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Microdeletion and mutation analysis of the *SHOX* gene in patients with idiopathic short stature with FISH and sequencing

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Background/aim: The aim of this study was to investigate the prevalence of the microdeletions and mutations of the *SHOX* gene in children with idiopathic short stature (ISS) by the usage of fluorescence in situ hybridization (FISH) and direct sequencing technique.

Materials and methods: Thirty-seven children referred to our clinic because of short stature were classified as having ISS after clinical examination. Chromosome analyses, FISH analysis of the *SHOX* gene, and direct sequencing of the coding exons of *SHOX*, through the second to the sixth exon, in 24 of the 37 patients were also performed.

Results: All children had normal karyotypes and the *SHOX* gene region was found to be intact in all. No mutation was detected in the exonic sequences and exon/intron boundaries of the *SHOX* gene in 24 children analyzed.

Conclusion: No mutation was detected in the exonic sequences and exon/intron boundaries of the *SHOX* gene and this indicated that the prevalence of the *SHOX* mutations can differ according to the selection criteria, used methods, sample size, and population.

Key words: Idiopathic short stature, microdeletion, mutation analyses, fluorescence in situ hybridization, *SHOX* gene

1. Introduction

Short stature, affecting 2%–3% of individuals in the population, is a multifactorial developmental condition with a strong genetic component and environmental factors (1,2). Several monogenic genetic causes are known in short stature. One of these genes is the short stature homeobox-containing gene (*SHOX*) encoding a homeodomain transcription factor (3,4). This gene, localized on pseudoautosomal region (PAR) 1 of the X and Y chromosomes, has been specifically associated with some cases of idiopathic short stature (ISS) and the short stature of patients with Turner syndrome (4). *SHOX* gene mutation prevalence varies between 2% and 14% in children with ISS (2–6). Additionally, Leri–Weill dyschondrosteosis (LWD, MIM 127300), Langer mesomelic dysplasia (LMD, MIM 249700), and familial short stature are also associated with *SHOX* gene mutations (1,3).

The *SHOX* gene, an important mediator of linear growth, is expressed in osteoblasts, especially in bone marrow fibroblasts and hypertrophic chondrocytes in the human embryo from the second month of gestation, and is also expressed in the growth plate in the postnatal period (1,7). Deficiency of *SHOX* in the developing skeleton during fetal life is associated with evident disorganization of chondrocyte proliferation (1). A dose-dependent association between the number of active copies of the *SHOX* gene and height are known, while *SHOX* gene haploinsufficiency is thought to be the cause of short stature like in Turner syndrome and *SHOX* gene overdose is associated with tall stature like in Klinefelter syndrome (1,8–10). In the studies done so far, although the phenotype of *SHOX* gene haploinsufficiency is highly variable, heterozygous intragenic mutations, total gene deletions that correspond to about 80% of all mutations, or microdeletions in the transcription unit have been shown to be responsible for haploinsufficiency of *SHOX* (10,11). These causes account for at least 5% of cases of ISS and for 50%–100% of cases of LWD according to the cohort studied (10).

The updated *SHOX* mutation database, which was established to provide clinicians and scientists access to a central source of information about *SHOX* mutations,

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currently contains 324 intragenic mutations, 188 of which are unique allelic variants. The variants detected in control individuals are pooled in 185 mutations, which are disease-related mutations. It is obvious that searching *SHOX* gene mutations has great value in clinical follow-up in patients with ISS. The present study aimed to investigate the microdeletions and mutations of the *SHOX* gene in children referred to our clinic because of ISS with fluorescence in situ hybridization (FISH) and direct sequencing techniques.

2. Materials and methods

2.1. Patients

We studied 37 children (age: 5–18 years, mean ± standard deviation (SD): 10.89 ± 2.83 years) seen in the pediatric endocrinology division and referred to our department for evaluating genetic etiology concordant with their clinical findings. Patients consisted of an entirely native unrelated Turkish population. Written informed consent was obtained from the patients' guardians for genetic analysis and the study was approved by the Gazi University Institutional Ethics Board Committee.

All the patients were diagnosed with ISS according to following criteria: 1) height below 3rd percentile or –2 SDs with normal body proportions; 2) normal growth hormone levels and normal response to growth hormone stimulation tests; 3) normal karyotype; 4) absence of congenital skeletal abnormalities or dysmorphic features; 5) absence of chronic, metabolic, and systemic diseases or nutritional problems; and 6) normal parental height (1–4,8,11–15). Appropriate written informed consent was obtained and study participants were screened for microdeletions and/or mutations in the *SHOX* gene.

2.2. Methods

A peripheral blood sample of 10 mL with heparin for karyotyping and FISH analyses and 5 mL of peripheral blood sample with ethylenediaminetetraacetic acid (EDTA) for DNA extraction were taken from each patient. After cultivation of the peripheral blood lymphocytes, G-banding with high-resolution band technique was performed and 20 metaphases were analyzed.

FISH analyses were performed according to Rao et al. (6). The cosmid LLYNOYCO3M34F5 specific DNA probe was optimized to detect copy numbers of the *SHOX* gene region at Xp22/Yp11. The *SHOX* (Xp22) specific DNA probe was directly labeled with PlatinumBright 550 provided in a 2× concentrated form. The *SHOX* probe was designed as a dual color assay to detect deletions at Xp22/Yp11. For each patient, at least 5 metaphases and 30 nuclei were examined.

2.3. PCR and DNA sequence analyses

Genomic DNAs were isolated from 200–250 µL of peripheral blood lymphocytes using a DNA isolation kit (Axygen Scientific, USA) and measured with a spectrophotometer (NanoDrop ND-1000, USA). Amplification of exons 2–6 of the *SHOX* gene was performed by using specific primers. Primer sequences and polymerase chain reaction (PCR) conditions are shown in the Table. PCR products were directly sequenced with both forward and reverse DNA strands with the Sanger method and the sequence data were analyzed with AB Sequence Scanner Software 2 v2.0 (Applied Biosystems, USA).

3. Results

We studied 33 female and 4 male patients with ISS. The height of patients varied between 98 and 147 cm (mean ± SD: 126.95 ± 13.59). All patients' heights were below –2 SDs and the mean SD value was –2.44. While four of the patients' height SD was below –3 SD, the lowest value was –3.51 SD. No microdeletion in the critical region of the *SHOX* gene was detected via FISH technique and the dye results of one patient are shown in Figure 1.

For further investigation, 24 patient of 37 whose DNAs were available were screened by direct sequencing for all coding exons (exons 2–6) and exon/intron boundaries of the *SHOX* gene. Because of insufficient DNA and refusal to give blood samples again (7 out of 13), or rejection of further molecular analyses (6 out of 13), 13 patients could not be screened for the relevant gene. The sequence analyses were performed on both DNA strands and this process did not identify any allelic variants in the annealing sites (Figure 2).

Table. The forward-reverse sequences and the characteristics of the primers for the *SHOX* gene.

Exons	Forward primer (5'-3')	Revers primer (5'-3')	Tm (**°C**)
Exon 2	GAGGTCGCCGCGTATAAATAG	GAGACGGGAGCTGCAAATGT	61
Exon 3	GTCAAAGCGCATTGGTTTTC	CGTCTCCAAAAGTCCAGGAAC	60
Exons 4 and 5*	ATTTCCAGTACTAGGTAGTCAGTGATG	CAAATAGGGGAAAGGGGAAGG	63
Exon 6	AGAAGAGGCACGTTGGAGGTTTC	TCCTCAGGCCTCTTGCAGGA	63

*Exons 4 and 5 were amplified together; Tm: annealing temperature.

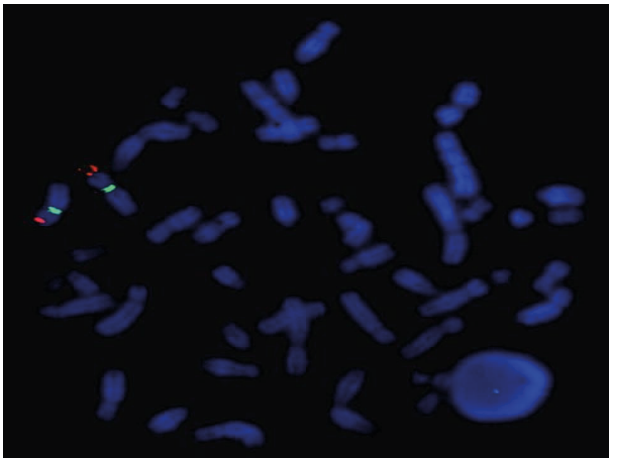

Figure 1. Fluorescence in situ hybridization results of a female patient. The centromeric region of chromosome X (DXZ1, CEPX, and control probe) was labeled with the green and the critical region (Xp22) of the *SHOX* gene was labeled with the red fluorescent signal.

4. Discussion

The *SHOX* gene is one of the genes escaping from the inactivation process held on chromosome X, and it is expressed on both the active and inactive copy of chromosome X and also on chromosome Y (3,4,6). In addition to the probable effects of other genetic factors such as modifier genes, the occurrence of two functional copies, and the pattern of pseudoautosomal inheritance, the *SHOX* gene has gene-dose effect, and deficiency of the gene causes a broad phenotypic spectrum such as short stature including ISS, and sometimes other skeletal abnormalities such as Madelung deformity (1–10,16–18).

In this study, patients with ISS who met the selection criteria based on physical examination were evaluated in consecutive genetic studies. In accordance with previous studies, to determine *SHOX* gene abnormalities, conventional cytogenetic analysis was performed in the first step to exclude numerical or structural chromosomal abnormalities including PAR1 (3,9). In the next step, microdeletions including the *SHOX* gene were searched via FISH because deletions are more frequently seen than point mutations in this region (1–4,8,9,11–13,15). Although several alternative techniques such as microsatellite marker analyses and multiplex ligation-dependent probe amplification exist to identify microdeletions, FISH is one of the most commonly used convenient methods and does not require any confirmation tests because of its high accuracy (1–3,8,13–15). In the present study, FISH analysis of both metaphases and nuclei from peripheral blood lymphocytes did not show any deletions in 37 patients. The results of microdeletion analyses by FISH are controversial in the literature. Stuppia et al. (13) reported that approximately 7% of patients with ISS had *SHOX* gene critical region deletion. However, Musebeck et al. (15) did not find any *SHOX* gene deletions in a series of 35 patients suffering from ISS, similar to our findings.

SHOX gene mutations distributed along the entire gene and the spectrum of intragenic mutations are highly heterogeneous (http://www.shox.uni-hd.de). Depending on the recommendation of the sequencing procedure, twenty-four patients with ISS who were admitted to give peripheral blood samples for this purpose were analyzed (exons 2, 3, 4, 5, and 6) and none of them revealed any mutation (1,3,4,13,14,19). The prevalence of the point mutations of the *SHOX* gene reported from different studies has high variability. Rao et al. (6) screened 91 patients with ISS and they identified a nonsense mutation in one of the patients. Since then, different studies have evaluated the frequency of point mutations and deletions in *SHOX* genes in children with ISS. Rappold et al. (1) studied 1608 patients with short stature from 14 different countries using a similar methodology. They reported that the rate of alteration of the gene was 2.2%. In our study, we did not identify any mutation or microdeletion in the patients with ISS. We analyzed all coding exons and exon/intron boundaries of the *SHOX* gene, but we could not screen enhancer, regulatory, and intronic regions because of the technical and the cost limitations.

Microdeletion mutation analyses of the *SHOX* gene, covering 69 patients with ISS, including 12 monozygotic and 6 dizygotic twins and 44 brothers, were previously reported from Turkey (19). That study used FISH analyses of the critical region of the *SHOX* gene of the selected eight shortest children and did not come across any deletions. The screening of exon 2 of all of the patients concluded a lack of mutations. In total, *SHOX* gene mutation prevalence is about 1% and the region of the mutations consists of almost all parts of the entire gene (19). Because of this, instead of searching only one or a small number of exons in a large number of cases, we aimed to cover all the probable mutations of all the coding exons and exon/intron boundaries in a more reasonable number of patients.

Searching the *SHOX* gene via high accuracy techniques like FISH and direct sequencing similar to ours could end with no mutations found because of variable selection criteria, sample sizes, and population diversity. Despite the contradictory results, it is valuable to search for gene alterations in ISS patients as there is a chance for additional height after treatment by growth hormone (GH) replacement in these children if the mutations of the *SHOX* gene could be verified (8,9,20). In a 2-year period, a gain in height of an average of 6.2 cm by GH therapy was reported (20). The molecular diagnosis of *SHOX* gene

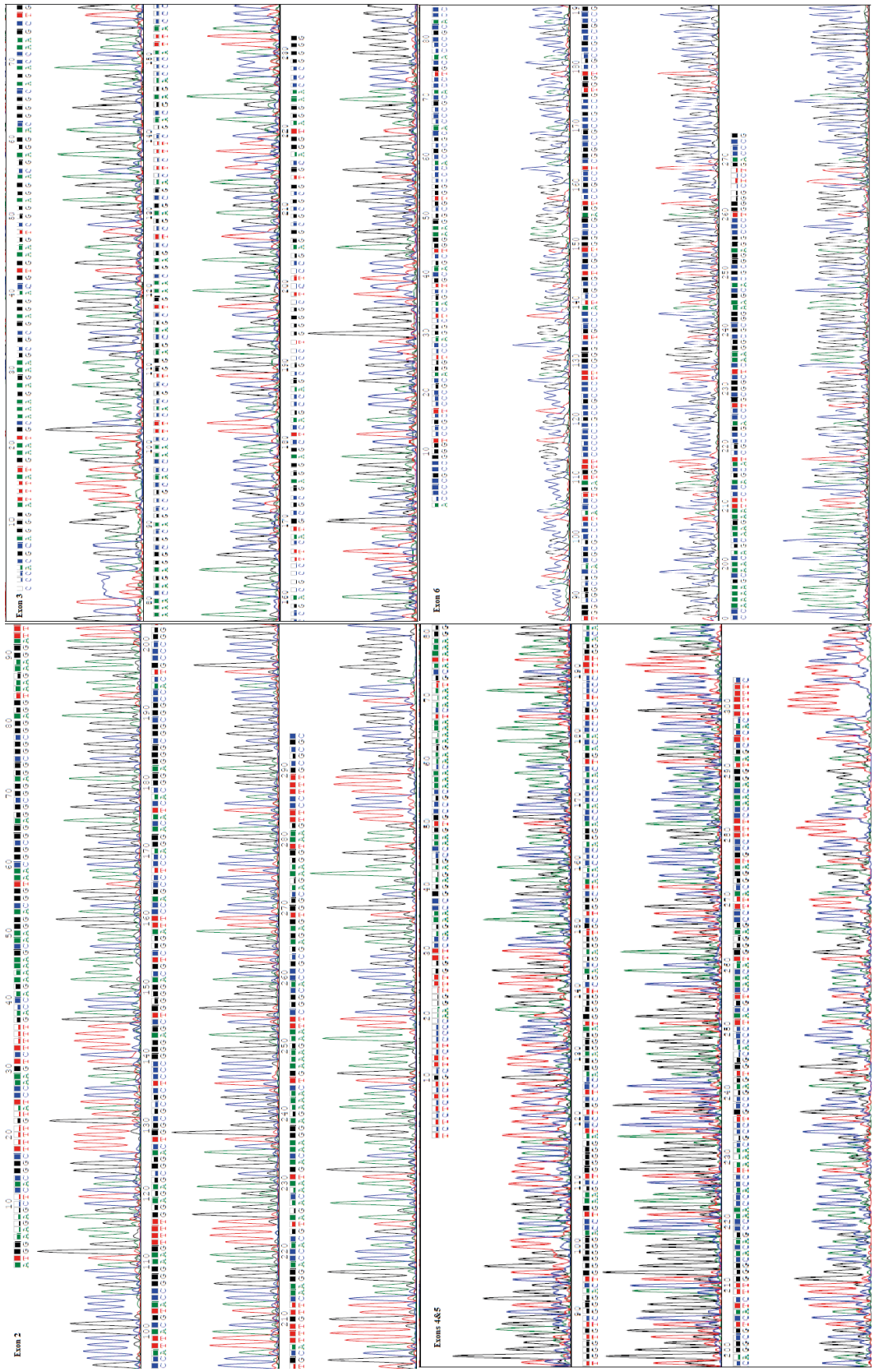

Figure 2. The electropherogram of the coding exons of the *SHOX* gene.

defects is likely to have therapeutic implications, as human recombinant growth hormone treatment associated with or without gonadotropin-releasing hormone analogues might improve the final height.

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