Background. The deficiency of SHOX gene (short stature homeobox-containing gene) has been recognized as the most frequent monogenetic cause of short stature. SHOX gene has been associated with short stature in Turner syndrome and Leri-Weill dyschondrosteosis as well as with non-syndromic idiopathic short stature. The aim of this study was to determine the frequency of SHOX deletions and mutations in a cohort of Slovenian children with short stature, and to delineate indications for routine SHOX gene mutation screening.

Methods and results. 40 selected subjects with idiopathic short stature were screened for entire SHOX gene deletion and for mutations in the SHOX gene coding region (exon 2 to 6), together with sequences flanking the exon-intron boundaries. FISH analysis on metaphase and interphase spreads revealed no entire gene deletion. Additionally, no pathogenic point mutations or smaller deletion/duplications were identified in this study group.

Conclusions. SHOX gene deletions and point mutations are not a common cause of idiopathic short stature in a cohort of Slovenian children with short stature. Therefore, the frequency of SHOX mutations must be much lower as expected based on the reported data.
1 INTRODUCTION

Short stature is a frequent childhood developmental condition with an incidence of 3 in 100 (1, 2). With the majority of these individuals, the underlying cause remains unknown and the condition is referred to as idiopathic short stature (ISS). It has been long known that human height follows a polygenic mode of inheritance. To date, there is about 50 genes and regions of the genome associated with height (3, 4). Among them, the deficiency of short stature homeobox-containing gene (SHOX gene) has been found as the most frequent monogenetic cause of short stature (5, 6).

Numerous studies in the last decade have indicated that syndromic short stature and idiopathic growth retardation are associated with SHOX deficiency. Nullzygosity of SHOX results in Langer mesomelic dysplasia (LMD), while haploinsufficiency of SHOX leads to Leri-Weill dyschondrosteosis (LWD) and short stature observed in Turner syndrome (TS). Heterozygous SHOX mutations or SHOX deletions were detected in 2-15% of individuals with idiopathic short stature (ISS) (6-8). The phenotypic outcome of SHOX deficiency is extremely variable: from most severe LMD, milder LWS, to isolated SHOX-related short stature at the mildest end of the spectrum. On the contrary, the over-expression of the gene is associated with tall stature in Klinefelter syndrome (Figure 1). It is important to emphasize that short stature can also be non-pathological in the case of familial short stature and constitutional delay (9).

1.1 SHOX Gene

The SHOX gene resides in pseudoautosomal region (PAR1) on the short arm of Xp and Yp, and has an important role in mediating linear growth. This telomeric PAR1 region spans over 2.7 Mb, and contains, so far, 29 known genes which escape X inactivation leading to the expression of SHOX from both sex chromosomes. The inheritance of pseudoautosomal region therefore mimics an autosomal dominant mode of inheritance (1, 11). The SHOX gene is composed of 6 exons: the last one has two alternatively spliced forms (exon 6a and exon 6b) encoding two different isoforms; SHOXa and its shortened version of SHOXb (11). Both isoforms function as transcriptional activators binding on specific regulatory region on DNA (7).

To date, there is about 250 SHOX gene mutations listed in Human Genome Mutation Database (HGMD Professional 2013.3), and an additional 1000 unique gene variants in SHOX database at www.shox.uni-hd.de. Among them, gross deletions and nucleotide substitutions are the most frequent ones. At the University Children’s Hospital Ljubljana, we have established a method using both cytogenetic and molecular genetic technique for the assessment of SHOX deficiency in Slovenian children with short stature.

2 PATIENTS AND METHODS

From 2011 to 2014, we have tested 107 short stature children (92 females, 15 males, mean age 10.5±4.4). They were all referred for cytogenetic testing to the Cytogenetic laboratory at the Unit for Special Laboratory Diagnostics at the University Children’s Hospital, University Medical Centre Ljubljana. After standard cytogenetic analyses, 16 girls were diagnosed with Turner syndrome or Turner syndrome variant. The subjects found to harbour structural chromosomal rearrangement were also excluded from further molecular analysis. The remaining patients were clinically examined at The Department of Endocrinology, Diabetes and Metabolism, University Children’s Hospital, University Medical Centre Ljubljana. Idiopathic short stature (ISS) was defined as the height below the 5th percentile for chronological age and sex and the absence of specific causative disorder. After thorough diagnostic work-up, overall 40 selected individuals (34 females, 6 males, mean age 9.1±4.1) were included in the further molecular investigation of short stature. All cytogenetics and molecular-genetics studies were undertaken with fully informed consent. The study followed the principles of the Declaration of Helsinki.
2.1 Cytogenetic Investigation and FISH Analysis
All patients were previously screened for chromosomal aneuploidy and/or structural rearrangement using standard cytogenetic analysis on stimulated lymphocytes. Only children with normal karyotype in 30 metaphases studied by GTG banding at the 500 band level were enrolled in subsequent fluorescence in situ hybridisation (FISH). FISH analysis was performed on metaphase and interphase chromosome spreads using a probe specific for the SHOX gene. We have selected BlueFish probe RP13-391G2 (BlueGnome) hybridizing to cytoband Xp22.33 starting from 562252 to 630112. For internal control we used chromosome X centromere probe (DXZ3 locus) and/or chromosome Y probe (DYZ1 locus) in parallel hybridisation.

2.2 DNA Sequencing Analysis
DNA was extracted directly from fixed cytogenetic cell suspensions with fast and simple isolation protocol using dedicated QiAmp DNA mini isolation kit, together with automated isolation system Qiacube (Qiagen, Hilden, Germany) (13). The amount of isolated DNA with concentrations between 10-15 ng/μl was suitable for PCR amplification of all SHOX exons. When available, genomic DNA was isolated from peripheral blood with the FlexiGene DNA Kit 250 (Qiagen, Hilden, Germany). SHOX gene coding region was PCR amplified using in-house designed sets of primers (sequences available upon request). Amplicons were sequenced using BigDye Terminator v.3.1 Cycle Sequencing Kit and 3500 Genetic Analyzer capillary electrophoresis system (Life Technologies, Foster City, CA, USA).

3 RESULTS
40 patients were diagnosed with ISS (no evidence of organic disease, normal wrist X-rays as indicator of bone age and presence of Madelung deformity, normal endocrine screen and normal growth hormone secretion assessed by growth hormone levels after provocative testing with Arginine or L-dopa). Short stature with disproportions of bodily parts (possible skeletal dysplasia) was present in 2 subjects, and 7 subjects had mild dysmorphic features that were not assigned to a known syndrome. None of the participants presented with the Madelung deformity, which was assessed clinically and with radiological imaging. Clinical characteristics of the selected participants are summarized in Table 1. FISH analysis on metaphase and interphase spreads revealed no entire gene deletion. Additionally, no pathogenic point mutations or smaller deletion/duplications were identified in any of the 40 participants.

| N | 40 |
|---|---|
| Age (years) | 9.1±4.1 |
| Gender | 34 female (85.0 %), 6 male (15.0 %) |
| Height SDS | -1.95±0.46 |
| Bone age SDS | -0.93±1.02 |
| Target height SDS | -0.20±0.65 |
| Possible skeletal dysplasia | 2 (5.0 %) |
| Dysmorphic features | 7 (17.5 %) |

N: number of patients; SDS: standard deviation score

4 DISCUSSION
The clinical phenotypes of SHOX haploinsufficiency disorders are extremely variable, from extremely short stature due to homozygous deletion or mutation on both SHOX alleles, to milder ISS without other clinical characteristic. We have conducted genetic screening study for the assessment of SHOX deletion/mutation prevalence in a group of 40 Slovenian children with ISS. The SHOX gene region was analysed using two independent methods with a different mutation detection range: fluorescence in situ hybridisation to identify large deletions, and direct DNA sequencing to identify point mutations and small deletions or insertions. FISH analysis appears as an easy, appropriate, and inexpensive method for the detection of SHOX deletion (14). Cytogenetic chromosomal investigations in our group of patients with ISS did not reveal SHOX deletion. Sequencing analysis also found no pathogenic point mutations in coding region of SHOX gene. In contrast to FISH, this method appears as time consuming and expensive, and, most importantly, in general, it is covering a lower percentage of causative gene defects, as point mutations are less frequent compared to SHOX gene deletions (8).

Our estimated prevalence of SHOX molecular defect was lower than previously reported. The largest published study on 1608 patients with short stature revealed SHOX deficiency in 4.2% of the analysed individuals: complete gene deletion in 70%, partial deletion in 5.9% and point mutation in 23.5% (8). General estimates for prevalence in children with ISS ranked from 2 to 15% (2, 5, 14, 15). In contrast, studies that were performed on smaller groups, show conflicting results. One of the first published studies on SHOX deficiency using FISH analysis detected no deletions in a cohort of 36 patients with unexplained short stature (16). There are several possibilities explaining the discrepancies between our and reported findings. Different clinical criteria for short
stature between studies with different methodological approaches are just one of them. During recent years, the discovery of deletion downstream the SHOX and its functional characterization led to the identification of several enhancer elements. To date, 4 enhancers located downstream and 3 enhancers upstream of the SHOX gene inside PAR1 have been described (17). Among them, the recurrent PAR1 deletion downstream of the SHOX spanning 47543 bp with identical breakpoints in several patients was characterized and confirmed as regulatory enhancer element for SHOX transcription (18).

With this additional enhancers recognized and higher frequency of SHOX deletion compared to point mutation, multiple ligation probe amplification (MLPA) seems as the most logical method of choice in investigation of ISS genetic defects. MLPA analysis was already recognized as fast, simple and high throughput screening method in the group of short children, and is recommended to be used for large scale screening of SHOX deletions (19).

An estimated heritability of human height is about 80-90% (20). Recent genome-wide association studies for copy number variations (CNV) and SNP arrays showed that rare CNV and SNP are also a common cause of short stature (4, 20). Especially SNP rs10427272 in HMG2 gene is a strong candidate for a height-associated allelic variant. Researchers analyzing large samples confirmed that rs10427272 C allele is associated strongly with increased human height (21, 22). Despite numerous height variations contributing only a small fraction under a polygenic model, finding the genetic cause in this frequent condition is very important.

5 CONCLUSIONS

Linear growth is one of the most sensitive indicators of health, and is affected by various pathophysiological mechanisms, including genetic variations in SHOX gene. In the present study of children with ISS, however, no deletions or pathogenic point mutation in the SHOX gene were identified. This data does not corroborate currently published results in other populations, where a higher incidence of genetic variations (especially deletions) in SHOX gene was determined. Therefore, we propose that, in children with ISS, first-tier genetic analysis should include one of molecular cytogenetic testing, either MLPA or FISH, and not SHOX mutations detection, since they are less frequent.

FUNDING

The study was supported in part by the Slovenian National Research Agency grants J3-6800, J3-6798 and P3-0343.

CONFLICTS OF INTEREST

The authors declare that no conflicts of interest exist.

ETHICAL APPROVAL

Written informed consent was obtained from all participants or their parents. The study was approved by the Slovene Medical Ethics Committee.

REFERENCES

1. D’haene B, Hellemans J, Craen M, De Schepper J, Devriendt K, Fryns JP, et al. Improved molecular diagnostics of idiopathic short stature and allied disorders: quantitative polymerase chain reaction-based copy number profiling of SHOX and pseudoautosomal region 1. J Clin Endocrinol Metab 2010; 95: 3010-8.
2. Rappold GA, Fukami M, Niesler B, Schiller S, Zumkeller W, Bettendorf M, et al. Deletions of the homeobox gene SHOX (short stature homeobox) are an important cause of growth failure in children with short stature. J Clin Endocrinol Metab 2002; 87: 1402-6.
3. Chial H. Polygenic inheritance and gene mapping. Nature Educ 2008; 1: 17.
4. van Buvenvoorde HA, Lui JC, Kant SG, Oostdijk W, Gijsbers AC, Hoffer MJ, et al. Copy number variants in patients with short stature. Eur J Hum Genet 2014; 22: 602-9.
5. Binder G. Short stature due to SHOX deficiency: genotype, phenotype, and therapy. Horm Res Paediatr 2011; 75: 81-9.
6. Rappold G, Blum WF, Shavrikova EP, Crowe BJ, Roeth R, Quigley CA, et al. Genotypes and phenotypes in children with short stature: clinical indicators of SHOX haploinsufficiency. J Med Genet 2007; 44: 306-13.
7. Stuppa L, Calabrese G, Gatta V, Pintor S, Morizio E, Fantasia D, et al. SHOX mutations detected by FISH and direct sequencing in patients with short stature. J Med Genet 2003; 40: E11.
8. Luchtmann N. Short stature in children. Continuing Med Educ 2006; 24: 372.
9. McEvoy BP, Vischer PM. Genetics of human height. Econ Hum Biol 2009; 7: 294-306.
10. Helena Mangs A, Morris BJ. The Human Pseudoautosomal Region (PAR): origin, function and future. Curr Genomics 2007; 8: 129-36.
11. Rao E, Weiss B, Fukami M, Rump A, Niesler B, Mertz A, et al. Pseudoautosomal deletions encompassing a novel homeobox gene cause growth failure in idiopathic short stature and Turner syndrome. Nat Genet 1997; 16: 54-63.
12. Amorim MR, Vargas FR, Llerena JC Jr. Pseudoautosomal region 1 DNA extraction from fixed cytogenetic cell suspensions. Genet Mol Res 2007; 6: 500-3.
13. Morizio E, Stuppa L, Gatta V, Fantasia D, Guanciali Franchi P, Rinaldi MM, et al. Deletion of the SHOX gene in patients with short stature of unknown cause. Am J Med Genet A 2003; 119: 293-6.
14. Huber C, Rosilio M, Munnich A, Cormier-Daire V, French SHOX GeneNIS Module. High incidence of SHOX anomalies in individuals with short stature. J Med Genet 2006; 43: 735-9.
15. Binder G, Schwarze CP, Ranke MB. Identification of short stature caused by SHOX defects and therapeutic effect of recombinant human growth hormone. J Clin Endocrinol Metab 2000; 85: 245-9.
16. Musebeck J, Mohnik K, Beye P, Tönnies H, Neitzel H, Schnabel D, et al. Short stature homeobox-containing gene deletion screening by fluorescence in situ hybridisation in patients with short stature. Eur J Pediatr 2001; 160: 561-5.
17. Benito-Sanz S, Aza-Carmona M, Rodríguez-Estevez A, Rica-Etxebarria I, Gracia R, Campos-Barros A. et al. Identification of the first PAR1 deletion encompassing upstream SHOX enhancers in a family with idiopathic short stature. Eur J Hum Genet 2012; 20: 125-7.

18. Benito-Sanz S, Royo JL, Barroso E, Paumard-Hernández B, Barreda-Bonis AC, Liu P. et al. Identification of the first recurrent PAR1 deletion in Léri-Weill dyschondrosteosis and idiopathic short stature reveals the presence of a novel SHOX enhancer. J Med Genet 2012; 49: 442-50.

19. Gatta V, Antonucci I, Morizio E, Palka C, Fischetto R, Mokini V. et al. Identification and characterization of different SHOX gene deletions in patients with Leri-Weill dyschondrosteosis by MLPA assay. J Hum Genet 2007; 52: 21-7.

20. Zahnleiter D, Uebe S, Ekici AB, Hoyer J, Wiesener A, Wieczorek D. et al. Rare copy number variants are a common cause of short stature. PLoS Genet 2013; 9: e1003365.

21. Weedon MN, Lettre G, Freathy RM, Lindgren CM, Voight BF, Perry JR. et al. Diabetes Genetics Initiative; Wellcome Trust Case Control Consortium, Davey Smith G, Groop LC, Hattersley AT, McCarthy MI, Hirschhorn JN, Frayling TM. A common variant of HMGA2 is associated with adult and childhood height in the general population. Nat Genet 2007; 39: 1245-50.

22. Yang TL, Guo Y, Zhang LS, Tian Q, Yan H, Guo YF, Deng HW. HMGA2 is confirmed to be associated with human adult height. Ann Hum Genet 2010; 74: 11-6.