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ORIGINAL ARTICLE

Systematic molecular analyses of *SHOX* in Japanese patients with idiopathic short stature and Leri–Weill dyschondrosteosis

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The etiology of idiopathic short stature (ISS) and Leri–Weill dyschondrosteosis (LWD) in European patients is known to include *SHOX* mutations and copy-number variations (CNVs) involving *SHOX* and/or the highly evolutionarily conserved non-coding DNA elements (CNEs) flanking the gene. However, the frequency and types of *SHOX* abnormalities in non-European patients and the clinical importance of mutations in the CNEs remains to be clarified. Here, we performed systematic molecular analyses of *SHOX* for 328 Japanese patients with ISS or LWD. *SHOX* abnormalities accounted for 3.8% of ISS and 50% of LWD cases. CNVs around *SHOX* were identified in 16 cases, although the ~47 kb deletion frequently reported in European patients was absent in our cases. Probably damaging mutations and benign/silent substitutions were detected in four cases, respectively. Although CNE-linked substitutions were detected in 15 cases, most of them affected poorly conserved nucleotides and were shared by unaffected individuals. These results suggest that the frequency and mutation spectrum of *SHOX* abnormalities are comparable between Asian and European patients, with the exception of a European-specific downstream deletion. Furthermore, this study highlights the clinical importance and genetic heterogeneity of the *SHOX*-flanking CNVs, and indicates a limited clinical significance of point mutations in the CNEs.
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INTRODUCTION

SHOX (NM_000451.3) is located in the short arm pseudoautosomal region of the sex chromosomes (PAR1), and encodes a homeobox-containing transcription factor that plays a critical role in skeletal growth.[1] *SHOX* haploinsufficiency leads to idiopathic short stature (ISS; OMIM #300582) without skeletal malformations and Leri–Weill dyschondrosteosis (LWD; OMIM #127300) characterized by Madelung deformity.[1–3] Madelung deformity is a cluster of anatomical changes in the wrist including bowing of the radius and dorsal dislocation of the distal ulna, which can result in wrist pain, deformation and/or limited joint mobility.[2, 3] Genetic defects underlying *SHOX* haploinsufficiency include several mutations in the coding region and various copy-number variations (CNVs) in PAR1.[2,4–11] The ISS/LWD-associated CNVs are predicted to involve *SHOX* exons and/or *cis*-acting enhancers. Although the precise positions of the *SHOX* enhancers remain to be determined, they likely reside within the highly evolutionarily conserved non-coding DNA elements (CNEs) around the gene.[10–14] Previous studies have identified seven

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CNEs (CNE-5, CNE-3, CNE-2, CNE4, CNE5, evolutionarily conserved region (ECR) 1 and evolutionarily conserved sequence (ECS) 4/CNE9) that exert *in vitro* or *in vivo cis*-regulatory activity.[10–14] *In vitro* assays confirmed physical interaction between these CNEs and the *SHOX* promoter.[10,15]

So far, mutation screening and copy-number analyses of *SHOX* have been performed mostly for patients of European origin.[4–10, 16–20] Previous studies indicated that *SHOX* haploinsufficiency is primarily caused by submicroscopic CNVs in PAR1, and accounts for 2–17% of ISS cases and 35–100% of LWD cases.[4–10,16–20] However, the applicability of these findings to non-European populations remains unclear. For example, although a ~47 kb deletion in the *SHOX* downstream region was frequently identified in European patients,[10,19,20] it is unknown whether this CNV is shared by patients of other ethnicities. More importantly, there is no report of sequence analysis of the CNEs in the patients with ISS or LWD. Thus, the clinical importance of point mutations in the known CNEs has yet to be studied. To address these unsolved issues, we performed systematic molecular analysis of *SHOX* in a large cohort of Japanese patients.

MATERIALS AND METHODS

Subjects

The study was approved by the Institutional Review Board Committee at the National Center for Child and Development, and performed after obtaining informed consent from the participants or their parents.

The study group consisted of 328 Japanese patients with short stature (164 males and 164 females; aged 0.5–17.9 years). The patients satisfied the following conditions: (i) referred to our pediatric endocrinology clinics between March 2013 and November 2015 for evaluation of short stature; (ii) short stature with standard deviation scores of < -2.0; (iii) no chronic diseases, such as growth hormone deficiency, congenital heart disease, achondroplasia or thyroid disease that may affect growth; and (iv) lack of cytogenetically detectable chromosomal abnormalities. The patients underwent radiological examinations of the hand. We examined the presence or absence of Madelung deformity-compatible features including narrowing of the ulnar portion of the distal radial physis, anterior bowing of the radial shaft and dorsal subluxation of the ulnar head.[3] Of the 328 patients, 16 with radiologically recognizable Madelung deformity were diagnosed with LWD, whereas the remaining 312 were diagnosed with ISS. Four patients were included from our previous study.[21] As controls, we used genomic DNA samples obtained from 100 healthy Japanese adults with normal height (50 males and 50 females).

Copy-number analysis

All patients were subjected to copy-number analysis. Genomic DNA was extracted from peripheral leukocytes. CNVs in PAR1 were analyzed by multiplex ligation-dependent probe amplification using a commercially available kit (SALSA P018-G1, MRC-Holland, Amsterdam, Netherlands) and further characterized by array-based comparative genomic hybridization using a custom-made microarray (8 × 60 k format, Agilent Technologies, Santa Clara, CA, USA). To exclude non-pathogenic variations, we referred to the Database of Genomic Variants (http://projects.tcag.ca/variation/).

Sequence analysis

Of the 312 patients with no pathogenic CNVs, 309 (300 ISS and 9 LWD) were subjected to sequence analysis of *SHOX* exons; 3 patients were excluded from this analysis because of insufficient amounts of DNA samples. We also carried out sequence analysis of the 7 CNEs for 83 patients (76 ISS and 7 LWD) whose DNA samples were sufficient for this experiment.

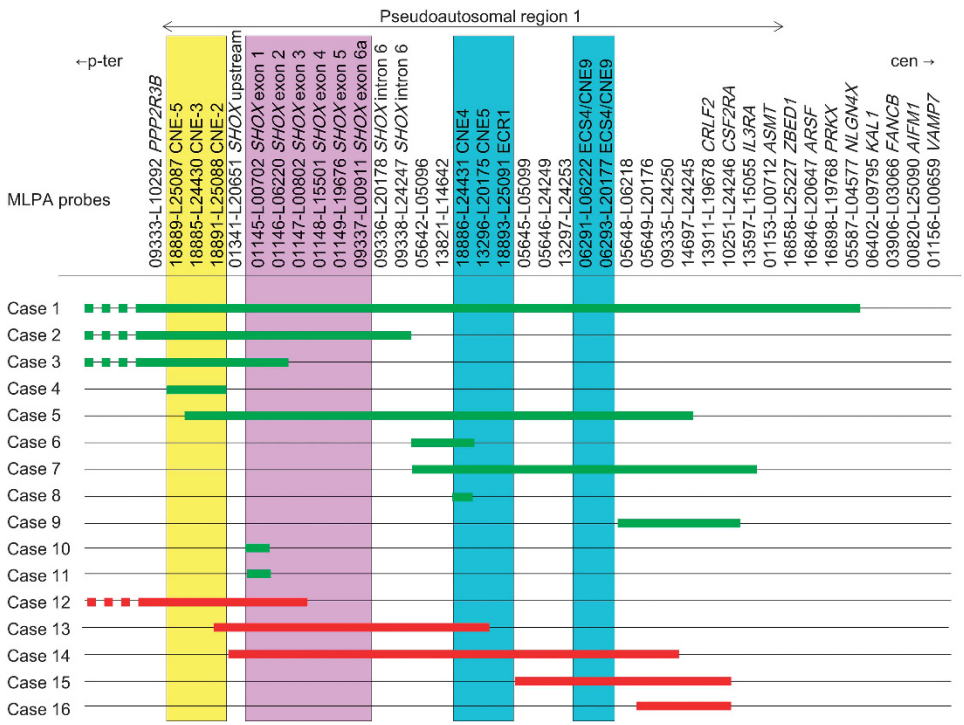

Figure 1 Copy-number variations (CNVs) identified in cases 1–16. The upper panel shows the names of multiplex ligation-dependent probe amplification (MLPA) probes. Probes of the *SHOX* exons are shaded in purple, and those of the putative enhancer regions, that is, highly evolutionarily conserved non-coding DNA elements (CNEs), evolutionally conserved region (ECR), or evolutionally conserved sequence (ECS), are shaded in blue or yellow. Probes in the pseudoautosomal region 1 are indicated with an arrow. The green and red lines depict copy-number loss and gain, respectively. The dotted lines indicate the copy-number unknown regions. p-ter: short arm telomere; cen, centromere.

Table 1 Nucleotide alterations identified in this study

Case	Genomic position[a]	Affected SHOX exon/CNE	Nucleotide change	Amino acid change	Predicted function: PolyPhen-2[d]	Predicted function: SIFT[e]	SHOX mutation database[b]	dbSNP[c]	Allele frequency in the general population: HGV[f]	ExAC[g]	1000Genome[h]	Our control[i]
Missense substitutions												
17	chrX: 595 440	exon 3	C365G	Thr122Ser	Probably damaging	Damaging	N.D.	N.D.	N.D.	1/114 528	N.D.	N.E.
18	chrX: 595 468	exon 3	G393C	Glu131Asp	Probably damaging	Damaging	N.D.	rs374188319	N.D.	2/100 146	N.D.	N.E.
19	chrX: 595 500	exon 3	C425G	Pro142Arg	Probably damaging	Damaging	ISS and LWD?	N.D.	N.D.	N.D.	N.D.	N.E.
20	chrX: 605 190	exon 6	C698T	Ala233Val	Probably damaging	Tolerated	ISS and LWD?	N.D.	0.008	2/12 752	N.D.	N.E.
21	chrX: 591 769	exon 2	C137T	Ser46Phe	Benign	Tolerated	N.D.	N.D.	N.D.	N.D.	N.D.	N.E.
Silent substitutions												
22	chrX: 595 387	exon 3	G312C	Val104Val	—	—	N.D.	N.D	N.D.	N.D.	N.D.	N.E.
23	chrX: 601 735	exon 5	C546T	Gly182Gly	—	—	Phenotype unknown	rs138998412	N.D.	3/121 402	N.D.	N.E.
24	chrX: 601 765	exon 5	C576T	Asp192Asp	—	—	Heart disease	rs200088460	N.D.	7/121 400	N.D.	N.E.
Nucleotide changes in CNE												
25	chrX: 398 441	CNE-5	A>G	None	—	—	N.D.	rs373482520	N.D.	N.D.	N.D.	3/200
26	chrX: 398 561	CNE-5	C>T	None	—	—	N.D.	rs183167540	N.D.	N.D.	7/5 008	N.E.
25	chrX: 516 836	CNE-2	C>A	None	—	—	N.D.	N.D	N.D.	N.D.	N.D.	0/200
27	chrX: 751 039	CNE5	T>G	None	—	—	N.D.	N.D	N.D.	N.D.	N.D.	1/200
26, 28–30	chrX: 751 072	CNE5	A>G	None	—	—	N.D.	rs139314781	N.D.	N.D.	7/5 008	N.E.
26, 27, 31–33	chrX: 751 205	CNE5	C>T	None	—	—	N.D.	rs73616298	N.D.	N.D.	215/5 008	N.E.
34, 35	chrX: 751 319	CNE5	A>G	None	—	—	N.D.	rs184927696	N.D.	N.D.	14/5 008	N.E.
36	chrX: 751 686	CNE5	G>A	None	—	—	N.D.	N.D	N.D.	N.D.	N.D.	1/200
37	chrX: 780 587	ECR1	C>G	None	—	—	N.D.	N.D	N.D.	N.D.	N.D.	0/200
38	chrX: 780 846	ECR1	T>G	None	—	—	N.D.	N.D	N.D.	N.D.	N.D.	2/200
39	chrX: 835 007	CNE9/ECS4	G>C	None	—	—	N.D.	N.D	N.D.	N.D.	N.D.	3/200

Abbreviations: CNE, highly conserved non-coding DNA elements; ECR, evolutionarily conserved region; ECS, evolutionarily conserved sequence; ISS, idiopathic short stature; LWD, Leri–Weill dyschondrosteosis; N.D., no data; N.E. not examined.
[a]Genomic positions referred to UCSC Human Genome Browser (February 2009, hg 19, Build 37).
[b]LOVD X-chromosome gene database short stature homeobox (http://grenada.lumc.nl/LOVD2/MR/home.php?select_db=SHOX).
[c]NCBI dbSNP web site (http://www.ncbi.nlm.nih.gov/SNP/).
[d]Based on the data of PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/).
[e]Based on the data of SIFT (http://sift.jcvi.org/).
[f]Human genetic variation browser (http://www.genome.med.kyoto-u.ac.jp/SnpDB/).
[g]ExAC Browser (http://exac.broadinstitute.org/).
[h]1000 Genomes Browser (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/)
[i]100 Japanese adults with normal stature.

DNA fragments corresponding to the target regions were amplified by multiplex-PCR or by using the Haloplex system (Agilent Technologies, Santa Clara, CA, USA) and sequenced on a MiSeq next-generation sequencer (Illumina, San Diego, CA, USA). The highly polymorphic oligonucleotide repeats in the CNEs were excluded from sequence analysis. Nucleotide changes indicated by next-generation sequencing were confirmed by the Sanger method. Primer sequences are available on request. Nucleotide substitutions whose allelic frequency in the databases (dbSNP, http://www.ncbi.nlm.nih.gov/snp/; or 1000 Genome Browser, http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/) was more than 0.05 were excluded as common polymorphisms.

The functional outcomes of missense substitutions were predicted by *in silico* analysis using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) and SIFT (http://sift.jcvi.org/). To examine the population frequency of exonic substitutions, we referred to the Exome Aggregation Consortium Browser (http://exac.broadinstitute.org/) and the Human Genetic Variation Browser (http://www.genome.med.kyoto-u.ac.jp/SnpDB). To evaluate the population frequency of novel substitutions, we analyzed DNA samples of control individuals.

To assess the functional significance of nucleotide changes in the CNEs, we examined whether these substitutions were located within the putative enhancer sequences. We referred to ENCODE Broad Chromatin State Segmentation in the UCSC browser (http://genome.ucsc.edu/). The evolutionary conservation of the affected nucleotides was analyzed using the UCSC browser.

RESULTS

Copy-number analysis

Submicroscopic CNVs in PAR1 were identified in 16 cases (cases 1–16; Figure 1 and Supplementary Figures 1 and 2). These CNVs consisted of 11 deletions and 5 duplications. The deletion in case 1 extended beyond PAR1, whereas CNVs in the remaining cases were located within PAR1. The deletion in case 9 affected a genomic interval downstream of the known CNEs. The ~47 kb deletion common in European patients,[10,19,20] was absent in our cases. The breakpoints of the CNVs in cases 2, 3, 5, 12 and 14 were located within or close to repetitive sequences (Supplementary Figure 2).

Sequence analysis

Sequence analysis of *SHOX* identified eight intragenic substitutions in eight cases (cases 17–24; Table 1 and Figure 2). Of these, five were missense substitutions, and three were silent (synonymous) changes. These eight substitutions were absent or extremely rare in the general population. Four of the five missense substitutions were assessed as 'probably damaging', whereas p.Ser46Phe in case 21 was scored as 'benign'. The substitutions in cases 17–19 were located within the homeobox.

Sequence analysis of the known CNEs detected 11 substitutions in 15 cases (cases 25–39; Table 1 and Figure 2). Of these, five were rare

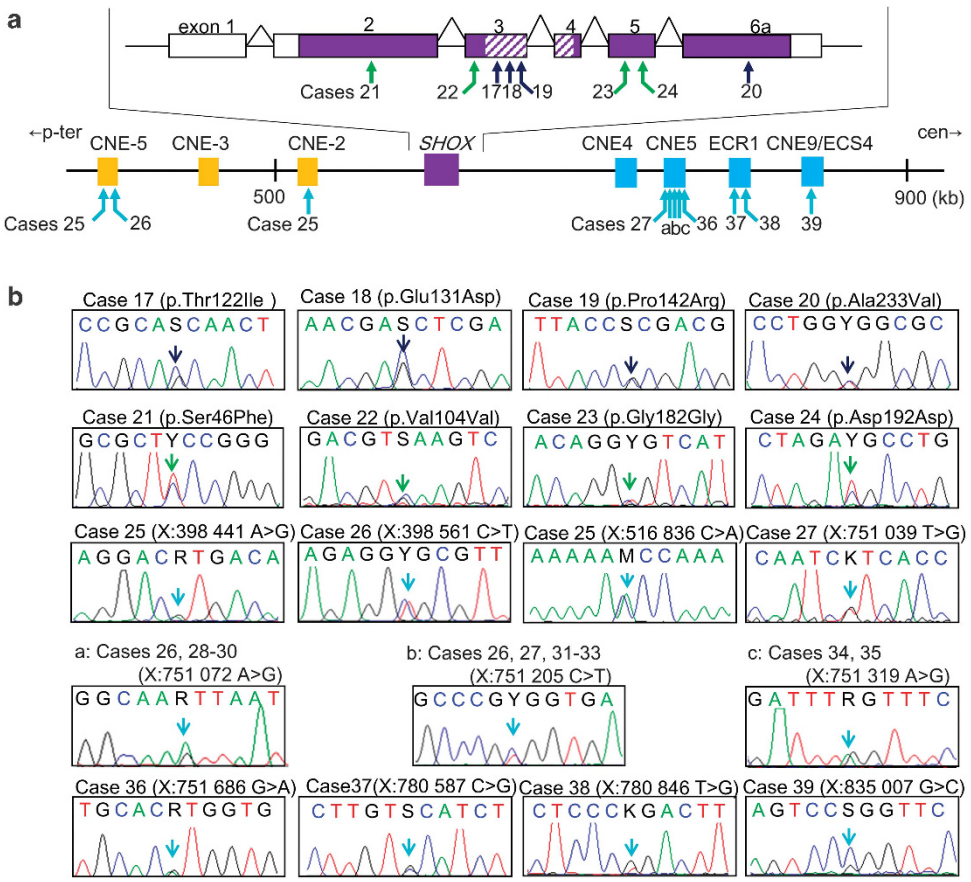

Figure 2 Nucleotide substitutions identified in cases 17–39. (**a**) Genomic structure of *SHOX* and its flanking region. Positions refer to the Human Genome (hg 19; NCBI Build 37). The white and purple boxes indicate the untranslated and translated exons, respectively. The striped boxes depict the homeobox. The yellow and light blue boxes denote the putative upstream and downstream enhancer regions, respectively. Probably damaging mutations and benign/silent substitutions are indicated by dark blue and green arrows, respectively. Substitutions in the putative enhancer regions are depicted by light blue arrows. (**b**) Electrochromatographs of the substitutions. Mutated nucleotides are indicated by arrows. Substitutions indicated by a, b, and c were identified in multiple patients. p-ter: short arm telomere; cen, centromere.

polymorphisms, and the others were first identified herein. Most of the novel substitutions were shared by the control individuals. Substitutions in CNE-5 and CNE5 resided within putative enhancer sequences (Supplementary Figure 3). Most of the altered nucleotides were not highly conserved among species (Supplementary Figure 4).

Clinical features of patients with *SHOX* abnormalities

Cases 1–16 with CNVs comprised ten ISS and six LWD patients, whereas cases 17–20 with probably damaging mutations included two ISS and two LWD patients (Table 2). Thus, apparent *SHOX* abnormalities accounted for 12 of 312 (3.8%) ISS patients and eight of 16 (50%) LWD patients. Cases with benign or silent substitutions (cases 21–24) and CNE-linked substitutions (cases 25–39) were all ISS.

DISCUSSION

Systematic molecular analysis identified probably pathogenic *SHOX* abnormalities in 3.8 and 50% of Japanese ISS and LWD cases, respectively. These results indicate that the frequency of *SHOX* haploinsufficiency is comparable between Japanese and European patients. On the other hand, none of our patients carried the well-known ~47 kb downstream deletion,[10,19,20] indicating that this CNV is a European-specific founder mutation. The lack of *SHOX*

Table 2 Clinical and molecular findings of patients with *SHOX* abnormalities

Cases	*Sex*	*Age*	*Clinical diagnosis*	*Stature (SDS)*	*Additional clinical features*	*Genetic defects*	*Affected SHOX exon*	*Affected CNE*
1	F	N.D.	LWD	N.D.	N.D.	Deletion	All	All
2	F	6.9	LWD	−3.6	None	Deletion	All	CNE-5, CNE-3, and CNE-2.
3	F	0.5	LWD	−1.7	None	Deletion	Exons 1–2	CNE-5, CNE-3, and CNE-2.
4	M	4.0	ISS	−5.0	Macrocephalus	Deletion	None	CNE-5, CNE-3, and CNE-2.
5	F	10.8	LWD	−1.4	CV	Deletion	All	CNE-3, CNE-2. CNE4, CNE5, ECR1 and ECS4/CNE9
6	F	4.0	ISS	−3.1	None	Deletion	Exon 6b	CNE4
7	M	16.0	LWD	−3.0	None	Deletion	None	CNE4, CNE5, ECR1 and ECS4/CNE9
8	F	11.0	ISS	−2.3	None	Deletion	None	CNE4
9	F	11.1	LWD	−1.4	None	Deletion	None	Downstream region
10	M	6.7	ISS	−2.3	None	Deletion	Exon 1	None
11	M	6.3	ISS	−4.1	None	Deletion	Exon 1	None
12	M	9.5	ISS	−2.5	None	Duplication	Exons 1–3	CNE-5, CNE-3, and CNE-2.
13	F	1.9	ISS	−2.7	None	Duplication	All	CNE-2, CNE4 and CNE5
14	M	3.2	ISS	−2.9	None	Duplication	All	CNE4, CNE5, ECR1 and CNE9/ECS4
15	F	4.6	ISS	−2.4	None	Duplication	None	CNE9/ECS4
16	M	6.4	ISS	−2.0	None	Duplication	None	Downstream region
17	F	8.6	LWD	−2.6	Webbed neck, CV	Missense substitution	Exon 3	None
18	F	4.4	ISS	−3.2	Mild CV	Missense substitution	Exon 3	None
19	F	5.5	LWD	−2.5	Mesomelia, CV	Missense substitution	Exon 3	None
20	M	9.8	ISS	−2.1	None	Missense substitution	Exon 6	None
21	M	10.8	ISS	−2.1	None	Missense substitution	Exon 2	None
22	F	3.2	ISS	−3.5	None	Silent substitution	Exon 3	None
23	M	3.5	ISS	−2.3	None	Silent substitution	Exon 5	None
24	F	2.9	ISS	−3.0	CV	Silent substitution	Exon 5	None
25	F	5.3	ISS	−2.8	None	Substitution in CNE	None	CNE-5 and CNE-2
26	F	5.2	ISS	−3.4	None	Substitution in CNE	None	CNE-5 and CNE5[a]
27	M	4.9	ISS	−3.2	None	Substitution in CNE	None	CNE5[b]
28	M	3.0	ISS	−4.7	Macrocephalus	Substitution in CNE	None	CNE5
29	F	4.6	ISS	−2.3	None	Substitution in CNE	None	CNE5
30	F	10.5	ISS	−2.5	None	Substitution in CNE	None	CNE5
31	M	14.0	ISS	−2.6	N.D.	Substitution in CNE	None	CNE5
32	F	6.1	ISS	−2.5	None	Substitution in CNE	None	CNE5
33	M	9.9	ISS	−2.9	None	Substitution in CNE	None	CNE5
34	F	10.0	ISS	−3.6	Micrognathia, high-arched palate	Substitution in CNE	None	CNE5
35	F	2.0	ISS	−3.5	Prominent forehead	Substitution in CNE	None	CNE5
36	M	6.0	ISS	−3.0	None	Substitution in CNE	None	CNE5
37	M	5.6	ISS	−2.9	Blepharoptosis	Substitution in CNE	None	ECR1
38	M	16.8	ISS	−2.6	None	Substitution in CNE	None	ECR1
39	F	N.D.	ISS	N.D.	None	Substitution in CNE	None	CNE9/ECS4

Abbreviations: CNE, highly conserved non-coding DNA elements; CV, cubitus valgus; ECR, evolutionarily conserved region; ECS, evolutionarily conserved sequence; F, female; ISS, idiopathic short stature; LWD, Leri-Weill dyschondrosteosis; M male; N.D., no data.
[a]Case 26 carried two nucleotide alterations in CNE5.
[b]Case 27 carried three nucleotide alterations in CNE5.

abnormalities in seven LWD patients provides further evidence for the allelic heterogeneity of LWD. As mutations in *NPR2*, a causative gene of Maroteaux-type acromesomelic dysplasia, have recently been identified in patients with LWD-compatible clinical features,[22] such mutations may be present in some of our patients with normal *SHOX*.

Submicroscopic CNVs in PAR1 were identified in cases 1–16. Our results highlight the importance of PAR1-linked CNVs as a cause of *SHOX* haploinsufficiency. Furthermore, the results for case 9 support the hypothesis that a hitherto unidentified *SHOX* enhancer resides within the ~500 kb region ~300 kb downstream of the exons.[23,24] Likewise, the results of case 16 are consistent with the recently proposed notion that the duplications in the downstream region of the known CNEs can underlie ISS and LWD.[25] The high frequency and heterogeneity of CNVs in cases 1–16 may reflect the genomic instability of PAR1; the recombination rate of PAR1 during male meiosis is ~17-times higher than the genomic average.[26] Indeed, the breakpoints of cases 2, 3, 5, 12 and 14 were located close to or within repetitive sequences, which provide substrates for non-allelic homologous recombination.[27] While the breakpoints of cases 1–16 were widely distributed in PAR1 and relatively frequent in the genomic regions adjacent to *SHOX*, this is consistent with the occurrence of male-specific crossover throughout PAR1 with hotspots in the ~0.8 Mb genomic interval around *SHOX*.[26]

Probably damaging intragenic mutations were detected in cases 17–20. These data confirm the results of the European studies that the pathogenic point mutations in *SHOX* are widely distributed in the coding region and relatively common in the homeobox.[2,28] On the other hand, the pathogenicity of four benign/silent substitutions in *SHOX* exons and ten substitutions in the CNEs remains unknown. As three of the ten substitutions in the CNEs resided within putative enhancer sequences, these substitutions may disturb *SHOX* expression. However, most of the affected nucleotides were not highly conserved among species, raising a question of the functional significance of the substitutions. Furthermore, most of these substitutions were shared by control individuals. Our data imply that the point mutations in the known CNEs account for only a minor fraction of the etiology of *SHOX* haploinsufficiency, if any. As additional *SHOX* enhancers are likely to reside in PAR1,[23,24] sequence analysis of these novel enhancer regions is needed.

In summary, our results indicate that the frequency and mutation spectrum of *SHOX* abnormalities are comparable between Asian and European patients, with the exception of a European-specific downstream deletion. Furthermore, this study highlights the clinical significance and genetic heterogeneity of PAR1-linked CNVs, and implies a limited role of CNE-linked mutations in the etiology of ISS and LWD.

CONFLICT OF INTEREST

Maki Fukami has received a research grant from JCR pharmaceuticals. The remaining authors declare no conflict of interest.

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