DNA Methylation Status of *SHOX*-Flanking CpG Islands in Healthy Individuals and Short Stature Patients with Pseudoautosomal Copy Number Variations

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Keywords

Copy number variation · CpG island · DNA methylation · Idiopathic short stature · Leri-Weill dyschondrosteosis

Abstract

SHOX resides in the short arm pseudoautosomal region (PAR1) of the sex chromosomes and escapes X inactivation. *SHOX* haploinsufficiency underlies idiopathic short stature (ISS) and Leri-Weill dyschondrosteosis (LWD). A substantial percentage of cases with *SHOX* haploinsufficiency arise from pseudoautosomal copy number variations (CNVs) involving putative enhancer regions of *SHOX*. Our previous study using peripheral blood samples showed that some CpG dinu-

cleotides adjacent to *SHOX* exon 1 were hypomethylated in a healthy woman and methylated in a woman with gross X chromosomal rearrangements. However, it remains unknown whether submicroscopic pseudoautosomal CNVs cause aberrant DNA methylation of *SHOX*-flanking CpG islands. In this study, we examined the DNA methylation status of *SHOX*-flanking CpG islands in 50 healthy individuals and 10 ISS/LWD patients with pseudoautosomal CNVs. In silico analysis detected 3 CpG islands within the 20-kb region from the translation start site of *SHOX*. Pyrosequencing and bisulfite sequencing of genomic DNA samples revealed that these CpG islands were barely methylated in peripheral blood cells and cultured chondrocytes of healthy individuals, as well as in peripheral blood cells of ISS/LWD patients

with pseudoautosomal CNVs. These results, in conjunction with our previous findings, indicate that the DNA methylation status of *SHOX*-flanking CpG islands can be affected by gross X-chromosomal abnormalities, but not by submicroscopic CNVs in PAR1. Such CNVs likely disturb *SHOX* expression through DNA methylation-independent mechanisms, which need to be determined in future studies.

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SHOX (NM_000451.3) is a growth-controlling gene located in the short arm pseudoautosomal region (PAR1) of the X and Y chromosomes [Rao et al., 1997]. *SHOX* escapes X chromosome inactivation (XCI) and exhibits biallelic expression in both males and females [Carrel and Willard, 2005]. *SHOX* haploinsufficiency accounts for 1.9–22.2% of idiopathic short stature (ISS; OMIM 300582) and 33.9–100% of Leri-Weill dyschondrosteosis (LWD; OMIM 127300) [Schneider et al., 2005; Funari et al., 2010; Benito-Sanz et al., 2012; Rosilio et al., 2012; Poggi et al., 2015]. The most common genetic cause of *SHOX* haploinsufficiency is copy number variations (CNVs) involving *SHOX* and/or its putative enhancer regions [Benito-Sanz et al., 2006; Chen et al., 2009; Rosilio et al., 2012]. To date, 7 highly conserved noncoding DNA elements (CNEs) with *cis*-regulatory activity have been reported as putative enhancer regions of *SHOX* [Fukami et al., 2006; Chen et al., 2009; Durand et al., 2010; Verdin et al., 2015]. Furthermore, since various pseudoautosomal CNVs outside of known CNEs were identified in ISS/LWD patients [Bunyan et al., 2014; Sandoval et al., 2014; Fukami et al., 2015], additional *SHOX* enhancers may reside in PAR1. Submicroscopic CNVs involving known CNEs lead to a phenotype similar to that of intragenic mutations [Flanagan et al., 2002; Benito-Sanz et al., 2012; Verdin et al., 2015], indicating that copy number changes of the enhancers can completely abolish *SHOX* expression. Indeed, Flanagan et al. [2002] documented monoallelic expression of *SHOX* in skeletal tissues of a patient with a *SHOX* downstream deletion.

CpG islands often reflect transcriptional levels of adjacent genes [Deaton and Bird, 2011]. Typically, genes escaping XCI are coupled with hypomethylated CpG islands in genomic DNA samples of both sexes, while genes subjected to XCI frequently exhibit substantial methylation of nearby CpG islands exclusively in female samples [Plath et al., 2002]. Since *SHOX* is an XCI escapee gene [Carrel and Willard, 2005], it may also be accompanied by hypomethylated CpG islands. Indeed, we have previously shown that 12 CpG dinucleotides adjacent to *SHOX*

exon 1 were hypomethylated in peripheral blood cells of a healthy woman [Suzuki et al., 2016]. Furthermore, in that study, we found that the same CpG dinucleotides were markedly methylated in peripheral blood cells of a woman with short stature and catastrophic X chromosomal rearrangements with a 46,X,der(X)(pter→p22.31::q22.1→q10::q10→q22.1::p22.31→pter) karyotype. These findings provided the first indication that X-chromosomal rearrangements can affect DNA methylation of *SHOX*-flanking CpG islands, which are otherwise hypomethylated. However, because only a small number of CpG dinucleotides in only 2 individuals were examined in that study, the results need to be validated. Moreover, our data raised an important question as to whether pseudoautosomal CNVs lead to ISS/LWD by causing abnormal DNA methylation of the CpG islands. In the present study, we analyzed the DNA methylation status in several healthy individuals and patients with ISS/LWD and submicroscopic CNVs in PAR1.

Materials and Methods

Identification of *SHOX*-Flanking CpG Islands

We performed *in silico* analysis to identify CpG islands in the *SHOX*-flanking region. A 20-kb genomic interval around the translation start site of *SHOX* (chrX:611,501–631,500) was analyzed using the default settings of the UCSC Genome Browser (www.genome.ucsc.edu/; hg38, build 38).

Clinical Samples

Peripheral blood samples were obtained from 9 healthy adults with normal height (4 males and 5 females) and 40 children without apparent short stature (20 boys and 20 girls). We also examined a genomic DNA sample obtained from cultured chondrocytes established from a surgical specimen of a boy with polydactyly [Nasu et al., 2015].

Furthermore, we obtained DNA samples from 10 patients with ISS or LWD and submicroscopic CNVs in PAR1 (Table 1). CNVs in these patients were detected by multiplex ligation-dependent amplification (SALSA P018-G1, MRC-Holland, Netherlands) or comparative genomic hybridization using a custom-made array (8 × 60 k format, Agilent Technologies, Santa Clara, CA, USA). CNVs in patients 1–5 encompassed 1 or more of the known CNEs, while those in patients 6–10 involved genomic regions outside of the CNEs (Fig. 1). Seven of the 10 patients were identified through our previous analysis of 328 patients with ISS or LWD [Shima et al., 2016].

DNA Methylation Analysis of the *SHOX*-Flanking CpG Islands

We examined the DNA methylation status of 3 *SHOX*-flanking CpG islands (CpG islands 1, 2, and 3). Genomic DNA samples were treated with bisulfite using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine, CA, USA). From each CpG island, a DNA fragment containing 6 or more CpG dinucleotides was PCR-

Fig. 1. Genomic position of copy number variations in 10 patients. The purple and gray bars indicate *SHOX* exons and CpG islands, respectively. The yellow and blue bars denote the putative upstream and downstream enhancer regions, respectively. The green arrows indicate deleted regions, while the red arrows denote amplified regions. pter, short arm telomere.

Table 1. Clinical and molecular findings of patients with pseudoautosomal CNVs

Patient	Sex	Age, years	Height, SDS	Skeletal deformity	CNV type	Size of CNV ^a , kb	Genomic position of CNV ^a	Affected <i>SHOX</i> enhancer ^b	Previous report ^c
1	M	4.0	-5.0	None	Deletion	128	428,003–556,839	CNE-5, CNE-3, CNE-2	Case 4
2	F	4.0	-2.5	None	Deletion	121	660,411–782,200	CNE4	Case 6
3	F	11.0	-2.4	None	Deletion	35	751,623–787,295	CNE4	Case 8
4	M	16.0	-3.0	Madelung	Deletion	920	682,821–1,603,243	CNE4, CNE5, ECR1, ECS4/CNE9	Case 7
5	F	4.7	-2.5	None	Duplication	517	838,550–1,355,761	ECS4/CNE9	Case 15
6	F	11.2	-1.4	Madelung	Deletion	462	884,358–1,347,158	None	Case 9
7	F	6.8	-3.3	None	Duplication	391	14,893–406,338	None	
8	F	7.2	-3.3	None	Duplication	23	894,185–918,040	None	
9	M	6.5	-2.0	None	Duplication	376	928,644–1,305,631	None	Case 16
10	F	13.0	-4.0	None	Duplication	171	1,592,201–1,763,328	None	

CNE, highly conserved noncoding DNA elements; CNV, copy number variation; ECR, evolutionarily conserved region; ECS, evolutionarily conserved sequence; F, female; M, male; SDS, standard deviation score.

^a Minimal size of each CNV estimated from the results of array-based comparative genomic hybridization (hg38, build 38).

^b Genomic position: CNE-5, chrX:437,622–438,171; CNE-3, chrX:499,544–499,929; CNE-2, chrX:555,875–556,494; CNE4, chrX:753,350–754,005; CNE5, chrX:790,090–791,115; ECR1, chrX:819,845–820,500; and ECS4/CNE9, chrX:874,011–874,813.

^c Previous report: Shima et al. [2016].

amplified (primer sequences are available upon request). The PCR products were subjected to pyrosequencing on PyroMark Q24 (QIAGEN, Valencia, CA, USA). We calculated the methylation index of each CpG dinucleotide by the PyroMark Q24 analysis software.

To confirm the results of pyrosequencing, we analyzed the methylation status of additional CpG dinucleotides in CpG island

1 by bisulfite sequencing. PCR products containing 12 CpG dinucleotides were subcloned using the TOPO TA Cloning Kit (Life Technologies, Carlsbad, CA, USA) and subjected to direct sequencing. The methods were reported previously [Suzuki et al., 2016]. The results of the 10 patients were compared with those of 2 healthy children. We also analyzed a sample obtained from chondrocytes of a healthy boy.

Fig. 2. Genomic structure of *SHOX* and its flanking CpG islands. The white and black boxes indicate untranslated and translated *SHOX* exons, respectively. Three CpG islands (CpG islands 1, 2, and 3; shown as gray boxes) are identified within a 20-kb region around the transcription start site of *SHOX*. CpG dinucleotides in each island are underlined. Red and blue lines depict the target re-

gions of pyrosequencing and bisulfite sequencing analyses, respectively. Analyzed cytosines are shown in red. Of these, 2 cytosines in CpG island 1 (shown in parentheses) are not included in the results, because of the poor reproducibility of the methylation analysis. The numbers indicate the genomic distance from pter (hg38, build 38). Tel, short arm telomere; Cen, centromere.

Results

Identification of *SHOX*-Flanking CpG Islands

Three CpG islands were identified in the *SHOX*-flanking region (CpG island 1 at chrX:619,008–620,527; CpG island 2 at chrX:623,829–624,591; and CpG island 3 at chrX:629,695–631,462) (Fig. 2). These islands were 763–1,768 bp in size and encompassed 66–163 CpG dinucleotides.

DNA Methylation Analysis of the *SHOX*-Flanking CpG Islands

We analyzed the DNA methylation indexes of 6 or 7 CpG dinucleotides from each CpG island (Fig. 3). CpG islands 1, 2, and 3 were hypomethylated in blood samples of control adults and children. Likewise, these islands were barely methylated in cultured chondrocytes. Methylation indexes of the chondrocytes were almost comparable to or slightly lower than those of blood samples in most CpG dinucleotides examined.

Furthermore, CpG islands 1, 2, and 3 were hypomethylated in patients 1–10 with pseudoautosomal CNVs. Actually, methylation indexes of all tested CpG dinucleo-

tides in patients 1–10 completely overlapped with those of control individuals.

The results of pyrosequencing were confirmed by bisulfite sequencing in CpG island 1 (Fig. 4). The 12 CpG dinucleotides were invariably hypomethylated in blood cell-derived genomic DNA samples obtained from healthy individuals and patients 1–10, as well as in the chondrocyte-derived sample obtained from a healthy boy.

Discussion

We identified 3 CpG islands around *SHOX*. Pyrosequencing and bisulfite sequencing of DNA samples revealed that these CpG islands were barely methylated in peripheral blood cells and cultured chondrocytes of healthy individuals. These results are consistent with the current understanding that *SHOX* escapes XCI [Carrel and Willard, 2005; Berletch et al., 2011]. Notably, DNA methylation indexes of blood cells and chondrocytes mostly overlapped, except for some CpG dinucleotides for which chondrocytes showed somewhat lower indexes

Fig. 3. Results of pyrosequencing. C1–C7 indicate the analyzed cytosines in CpG island 1 (upper panel), CpG island 2 (middle panel), and CpG island 3 (lower panel). Two cytosines in CpG island 1 (C4 and C5) are not included in the results because of the

poor reproducibility of the methylation analysis. White, striped, and gray circles indicate healthy adults, healthy children, and chondrocytes, respectively. Black circles depict short stature patients with pseudoautosomal copy number variations.

than blood cells did (Fig. 3, 4). It is known that *SHOX* is strongly expressed in osteogenic tissues and weakly expressed in most other tissues [Clement-Jones et al., 2000]. Similar methylation patterns in peripheral blood cells and chondrocytes suggest that tissue-specific expression of *SHOX* is not dependent on the DNA methylation status of adjacent CpG islands.

Furthermore, methylation indexes of *SHOX*-flanking CpG islands were comparable between unaffected individuals and patients with ISS/LWD and pseudoautosomal CNVs. It is worth mentioning that our subjects included patients 1–5 with CNE-containing CNVs. In particular, patient 4 carried a deletion downstream of *SHOX*, which is predicted to abolish *SHOX* expression [Flanagan et al., 2002]. The results of the present study indicate that copy number changes of *SHOX* enhancers do not alter the DNA methylation status of *SHOX*-flanking CpG islands. Thus, pseudoautosomal CNVs may cause *SHOX* haploinsufficiency by some other mechanisms, such as defective binding of transcription factors to *SHOX* enhancers

or perturbed physical interaction between the *SHOX* promoter and enhancers.

In our previous study, we detected marked hypermethylation of several CpG dinucleotides in CpG island 1 in peripheral blood cells of a woman with gross X-chromosomal rearrangements [Suzuki et al., 2016]. The woman carried a 48-Mb duplication involving *SHOX* and the X inactivation-specific transcript (*XIST*) and other genes, and a 107-Mb deletion involving several genes. Increased DNA methylation in this woman may reflect abnormal XCI for the entire X chromosome, because she had an additional copy of *XIST* [Deaton and Bird, 2011]. Alternatively, the decreased physical distance between *SHOX*-flanking CpG islands and *XIST* may have induced aberrant methylation of these islands. Further analyses of women with various types of X-chromosomal rearrangements will clarify the factors that affect the DNA methylation status of *SHOX*-flanking CpG islands.

Fig. 4. Results of bisulfite sequencing. Twelve CpG dinucleotides in CpG island 1 were examined by Sanger sequencing. The results of genomic DNA samples obtained from blood cells of 2 healthy children and patients 1–10 and a DNA sample from chondrocytes of a healthy boy are shown.

In conclusion, the present study, in conjunction with our previous work, indicates that CpG islands in the *SHOX*-flanking region remain hypomethylated in healthy individuals as well as in ISS/LWD patients with pseudo-autosomal CNVs, but can be methylated in women with extensive X-chromosomal rearrangements. Copy number changes of *SHOX* enhancers likely disturb *SHOX* expression through DNA methylation-independent mechanisms, which need to be determined in future studies.

Statement of Ethics

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.

Disclosure Statement

The authors declare no conflicts of interest.

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