CYTOGENETIC AND MOLECULAR GENETIC CHARACTERIZATION OF CHILDREN WITH SHORT STATURE

CITOGENETSKA IN MOLEKULARNO GENETSKA OPREDELITEV NIZKE RASTI PRI OTROCIH

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ABSTRACT

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 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.

Keywords:
SHOX gene, idiopathic short stature, FISH analysis, DNA sequnecing

IZVLEČEK

Izhodišča. *Razlike v številu aktivnih kopij gena SHOX (short stature homeoboxcontaining gen) so najpomembnejši monogenetski vzrok nizke rasti. Vpliv gena SHOX je opredeljen pri razvoju nizke rasti v sklopu Turnerjevega sindroma ali Leri-Weillove dishondrosteoze ter je lahko vzrok nesindromske idiopatske nizke rasti. Namen raziskave je določiti frekvenco delecij in mutacij gena SHOX v skupini slovenskih otrok z nizko rastjo ter opredeliti smernice za presejalno testiranje mutacij v genu SHOX.*

Metode in rezultati. *Izbranih 40 preiskovancev z nizko rastjo smo genetsko testirali za delecijo celotnega gena SHOX ter pojavnost mutacij v celotnem kodirajočem področju gena (eksoni 2 do 6) skupaj z mejami med eksoni in introni. Z analizo FISH na metafaznih in interfaznih kromosomih pri nobenem izmed preiskovancev nismo odkrili delecije. Prav tako pri nobenem izmed 40 preiskovancev nismo odkrili točkovne mutacije ali manjše delecije/duplikcije.*

Zaključki. *Delecije in točkovne mutacije gena SHOX v izbrani skupini slovenskih otrok z nizko rastjo niso pogost razlog za idiopatsko nizko rast. Zato predvidevamo, da je frekvenca mutacij gena SHOX nižja, kot bi pričakovali glede na podatke iz literature.*

Ključne besede:
gen SHOX, idiopatska nizka rast, analiza FISH, sekveniranje DNK

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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).

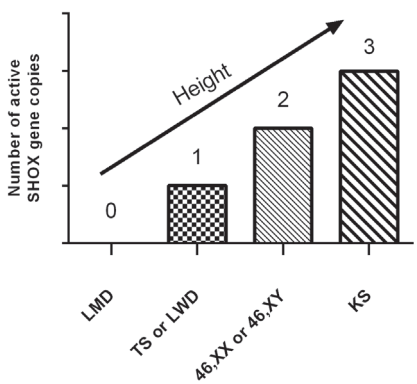

Figure 1. Number of active copies of SHOX gene associated with human height. LMD, Langer mesomelic dysplasia; TS, Turner syndrome; LWD, Leri-Weill dyschondrosteosis; KS, Klinefelter syndrome.

The evolutionary and biological basis of human height is not fully understood, especially its links to disease (10). The aim of this study was to determine the frequency of *SHOX* deletions and mutations in a cohort of Slovenian children with ISS, and to delineate indications for routine *SHOX* gene mutation screening.

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 isoformes; *SHOX*a and its shortened version of *SHOX*b (11). Both isoformes 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.

Table 1. Clinical features of children with idiopathic short stature screened for SHOX gene mutations.

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 rs1042725 in HMGA2 gene is a strong candidate for a height-associated allelic variant. Researchers analyzing large samples confirmed that rs1042725 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

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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.

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