



Screening of *SHOX* gene sequence variants in Saudi Arabian children with idiopathic short stature

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Purpose: Short stature affects approximately 2%–3% of children, representing one of the most frequent disorders for which clinical attention is sought during childhood. Despite assumed genetic heterogeneity, mutations or deletions in the short stature homeobox-containing gene (*SHOX*) are frequently detected in subjects with short stature. Idiopathic short stature (ISS) refers to patients with short stature for various unknown reasons. The goal of this study was to screen all the exons of *SHOX* to identify related mutations.

Methods: We screened all the exons of *SHOX* for mutations analysis in 105 ISS children patients (57 girls and 48 boys) living in Taif governorate, KSA using a direct DNA sequencing method. Height, arm span, and sitting height were recorded, and subischial leg length was calculated.

Results: A total of 30 of 105 ISS patients (28%) contained six polymorphic variants in exons 1, 2, 4, and 6. One mutation was found in the DNA domain binding region of exon 4. Three of these polymorphic variants were novel, while the others were reported previously. There were no significant differences in anthropometric measures in ISS patients with and without identifiable polymorphic variants in *SHOX*.

Conclusion: In Saudi Arabia ISS patients, rather than *SHOX*, it is possible that new genes are involved in longitudinal growth. Additional molecular analysis is required to diagnose and understand the etiology of this disease.

Key words: Molecular sequence, Idiopathic short stature, *SHOX* gene, Anthropometric measures

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Introduction

Growth is a very important feature of development of human in which several environmental and genetic factors involved¹. Short stature is defined as a height more than 2 standard deviations (SDs) below the mean for age and sex, comparing to the national height standards or one that is below the third percentile for the age and gender in a population². About 2%–3% of the children in the world have short stature³. Eighty percent (80%) of the cases have no history of small for gestational age, growth hormone (GH) deficiency, or other pathologies, therefore, this group of patients defined as idiopathic short stature (ISS)². A small proportion of genes are concerning to short stature⁴. Short stature homeobox (*SHOX* gene MIM 31286⁵) is one of the major genetic contributors to human growth. *SHOX* mutations can cause Leri Weill dyschondrosteosis and Langer mesomelic dysplasia. Moreover, Short stature in Turner syndrome is the result of *SHOX* gene haploinsufficiency³. In 1997, Rao et al.⁴ were the first to describe *SHOX* gene mutations in individuals with ISS (ISS MIM 300582). *SHOX* gene is found in the pseudoautosomal region 1 of the short arms of both human sex chromosomes

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at the position Xp22.3–Yp11.3 and consists of six exons, the first is noncoding and the others coding⁴. Several clinical and molecular studies identified the *SHOX* gene as the cause of the short stature phenotype⁶. Intragenic mutations are described in 2%–15% of cases⁷. Previously, it has been proven that the conserved noncoding elements downstream of the *SHOX* gene regulate gene function⁸. These conserved elements present a 250-kb downstream regulatory domain that may be deleted in patients with ISS and in other *SHOX* gene defects with a frequency of 22%³.

Some limited studies were done based on clinical features to evaluate the scale of regional difference in prevalence of short stature in Saudi Arabia patients^{9–11}. Therefore, to the best of our knowledge this is the first study for molecular screening of children with ISS in Saudi Arabia.

Materials and methods

Prospective study of 105 children (57 female and 48 male children) were diagnosed clinically as having ISS and screened for mutations of the *SHOX* gene. Their ages ranged from 2 to 18 years with mean of 11 years. They were enrolled between September 2013 and December 2015 and referred by the pediatric endocrinology outpatient services of Armed Forces Hospital, Taif region, KSA, under the prior approval in accordance with the principles of the Regional Research and Ethics Committee of Hospitals (IRB, H-02-T-001).

Patients with normal thyroid function tests, normal karyotype in females, negative celiac screening, negative chronic organic/psychological or syndromic diseases, normal 2 GH provocation test with peak value ≥ 10 $\mu\text{g/L}$ (to exclude GH deficiency)¹² and lack of clear signs of chondrodysplasias were entered this study. These inclusive criteria were chosen according to the proposed consensus on the definition of ISS², as it is a diagnosis of exclusion. For every patient; measurement of weight (kg), standing height (cm), arm span (cm) (which is the length from one end of an individual's arms (measured at the fingertips) to the other when raised parallel to the ground at shoulder height at a 90°¹³), sitting height (cm) (which is

the distance from the highest point on the head to the base sitting surface)¹³ and calculation of subischial leg length (cm) (which is the difference between height and sitting height)¹³ was performed. Also, paternal height (cm) and maternal height (cm) were measured for every patient, to calculate the mid parental height (for boys: [paternal height+ {maternal height+ 13 cm}]/2, for girls: [maternal height+ {paternal height– 13 cm}]/2). X-ray of the left hand was evaluated for bone age and peripheral blood samples were collected from the patients for DNA extraction.

Genomic DNA was extracted from peripheral blood leucocytes using an extraction kit (Thermo Fisher, Waltham, MA, USA) and used for polymerase chain reaction (PCR) amplification. The entire regions of exons of the *SHOX* gene were amplified by PCR using the newly designed specific primers. The PCR products were confirmed by agarose gel electrophoresis.

Direct sequencing of purified products using the same primers (Table 1) was performed on both strands using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit on a 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) and the raw sequencing results were collected using the Data collection and analyzed using sequencing analysis software version 3.1 (Life Technologies, Grand Island, NY, USA). Seqscape software version 2.7 (Applied Biosystems) was used for base-calling and mutation detection. All *SHOX* variants reported in this study were submitted to the Human Variation Database of the National Center for Biotechnology Information.

Results

Clinical characteristics and, mean of all anthropometric measures of ISS patients included in the study (both mutated and nonmutated) are shown in Table 2. Of all ISS children analyzed 17 girls and 13 boys have variants indicate that females more affected than males with ratio of 57% females and 43% males in ISS patients with variants. These results revealed a 28% frequency of alterations to the *SHOX* gene in Saudi Arabia children with ISS. Anthropometry of ISS patients with *SHOX* variants and those without revealed that there are no big differences between mean height, mean weight,

Table 1. Primers and PCR conditions used to amplify genomic DNA segments of the *SHOX* gene

Primer	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temperature (°C)/time	Product size (bp)
SX-1	GGAGCCAATAGGGGTCTTCG	GCTTGGTGAGCTCGGTTTTG	55/30 sec	308
SX-2	CTCTCTCCAGCCGTGAATC	CGAGAGTGGCAGAAGGTAA	55/45 sec	758
SX-3	CACGTTGCGCAAACCTCC	GGAGCCATCGCCTGGTC	55/30 sec	312
SX-4+5	TTCACAGGGCTCTTCACATC	CTTCCCCTTTCCCCTATTTGC	55/30 sec	376
SX-6a-1	CCCCCAGTCCCCATCCTG	CCTCCCAGGCAGCAATAAGG	62/50 sec	871
SX-6a-2	CCTCCCAGGCAGCAATAAGG	CATCTTCTGCGTTCCCCCAG	55/45 sec	696
SX-6a-3	CTCCATCTTCTGCGTTCCCCCAG	TGGGAAGTAGGTTCAATTCATCAG	55/1 min	1,034
SX-6b	CAGCCTCCCAAAGTGCTGGGACTAC	TGTACAGTTTGAGGGTGTGTGTGC	60/45 sec	752

Table 2. Evaluation of anthropometric measurements in the mutant and nonmutant groups within Idiopathic short stature patients

Variable	Girls		Boys	
	Mutated (n=17)	Nonmutated (n=40)	Mutated (n=13)	Nonmutated (n=35)
Age (yr)	9.2±1.3	10.7±3.5	11.6±3.2	11.2±3.7
Height (cm)	117.3±17.5	119.4±17.1	125.3±14	122.9±15.9
Height SDS	-3.27±0.52	-2.99±0.46	-3.01±0.21	-2.94±0.33
Weight (kg)	23.2±10.2	24.3±10.7	27.2±9.4	27.4±9.8
Weight SDS	-2.81±0.72	-2.71±0.69	-2.48±0.4	-2.25±0.57
Arm span (cm)	112±16.9	114.7±16.6	120±13.2	117.3±15.2
Sitting height (cm)	66.4±9.4	66.6±9.7	70.3±7.8	69.4±8.8
Subsiceal leg length (cm)	51.2±8.2	52.7±8	54.9±6	53.5±7.5
Maternal height (cm)	151±6.38	150±5.88	155.5±6	151±6.94
Paternal height (cm)	166±7.65	165±8.29	165.25±5.96	167±7.49

Values are represented as mean±standard deviation.

SDS, standard deviation score.

z score was calculated using standardized height and weight calculator used by National Health Nutrition Examination Survey - NHANES, and P values were calculated according to t test and found to be not significant.

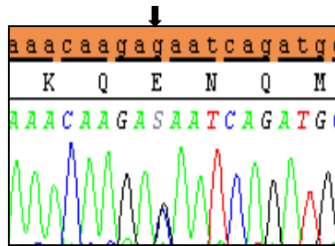


Fig. 1. Polymorphic variant detected in exon 4 in homeodomain of *SHOX* in Idiopathic short stature patients. Missense mutation in heterozygous genotype form c.528 G>C, p.E176D. Black arrow indicates the site of mutation.

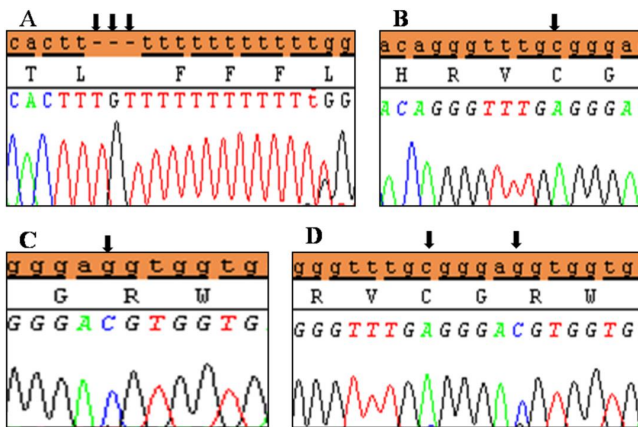


Fig. 2. Polymorphic variants detected in noncoding region exon 1 of *SHOX* in Idiopathic short stature (ISS) patients. (A) novel insertion variant c.-645_-646 insTGT, (B) polymorphic variant c.-512 C>A, (C) polymorphic variant c.-507 G>C, and (D) both variants in the same ISS patients. Black arrows indicate the site of the variant.

arm span, sitting height and subsiceal leg length in both groups within males gender while big differences were noted in these para-

eters in females, suggesting that females more to be affected by *SHOX* variation. Direct DNA sequencing of purified PCR products resulted in overall with 1 mutation and 6 polymorphic variants. Four variants were previously reported in the *SHOX* database and the others have not been described and defined as novel. These mutation and variants are located in exons 1, 2, 4 and 6, while, no variants were detected in exons 3 and 5. Interestingly, we found a missense mutation (c.528G>C, p. E176D) in the DNA homeodomain binding site of exon-4 at the position 528. This mutation was detected in 1 girl patient aged 5.5 years as a heterozygous form (Fig. 1). According to the PolyPhen-2 website program, this mutation is predicted to be probably damaging and considered as pathogenic one. Three polymorphic variants identified in noncoding region of exon-1. First variant is a novel and found as insertion of 3 nucleotides between the position c.-646_-645insTGT (Fig. 2A). The second and third variants were reported previously¹⁴⁾ and detected in positions c.-512C>A and c.-507G>C, respectively, as a homozygous genotype forms (Fig. 2B, C). Interestingly, 6 patients were carried both variants (c.512C>A and c.-507G>C) as a compound homozygous (Fig. 2D). Next, 1 polymorphic variant was identified in 5'UTR (untranslated region) of exon-2, in position c.-372 G>A as homozygous form in five patients and heterozygous form in 9 cases (Fig. 3A, B). Two novel polymorphic variants were identified in exon-6a. First one was detected in position c.*41C>A in the 6a- 3'UTR as homozygous in 1 patient and heterozygous genotype forms in the other one (Fig. 4A1, A2). Second variant was found as an insertion variant detected in position c.*284_285insAG in 6a- 3'UTR (Fig. 4B). Table 3 summarized the phenotypically characteristics and polymorphic variants frequencies that found in this study. There is no significant differences in the phenotypic parameters between ISS patients that having DNA variations and those without DNA variations.

Discussion

Longitudinal growth is a complicated process that is controlled

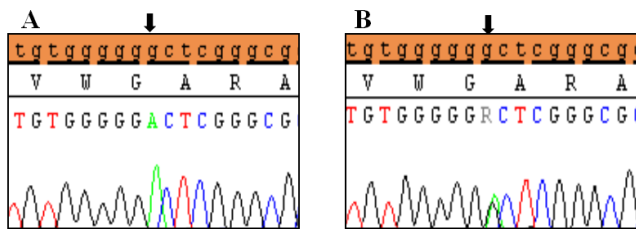


Fig. 3. Polymorphic variant detected in 5'UTR region of exon 2 of *SHOX* in Idiopathic short stature patients. (A) Homozygous genotype form c.-372 G>A and (B) heterozygous genotype form c.-372 G>A. Black arrows indicate the site of the variant.

by genetic factors and regulated by both permissive and regulatory factors¹⁵. *SHOX* gene belongs to the transcriptional regulators family that has an essential role in development¹⁶. *SHOX* gene variants expected to be responsible for 2% of patients with ISS¹⁷ that has increased to 23% by time due to better clinical attention to such gene¹⁸. Our study of 105 children with ISS showed 30 patients (28%) of the children had at least 1 variant that explained their short stature. The higher frequency in our study when compared to published studies that reported this association with a very broad frequency range varying from 1.5% to 15%¹⁹, which may be explained by ascertainment bias or by a true increased frequency of *SHOX* variants as a result of high rate of consanguineous marriage in Saudi Arabia as reported by el-Hazmi et al.²⁰.

Comparison of mutation prevalence in prior studies is difficult

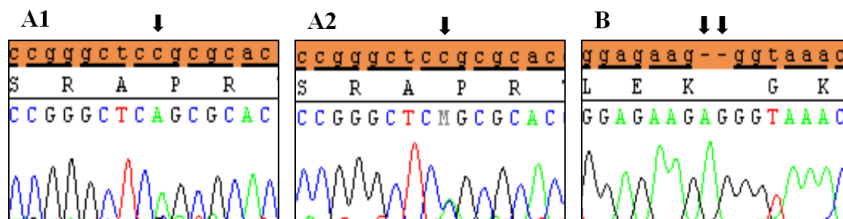


Fig. 4. Polymorphic variants detected in exon 6a of *SHOX* in idiopathic short stature patients. (A) Novel missense variant (c.*41C>A) in homozygous form (A1) and heterozygous form (A2), (B) Novel variant (c.*284_285 Ins(dup)AG). Black arrows indicate the site of the variant.

Table 3. Phenotypic characters, frequency, and localization of the seven *SHOX* gene polymorphic sites in the Idiopathic short stature group sample

Parameter	Exon-1		Exon-2		Exon-4	Exon-6	
	c.-646_-645 InsTGT	c. -512C>A	c. -507G>C	c. -372G>A	c. 528G>C	*41C>A	*284-285 InsAG
Age (yr)	10.8±2.8	11.16±2	11.3±1.9	11.2±2.7	5.5	14.9±2.5	13
Sex							
Male	3	5	8	6	-	-	1
Female	5	1	10	8	1	2	-
Frequency	7%	5%	17%	13%	1%	2%	1%
Weight (kg)	23.65±5.4	26.86±8.6	23.44±9.9	26.97±10	12.8	32.95±11.1	27
Height (cm)	122.8±12.9	123±10.3	116.7±17.5	123.3±10.8	92.5	135.1±6.9	132.8
Arm span (cm)	117.9±12.2	118.1±10.1	111.6±17.3	118.2±10.2	88.5	129.1±7	127.7
Sitting height (cm)	69.05±7.4	68.7±5.9	65.7±9.2	69.3±6.1	52.8	76.3±3.3	74
Subischeal leg length (cm)	53.8±5.6	54.3±4.4	50.9±8.4	53.9±4.7	39.7	58.8±3.6	58.8
Paternal height (cm)	163.6±6.5	163.4±5.3	163.2±6.9	164.3±7.3	170	165±9	170
Maternal height (cm)	152.3±3.9	151.5±4	153±5.2	152.4±6.6	152	152±4	146.5
Localization	Promoter	Promoter	Promoter	5'UTR	Homeobox	6a- 3'UTR	6a- 3'UTR
Amino acid	-	-	-	-	p.(Glu176Asp) Homeodomain	-	-
SHOX database ID	Novel	SHOX_00365	SHOX_00366	SHOX_00064	SHOX_00102	Novel	Novel
RefSNP	Novel	Solc et al. ¹⁴ (2014)	Solc et al. ¹⁴ (2014)	Solc et al. ¹⁴ (2014)	rs778921118	Novel	Novel
Submission number	ss1753009960	ss1753009945	ss1753009557	SCV000301477	SCV000301479	SCV000301480	SCV000301481

Values are presented as mean±standard deviation.

Sequence variants are described according to Human Genome Variation Society recommendations based on the coding DNA reference sequence (NM_006883.2).

due to varying definitions of ISS, the inclusion of patients with skeletal abnormalities, different mutational analyses (single-strand conformational polymorphism vs. direct sequencing), differences in the regions of the *SHOX* gene analyzed, and population diversity. Our variants rate is higher than the mutation range as previously reported by Rappold et al.²¹⁾ of a large cohort of 1,534 patients with ISS, a mutational frequency of 2.2% was documented. Huber et al.⁶⁾ found a frequency of 15% in 84 patients, Sandoval et al.²²⁾ found a frequency of 8.1% in 62 ISS Colombian patients, while Chen et al.³⁾ found a frequency of 22%. Interestingly, in this study, most of our male patients present at school age, while females presented earlier and shorter. This result was in agreement with Binder, who noted that although the penetrance of *SHOX* deficiency is high, but its clinical expression is very variable seemingly more marked with age²³⁾. Characteristic signs are more frequent and more severe in girls, a finding which may be explained by the presence of higher estrogen levels in females²⁴⁾. Previous studies showed several mutations in *SHOX* gene in ISS patients in some countries^{22,25)}.

Only few clinical studies related to pattern, diagnosis and frequency of short stature in Saudi Arabia children were done⁹⁻¹¹⁾. The molecular genetics techniques allowed the opportunity to improve the diagnoses of disease especially in the field of endocrinology science. With respect to variants found in the present study and those previously reported in literature, 2 polymorphic variants in noncoding region of exon-1 (c.-507G>C, c.-512C>A) and one polymorphic variant was identified in 5'UTR of exon-2 (c.-372G>A) were already reported¹⁴⁾.

According to the hypothesis of the ability of regulatory elements to modulate gene expression²⁶⁾, and as reported previously, the 5' UTR has an important role in posttranscriptional regulatory pathways that control mRNA localization, stability and translation efficiency²⁷⁾, the high frequency of variants that identified in noncoding regions of exons 1 and 2 as a promoter region could be affecting the expression of *SHOX* protein.

Interestingly, we detect a pathogenic mutation in the homeodomain DNA binding site of *SHOX* gene in exon 4 (c. 528G>C). This homeodomain play important role in DNA binding, protein transactivation, cell cycle and growth regulation²⁸⁾. Clement-Jones et al.¹⁶⁾ stated that the homeodomain proteins have an important physiological functions in regulating the embryonic development in vertebrates, suggesting that the mutations in *SHOX* gene especially in homeodomain region may lead to developmental abnormalities. Other studies revealed the functional consequences for the mutants R168W, L132V, A170P and R173H that found in homeodomain region of *SHOX* gene with respect to DNA binding, dimerization or nuclear translocation of the *SHOX* protein^{29,30)}.

In this study, 3 novel variants were found, one in exon 1 and 2 variants in the 3' UTR of exon-6a region. Thomas et al.³¹⁾ reported that there is a high phenotypic variability related to gain or loss of *SHOX* gene sequences and their regulatory regions. The analysis of

a region located at 3' of the gene has been included in the screening of short stature, since some studies suggested the presence of distal regulatory elements of *SHOX* transcription in this region^{8,32)}. Recently, Solc et al.¹⁴⁾ documented the common variants in the coding and noncoding sequences including 5' region of *SHOX* gene.

Several studies, including the present one, employed similar measures criteria to identify ISS^{33,34)}. No significant differences were found regarding the SD of height, mid parental height, arm span, sitting height, subischial leg length, and age within ISS patients. This can be explained by the suggestion of Rosilio et al.³⁵⁾ that anomalies in *SHOX* enhancer regions (i.e., noncoding region) seemed to be linked to a milder phenotype. This approves the fact that, the correlation between phenotype-genotype in patients with ISS is hard to detect. It might have been expected that children with variants in *SHOX* gene would had more severe short stature than those without variants in *SHOX* gene, however, we found no remarkable differences in the degree of short stature between those with or without identifiable *SHOX* gene polymorphic variants. Thus, there is no clinical parameter that serves to distinguish ISS patients with *SHOX* mutation from that idiopathic nonendocrine short stature without *SHOX* mutation.

This results was in agreement with finding of Rappold et al.³⁰⁾ as they reported that no significant difference of the mutations frequency was detected when comparing males and females in ISS pediatric patients. Similarly, L05101 deletion frequency between men and women in ISS patients are not significantly difference¹⁴⁾.

Conflicts of interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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