a further confirmation for the *SHOX* containing deletions. MLPA analysis was also carried out in DNA of 58 LWS patients who had been previously tested for the presence or absence of the *SHOX* gene and in DNA of 100 control individuals.

In total, MLPA analysis demonstrated a deletion in the pseudoautosomal region of 33/201 tested ISS patients, with peaks at a ratio <0.5 as compared to normal controls (Figure 2). Deletion sizes ranged between 52 kb and several Megabases of DNA and no duplications were detected. Twenty-three of the 33 microdeletions included the *SHOX* gene. A comparison of the results to the control cohort showed that two of the deletions (no.27 and 28; see Figure 2) represent polymorphisms as they were also detected in two control individuals (Figure 2). Among the 31 remaining microdeletions of the PAR region, eight ISS patients presented deletions including

only the enhancer sequences, CNE7, CNE8 and/or CNE9 (no.24-26,29-33; Figure 2). Three of the eight patients (no.29-31) presented very small deletions of only the enhancer intervals CNE7 and CNE8, and in proband 32 only the CNE8 interval was deleted (CNEs as defined by Sabherwal *et al*, 2007).

MLPA analysis also demonstrated a heterozygous deletion in the PAR region in 30/58 LWS patients, showing all the peaks at a ratio <0.5 as compared to normal controls. Among the 30 microdeletions, 16 deletions encompassed the *SHOX* gene, whereas 14 deletions were located downstream of the *SHOX* gene. The smallest deletion (no.49, Figure 2) included exons 4–5 of the *SHOX* gene (10 kb) and was only detectable by MLPA. A comparison with the controls demonstrated that the deletion in one of the patients (no.50) represents a polymorphism. Among the 29 microdeletions of the PAR1 region in LWS patients, one patient (no.62) included only the enhancer interval CNE7 and CNE8.

Altogether, we identified 63 persons with copy number variations in the PAR region by screening 793 patients with short stature (735 with ISS and 58 with LWS). Sixty of these deletions were absent in a series of 100 control persons, while three were also found in the control group and had to be judged as normal variants (no.27, 28, 50). To confirm these results with an independent method, we carried out fluorescence *in situ* hybridization (FISH) on chromosomes of several patients with small deletions, where metaphase spreads were available (no.24-26, 29-33, 62). In all cases, deletions were confirmed by FISH analysis using the cosmids 51D11, 61E05 and 10D05 with a map location between 670 and 780 kb from the telomere (Figure 3).

To further correlate the significance of one or more of the enhancer CNE elements in *SHOX* downstream deletions, a detailed analysis of the deletion boundaries was carried out in seven ISS cases (no.27-33) and one LWS case (no.62) by SNP and microsatellite analysis (Suppl.Figure). The smallest deletion was confined to ≤ 2 kb in proband 27 and 28 and represents a polymorphism, proband 33 had a deletion of ≤ 80 kb. In three individuals with ISS and one individual with LWS, a deletion interval smaller than 60 kb was detected that included the CNE7 and CNE8 enhancer interval. The deletion in ISS proband 32 was the smallest detectable functional variant in this

series and included only the CNE8 interval.

Discussion

This study was performed to establish a comprehensive view of copy-number variations in the pseudoautosomal region of the human sex chromosomes in 735 patients with idiopathic short stature. The pseudoautosomal region represents a block of sequence identity shared between the X and Y chromosomes, flanked by the telomere. It is characterized by an elevated CG content, abundant *Alu* repeats and one of the highest recombination rates in the human genome.[17] Deletions in the pseudoautosomal region were identified in 33 of the 735 screened ISS patients (4.5%), 31 of these were not found in the screened controls. Deletions in the PAR1 region were also identified in 30 of the 58 screened LWS cases and in three of the analyzed controls. As we were interested in the level and significance of enhancer deletions in patients with idiopathic short stature with regard to the overall frequency of detected mutations/deletions, the incidence of enhancer deletions of 22% was an unexpected finding, while this proportion was even higher at 34% in patients with LWS. Figure 4 gives a summary of all the SHOX gene deletions, the intragenic mutations and downstream enhancer deletions in the analysed patients with ISS and LWS. It is very likely that these high incidences are still an underestimate, as we cannot rule out the possibility that some cases have been overlooked due to point mutations in enhancer sequences or deletions in other parts of this region.

Do cis-regulatory mutations in ISS or LWS make a qualitatively different contribution to disease? Leri-Weill syndrome is clinically defined by a shortened middle portion of the limb (mesomelia) leading to short stature and an abnormal alignment of the radius and ulna at the wrist. The Madelung deformity typically develops at puberty and females are generally more severely affected than males. Other clinical features include a high muscle mass and high body mass index.[14] [18] [19] As cis-regulatory deletions in our analysis occur more frequently in patients with LWS compared to ISS,

it is possible that enhancer deletions may lead to more pronounced phenotypes, as exemplified in the more striking phenotype of Leri-Weill syndrome.

The deletion of the limb-specific enhancer CNE9 has been previously shown to be an important cis-controlling element in SHOX haploinsufficiency. [11-13 15] This study confirms that CNE9 probably represents the most important enhancer in the proximal SHOX region, but has also has revealed that CNE8 together with CNE7, but also the deletion of the CNE8 interval alone, can be identified in patients with short stature as well as skeletal defects (no.29-32; 62; Suppl.Figure). Alternatively, it also can not be totally ruled out that the deletion of the CNE7 and CNE8 intervals may have only a minor effect, as this interval was absent in the sequence data of one recently sequenced individual, however pedigree data were not available and SNP and microsatellite analysis, MLPA and FISH was not carried out. [20] It is also possible that this part of the sequence is still inaccurate or incomplete. We also know that the phenotype in patients can be very variable (intra- and interfamiliar) and that there are individuals with SHOX deficiency and normal stature. [12,14] Long-range transcriptional control by enhancer sequences have been identified mostly through the analysis of patients with genetic malformations and found to reside up to hundred of kilobases away from the genes they control. [10] Three examples of long-range control in limb development include enhancers in the vicinity of the mammalian *Hoxd* cluster [21] and the *Sox9* gene, defects in which cause campomelic dysplasia, a dominant skeletal malformation syndrome often associated with XY sex reversal, or Pierre Robin sequence, an important subgroup of cleft palate. [22-24] Chromosome rearrangements which separate the genes from their conserved non-coding DNA elements have only been reported in a small minority of cases. The *PAX6* and *SHH* position effect cases are further good examples of the separation of cis-regulatory elements. [10] Given the findings on enhancer significance on key regulatory master

genes in the literature, the high level of enhancer deletions in the *SHOX* gene is to our knowledge unprecedented in the human genome.

The conserved non-coding elements in the vicinity of the *SHOX* gene are embedded in a region with characteristics of a gene desert. Deletions encompassing more proximal or more distal pseudoautosomal genes have been detected in a number of patients in our study, but this does not lead to obvious phenotypic consequences. The same holds true for a duplication of the *ASMT* gene which was found in two normal controls. An important diagnostic issue has however been raised, related to the observation that five female probands carry deletions extending into the X-specific region of the X chromosome. While these females only present short stature, their male babies could also suffer from mental retardation, Chondrodysplasia punctata, Ichthyosis and Kallmann Syndrome, [25] depending on their exact deletion size and which X-chromosomal genes are missing. For these individuals, prenatal diagnosis might represent an option.

In summary, we have applied DNA sequencing, microsatellite, FISH and MLPA analysis to detect DNA rearrangements in a large population of patients with idiopathic short stature of different ethnic origin. We have provided strong evidence that cis-regulatory deletions can contribute to the patient´s condition to an unexpectedly high degree. These data also re-emphasize that regulatory input can be crucial for developmental genes, most notably genes with a dynamic expression pattern. Lastly, our findings also help to optimise diagnostic options for individuals with SHOX haploinsufficiency and thus may have immediate value for patients with idiopathic short stature.

Acknowledgements

We thank Eva Decker, Claudia Durand and Claire Bacon for reading of this manuscript. This work was supported by the Deutsche Forschungsgemeinschaft and the Land Baden-Württemberg. Jianjun Chen was supported by a grant of the postdoctoral fellowship program of the Faculty of Medicine, Heidelberg.

References

1. Gudbjartsson DF, Walters GB, Thorleifsson G, Stefansson H, Halldorsson BV, Zusmanovich P, Sulem P, Thorlacius S, Gylfason A, Steinberg S, Helgadottir A, Ingason A, Steinthorsdottir V, Olafsdottir EJ, Olafsdottir GH, Jonsson T, Borch-Johnsen K, Hansen T, Andersen G, Jorgensen T, Pedersen O, Aben KK, Witjes JA, Swinkels DW, den Heijer M, Franke B, Verbeek AL, Becker DM, Yanek LR, Becker LC, Tryggvadottir L, Rafnar T, Gulcher J, Kiemeney LA, Kong A, Thorsteinsdottir U, Stefansson K. Many sequence variants affecting diversity of adult human height. *Nat Genet* 2008; 40:609-15.
2. Lettre G, Jackson AU, Gieger C, Schumacher FR, Berndt SI, Sanna S, Eyheramendy S, Voight BF, Butler JL, Guiducci C, Illig T, Hackett R, Heid IM, Jacobs KB, Lyssenko V, Uda M; Diabetes Genetics Initiative; FUSION; KORA; Prostate, Lung Colorectal and Ovarian Cancer Screening Trial; Nurses' Health Study; SardiNIA, Boehnke M, Chanock SJ, Groop LC, Hu FB, Isomaa B, Kraft P, Peltonen L, Salomaa V, Schlessinger D, Hunter DJ, Hayes RB, Abecasis GR, Wichmann HE, Mohlke KL, Hirschhorn JN. Identification of ten loci associated with height highlights new biological pathways in human growth. *Nat Genet* 2008; 40:584-91.
3. Weedon MN, Lango H, Lindgren CM, Wallace C, Evans DM, Mangino M, Freathy RM, Perry JR, Stevens S, Hall AS, Samani NJ, Shields B, Prokopenko I, Farrall M, Dominiczak A; Diabetes Genetics Initiative; Wellcome Trust Case Control Consortium, Johnson T, Bergmann S, Beckmann JS, Vollenweider P, Waterworth DM, Mooser V, Palmer CN, Morris AD, Ouwehand WH; Cambridge GEM Consortium, Zhao JH, Li S, Loos RJ, Barroso I, Deloukas P, Sandhu MS, Wheeler E, Soranzo N, Inouye M, Wareham NJ, Caulfield M, Munroe PB, Hattersley AT, McCarthy MI, Frayling TM. Genome-wide association analysis identifies 20 loci that influence adult height. *Nat Genet* 2008; 40:575-83.
4. Cohen P, Rogol AD, Deal CL, Saenger P, Reiter EO, Ross JL, Chernausek SD, Savage MO, Wit JM; 2007 ISS Consensus Workshop participants. Consensus statement on the diagnosis and treatment of children with idiopathic short stature. *J Clin Endocrinol Metab* 2008; 93:4210-7.
5. Ellison JW, Wardak Z, Young MF, Gehron Robey P, Laig-Webster M, Chiong W. PHOG, a candidate gene for involvement in the short stature of Turner syndrome. *Hum Mol Genet* 1997; 6:1341-7.
6. Belin V, Cusin V, Viot G, Girlich D, Toutain A, Moncla A, Vekemans M, Le Merrer M, Munnich A, Cormier-Daire V. *SHOX* mutations in dyschondrosteosis (Leri-Weill syndrome).*Nat Genet* 1998;19:67-9.
7. Shears DJ, Vassal HJ, Goodman FR, Palmer RW, Reardon W, Superti-Furga A, Scambler PJ, Winter RM. Mutation and deletion of the pseudoautosomal gene SHOX cause Leri-Weill dyschondrosteosis. *Nat Genet* 1998; 19:70-3.
8. Kosho T, Muroya K, Nagai T, Fujimoto M, Yokoya S, Sakamoto H, Hirano T, Terasaki H, Ohashi H, Nishimura G, Sato S, Matsuo N, Ogata T. Skeletal features and growth patterns in 14 patients with haploinsufficiency of SHOX: implications for the development of Turner syndrome. *J Clin Endocrinol Metab* 1999; 84:4613-21.
9. Rao E, Weiss B, Fukami M, Rump A, Niesler B, Mertz A, Muroya K, Binder G, Kirsch S, Winkelmann M, Nordsiek G, Heinrich U, Breuning MH, Ranke MB, Rosenthal A, Ogata T, Rappold GA. Pseudoautosomal deletions encompassing a novel homeobox gene cause growth failure in idiopathic short stature and Turner syndrome. *Nat Genet* 1997; 16:54-63.
10. Kleinjan D.A. and van Heyningen V. Long-range control of gene expression: emerging mechanisms and disruption in disease. *Am J Hum Genet* 2005; 76:8–32.

11. Benito-Sanz S, Thomas NS, Huber C, Gorbenko del Blanco D, Aza-Carmona M, Crolla JA, Maloney V, Rappold G, Argente J, Campos-Barros A, Cormier-Daire V, Heath KE. A novel class of Pseudoautosomal region 1 deletions downstream of SHOX is associated with Leri-Weill dyschondrosteosis. *Am J Hum Genet* 2005; 77:533-44.
12. Huber C, Rosilio M, Munnich A, Cormier-Daire V; French SHOX GeNeSIS Module. High incidence of SHOX anomalies in individuals with short stature. *J Med Genet* 2006; 43:735-9.
13. Fukami M, Kato F, Tajima T, Yokoya S, Ogata T. Transactivation function of an approximately 800-bp evolutionarily conserved sequence at the SHOX 3' region: implication for the downstream enhancer. *Am J Hum Genet* 2006; 78:167-70.
14. Rappold G, Blum WF, Shavrikova EP, Crowe BJ, Roeth R, Quigley CA, Ross JL, Niesler B. Genotypes and phenotypes in children with short stature: clinical indicators of SHOX haploinsufficiency. *J Med Genet* 2007; 44:306-13.
15. Sabherwal N, Bangs F, Röth R, Weiss B, Jantz K, Tiecke E, Hinkel GK, Spaich C, Hauffa BP, van der Kamp H, Kapeller J, Tickle C, Rappold G. Long-range conserved non-coding SHOX sequences regulate expression in developing chicken limb and are associated with short stature phenotypes in human patients. *Hum Mol Genet* 2007; 16:210-22.
16. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. *Nucleic Acids Res* 2002; 30:e57.
17. Blaschke RJ, Rappold G. The pseudoautosomal regions, SHOX and disease. *Curr Opin Genet Dev* 2006; 16:233-9.
18. Ross JL, Scott C Jr, Marttila P, Kowal K, Nass A, Papenhausen P, Abboudi J, Osterman L, Kushner H, Carter P, Ezaki M, Elder F, Wei F, Chen H, Zinn AR. Phenotypes Associated with SHOX Deficiency. *J Clin Endocrinol Metab* 2001; 86:5674-80.
19. Flanagan SF, Munns CF, Hayes M, Williams B, Berry M, Vickers D, Rao E, Rappold GA, Batch JA, Hyland VJ, Glass IA. Prevalence of mutations in the short stature homeobox containing gene (SHOX) in Madelung deformity of childhood. *J Med Genet* 2002; 39:758-63.
20. Levy S, Sutton G, Ng PC, Feuk L, Halpern AL, Walenz BP, Axelrod N, Huang J, Kirkness EF, Denisov G, Lin Y, MacDonald JR, Pang AW, Shago M, Stockwell TB, Tsiamouri A, Bafna V, Bansal V, Kravitz SA, Busam DA, Beeson KY, McIntosh TC, Remington KA, Abril JF, Gill J, Borman J, Rogers YH, Frazier ME, Scherer SW, Strausberg RL, Venter JC. The diploid genome sequence of an individual human. *PLoS Biol* 2007; 5:e254.
21. Spitz F, Gonzalez F, Duboule D. A global control region defines a chromosomal regulatory landscape containing the HoxD cluster. *Cell* 2003; 113:405-17.
22. Bagheri-Fam S, Barrionuevo F, Dohrmann U, Günther T, Schüle R, Kemler R, Mallo M, Kanzler B, Scherer G. Long-range upstream and downstream enhancers control distinct subsets of the complex spatiotemporal Sox9 expression pattern. *Dev Biol* 2006; 291:382-97.
23. Velagelati G.V., Bien-Willner G.A., Northup J.K., Lockhart L.H., Hawkins J.C., Jalal S.M., Withers M., Lupski J.R., Stankiewicz P. Position effects due to chromosome breakpoints that map approximately 900 kb upstream and approximately 1.3 Mb downstream of SOX9 in two patients with campomelic dysplasia. *Am J Hum Genet* 2005; 76:652–62.
24. Benko S, Fantes JA, Amiel J, Kleinjan DJ, Thomas S, Ramsay J, Jamshidi N, Essafi A, Heaney S, Gordon CT, McBride D, Golzio C, Fisher M, Perry P, Abadie V, Ayuso C, Holder-Espinasse M, Kilpatrick N, Lees MM, Picard A, Temple IK, Thomas P, Vazquez MP, Vekemans M, Crollius HR, Hastie ND, Munnich A, Etchevers HC, Pelet A, Farlie PG, Fitzpatrick DR, Lyonnet S. Highly conserved non-coding elements on either side of

SOX9 associated with Pierre Robin sequence. *Nat Genet* 2009; 41:359-64.

25. Ballabio A, Bardoni B, Carrozzo R, Andria G, Bick D, Campbell L, Hamel B, Ferguson-Smith MA, Gimelli G, Fraccaro M, et al. Contiguous gene syndromes due to deletions in the distal short arm of the human X chromosome. *Proc Natl Acad Sci U S A* 1989; 86:10001-5.

The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd to permit this article (if accepted) to be published in JMG and any other BMJPGL products and sublicences such use and exploit all subsidiary rights, as set out in our licence (http://jmg.bmj.com/ifora/licence.pdf).

G.W., D.S. and J.D. are (or were) employees of the Bioscientia Institute of Medical Genetics, W.B. is an employee of Eli Lilly and company. G.R. is a patent owner and has been reimbursed by Eli Lilly for attending and speaking at several conferences. J.C., Z.Z., R.R., B.W. have no competing interests.

Figures legends

Figure 1. Schematic representation of the PAR1 region showing the position of evolutionarily conserved non-coding DNA elements (CNEs), microsatellites and cosmids that were used for deletion mapping.[15] The *SHOX* gene is indicated in red and resides between 505.1 and 540.1 kb from the telomere (NCBI build 125, March 2006). The CNEs and microsatellites used for deletion mapping are shown above the scale bar. CNEs are indicated in green; CNE4, CNE5 and CNE9 with a star symbol have shown enhancer activity in the developing chicken limb bud.[15] CNE3, CNE4, CNE5, CNE7 and CNE9 have previously shown enhancer activity in the chicken neural tube.[15] Microsatellites are indicated in blue. The *SHOX* exons and the cosmids contig used for deletion mapping are shown below the scale bar. Cosmid 34F05 (LLN0YCO3´M´34F05) includes the *SHOX* gene; Cosmid 51D11 (LLN0YCO3'M'51D11) includes CNE6, CNE7 and markers *DXYS10085*; Cosmid 61E05 (LLN0YCO3'M'61E05) includes CNE8 and markers *DXYS10087* and *DXYS10096*; Cosmid 10D05 (LLN0YCO3'M'10D05) includes CNE9. *TEL*, telomere; *CEN*, centromere.

Figure 2. Mapping of 63 PAR deletions in patients with ISS and LWS using the SALSA P018C and P018D-1 *SHOX* MLPA kit. Maximum deletion sizes are according to Ensemble Genome Browser coordinates (in kb) and are given on the right side. Blackened areas indicate the presence of two copies of the MLPA probe; white areas indicate the deletion of one allele; green areas indicate the presence of more than two copies of the MLPA probe; grey areas indicate the common minimal deletion interval in patients, and solidus areas indicate that the analyzed marker was non-informative. The ISS 24-33 and LWS 50-63 patients present cases with an intact *SHOX* region and downstream deletions. In the control population, two deletions of the locus at 685 Xptel, a duplication of locus at 818 Xptel, as well as two duplications

of the *ASMT* gene at 1711 Xptel were found. Note also that in one patient (no.9) a deletion of the *STS* gene was identified.

Figure 3. FISH analyses of patients with idiopathic short stature

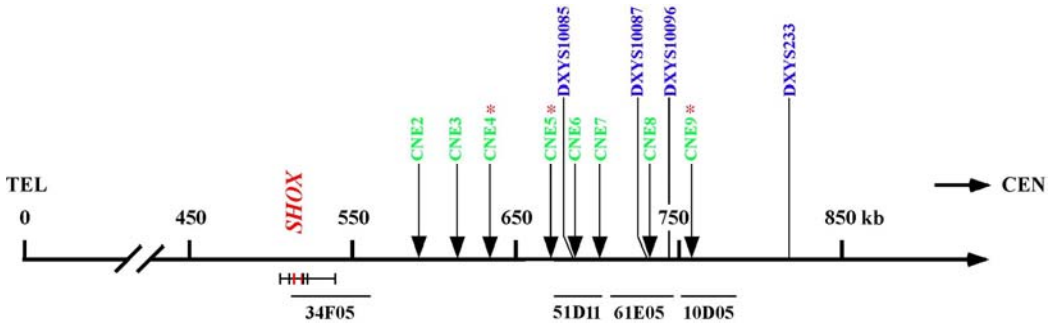
The CNE9-containing cosmid 10D05 (LLN0YCO3'M'10D05) was used as a probe for FISH analysis. A) Metaphase chromosomes from a female control with FISH signals on both X chromosomes. B) Metaphase chromosomes from a female patient (no. 24) with a deletion on one of the chromosomes (arrows). C) Metaphase from a male control with FISH signals on both X and Y chromosomes. D) Metaphase from a male patient (no. 33) with a deletion on the Y chromosome (arrows) but not on the X chromosome. A reference probe residing on the long arm of the X-chromosome (cosmid P9) and a Y centromeric probe (*DYZ3*) were used as controls. The green signals indicate cosmids 10D05 and P9 and hybridised to opposite ends of the X chromosome; the red signal indicates probe *DYZ3*.

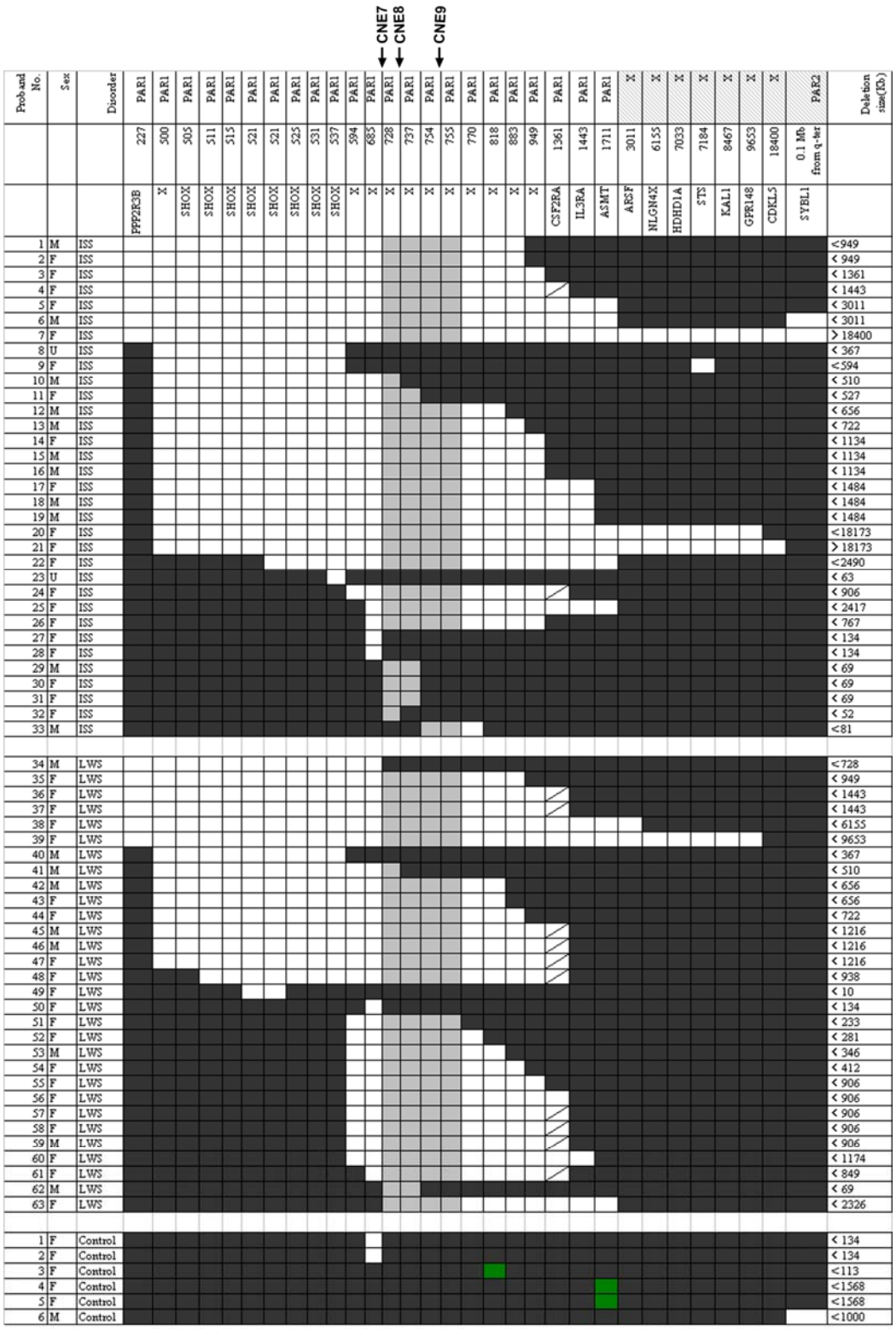
Figure 4. Summary of the spectrum of different *SHOX* mutations in patients with ISS and LWS

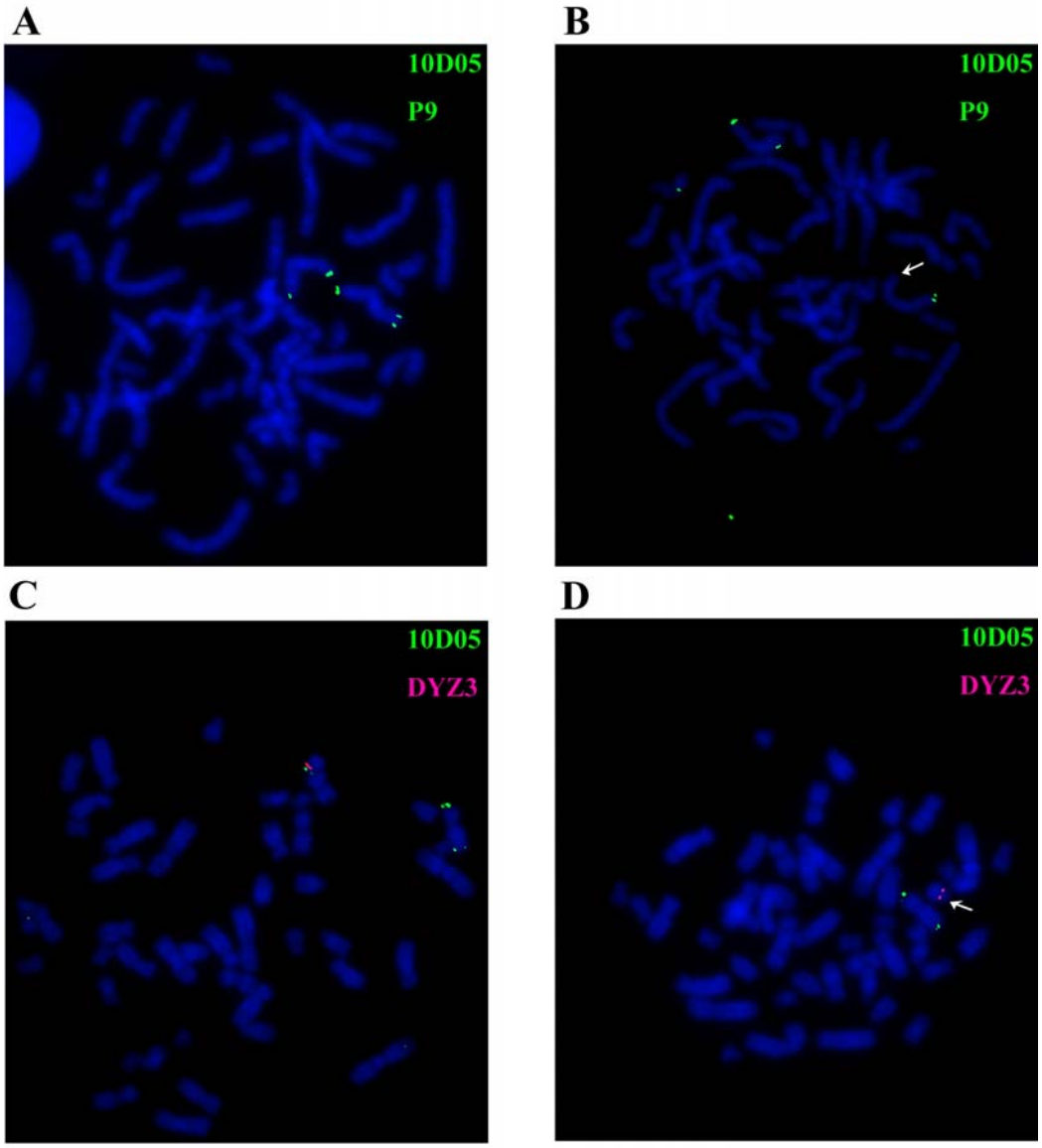
From all studied 735 unrelated patients with ISS, 31 are caused by *SHOX*-related deletions. Eight presented deletions proximal to *SHOX* (black) and 23 presented a *SHOX* gene deletion (hatched). A further five patients with ISS presented an intragenic mutation (white; 4 missense, 1 frameshift mutation; data not shown), which were initially excluded from the deletion study (total number of patients with ISS: 740). In 67 unrelated LWS, 38 are caused by *SHOX*- related defects: 13 presented a deletion downstream of *SHOX* (black), 16 presented a *SHOX* gene deletion (hatched) and nine presented an intragenic mutation (white; 5 missense, 3 nonsense, 1 frameshift mutation). The nine patients with intragenic mutations were initially excluded from the deletion study. In total, 4.9 % of patients with ISS and 56.7 % of patients with LWS are caused by point mutations or deletions of *SHOX* or its regulatory regions.

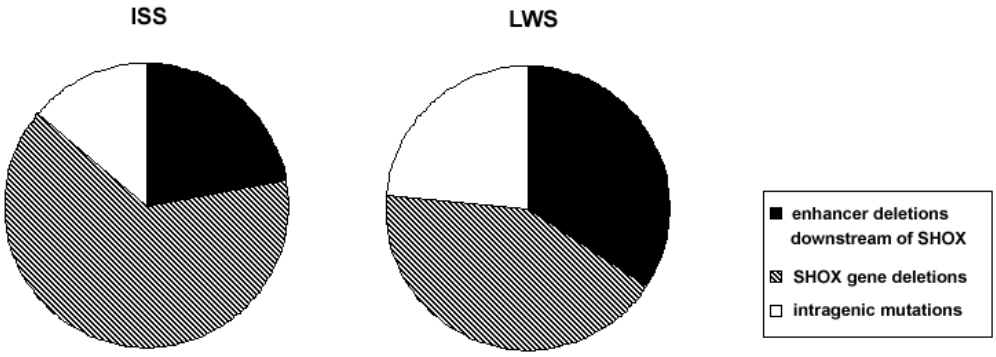
Suppl. Figure 1. Summary of the fine mapping by SNP, Microsatellite, and MLPA

analysis in 7 ISS (no. 27-33) and 1 LWS case (no. 62) with smallest *SHOX* downstream deletions to pinpoint the affected CNEs. Numbers indicate PCR fragments containing SNPs, named according to their distance (kilobases) from the telomere (build 36.1). Blacked areas indicate the presence of two copies of MLPA probe or heterozygosity of a SNP marker; white areas indicate one copy of MLPA probe (deleted interval) or homozygosity of a SNP marker.


TEL
0
450
SHOX
550
CNE2
CNE3
CNE4*
650
CNE5*
DXYS10085
CNE6
CNE7
DXYS10087
CNE8
DXYS10096
750
CNE9*
DXYS233
850 kb
CEN
34F05
51D11
61E05
10D05


CNE7
CNE8
CNE9
Proband No.
Sex
Disorder
Deletion size(Kb)
PAR1
PAR2
227
500
505
511
515
521
521
525
531
537
594
685
728
737
754
755
770
818
883
949
1361
1443
1711
3011
6155
7033
7184
8467
9653
18400
0.1 Mb from q-ter
PPP2R3B
X
SHOX
CSF2RA
IL3RA
ASMT
ARSF
NLGN4X
HDHD1A
STS
KAL1
GPR148
CDKL5
SYBL1
ISS
LWS
Control


A
10D05
P9
B
10D05
P9
C
10D05
DYZ3
D
10D05
DYZ3


ISS
LWS
enhancer deletions downstream of SHOX
SHOX gene deletions
intragenic mutations

Proband	Sex	Disorder	667	671	676	685	686	686	687	688	690	691	693	701	701	711	724	728	730	731	732	732	735	735	737	737	737	742	743	747	748	754	755	755	758	766	770	775	784	796	801	817	818	818	Deletion
No.			snp	CNE	snp	DXYS	CNE	MLPA	snp	snp	snp	snp	snp	snp	CNE	snp	snp	MLPA	DXYS	DXYS	snp	CNE	snp	snp	snp	DXYS	MLPA	snp	DXYS	snp	snp	MLPA	CNE	MLPA	snp	snp	MLPA	snp	snp	snp	snp	snp	DXYS	MLPA	Size (kb)
			717	5	726	10085	6	5098	737	738	740	741	742	751	7	761	773	5099	10086	10087	S6	8	C1	785	787	6797	5100	C2	10096	S8	798	6222	9	6219	S2	S11	6218	825	834	846	851	867	233	5103	
27	F	ISS																																											< 2
28	F	ISS																																											< 2
29	M	ISS																																											< 61
30	F	ISS																																											< 61
31	F	ISS																																											< 62
32	F	ISS																																											< 37
33	M	ISS																																											< 80
62	M	LWS																																											< 61