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SHORT COMMUNICATION

Rare pseudoautosomal copy-number variations involving *SHOX* and/or its flanking regions in individuals with and without short stature

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Pseudoautosomal region 1 (PAR1) contains *SHOX*, in addition to seven highly conserved non-coding DNA elements (CNEs) with cis-regulatory activity. Microdeletions involving *SHOX* exons 1–6a and/or the CNEs result in idiopathic short stature (ISS) and Leri–Weill dyschondrosteosis (LWD). Here, we report six rare copy-number variations (CNVs) in PAR1 identified through copy-number analyzes of 245 ISS/LWD patients and 15 unaffected individuals. The six CNVs consisted of three microduplications encompassing *SHOX* and some of the CNEs, two microduplications in the *SHOX* 3′-region affecting one or four of the downstream CNEs, and a microdeletion involving *SHOX* exon 6b and its neighboring CNE. The amplified DNA fragments of two *SHOX*-containing duplications were detected at chromosomal regions adjacent to the original positions. The breakpoints of a *SHOX*-containing duplication resided within *Alu* repeats. A microduplication encompassing four downstream CNEs was identified in an unaffected father–daughter pair, whereas the other five CNVs were detected in ISS patients. These results suggest that microduplications involving *SHOX* cause ISS by disrupting the cis-regulatory machinery of this gene and that at least some of microduplications in PAR1 arise from *Alu*-mediated non-allelic homologous recombination. The pathogenicity of other rare PAR1-linked CNVs, such as CNE-containing microduplications and exon 6b-flanking microdeletions, merits further investigation.
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INTRODUCTION

SHOX located in the pseudoautosomal region 1 (PAR1) regulates skeletal development.[1] *SHOX* haploinsufficiency underlies idiopathic short stature (ISS; MIM no. 300582), Leri–Weill dyschondrosteosis (LWD) characterized by Madelung deformity (MIM no. 2 49 700) and less-specific skeletal changes including micrognathia.[1–4] *SHOX* transcripts include *SHOXa* encoded by exons 1–5 and 6a, and *SHOXb* encoded by exons 1–5 and 6b.[1] *SHOX* expression requires multiple cis-acting elements in PAR1;[5–11] previous studies have identified highly conserved non-coding DNA elements (CNEs) with cis-regulatory activity in the upstream (CNE-2, CNE-3 and CNE-5) and downstream regions (CNE4, CNE5, ECR1 and CNE9/ECS4) of *SHOX* (Supplementary Figure S1).[7,8,10,11]

SHOX haploinsufficiency in cytogenetically normal individuals is mainly caused by microdeletions involving *SHOX* exons 1–6a and/or the CNEs.[2,3,5–9] Recently, microduplications encompassing *SHOX* were also identified in patients with ISS and LWD.[12,13] However, the pathogenicity of these copy-number variations (CNVs) remains controversial, because *SHOX* overdosage has been associated with tall or normal stature.[14–17] Likewise, although a deletion in *SHOX* intron 6b was identified in an ISS family, its relevance to the disease phenotype remains unclear.[18] Furthermore, the genomic origins of PAR1-linked CNVs have been poorly investigated.

SUBJECTS AND METHODS

Detailed methods are provided in the Supporting Information (Supplementary Methods). This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development and performed after obtaining informed consent. We searched for PAR1-linked CNVs in 245 patients with ISS or LWD and in 15 unaffected individuals. All patients had short stature with s.d. scores of <-2.0. Diagnosis of LWD was based on radiological findings of Madelung deformity.[3] Individuals with chronic diseases or apparent chromosomal alterations were excluded. When possible, we analyzed participants' parental samples. Genomic DNA samples were subjected to multiplex-ligation-dependent probe amplification.

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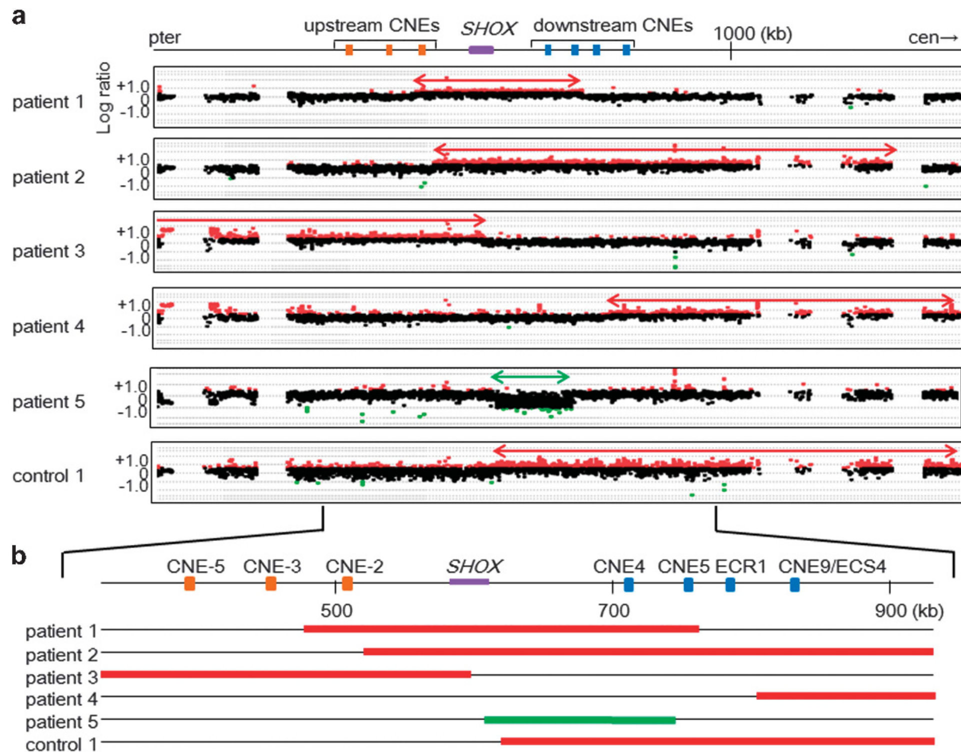

Figure 1 (**a**)Array-based comparative genomic hybridization. The upper horizontal line indicates the physical distance from the Xp/Yp telomere (pter; hg19, Build 37). The purple box depicts *SHOX*. Orange and blue boxes denote highly evolutionarily conserved non-coding DNA elements (CNEs) in the *SHOX* upstream and downstream regions, respectively. The black, red and green dots denote normal, increased (log ratio ⩾ +0.5) and decreased (log ratio ⩽ −1.0) copy-numbers, respectively. The red and green arrows indicate duplicated and deleted regions, respectively. cen, centromere. (**b**) Schematic representation of the *SHOX*-flanking region. The upper horizontal line indicates the physical distance from the Xp/Yp telomere. The red and green lines denote the duplicated and deleted regions, respectively.

All CNVs, except for well-documented microdeletions involving *SHOX* and/or downstream CNEs,[2,3,9] were further characterized by array-based comparative genomic hybridization. We also investigated the genomic structures of CNVs.

RESULTS

Rare CNVs were identified in patients 1–5 and control 1 (Figure 1, Table 1, and Supplementary Figure S2). Patients 1–3 carried microduplications involving *SHOX* and three or four of the seven CNEs. Patient 4 and control 1 had microduplications in the *SHOX* 3′-region encompassing one and all downstream CNEs, respectively. Patient 5 had a microdeletion involving exon 6b and CNE4. The CNV in patient 1 was a 270 737 bp tandem duplication, whose breakpoints resided within *Alu* repeats and shared a 12 bp overlap (Figure 2a). Fusion junctions of other CNVs could not be determined. Fluorescence *in situ* hybridization analysis of patient 2 using a *SHOX*-containing cosmid generated a signal only at Xp22.3 (Figure 2b), indicating that the amplified DNA fragment was probably inserted into a genomic interval adjacent to the original position. Patients 1–5 manifested mild or moderate short stature (Table 1). None of the five patients had Madelung deformity, whereas patient 3 showed micrognathia. The phenotypically normal father of control 1 had the same CNV as the proband.

DISCUSSION

This study provides further evidence that *SHOX*-containing microduplications account for a small fraction of the etiology of ISS. Our findings contradict prior observations that trisomy of PAR1 owing to chromosomal rearrangements leads to tall or normal stature.[13–17] These conflicting data can be explained by assuming that relatively large duplications containing all *SHOX* exons and cis-acting enhancers lead to gene overexpression and resultant tall stature, whereas small duplications encompassing only a part of these components attenuate *SHOX* expression by disrupting the cis-regulatory machinery. Consistent with this, the microduplication in patient 1 increases the distance between *SHOX* exons and the CNEs, and the duplicated DNA fragment in patient 2 was detected at a genomic region adjacent to the original position. On the other hand, the results of patient 4 and control 1 question the pathogenicity of duplications affecting only the CNEs. To our knowledge, no previous study has shown that enhancer overdosage impairs gene expression. Nevertheless, PAR1 microduplications apart from *SHOX* may disrupt the cis-regulatory machinery. Indeed, CNE-containing microduplications were identified in multiple ISS patients.[12,19] Furthermore, the pathogenicity of the exon 6b-containing microdeletion in patient 5 remains unclear. *SHOX* exon 6b has not been implicated in skeletal development; however, elimination of SHOXb may affect the function of SHOXa isoform, because SHOXb dimerizes with SHOXa.[20] Alternatively, the ISS of patient 5 may be ascribed to CNE4 deletion. Moreover, as a deletion in intron 6b has been identified in an ISS family,[18] exon 6b-flainking regions may contain another enhancer.

Our data imply that the duplication in patient 1 arose from *Alu*-mediated non-allelic homologous recombination (NAHR). Because PAR1 is enriched with *Alu* repeats[4] and *Alu*-mediated NAHR has been

Table 1 Clinical and molecular findings of individuals with pseudoautosomal copy-number variations

	Patient 1	*Patient 2*	*Patient 3*	*Patient 4*	*Patient 5*	*Control 1*	*Father of control 1*
Clinical findings[a]							
Clinical diagnosis	ISS	ISS	ISS	ISS	ISS	Unaffected	Unaffected
Age at exam (years)	1.9	3.2	7.8	4.6	4	22	63
Sex	Female	Male	Female	Female	Female	Female	Male
Height	− 2.7 s.d.	− 2.9 s.d.	− 2.8 s.d.	− 2.4 s.d.	− 3.1 s.d.	+ 0.1 s.d.	+ 0.4 s.d.
Skeletal deformity	No	No	No	No	No	No	No
Other clinical features	None	None	Micrognathia	None	None	None	None
Family history of ISS/LWD	Borderline ISS (mother)	No	No	ISS (mother)	No	No	No
Molecular findings							
Type of CNV	Duplication	Duplication	Duplication	Duplication	Deletion	Duplication	Duplication
Maximum interval (chrX)[b]	486 700–757 437[d]	520 681–1 314 734	1–596 006	798 435–1 474 970	619 112–743 611	617 949–1 497 274	617 949–1 497 274
Minimum interval (chrX)[c]		521 908–1 262 229	1–595 730	799 285–1 474 654	621 144–742 875	621 145–1 473 010	621 145–1 473 010
Affected SHOX elements							
Upstream enhancer sequences	CNE-2	None	CNE-2, CNE-3 and CNE-5	None	None	None	None
Exon	Exons 1–6b	Exons 1–6b	Exons 1–3	None	Exon 6b	None	None
Downstream enhancer sequences	CNE4 and CNE5	CNE4, CNE5, ECR1 and CNE9/ECS4	None	CNE9	CNE4	CNE4, CNE5, ECR1 and CNE9/ECS4	CNE4, CNE5, ECR1 and CNE9/ECS4

Abbreviations: CNE: highly evolutionarily conserved non-coding DNA elements (Chen *et al.*[10] and Durand *et al.*[11]); ECR: evolutionarily conserved region (Benito Sanz *et al.*[7]); ECS: evolutionarily conserved sequence (Fukami *et al.*[8]); ISS, idiopathic short stature; LWD, Leri–Weill dyschondrosteosis.
[a]These data were obtained before therapeutic interventions.
[b]The genomic interval between the first copy-number neutral probes at both sides of the CNV (hg19, build 37).
[c]The genomic interval between the two most distant probes within the CNV.
[d]Precise position of the breakpoints was determined in this patient.

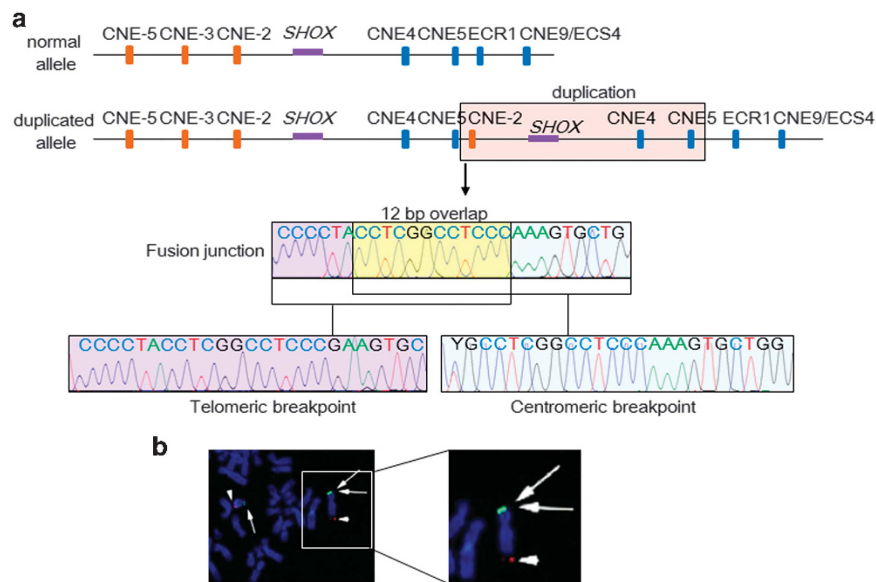

Figure 2 Characterization of copy-number variations (CNVs) in patients 1 and 2. (**a**) Upper panel: schematic representation of the duplication in patient 1. The purple boxes depict *SHOX*. Orange and blue boxes denote highly conserved non-coding DNA elements (CNEs) in the *SHOX* upstream and downstream regions, respectively. The red shaded area indicates the duplicated region. The genomic structure is not drawn to scale. Lower panel: DNA sequences at the fusion junction of the duplication in patient 1. The PCR product containing the junction was amplified using a primer pair, 5′-CCTCCAAAATAGCTGGCAATA-3′ and 5′-AGCATAAAATTCCCCATCTGA-3′. The CNV was a tandem duplication of 270 737 bp (chrX:486,700–757,437; hg19, Build 37). (**b**) Fluorescence *in situ* hybridization analysis of patient 2. The *SHOX*-containing cosmid probe (green) generated signals only at Xp22.3 (arrows). The red signals (arrowheads) indicate the control probe for the Xq/Yq telomere.

implicated in the development of a microdeletion involving *SHOX*,[9] it is possible that NAHR underlies various PAR1-linked CNVs. However, other mechanisms such as non-homologous end-joining may also underlie these CNVs.

Patients 1–5 showed no signs of Madelung deformity, indicating that PAR1-linked duplications and exon 6b-containing deletions result in relatively mild phenotypes. However, the mild phenotypes in our patients may be related to their young ages, because skeletal features in patients with *SHOX* abnormalities usually ameliorate after puberty.[3] Notably, patient 3 with a duplication encompassing *SHOX* exons 1–3 manifested micrognathia, which may be associated with SHOX dysfunction,[2] whereas patients 1–2 with duplications of all *SHOX* exons manifested no skeletal deformities. These findings are consistent with previous findings that partial *SHOX* duplications exert more significant effects on skeletal development than complete duplications.[12] Alternatively, partial *SHOX* duplications may have a broad phenotypic spectrum, because these CNVs were identified in several unaffected relatives of the patients.[12]

Collectively, our results indicate that *SHOX*-containing microduplications cause ISS by disrupting the cis-regulatory machinery of *SHOX* and that *Alu*-mediated NAHR underlies at least some of these microduplications. Further studies are necessary to clarify the pathogenicity of other rare PAR1-linked CNVs.

CONFLICT OF INTEREST

MF has received a research grant from JCR Pharmaceuticals. The remaining authors declare no conflict of interest.

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DISCLAIMER

The sponsor had no role in the study design, in the collection, analysis or interpretation of data, in the writing of the report or in the decision to submit the report for publication.

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Supplementary Information accompanies the paper on Journal of Human Genetics website (http://www.nature.com/jhg)