# RESEARCH

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# Rare dosage abnormalities flanking the *SHOX* gene



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# Abstract

**Background:** Transcriptional regulation of the *SHOX* gene is highly complex. Much of our understanding has come from the study of copy number changes of conserved non-coding sequences both upstream and downstream of the gene. Downstream deletions have been frequently reported in patients with Leri–Weill dyschondrosteosis or idiopathic short stature. In contrast, there are only four cases in the literature of upstream deletions that remove regulatory elements. Although duplications flanking the *SHOX* gene have also been reported, their pathogenicity is more difficult to establish. To further evaluate the role of flanking copy number variants in *SHOX*-related disorders, we describe nine additional patients from a large *SHOX* diagnostic cohort.

**Results:** The nine cases presented here include five with duplications (two upstream of *SHOX* and three downstream), one with a downstream triplication and three with upstream deletions. Two of the deletions remove a single conserved non-coding element (CNE-3) while the third does not remove any known regulatory element but is just 4 kb upstream of *SHOX*, and the deleted region may be important in limb bud development. We also describe six families with novel sequence gains flanking *SHOX*. Three families had increased dosage of a proposed regulatory element approximately 380 kb downstream of *SHOX* (X:970,000), including one family with the first ever reported triplication of this region. One family had two in *cis* downstream duplications co-segregating with LWD, and the two others had a duplication of just the upstream *SHOX* regulatory element CNE-5.

**Conclusions:** This study further extends our knowledge of the range of variants that may potentially cause *SHOX*-related phenotypes and may aid in determining the clinical significance of similar variants.

Keywords: SHOX, Regulatory element, Deletion, Duplication

# Background

Leri–Weill dyschondrosteosis (LWD; MIM ID # 127300) [1], characterised by mesomelic disproportionate short stature and Madelung deformity of the wrist, is a pseudoautosomal dominantly inherited skeletal dysplasia. Heterozygous deletions or sequence variants resulting in haploinsufficiency for the short-stature homeobox-containing gene (*SHOX*;MIM ID # 312865) are the only known genetic cause of LWD [2, 3] and have also been reported in 1.5–15% of cases of idiopathic short stature (ISS; MIM ID # 300582), defined as a height below the 3rd centile or two standard deviations below the mean (-2 SD) in the absence of known specific causative disorders [4–8]. Homozygosity or compound heterozygosity for *SHOX* variants results in Langer mesomelic dysplasia (LMD; MIM ID # 249700), the most severe end of the spectrum of *SHOX*-related disorders with significant short stature, limb aplasia, a small ulna and fibula,



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a small mandible plus thickening and curvature of the radius and tibia [2, 3, 9-11].

The characterisation of deletions downstream of SHOX identified putative DNA elements that act as long-range enhancers [12, 13]. Parallel comparative genomic studies identified multiple conserved non-coding DNA elements (CNEs) downstream of SHOX, four of which have been demonstrated to have transcriptional activity, namely CNE4 (X:714,085-714,740 (hg19)), CNE5 (X:750,825-751,850), CNE7 (ECR1; X:780,700-781,220) and CNE9 (ECS4; X:834,746-835,567) [14-17]. However, regions with regulatory activity are not always highly conserved [18, 19]; therefore, characterising novel copy number variants (CNVs) in individuals with a SHOX-related phenotype is important to try and identify further potential regulatory elements. This approach identified a potential additional regulatory element at approximately X:970,000 [20, 21]. The most recent *cis*-regulatory element to be identified was ZED (Zeugopodal Enhancer Downstream of SHOX) located at X:827,128-827,691 [19] which was shown to be the critical region within the common downstream 47.5 kb X:780,550-828,092 deletion [17, 22].

There are three known CNEs upstream of *SHOX* which have transcriptional activity: CNE-5 (X:398,357–398,906), CNE-3 (X:460,279–460,664) and CNE-2 (516,610–517,229) [23]. Deletions upstream of *SHOX* are infrequently reported compared to downstream deletions with only four previously reported probands with upstream CNEs deletions, all in individuals with ISS [24–26]. Upstream *SHOX* duplications are also comparatively rare with only four reported to date, all in patients with ISS. One of these duplications involved all three upstream CNEs [27] while two other reported cases involved two of the upstream elements (CNE-5/CNE-3 and CNE-3/CNE-2) [28]. The only patient reported to date with a duplication of a single upstream CNE had a duplication of CNE-2 [29].

It is accepted that SHOX downstream deletions that remove regulatory elements can result in SHOX haploinsufficiency, but other, rarer flanking dosage abnormalities are more difficult to interpret, in part due to their rarity in the literature. In this paper, we describe five individuals with a SHOX upstream dosage abnormality from a large SHOX cohort, including two deletions and two duplications of a single upstream CNE, plus an additional upstream deletion that contains no known CNE but removes part of a region that may be involved in limb bud development. We also present four probands with SHOX downstream dosage abnormalities: three short stature probands with unique CNV gains containing the proposed X:970,000 regulatory element, including the first reported case of a triplication of this region, and an LWD family with two in *cis* downstream duplications.

# Methods

# Study cohort

Patients 1–8 were identified from a *SHOX* cohort of 1963 referrals to the Wessex Regional Genetics Laboratory (WRGL). All patients referred specifically for *SHOX* analysis were included in the cohort, regardless of the referral reason. The deletions, duplications or triplication identified below were not detected in 22,017 array comparative genome hybridisation (aCGH) referrals to the WRGL (predominantly referred with developmental delay) or two cohorts of anonymised healthy controls totalling over 800 individuals [22, 27]. Patient 9 was identified after referral to the Switzerland Health Service. The probands presented here were consented for *SHOX* analysis as part of their routine clinical care within the UK National Health Service (Patients 1–8) and the Switzerland Health Service (Patient 9).

Purified genomic DNA obtained from blood samples was extracted according to standard protocols. Analysis of *SHOX* and its flanking regions was carried out using the following techniques:

# SHOX multiplex ligation-dependent probe amplification (MLPA) analysis

MLPA [30] was performed using the current *SHOX* kit at the time of testing according to the manufacturer's protocol (P018-G1; MRC-Holland, Amsterdam, The Netherlands). Abnormal MLPA data were repeated on a separate run in order to confirm the results.

## Sequencing of the SHOX coding region

Direct sequencing of all the coding exons (isoform A, NM\_000451.3, exons 2 to 6a) was carried out to exclude the presence of single-nucleotide variants and small deletions/insertions in the *SHOX* coding sequences and intron/exon boundaries (primer sequences available upon request).

# aCGH analysis

The sizes of the *SHOX* flanking region duplications in the probands 1–4 and 6 were further defined in the Salisbury laboratory using aCGH analysis. The aCGH was performed using Oxford Gene Technologies (OGT, Oxford, UK) 60-mer oligo-array printed in  $8 \times 60$  K International Standard Cytogenomic Array (ISCA) Consortium configuration, according to manufacturer's instructions, using Kreatech's pooled control DNA as a reference (Kreatech Diagnostics, Amsterdam, Holland). Slides were scanned using a G2539A Agilent microarray scanner (Agilent Technologies, Wokingham, UK) and analysed using OGT's CytoSure Interpret (v3.6) microarray software. The duplications in Patient 5 and 9 were confirmed and

sized using single-nucleotide polymorphism array (SNP array) testing in external laboratories.

#### Long-range PCR

As Patient 3 had a deletion below the detection limits of aCGH, long-range PCR of the deletion region was carried out in order to define the breakpoints. Essentially, PCR forward primers were designed at approximately 1-kb intervals across the potentially deleted region, and these were used in conjunction with a reverse primer situated immediately downstream of the maximum extent of the deletion as defined by aCGH. One primer pair produced a PCR product that was not present in normal controls, and this was subsequently Sanger sequenced to determine the breakpoints. Long-range PCR was also required for patient 2, but that involved a single pre-determined primer pair.

All chromosomal location data are based on the Ensembl hg19 assembly. As the *SHOX* gene lies within the pseudo-autosomal region, the dosage abnormalities may be on the X or the Y chromosome in males, but the locations given here refer to the X chromosome for all individuals to aid comparison.

#### Results

All patients underwent *SHOX* diagnostic testing, comprising sequencing of the *SHOX* coding exons and MLPA. All variants were initially detected by MLPA and then further defined using aCGH, SNP array testing and Sanger sequencing where applicable. The minimum and maximum breakpoints of the CNVs are shown in Fig. 1 and Table 1. We cannot rule out that the additional material is located elsewhere in the genome, and the exact location and orientation of the triplicated and duplicated segments could not be determined.

Sanger sequencing excluded the presence of a *SHOX* pathogenic coding sequence variant in all individuals. Although some individuals had additional molecular

or biochemical testing, none underwent whole exome sequencing so other potential causes of short stature have not been excluded. The Z-score (standard deviation) given for all patient heights is based on the relevant World Health Organisation length-for-age charts.

The clinical information, inheritance patterns and molecular results are summarised in Table 1, and the relevant pedigrees of the four families where samples from additional family members were available are shown in Fig. 2.

#### Discussion

Deletions flanking the *SHOX* gene are an established cause of *SHOX*-related phenotypes. However, CNVs near the *SHOX* gene should be interpreted with caution as the PAR1 region is highly repetitive and prone to structural rearrangements. The difficulty in distinguishing between a benign CNV and a clinically relevant CNV with variable phenotypic expression is demonstrated by the common 47.5 kb *SHOX* downstream deletion where six of the 14 probands with the X:780,550–828,092 deletion (43%) in one cohort had inherited the variant from a phenotypically normal parent [22]. Nevertheless, the study of such cases is important to increase our understanding of *SHOX* regulation and to try to identify the aetiology of these rearrangements.

We present nine novel CNVs flanking the *SHOX* gene in probands referred specifically for diagnostic *SHOX* testing; the frequency was 8/1963 for patients tested in the WRGL SHOX cohort. None of these CNVs were identified in 22,017 patients referred to the WRGL for diagnostic aCGH testing (although only eight of the nine variants are detectable by aCGH), and no comparable deletion or duplication has been reported in the Database of Genomic Variants (http://projects.tcag.ca/variation/) or in over 800 anonymised healthy controls analysed by MLPA [22, 27]. While it is not possible to unequivocally demonstrate causality, the identification of rare novel



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Proband	Referral centre	Proband details	Inheritance	Variant type	Variant size	
					Minimum	Maximum
Patient 1	Paediatric endocrinology	14-year-old female of Bangladeshi ori- gin with ISS (height 141.7 cm; – 2.84 SD) and poor weight gain. Karyotyp- ing, thyroid function, coeliac screen, full blood count, renal liver, bone profile and IGF1 were all normal	Unknown	Deletion of CNE-3	X:449,110–498,890	X:446,596-499,211
Patient 2	Paediatric endocrinology	7-year-old British Caucasian male with ISS (height 106.4 cm; – 3.27 SD). Skeletal survey, investigations for bone age, IGF1 and growth hormone provocation were all normal. His mother's height is 160.8 cm (– 0.36 SD)	Maternal	Deletion of CNE-3	Not applicable (sequenced)	X:458,739-460,800
Patient 3	Clinical genetics	5-year-old female with "a diagnosis of ISS". The family did not consent to further assessment	Unknown	Deletion	Not applicable (sequenced)	X:579,123–580,912
Patient 4	Paediatric endocrinology	12-year-old British Caucasian female. Normal weight, length, hearing, vision and development at birth. Had ISs at age 5 (height < $-2$ SD) but height at age 12 was 138.5 cm ( $-1.87$ SD). IGF1, karyotype and celiac investigations were all normal. Mother's height is 157 cm ( $-1.13$ SD). Her 7-year-old brother has a height of 119 cm ( $-1.22$ SD)	Maternal, and also present in her brother	Triplication of X:970,000	X:907,457-1,232,802	X:899,389–1,327,688
Patient 5	Paediatrics	6-year-old female of South Asian origin with ISS (height 101.2 cm; – 2.48 SD)	Unknown	Duplication of CNE-5	X:307,417–399,032	X:300,000-400,000
Patient 6	Paediatrics	8-year-old British Caucasian female. Short stature compared to parental heights (121.1 cm; – 1.1 SD). Karyo- typing, growth hormone stimulation, cortisol, prolactin, IGF1, IGFBP-3 and parathyroid hormone investigations were all normal. Her bone age was 1.5 years delayed	Unknown	Duplication of CNE-5	X:387,593-446,596	X:380,503-449,110
Patient 7	Paediatric endocrinology	<ul> <li>11-year-old Caucasian male of Eastern European origin with ISS (132.9 cm;</li> <li>2.03 SD). His bone age was &lt;1 year delayed. Baseline endocrinology tests were normal</li> </ul>	Unknown	Duplication of X:970,000	X:845,095-1,110,229	X:836,131–1,113,655

					Minimum	Maximum
Patient 8 Clinical gei	letics	9-year-old Caucasian female of mixed British and South American origin with LWD (height 124,5 cm; – 1.53 SD, and bilateral Madelung deformi- ties with a typical thickened Vicker's ligament). Father has LWD (bilateral Madelung deformities, height 175 cm; – 0.21 SD) as does her 11 year-old sister (bilateral Madelung deformities, height 140 cm; – 1 SD)	Both paternal, and both also present in her sister	Duplication	X:694,790–694,862	X:675,012–714,223
				Duplication of ZED and CNE9	X:809,260–963,755	X:781,231–970,703
Patient 9 Paediatric (	indocrinology	<ul> <li>4-year-old Caucasian female of Central European origin with ISS (height 98.1 cm; - 2 SD). Karyotype, glucose, cortisol and somatortropin investigations were all normal. Her bone age was delayed by 1 year and 3 months. Her father has a height of 175 cm (- 0.2 SD) and her paternal grandmother has a height of 170 cm (+1 SD)</li> </ul>	Paternal, and also present in her paternal grandmother	Duplication of X:970,000	X:963,670-1,401,665	X:899,389–1,471,351

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Table 1 (continued)





CNVs provides evidence for the importance of this class of abnormality and may help to define critical regulatory regions.

In this study, we describe three patients with small deletions upstream of *SHOX*, all in individuals with ISS. All of the four previously reported upstream deletions were also seen in patients with ISS [24–26]; therefore, upstream deletions may be associated with a more subtle phenotype than downstream deletions which can often result in LWD. The deletions in Patients 1

and 2 remove part or all of CNE-3. All four previously reported upstream deletions involved the loss of CNE-3, plus CNE-2 and/or CNE-5 [24–26]. Therefore, CNE-3 appears to be the critical upstream element with the loss of CNE-3 sufficient to cause ISS.

In contrast, the deletion in Patient 3 does not contain any known regulatory elements. However, the deleted interval contains a segment strongly conserved down to zebrafish, and a predicted layered H3K27Ac mark, often found near active regulatory elements or chromatin loops [31], is located close to the deletion. Figure 3 shows the UCSC genome browser output (http://genome-euro. ucsc.edu) from CNE-5 to X:970,000 including sequence conservation and predicted layered H3K27Ac marks. The layered H3K27Ac mark approximately 10 kb upstream of the deletion in Patient 3 does not correspond to any known regulatory element. Enriched H3K27 acetylated regions in developing human limbs [31] are shown in greater detail in the lower half of Fig. 3. The deleted region removes part of region that is acetylated at three of the four developmental stages for which data are available (E33, E41, E44 and E47 datasets) [31]. Therefore, the deleted interval may be essential for normal SHOX expression.

The two duplications in Patient 8 segregate with LWD in the proband and two other affected family members. In contrast, assignation of causality for the duplications in Patients 4–7 and 9 is more difficult; they were



ascertained through their short stature which is a common phenotype with multiple causes. Since flanking CNVs are generally associated with mild SHOX-related phenotypes, they could result in reduced expression rather than complete haploinsufficiency. Within SHOX cohorts, duplications of upstream CNEs have previously been reported exclusively in individuals with ISS [25, 28], although a duplication of all three upstream CNEs has been reported in one control individual from a study of the SHOX region [29] and one individual from a study of genome-wide CNVs [32]. Similarly, duplication of the proposed X:970,000 regulatory element [20] has also been reported as a copy number variant in another large study [33]. However, as the common 47.5 kb downstream deletion [17, 22] is associated with a highly variable phenotypic effect ranging from normal to Leri-Weill dyschondrosteosis (with 43% of carrier parents being phenotypically normal [22]), the presence of a SHOX flanking variant in a phenotypically normal individual does not preclude that variant from being causative but with a variable phenotype. SHOX phenotypic variability may also be influenced by modifier genes such as CYP26C1 [34].

In contrast with deletions, the causative mechanism(s) for duplications is less clear as there is no loss of sequence material. However, there is evidence to suggest that the duplication of only the downstream CNE9 regulatory element is sufficient to cause ISS [18, 28, 29]. There are a number of potential explanations that can be considered. Firstly, gene regulation depends not only on the presence of regulatory elements and associated complexes but also on the nuclear positioning, chromatin conformation and integrity of the flanking chromosomal segments [35, 36]. Insertion of duplicated material between SHOX and a given CNE would alter the normal chromatin structure and potentially affect gene expression. Similarly, the CNVs in patients 3, 4, 7 and 9 contain, or are close to, a predicted active H3K27Ac mark and could disrupt normal chromatin loop formation that links enhancers and promotors [37, 38].

Secondly, the presence of additional copies of a regulatory element may act to reduce the availability of transcription factors that bind to these elements. For example, as three copies of the X:970,000 region exist on one chromosome in Patient 4, transcription factors that bind to this region will only bind in the "optimal place" one third of the time. A similar mechanism could be postulated for Patients 5, 7 and 9.

Thirdly, although approximately 95% of large duplications genome-wide are reported to be tandem [39], there could be undetected complexity, particularly in Patient 8 who has two *in cis* downstream duplications. An inverted tandem duplication may allow single-stranded DNA to form a quasipalindromic loop which could block any enhancers contained within the region.

Fourthly, downstream duplications could disrupt the adjacent topologically associated chromatin domain (TAD) boundary. TADs are discrete compartments of approximately 1 Mb in size, which restrict regulatory chromosomal interactions [38, 40]. TAD boundary regions contain insulators that block interactions across adjacent TADs [41] and variants that disrupt TAD structures can cause malformation syndromes through de novo enhancer-promoter interactions and mis-expression [40]. The SHOX 3' TAD boundary has been mapped immediately upstream of the CRLF2 gene (i.e. close to X:1,314,890) [38], although other publications describe the SHOX TAD as covering an approximate region of either X:284,600-1,355,600 [25] or X:350,001-1,035,000 [37]. The duplication in Patient 9, and possibly the triplication in Patient 4, spans the SHOX TAD and would create an extra copy of the TAD boundary. In addition, the duplications seen in Patients 7 and 8 are contained within the SHOX TAD, so they would move the TAD boundary further from SHOX which may alter essential 3-D genomic architecture.

#### Conclusions

Although the dosage abnormalities presented here were all formally classified and reported as variants of uncertain clinical significance, they are rare or absent in control populations. The results from Patient 3 suggest an additional *SHOX* critical region near X:581,000, and the deletions in Patients 1 and 2 provide further evidence that the loss of CNE-3 can cause ISS. Also, the triplication seen in Patient 4 and the two novel duplications in Patients 7 and 9 involving the X:970,000 region provide further evidence that copy number gains containing X:970,000 may be a cause of short stature. Therefore, this study further extends our knowledge of the range of variants that may potentially cause *SHOX*-related phenotypes.

#### Abbreviations

aCGH: Array comparative genome hybridisation; CNEs: Conserved non-coding DNA elements; CNVs: Copy number variants; ISS: Idiopathic short stature; LWD: Leri–Weill dyschondrosteosis; MLPA: Multiplex ligation-dependent probe amplification; PAR1: Pseudoautosomal region 1; SNP array: Single-nucleotide polymorphism array; WRGL: Wessex Regional Genetics Laboratory.

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#### Authors' contributions

DJB and NST took part in conceptualisation; DJB, JIH, PJDF, RJH, LL, NST involved in validation; DJB, EG, JIH, PJDF, RJH, MHE, PK, RVH, LL, MTCR, ZM, AG, CRH, MA took part in investigation; EG, MHE, PK, RVH, MTCR, ZM, AG, CRH, MA involved in resources; DJB and NST involved in writing—original draft; DJB and NST participated in writing—review & editing; NST took part in supervision. All authors read and approved the manuscript.

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#### Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

#### Declarations

#### Ethics approval and consent to participate

The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki. The probands presented here were consented for *SHOX* analysis as part of their routine clinical care within the UK National Health Service (Patients 1–8) and the Switzerland National Health Service (Patient 9), so research ethics committee approval is not applicable for this study according to the NHS Health Research Authority guidelines (hra.nhs. uk). Written informed consent was obtained from a parent or legal guardian of any participants under 16 years old prior to sample collection.

#### **Consent for publication**

Not applicable.

#### **Competing interests**

The authors have no relevant financial or non-financial interests to disclose.

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