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A novel intronic mutation in *SHOX* causes short stature by disrupting a splice acceptor site: direct demonstration of aberrant splicing by expression of a minigene in HEK-293T cells

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Abstract

SHOX, the short stature homeobox-containing gene, encodes a critical regulatory protein controlling long bone growth. We examined patients in one family, identified an intronic mutation, and expressed *SHOX* minigenes in HEK293T cells to characterize the effect on gene splicing. We identified a novel mutation at position -3 (c.-432-3C>A;g.6120C>A) of the intron 1 splice acceptor site; three short (height Z-score -2.4 to -1.7) children were heterozygous and the father (height Z-score -3.4) was homozygous. A wild-type minigene produced alternative transcripts; one utilized the normal splice site between intron 1 and exon 2, the other a cryptic splice site in exon 2. Mutant *SHOX* minigene generated only the smaller transcript. The exon 2 acceptor splice site is weak; an alternative transcript is normally produced using a downstream cryptic splice site. The c.-432-3C>A mutation causes further weakening, and the cryptic splice site is preferentially utilized, resulting in *SHOX* deficiency and short stature.

Keywords

short stature; *SHOX*; splice site mutation

Introduction

SHOX, the short stature homeobox-containing gene, is located in the pseudoautosomal regions on the X and Y chromosomes and encodes a critical regulatory protein that controls

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Conflict of interest statement

Declaration of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

bone growth (1). Its location in the pseudoautosomal region allows *SHOX* to escape X inactivation, and expression of copies of *SHOX* on both the X and Y chromosomes (2) is required for normal skeletal development. The *SHOX* gene spans seven exons and includes four exons that have only recently been characterized (3). Two Shox protein isoforms, Shoxa and Shoxb, are generated through the use of alternative 5'-untranslated region (5'-UTR) sequences and final exons (4). Expression of the *SHOX* gene is regulated by a complex mechanism that utilizes sequential transcriptional and translational control mechanisms (5). Alterations in *SHOX* expression are associated with several disorders of growth, stature, and skeletal development. Homozygous loss of function of *SHOX* is the basis for Langer mesomelic dysplasia, characterized by extreme short stature and limb aplasia or hypoplasia of the ulna and fibula (6). Loss of one copy of *SHOX* or deletions in the pseudoautosomal regions (7) are also the cause of growth failure and skeletal defects in Turner syndrome (1) and Leri Weill dyschondroplasia (8). In both of these conditions, *SHOX* haploinsufficiency is associated with short stature and the Madelung deformity, a condition in which arrest of epiphyseal growth of the medial and volar portions of the distal radius leads to shortening of the radius and relative overgrowth of the ulna. Deletions and mutations in the *SHOX* gene have also been identified in patients with idiopathic short stature who lack apparent skeletal deformities (9). By contrast, duplication of Xp, which results in *SHOX* polyploidy, is associated with tall stature (10) and some cases of type I Mayer-Rokitansky-Kuster-Hauser syndrome (11). Overall, deficiency of *SHOX* is associated with considerable phenotypic heterogeneity. The variable skeletal phenotypes in patients with *SHOX* mutations and duplications suggest a quantitative relation of functional protein to bone development and growth.

Most *SHOX* mutations are due to large deletions that result in haploinsufficiency. However, a variety of point mutations and small deletions have been identified in *SHOX* that have been implicated in short stature. It is estimated that *SHOX* mutations are found with a frequency of 1:1000 in the normal population and in up to 2%–15% of children with idiopathic short stature (4).

We report the identification and molecular characterization of a novel *SHOX* gene intronic mutation at position –3 of the splice acceptor site of exon 2 leading to replacement of the conserved cytosine by adenine (c.–432–3C>A; g.6120C>A). This nucleotide replacement impairs normal splicing and leads to exclusive use of a cryptic splice acceptor site in exon 2 that predicts loss of the homeodomain of the *SHOX* protein.

Materials and methods

Case reports

The proband (II:2 in pedigree, Figure 1) was born full-term after an un-complicated pregnancy to healthy, non-consanguineous parents of Ashkenazi Jewish descent. Birth weight (3289 g) and length (50.8 cm) were normal. Her developmental milestones were appropriate, and her parents noted declining growth and short stature at the age of 1–2 years. At age 9 years, breast development and pubic hair were first noted, and she was begun on leuprolide and growth hormone by another provider. Breast development regressed on leuprolide but her growth velocity was unchanged (4.4 cm/year) on this combined therapy

and her growth remained less than the first percentile. At chronological age 11 years and 5 months, her bone age according to the standards of Greulich and Pyle was 9 years and 5 months and her serum IGF1 level ranged from 55.4 to 74.0 nmol/L. Her physical examination revealed proportionate short stature (−2.4 SDS) with a normal seated height/height SDS of 0.81 (12). Pubertal exam revealed Tanner 1 breasts and Tanner 3 pubic hair. She had a normal female karyotype (46, XX). A skeletal survey was normal and no Madelung deformity was present. At 12 years and 5 months, she was noted to have a mildly elevated serum level of thyroid-stimulating hormone at 8.2 μ IU/mL (reference range 0.5–3.8 μ IU/mL) and treatment with levothyroxine 50 μ g daily was initiated. Leuprolide was discontinued.

The proband's parents and two brothers were also evaluated (Table 1, Figure 1). The father (I:1) is the smallest member of his extended family, with a height of 152.4 cm (−3.4 SDS) and normal (SDS 1.2) seated height/height ratio (12). He was proportionate without skeletal deformities. Her two brothers (II:1 and II:3), ages 16 and 7 years, had proportionate short stature (height −2.3 and −1.7 SDS, respectively) with normal seated height/height ratios (0.64 and 0.60 SDS, respectively) (12). The mother (I:2) had a normal height (155 cm, −1.3 SDS). The proband's paternal grandmother was 155 cm tall and the paternal grandfather was 160 cm tall; both were proportionate without skeletal deformities. The maternal grandmother was 152 cm tall and the maternal grandfather was 155 cm tall. No Madelung deformity was present in any family members. Stature is expressed as SDS according to the population standards for age and gender (<http://www.magicfoundation.org/>). The protocol was approved by the appropriate institutional review boards, and informed consent and/or assent was obtained from all subjects.

Mutational analysis of *SHOX*

Genomic DNA was isolated from peripheral blood mononuclear cells of all subjects and the seven exons and flanking intronic sequences of the *SHOX* gene were amplified by PCR. The sequences were then screened for point mutations, small deletions and insertions within the *SHOX* coding sequences, and intron/exon boundaries by denaturing high-performance liquid chromatography (Transgenomic Wave 3500HT, Omaha, NE, USA), and abnormal heteroduplexes were analyzed by direct sequencing of amplicons using previously described techniques (primer sequences and PCR conditions are available upon request) (7). DNA from subject I-1 was also analyzed for larger *SHOX* gene deletions by multiplex probe ligation amplification using standard techniques (13). DNA mutation numbering is based on cDNA sequence (Accession no. NM_000451.3) and gDNA sequence (NCBI Reference Sequence: NC_000023.10) and expressed using HGVS V2.0 nomenclature.

In silico splice site prediction

The effect of the single base substitution identified in the splice acceptor site of intron 1 was evaluated using several splice site prediction algorithms (14, 15).

Synthesis of hybrid minigenes

Because we were unable to detect *SHOX* transcripts in peripheral blood mononuclear cells by reverse transcription (RT)-PCR, we synthesized hybrid *SHOX* minigenes to determine

the effect of this mutation on pre-mRNA splicing efficiency. We created wild-type and mutant *SHOX* minigenes in the pTARGET mammalian expression vector (Promega, Madison, WI, USA) using PCR to amplify and assemble exons 1–3 plus short intronic flanking regions of *SHOX* from normal human genomic DNA (Figure 2). The PCR products were cloned into pTARGET, and the *SHOX* sequences were verified by direct sequencing using ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA).

Characterization of *SHOX* expression

Wild-type or mutant *SHOX* minigenes were transiently transfected into HEK293T cells using Fugene 6 Transfection Reagent (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's recommendation. Approximately 48 h after transfection, total RNA was extracted from the cells using RNeasy Mini kit (Qiagen Inc., Valencia, CA, USA). The concentration of RNA obtained was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), and RNA integrity was determined by denaturing agarose gel electrophoresis. Aliquots of total RNA (1.5 µg) were used to generate first-strand cDNA using a cDNA synthesis kit (iScript; BioRad, Hercules, CA), using a blend of oligo(dT) and random primers. After first-strand cDNA synthesis, the reaction was diluted 1/10. For PCR, 5 µL of the diluted cDNA was amplified into a total volume of 20 µL using 10 µM primers located in exon 1 (5' - TCTTTCTACTGCAAACAGAAATGG-3') and exon 3 (5' - ATTCATAAATCCCTTCTGCCACT-3') and Amplitaq Gold DNA polymerase (Applied Biosystems) with the following parameters: an initial denaturation at 94°C for 3 min, followed by 40 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were analyzed by agarose gel electrophoresis and were sequenced on both strands. Total RNA was extracted from discarded human skull bone fragments and RT-PCR was performed using primers (5' - ATTCGTTTCCGCGCTCTTTTC-3' and 5' - GCCGCAATTGGCTTTCTTCTCAT-3') that were designed to anneal in exons 1 and 2 and conditions described above.

Results

Molecular analysis of *SHOX*

The proband was found to be heterozygous for a single nucleotide change (C to A) located three nucleotides upstream of the splice acceptor site in intron 1 (c.-432-3C>A). Both of her brothers were also heterozygous for this variant, while their father's DNA showed only the adenine nucleotide at position c.-432-3. Multiplex ligation-dependent probe amplification (MLPA) analysis of the father's DNA indicated that he had two copies of the *SHOX* gene without evidence for an intragenic deletion, thus confirming that he was homozygous for the c.-432-3C>A mutation. The mother carried two wild-type *SHOX* alleles. Review of a database that contains *SHOX* gene sequences from >6100 patients with short stature revealed this substitution in only one additional unrelated patient, and no patients with normal stature are reported to carry this variant (personal communication, Frank K Fujimura, PhD., Esoterix Inc., Calabasas Hills, CA). In addition, inspection of the

online *SHOX* gene mutation database (www.hd-lovd.uni-hd.de) similarly failed to disclose this mutation (16, 17).

In silico results

In silico analysis was used to determine the effect of the splice acceptor site mutation on splicing by utilizing splice prediction web interfaces. GeneSplicer (18) (<http://www.cbcb.umd.edu/software/GeneSplicer>) predicted a reduction in splice site strength from 84% for the wild-type sequence to 14% for the mutant splice site. We used the Cryp Skip web interface (19) (<http://cryp-skip.img.cas.cz>) to determine whether the C>A mutation might activate a cryptic splice site. This program indicated a 91% probability of cryptic splice site activation.

Splicing efficiency

The *SHOX* wild-type hybrid minigene produced two alternative transcripts (Figures 3 and 4). The larger and far more abundant transcript, 873 bp, results from the predicted splicing of exon 1 to exon 2 at the native splice acceptor site at position c.-432-3. The smaller transcript, 410 bp, is generated by use of a cryptic splice site in exon 2 at position c.47. However, we were unable to confirm the existence of an alternative transcript that utilizes this cryptic splice site by RT-PCR of normal bone (data not shown). Similarly, a recent comprehensive analysis of *SHOX* transcripts also failed to identify corresponding transcripts (3). Thus, use of this internal splice site by wild-type alleles in vivo remains speculative; however, utilization appears to require mutation of the normal splice site.

The mutant *SHOX* minigene prevented production of any normal transcript and generated a single, small transcript of 410 bp that was identical to the smaller transcript produced by the wild-type minigene. This transcript contains an in-frame ATG at position c.457 (based on wild-type transcript numbering) in exon 3 that has a strong Kozak sequence. The theoretical use of this alternative initiation codon would produce a truncated Shox protein missing the first 145 amino acids and consequently lacking most of the homeobox domain.

Genotype-phenotype

The father in this family (I-1) was homozygous for the *SHOX* mutation. Haplotype analysis of the father's DNA showed homozygosity for microsatellite markers that are linked to *SHOX*, thereby confirming identity by descent for the *SHOX* alleles (Figure 1).

Discussion

This case illustrates an example of familial short stature due to a novel acceptor splice site mutation in the *SHOX* gene. Splice site mutations result in either exon skipping, which utilizes the next available splice site, retention of the intron, or the use of a cryptic splice site (20, 21). Donor splice site mutations occur more frequently than acceptor splice site mutations (20).

Heterozygous loss-of-function mutations in the *SHOX* gene represent one of the best characterized genetic causes of idiopathic short stature. Although haploinsufficiency of

SHOX is frequently associated with skeletal abnormalities and disproportionate seated height (22), there is remarkable phenotypic heterogeneity (23) and many patients with *SHOX* mutations have proportionate short stature and normal skeletal development. Transmission of *SHOX* mutations leads to familial short stature, and the father in this family transmitted defective alleles to both his sons and daughter. The unique location of *SHOX* renders the gene susceptible to pseudoautosomal recombination during paternal meiosis. Genes in the pseudoautosomal region of the X chromosome are believed to escape X inactivation (24), and as a result, active copies of *SHOX* present on both the X and Y chromosome and both copies are required for normal phenotypic expression. If recombination occurs during meiosis, altered gene expression may result and can be associated with short stature or dysmorphic skeletal features (25). Males have a high frequency of recombination in this region, and several examples of paternal transmission of a heterozygous *SHOX* mutation to both sons and daughters have been recently described (26), presumably as a result of mutations being transmitted from a paternal X chromosome to a proband's Y chromosome. However, several lines of evidence indicate that the father of this family was homozygous for the missense mutation, and thereby transmitted mutant X and Y alleles to his affected children. First, haplotype analysis of the father's DNA using microsatellite markers linked to *SHOX* (Figure 1) revealed homozygosity across a 7-Mb region that includes *SHOX*, consistent with identity by descent and a likely previous pseudoautosomal crossover of the mutation. Second, MLPA analysis of the father's DNA gene failed to identify an intragenic *SHOX* deletion, indicating that the father had two copies of the *SHOX* gene and that both alleles carried the same missense mutation. There has been one previous report of a *SHOX* mutation being transmitted from a paternal X chromosome to a proband Y chromosome during pseudoautosomal recombination during male meiosis (10). Presumed consanguinity likely led to homozygosity of the mutation. This rare recombination event within the pseudoautosomal region allows for male transmission of mutant alleles to sons and a daughter.

We identified a dose effect of *SHOX* insufficiency in this family, as the homozygous father had more extreme short stature than his heterozygous children. However, despite having two copies of the abnormal *SHOX* gene, the father lacked features of Langer mesomelic dysplasia. This observation suggests that some functional Shox protein is produced despite the homozygous mutation. Although it is possible that the abnormally spliced *SHOX* transcript is translated, this is predicted to produce a Shox protein that lacks the homeobox domain, and thus would have very little if any biological activity. Alternatively, some normal transcripts may be produced through the use of the mutated acceptor site, although our in vitro studies did not demonstrate this. A more likely explanation for the phenotypic heterogeneity in this family is the use of alternative transcriptional mechanisms. The principal promoter for *SHOX* transcription (P_1) is located upstream of exon 1 and produces a transcript with a long 5'-UTR. However, *SHOX* has an alternative (P_2) promoter (5) that is located within exon 2 (Figure 5), and which produces a shorter transcript with a truncated 5'-UTR. This nascent *SHOX* transcript should be unaffected by the splice acceptor site mutation that we have identified in intron 1 and thus should generate a mature transcript that is translated into a fully functional Shox protein. It is unknown how much expression derives from use of the P_2 promoter in various cells or at different stages of development; however,

given the different translational efficiencies of the two transcripts, it has been suggested that P₂ is used in situations with immediate needs of high Shox protein amounts, whereas use of the P₁ promoter produces transcripts that facilitate regulation of Shox protein levels by translational control mechanisms (5).

Finally, although single base pair substitutions at splice sites are thought to be responsible for approximately 10% of all inherited, disease-causing mutations (27), our report emphasizes how uncommon splice site mutations are as a basis of *SHOX* deficiency. Review of the Human Short Stature Gene Allelic Variant Database (www.hd-lovd.uni-hd.de), which contains a registry of 202 unique disease-related allelic variants in the *SHOX* gene (16, 17), and the published literature, revealed only one other splice site mutation in *SHOX* that led to familial short stature (28).

In conclusion, we report a novel *SHOX* gene splice site mutation in a family with idiopathic short stature. Affected family members demonstrate variable heights with a dose effect that is consistent with a quantitative threshold of functional Shox protein that is necessary for normal development. The affected patients in our studied kindred have normal skeletal development, and therefore it is likely that some residual Shox activity accounts for the mild phenotype in patients carrying one or two mutant *SHOX* alleles.

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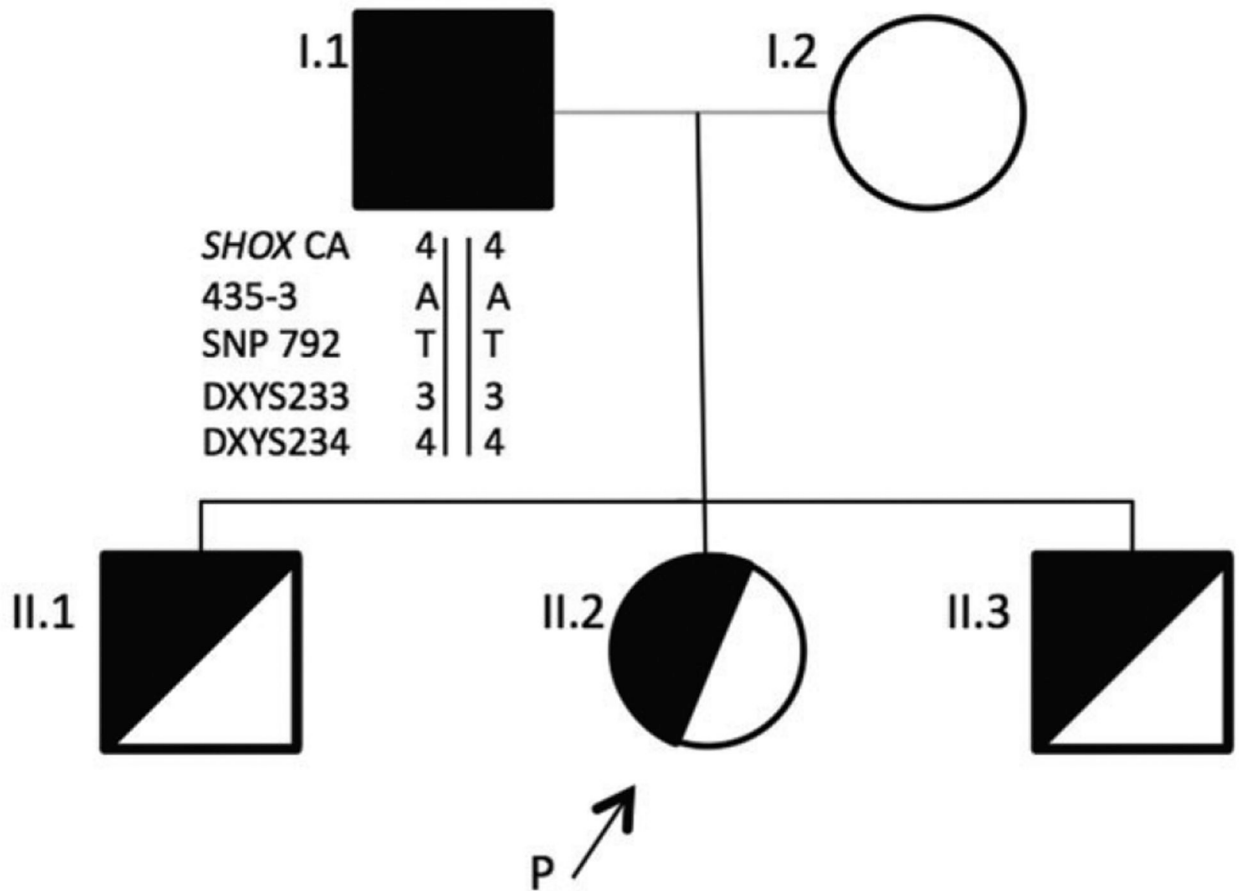


Figure 1.

Pedigree of the family described in this report.

The proband (II.2) is indicated with the arrow. The proband and her two brothers (II.1 and II.3) were found to be heterozygous for the *SHOX* mutation. Her father, (I.1) was homozygous; results of his single nucleotide polymorphism analysis are indicated. Her mother (I.2) had normal *SHOX* alleles.

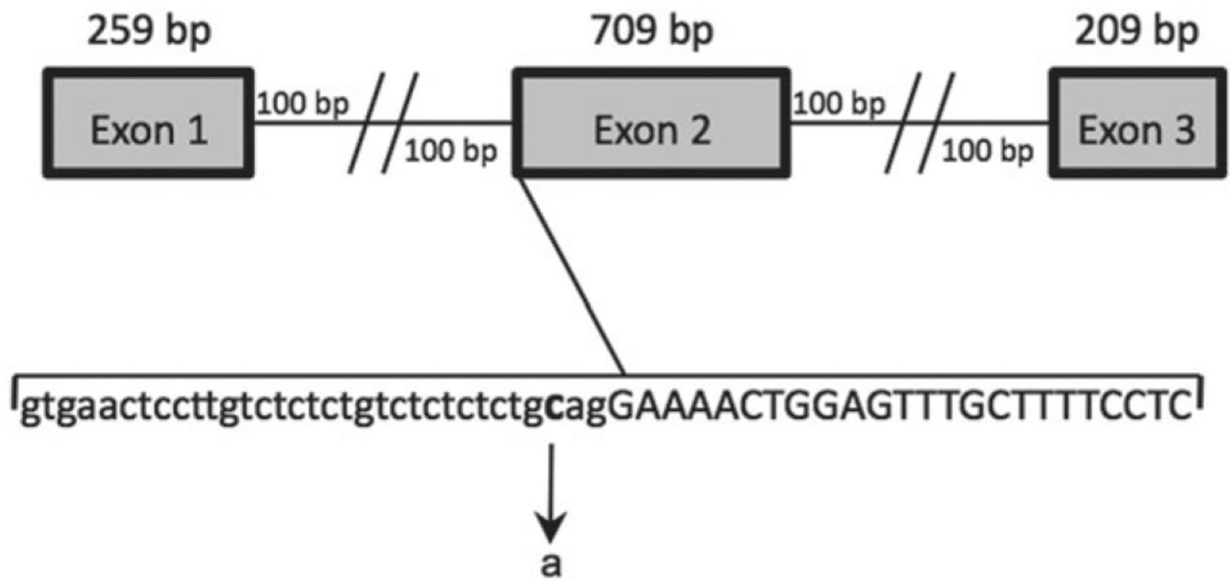


Figure 2.

SHOX minigene.

Minigenes were created using exons 1 – 3 and short intronic flanking regions. The wild-type sequence is shown above; the arrow indicates the replacement of cytosine by adenine at position –3 of the acceptor splice site in the mutant sequence.

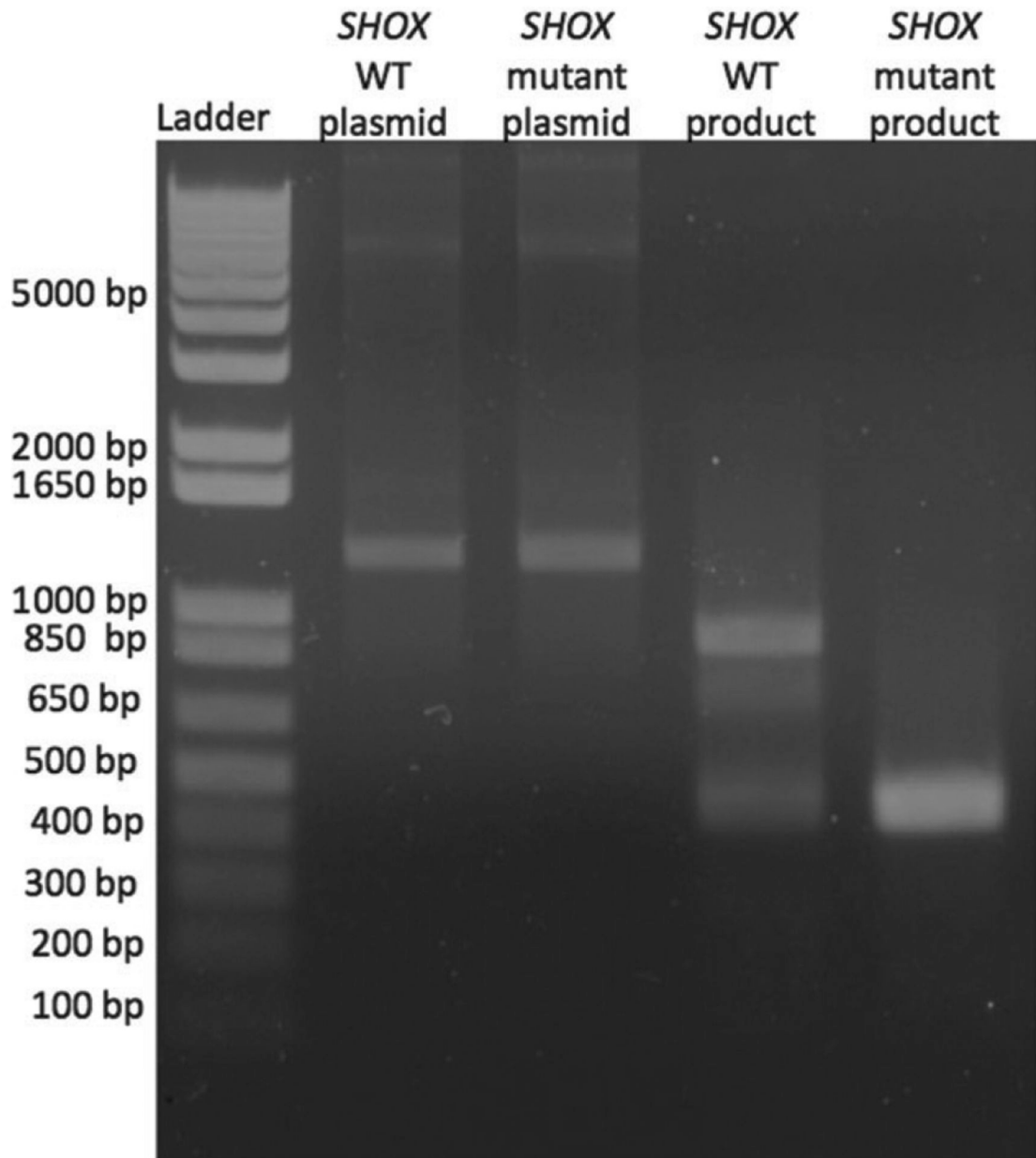


Figure 3.

Agarose gel electrophoresis showing RT-PCR products produced by *SHOX* minigenes. Lane 1 indicates DNA ladder with molecular sizes shown. Lanes 2 and 3 show PCR products generated from the *SHOX* wild-type and mutant plasmids, with amplicons of the expected size of 1200 bp. Lane 4 shows the RT-PCR product from transfection of HEK293T cells with the *SHOX* wild-type plasmid, with the expected amplicons of 893 bp and an additional smaller band of 410 bp. Lane 5 shows the RT-PCR product from HEK293T cells that had been transiently transfected with the *SHOX* mutant plasmid, which generates only the 410 bp using the cryptic splice site. A water control was negative, and is not shown in this figure.

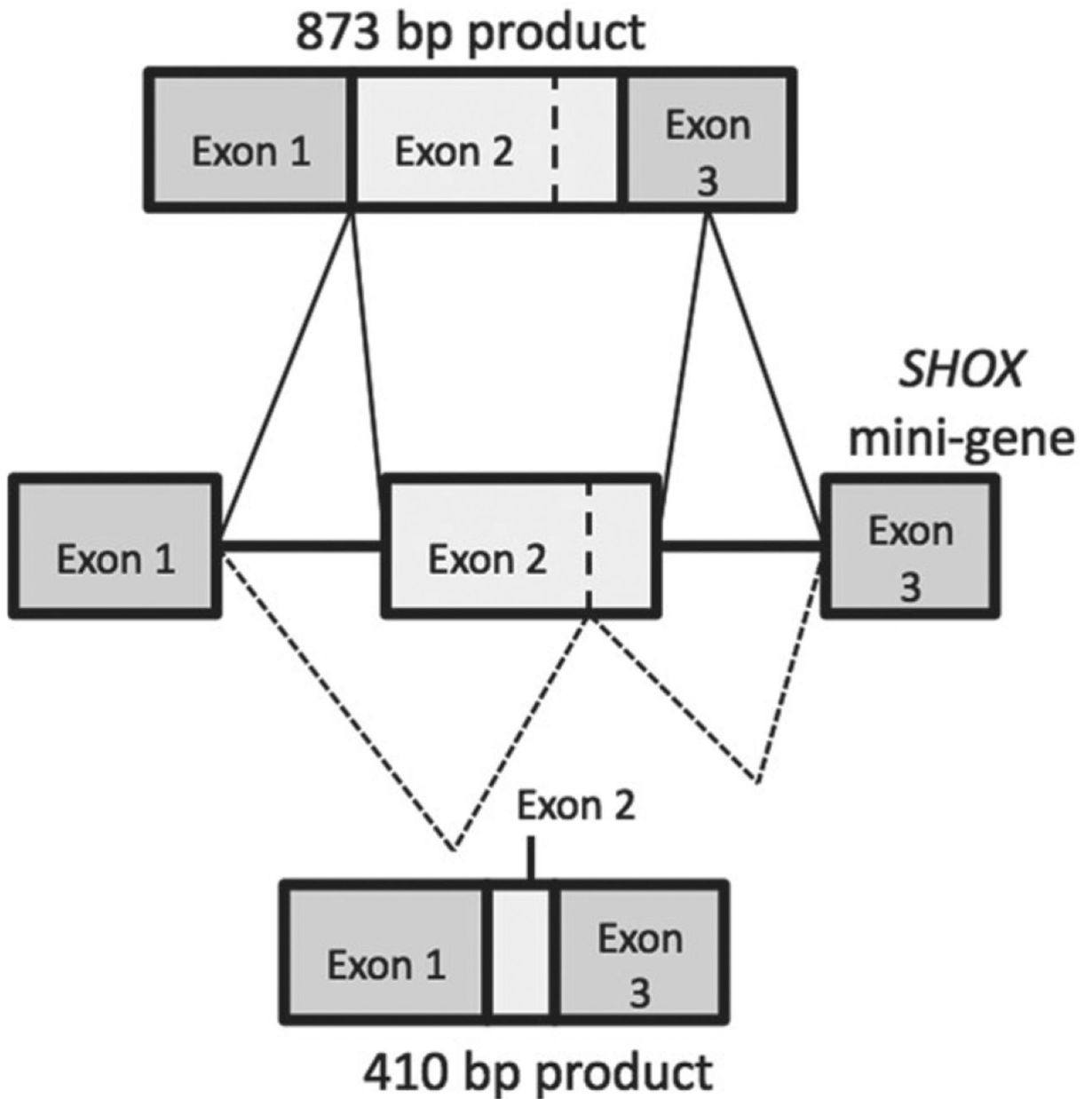


Figure 4.

Schematic of *SHOX* transcripts.

The *SHOX* minigene is indicated in the center of the figure with the exons and truncated flanking intronic sequences. The top product indicates normal splicing, with removal of the introns at the appropriate splice sites, and results in an 893-bp product, as seen on the gel in Figure 4. The dashed lines indicate the use of the cryptic splice site exon 2, which results in the 410-bp product.

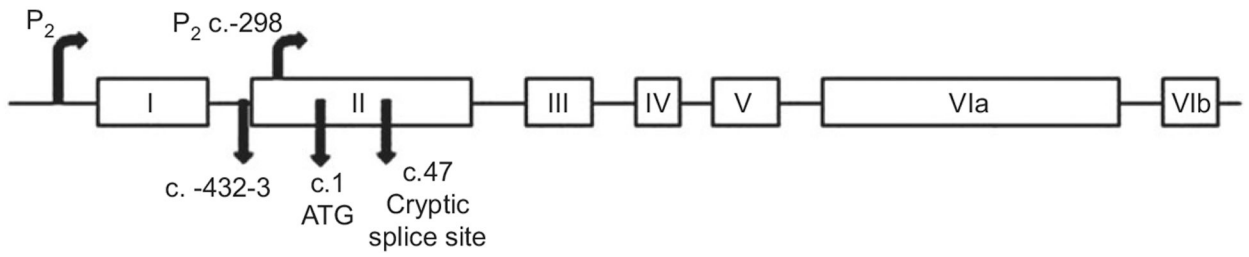


Figure 5.

Organization of *SHOX* gene.

The *SHOX* gene is shown above with exons numbered. (Two isoforms differ in exon VI.)

Promoter 1 (P₁) is within the 5'-UTR. Promoter 2 (P₂) is within exon 2 at position c.-298.

The normal AUG initiator codon is shown at position c.1. The location of the splice site

mutation is indicated at c.-432-3. The cryptic splice site is shown at position c.47.

Table 1

Baseline characteristics of family members described in this report.

Individual	Height, cm	Height SDS	Seated height/height ratio
Proband (age 11 years and 5 months)	129	- 2.4	0.81
Proband's brother (age 16 years)	155	- 2.3	0.64
Proband's brother (age 7 years)	113	- 1.7	0.60
Father	152.4	- 3.4	1.2
Mother	155	- 1.3	<i>a</i>
Paternal grandmother	155	- 1.3	<i>a</i>
Paternal grandfather	160	- 2.3	<i>a</i>
Maternal grandmother	152	- 1.7	<i>a</i>
Maternal grandfather	155	- 3.0	<i>a</i>

^aData not obtained.

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