RESEARCH ARTICLE

Identification of a novel 15.5 kb *SHOX* deletion associated with marked intrafamilial phenotypic variability and analysis of its molecular origin

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Abstract

Haploinsufficiency of the short stature homeobox contaning *SHOX* gene has been shown to result in a spectrum of phenotypes ranging from Leri–Weill dyschondrosteosis (LWD) at the more severe end to *SHOX*-related short stature at the milder end of the spectrum. Most alterations are whole gene deletions, point mutations within the coding region, or microdeletions in its flanking sequences. Here, we present the clinical and molecular data as well as the potential molecular mechanism underlying a novel microdeletion, causing a variable *SHOX*-related haploinsufficiency disorder in a three-generation family. The phenotype resembles that of LWD in females, in males, however, the phenotypic expression is milder. The 15523-bp *SHOX* intragenic deletion, encompassing exons 3–6, was initially detected by array-CGH, followed by MLPA analysis. Sequencing of the breakpoints indicated an *Alu* recombination-mediated deletion (ARMD) as the potential causative mechanism.

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Introduction

The short stature homeobox (*SHOX*) gene (also known as PHOG) is localized in 2.6 Mb pseudoautosomal 1 (PAR1) regions of Xp and Yp, hence escaping X-inactivation leads to an autosomal form of inheritance (Ellison *et al*. 1997; Rao *et al*. 1997; Shears *et al*. 1998). The gene undergoes alternative splicing resulting in two different mRNA transcripts (encoding SHOXa and SHOXb), comprising of a total of seven exons (1–5, 6a and 6b) spanning about 40 kb (Rao *et al*. 1997). The *SHOX* gene codes for a homeodomain protein that acts as a transcription factor, which is highly expressed in osteogenic cells and is implicated in limb development (Ellison *et al*. 1997; Marchini *et al*. 2004; Munns *et al*. 2004).

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SHOX deficiency has been found to be associated with a variety of phenotypic conditions which include Turner syndrome, isolated short stature, Leri–Weill dyschondrosteosis (LWD) and Langer mesomelic dysplasia (LMD) (Binder and Rappold 2005; Ellison *et al*. 1997; Rao *et al*. 1997; Belin *et al*. 1998; Shears *et al*. 1998). *SHOX*-related haplo insufficiency causes a phenotypic spectrum ranging from LWD at the more severe end to *SHOX*-related short stature at the milder end. LWD is a pseudoautosomal dominant disorder typically characterized by disproportionate short stature, mesomelic shortening of the forearms and forelegs and Madelung wrist deformity which occurs more commonly and more severely in affected females (Binder and Rappold 2005; Rao *et al.* 1997; Belin *et al.* 1998; Shears *et al.* 1998). At the heterozygous level, about 50–90% of LWD patients are found to carry different size deletions, less frequently point

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mutations within the coding sequence, or microdeletions in regulatory enhancers downstream of the *SHOX* gene (Cormier-Daire *et al.* 2001; Ross *et al.* 2001; Falcinelli *et al.* 2002; Flanagan *et al.* 2002; Chen *et al.* 2009). In addition, genomic alterations involving *SHOX* gene are independent of position and length in LWD patients and may result in a broader phenotypic traits even among family members, exhibiting a significant interfamilial and intrafamilial variabilities with a high severity in females (Belin *et al.* 1998; Shears *et al.* 1998; Schiller *et al.* 2000; Marchini *et al.* 2004; Munns *et al.* 2004).

Deletions disrupting the function of the *SHOX* gene were ascribed to the high incidence of repeated sequences such as *Alu* elements, and the GC-rich content within the PAR1 that makes the region a hot spot of recombination events between X and Y chromosomes (Lien *et al.* 2000; Blaschke and Rappold 2006). Currently, there are limited studies on the molecular mechanisms leading to rearrangements involving the *SHOX* gene. The study by Fukami *et al.* (2008) has supported the underlying mechanism of such derived deletions and showed that cryptic intragenic *SHOX* microdeletions can be generated both by the abundant *Alu* repeat-sequences mediating aberrant homologous recombination and by non-homologous end joining (NHEJ) (Fukami *et al.* 2008).

Here, we present a Greek–Cypriot family with a novel 15.5 kb *SHOX* intragenic deletion removing exons 3–6a identified by array-CGH. Based on the identification of the exact breakpoints of the deletion, we propose an *Alu* recombination-mediated deletion (ARMD) as the underlying mechanism of the observed genomic rearrangement.

Materials and methods

Clinical description

The proband, a female, second child of four children was born to nonconsanguineous parents of Greek–Cypriot descent. Her father was not evaluated clinically; however, he had a degree of short stature. The proband presented with short stature and a long standing history of head tremor. In addition, she complained of generalized joint aches along with pains and neck movement restriction. On examination at the age of 43 years, she had an occipitofrontal circumference (ofc) of 55.5 cm (>90th centile), her height was 151 cm (<5th centile) and her weight was 56 kg (25th–50th centile). She had disproportionate short stature with a reduced span to height ratio at 0.96, evidence of mesomelic limb shortening and a muscular body habitus. Clinically, the proband had normal appearance of wrist contour bilaterally. She had a Beighton score of 3/9 and mild skin hyperextensibility with no evidence of tissue fragility. Cardiovascular examination was unremarkable. She had right-sided torticollis and postural tremor mainly involving the head and upper limbs. There was no evidence of neck hypertonia. Extraocular movements were full. Pupils were equal and reactive. Visual fields were full to confrontation. There was no objective hearing loss. Sensation over the face and limbs was not impaired. Reflexes were 2+ and symmetric. There was no focal weakness or atrophy. Plantar responses were flexor bilaterally. There was no cerebellar dysfunction. Her gait was normal and Romberg test was negative. Her c-spine CT scan revealed several osteophytes. She had a normal ophthalmology and cardiology evaluations including echocardiography. Abdominal and uterine/ovarian ultrasound scans were unremarkable. There was no radiological evidence of a Madelung deformity.

The proband had four offspring and all of them were clinically evaluated for signs of the disorder. Two offspring had an unremarkable evaluation (III:2 and III:3; figure 1). Her 26 year old daughter (III:1) had disproportionate short stature (height of 152.4 cm ∼ 5th centile) with a span to height ratio of 0.97 and an upper to lower segment ratio of 1.07. Her wrists were clinically unremarkable as was the remainder of her examination. One of the proband's son (III:4), at the age of 19 years had a height of 173.4 cm (25th–50th centile), an extensive muscular build with unusually long arms and an increased span to height ratio at 1.06. He had very broad shoulders. He had mild skin hyperextensibility and a Beighton score was 2/9. Cardiovascular and neurological examinations were otherwise unremarkable. The proband's sister (II:4), at the age of 54 years, was disproportionately short with mesomelic limb shortening. Her height was 154 cm (2nd–9th centile), and arm span was 144.5 cm giving a reduced span to height ratio of 0.94. The remainder of her examination was unremarkable. Her daughter (III:5), examined at the age of 35 years, had marked mesomelic shortening with a height of 133.2 cm (<0.4th centile) and an arm span of 118 cm giving a reduced span to height ratio of 0.88. She had abnormal contours of her wrists bilaterally with restricted wrist movement. There was no evidence of scoliosis and the remainder of her examination was unremarkable. The proband's brothers (II:1 and II:6) were not reported as having short stature and they were never consented to clinical evaluation. Summarized clinical characteristics of affected family members are provided in table 1.

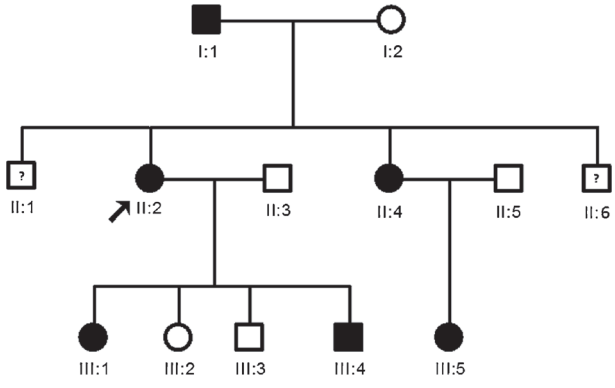

Figure 1. Pedigree of the family. The proband is indicated by an arrow. Clinically affected individuals with the deletion are indicated by the shaded symbols. Clinically unaffected individuals without the deletion are illustrated by the clear symbols. Members with unknown disease status are indicated with a question mark.

Table 1. Clinical characteristics of individual family members.

Subject no.	Age at examination (Y)	Gender	OFC (cm)	Arm span (cm)	Height (cm)	Arm span/ height ratio	US/LS	Madelung deformity	Other clinical characteristics
II:2	43	F	55.5 (90th centile)	145.5	151 (<5th centile)	0.96	1.04	No	Tremor, torticollis
II:4	54	F	56 (75th–90th centile)	144.5	154 (2nd–9th centile)	0.94	1.05	No clinical evidence, no X-ray available	NA
III:1	26	F	54.9 (75th centile)	148	152.4 (~5th centile)	0.97	1.07	No clinical evidence, no X-ray available	NA
III:4	19	M	57.4 (50th–75th centile)	184.5	173.4 (25th–50th centile)	1.06	1.03	No clinical evidence, no X-ray available	Very broad shoulder, muscular build
III:5	35	F	51 (3rd centile)	118	133.2 (<0.4th centile)	0.88	NA	Clinically yes	Mesomelic shortening, restricted wrist pronation/supination

Y, years; F, female; M, male; OFC, occipitofrontal circumference; cm, centimetres; US, upper segment; LS, lower segment; NA, not available.

Chromosomal analysis

Chromosomal G-banding analysis of the proband and her offspring (III-1 and III-2) was carried out from peripheral blood at 550 kb band level using conventional methodologies.

DNA extraction and SHOX gene sequencing

DNA was isolated from peripheral blood using the QIAamp DNA Mini kit (Qiagen, Hidden, Germany) according to the supplier's protocol. The *SHOX* gene was amplified in six fragments (primer sequences available upon request). PCR products were first verified for correct size on agarose gel, then purified using PCR columns (EdgeBio, Santafe, Spain), sequenced using the ABI Prism Dye terminator sequencing kit and finally analysed on an ABI 3130XL apparatus (Applied Biosystems, Foster City, USA).

Array-CGH

Array-CGH analysis of the proband was performed using the Cytochip ISCA array (BlueGnome ver. 1.0) with 180,000 oligos as described elsewhere (Tanteles *et al.* 2015). Fluorescent ratios were calculated using the BlueFuse Multi software ver. 4.2 (BlueGnome, Cambridge, UK).

Multiplex ligation probe amplification analysis

MLPA analyses of the proband and individuals I:1, I:2, II:4, III:1, III:2, III:3, III:4 and III:5, were carried out using the SALSA P018 MLPA kit (MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer recommendations. The PCR products were separated by capillary electrophoresis on the 3110XL Genetic Analyser (Applied Biosystems, Foster City, USA). Relative peak area for each *SHOX* probe was calculated by dividing the peak area value of each probe over the sum of the peak area value of reference probes. Subsequently, the average relative peak area of the control cases was calculated for each probe and then each probe value was divided by the control probe specific average sample. Probe relative peak ratios above 0.7 and below 1.3 were regarded as normal and below 0.7 were regarded as indicative for heterozygous deletions.

Quantitative real time PCR (qRT-PCR) and Sanger sequencing for breakpoint mapping

To identify the exact breakpoints of the deletion, qRT-PCR was carried out using multiple primer pairs (Metabion, Germany), which were designed to target the flanking regions of the array-CGH identified deletion breakpoints. qRT-PCR was performed as described elsewhere (Tanteles *et al.* 2015). PCR (primer sequences are available upon request), detection and fluorescent data analysis were carried out on the CFX96 real-time C1000 thermal cycler (Biorad, Hercules, USA) using the Sso Fast Evagreen Supermix (Biorad).

Upon qRT-PCR completion, the forward sequence of the primer pair nearest to the proximal deletion breakpoint as well as the reverse sequence of the primer pair nearest to the distal breakpoint that showed normal copy number state were subjected to PCR with sequences P-F (5′-tacgtaccgggaagttttgg-3′) and P-R (5′-tcccaccctcacagttctct-3′) (chrX: 591731–609409) (chrY: 541731–559409) using Crimson Taq DNA polymerase following manufacturer's instructions (New England BioLabs, Ipswich, USA). PCR amplification was carried out according to the following protocol: 5 min at 95°C for initial denaturation, followed by 30 cycles of 40 s at 95°C for denaturation, 40 s at 60°C for annealing, 3 min at 72°C for extension and 10 min at 72°C for final extension. PCR product was subsequently sequenced using Big Dye Terminator Cycle Sequencing kit V3.1 on the 3110XL Genetic Analyser (Applied Biosystems). The obtained fasta sequences were aligned to chromosome X using MAFFT Geneious ver. 6 plugin with default settings (Katoh *et al.* 2002; Kearse *et al.* 2012). The resulting alignment was used to identify the exact breakpoint location. The identified breakpoint locations were further verified with UCSC genome browser blat search (Kent 2002). Repeatmasker and USCS human genome browser tracks were used to identify repetitive elements in the region of interest (Karolchik *et al.* 2004; Tempel 2012).

Results

Chromosomal G-banding analysis at the 550 kb band level was carried out in the proband and revealed a low mosaic female karyotype with 10% 45, X and 90% 46, XX. Chromosomal studies of the proband's offspring, III-1 and III-2, showed normal results.

Initially *SHOX* gene sequencing was performed on the proband and normal results were obtained. Subsequently, targeted interrogation of the *SHOX* locus by array-CGH analysis of the proband using the 180K array (Cytochip ISCA array-BlueGnome) revealed a deletion within the PAR1 region at chromosomal band Xp22.33 of a minimal size of ∼15 kb (hg19: start 592,782 and stop 607,486) and a maximal size of ∼17.4 kb (hg19: start 592,064 and stop 609,426), (hg19: (592,064–592,782)–(607,486–609,426) del. build GRCh37). The deletion was mapped within the coding region of the *SHOX* gene encompassing exons 3–6 (figure 2).

MLPA analysis carried out in the proband (II:2) confirmed the heterozygous deletion involving exons 3–6 as probes 01147-L00802, exon 3, 01148-L15501, exon 4, 01149-L19676, exon 5 and 09337-L00911, exon 6 exhibited a ratio of <0.7. Subsequently, MLPA analysis of other family members identified the same deletion in the affected family members I:1, II:4, III:1, III:4 and III:5 (data available upon request).

To further refine the deletion breakpoints, qRT-PCR was carried out which identified the primer sequences nearest to the proximal and distal breakpoints of the deletion.

PCR was performed using the primer sequences selected by qRT-PCR using the primer sequences nearest to the deletion breakpoints encompassing the deletion. A PCR product of ∼1.4 kb was obtained, and Sanger sequenced to identify the exact location of the deletion breakpoint. Resulting sequence was aligned to *SHOX* gene indicating that the breakpoint was in the proximal position 592,343 and distal position 607,866. The breakpoint junction was located within two *Alu* repetitive elements with 12 bp common fusion segment (figure 3, a & b).

After sequencing and accurate delineation of the breakpoints, the size of the deletion was determined to be 15523 bp.

Discussion

In the current study, we present a three generation family with a *SHOX*-related haploinsufficiency phenotype caused

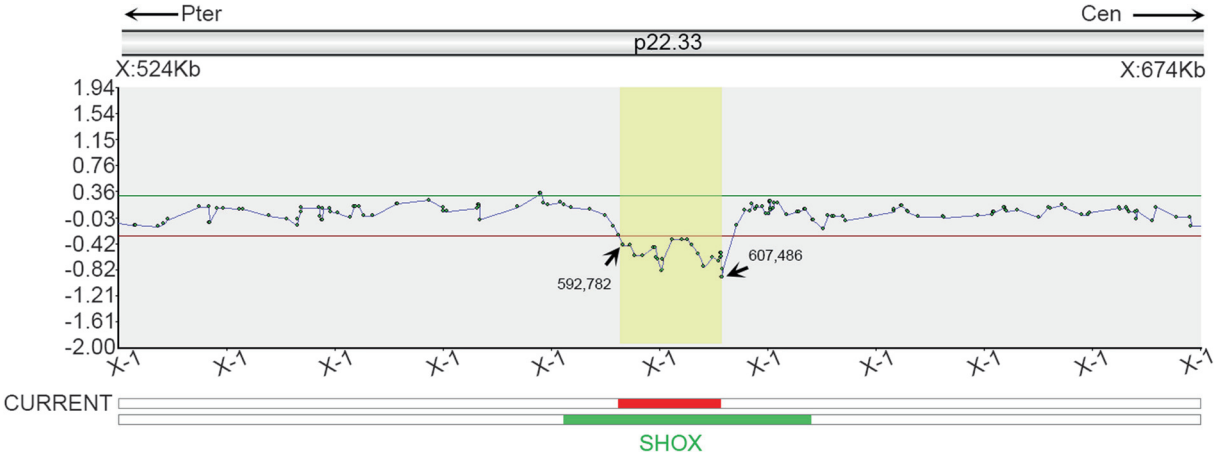

Figure 2. Array-CGH analysis of the proband indicated an Xp22.33 deletion. The presence and the breakpoints of the deletion are highlighted and underlined in red which correspond to ∼15 kb (pos. 592,782–607,486) residing in the coding region of the *SHOX* gene encompassing exons 3–6. *SHOX* gene is indicated by the green line.

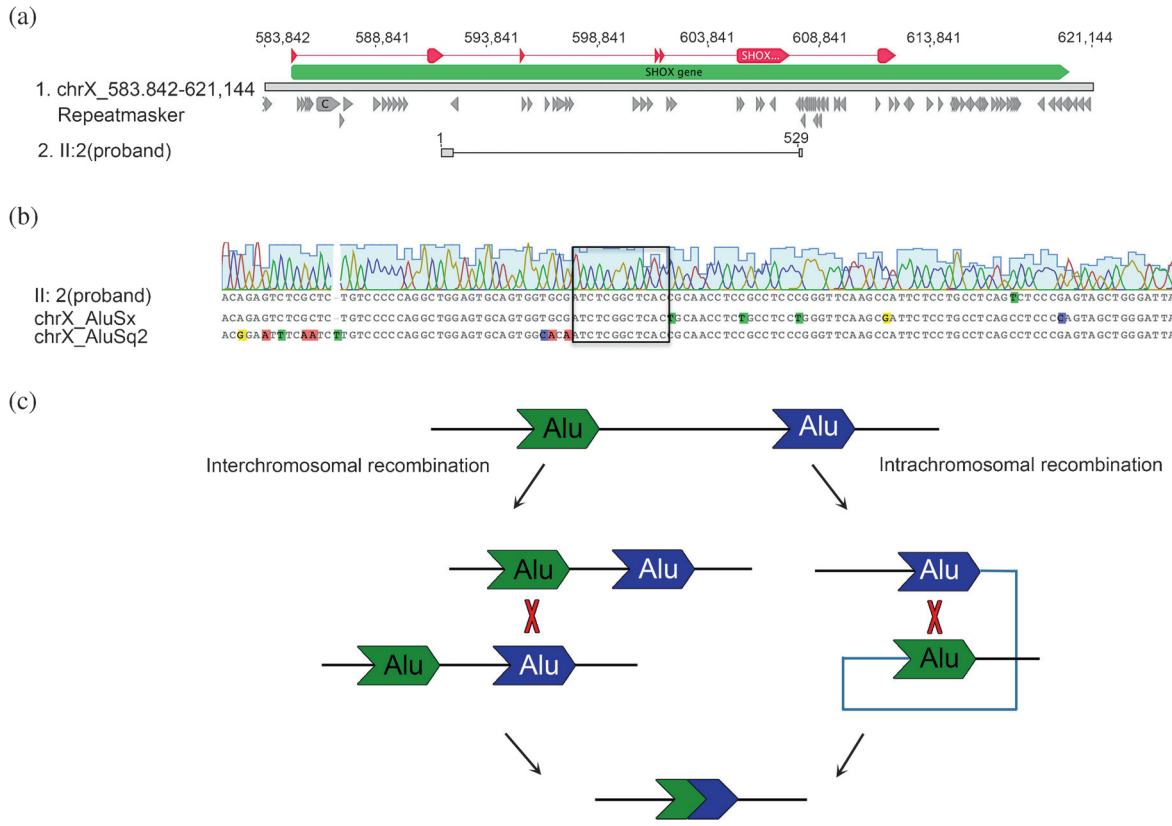

Figure 3. (a) Illustration of the *SHOX* gene deletion. The schematic illustrates the *SHOX* gene and proband sequence alignment; repetitive elements that span the region are annotated in grey colour, while exons are annotated in red. Black straight line that spans the proband sequence illustrates the identified 15.5-kb deletion that was observed. (b) Chromatogram of the proband aligned with the two *Alu* elements which are involved in the deletion. Highlighted region indicates the 12 bp segment which is shared by the distal and proximal breakpoints. (c) Schematic representation of the suggested mechanisms that can lead to the generation of the observed microdeletion. Interchromosomal (left) and intrachromosomal (right) recombination mechanisms are illustrated between *Alu* elements. Both recombination mechanisms result in sequence deletion and generation of chimeric *Alu* element.

by a rare 15.5 kb cryptic deletion residing within the flanking coding sequences of *SHOX* gene in the PAR1 region. The current molecular defect was identified following targeted interrogation of the *SHOX* locus by array-CGH, which demonstrated an intragenic deletion removing exons 3–6 of the *SHOX* gene. Microdeletions involving *SHOX* gene and its regulatory enhancers have been shown to be associated with *SHOX*-related haploinsufficiency disorders and in particular LWD (Schiller *et al*. 2000; Falcinelli *et al*. 2002; Gatta *et al*. 2014). *SHOX* deletions represent the majority of genomic alterations in LWD patients compared to point mutations in the region. This can be attributed to the complex architect and the high recombination meiotic events within the PAR1 region (Shears *et al.* 1998; Rappold *et al.* 2002).

Remarkably, deletions encompassing the entire *SHOX* gene are more common compared to single-exonic or multi-exonic cryptic deletions, which are very rarely detected and reported (Schiller *et al.* 2000; Falcinelli *et al.* 2002; Benito-Sanz *et al.* 2006; Gatta *et al.* 2007; Fukami *et al.* 2008). We report a novel 3–6a exonic deletion in a three-generation family with a phenotype resembling that of LWD in females and milder phenotypic expression in males. To the best of our knowledge, there are very few who reported small intragenic deletions among patients with a phenotype resembling that of LWD. Benito-Sanz *et al.* (2006) identified and characterized the breakpoints in a cohort of 47 LWD patients bearing deletions, and the great majority of the deletions (45/47) were encompassing the entire *SHOX* gene and only two were pure intragenic deletions, removing exons 4–6a and exons 2–6b, respectively. In addition, Chen *et al.* (2009) screened a cohort of 58 LWD patients and identified only a single patient with a 4–5 exonic deletion. Fukami *et al.* (2008) identified and characterized three intragenic cryptic deletions in LWD patients removing exons 4–5, exons 4–6a and 4–6b, after screening 29 families with at least one patient with LWD. Recently, Fukami *et al.* (2015) screened 245 ISS/LWD patients and identified only a single microdeletion removing exon 6b and its neighbouring enhancer sequence. The above studies further support the rarity of small exonic deletions of *SHOX* gene among LWD patients.

Few pure intragenic cryptic deletions were also reported through studies of single families such as the study by Funari *et al.* (2008), who reported a family with a deletion removing exons 4–6a.

Interestingly, most pure intragenic deletions are reported, which appear to share a common proximal breakpoint flanking exon 4. However, most studies have not performed accurate breakpoint mapping, therefore we cannot conclude whether they indeed share the same breakpoint at the base pair level. The deletion presented in this study is novel and a breakpoint flanking exon 3 has not been previously reported.

Nevertheless, mutations or deletions involving the *SHOX* gene and its enhancer sequences independent of position and length in LWD patients result in a highly variable phenotype from full expression of the phenotype to individuals showing normal height and no evidence of a classic Madelung deformity (Rappold *et al.* 2002; Binder 2011). In addition, it has been previously shown that members of the same family, sharing identical deletions, exhibit a great phenotypic variability with females having more pronounced phenotype (Binder 2011).

The clinical data presented in this study further support the phenotypic heterogeneity of LWD caused by *SHOX* haploinsufficiency (Shears *et al.* 1998; Schiller *et al.* 2000). In addition, the current cases provide additional evidence that different size intragenic deletions can also cause LWD and that accurate phenotype–genotype correlations cannot be established (Schiller *et al.* 2000; Falcinelli *et al.* 2002; Rappold *et al.* 2002). It is important to point out that the proband also has a low level mosaic karyotype with 10% 45, X identified in peripheral blood. However, as there is no clear consensus regarding the definition of low level mosaicism for chromosomal anomaly and due to the fact that chromosome X loss increases with age, one can argue that the 10% 45, X is low and may not be associated with the phenotype (Russell *et al.* 2007). On the other hand, as no other tissues were examined and the mosaicism may be higher in other tissue, it could also contribute to the patient's phenotype.

The molecular mechanism underlying CNV's involving *SHOX* gene and/or its flanking regions have been very poorly studied to date. To our knowledge, only Fukami *et al*. (2008, 2015) characterized the molecular mechanism underlying deletions and duplications in ISS/LWD patients and suggested that rare intragenic microdeletions or microduplications can be generated by both homologous repeat-sequence-mediated aberrant recombinations and nonhomologous end joining mechanisms.

The complex architecture of the PAR1 region is prone to genomic rearrangements that can possibly mediate different mechanisms resulting in deletions affecting the *SHOX* gene. The fact that breakpoints of intragenic deletions are scattered along the flanking sequences of the *SHOX* exons and no recurrent proximal and distal breakpoints have been reported, and provides further evidence that may be more than one mechanism is involved. Our study provides strong evidence that repeat sequences such as *Alu* elements can mediate such cryptic *SHOX* intragenic microdeletions. *Alu* elements are known for their ability to create genomic instability that can cause intragenic deletions using ARMD mechanisms (Sen *et al.* 2006). In this study, we report a novel deletion that removes exons 3–6a of the *SHOX* gene. The breakpoint junction of the deletion is located within two *Alu* repetitive elements of the AluS subfamily. The intragenic deletion described produces an uninterrupted chimeric *Alu* element most likely attributed to the intrachromosomal or interchromosomal ARMD mechanism (figure 3c) (Sen *et al.* 2006). Transposable elements are estimated to occupy ∼45% of the human genome, with nonLTR retrotransposons (L1, Alu, and SVA) being the majority of those elements (Mills *et al.* 2007; Cordaux and Batzer 2009). NonLTR retrotransposable elements are considered responsible for 0.3% of all human genetic disorders. The *SHOX* gene is located in one of the most *Alu*-rich areas of chromosome X making the gene prominent in genomic alteration mechanisms like ARMD deletion observed (Mills *et al.* 2007; Cordaux and Batzer 2009). Currently, mechanisms mediating rearrangements in the PAR regions are poorly studied and further investigations of similar cases are important to shed more light on the exact mechanism(s) involved.

In conclusion, we have identified a novel intragenic *SHOX* deletion encompassing exons 3–6a in a three generation LWD family of Cypriot descent with marked intrafamiliar phenotypic variability. Our results further support the variable clinical manifestation of the syndrome emphasizing severity in affected females. In addition, based on the identification of the deletion's exact breakpoints, we propose ARMD as the underlying mechanism of the observed genomic rearrangement.

Acknowledgement

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