very-long-range enhancer(s) of *SOX9* causes isolated PRS. However, there is no other report of patients with a molecular defect in the far upstream or the far downstream region of *SOX9*. Here, we report on a male patient with a complex genomic rearrangement in the 5′ region of *SOX9*. Clinical and molecular analyses of this patient provide further information on tissue-specific regulation of *SOX9*.

CLINICAL REPORT

This Japanese male patient was born at 38 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, his length was 48.0 cm (-0.48 SD), weight 2.83 kg (-0.55 SD) and head circumference 32.0 cm (± 0 SD). Immediately after birth, he was referred to our clinic because of respiratory distress and facial anomalies. He had hypoplastic mandible, cleft palate and glossoptosis and was therefore diagnosed as having PRS. In addition, he showed bilateral clubfeet. Campomelia and tibial skin dimples were not observed. He manifested normal male external genitalia with bilateral descended testes. On skeletal survey, dolichocephaly with hypoplasia of the facial bone, micrognathia and hypoplasia of the left scapula were evident (Fig. 1). The right scapula was unremarkable. The ischia appeared somewhat broad, and the ischiopubic synchondroses wide; yet, these findings were too mild to be distinguishable from the normal range. Other radiological hallmarks in campomelic dysplasia, such as cervical kyphosis, hypoplastic pedicles of the thoracic spine and narrow ilia, were not discernible. G-banding chromosome analysis showed a normal 46,XY karyotype. Direct sequence analysis for *SOX9* detected no mutation in the coding region [Wada et al., 2009].

During several months after birth, he continually required medical intervention for respiratory and feeding difficulties. He underwent a tracheotomy at 8 months of age. He showed no obvious developmental delay; he was able to stand and walk along the wall at 1 year of age and was able to indicate his desires and needs by pointing at 1 year and 7 months of age. On his last examination at 1 year and 7 months of age, he measured 76.3 cm (-1.77 SD) and weighed 9.2 Kg (-1.31 SD). His parents and sister were clinically normal.

MOLECULAR ANALYSES

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development. After obtaining written informed consent from the parents, a peripheral blood sample was taken from the patient. Parental samples were not available for molecular analysis.

High-resolution chromosomal banding revealed a karyotype of 46,XY,der(17)inv(17)(q21.31q24.3)del(17)(q24.3q23?) (Fig. 2A). Fluorescence *in situ* hybridization (FISH) analysis using RP11-84E24-BAC containing *SOX9* and RP11-20N01-BAC on 17q21.31 indicated a paracentric inversion on one of the two chromosome 17 (Fig 2B and 2C). Signals for *SOX9* were detected on two chromosome 17. Comparative genomic hybridization (CGH) analysis using a human genome oligoarray (1x1 M format, G4447A, Agilent Technologies Palo Alto, CA) indicated a heterozygous deletion in the *SOX9* upstream region (Fig. 3A). *In silico* analysis using UCSC genome browser (http://genome.ucsc.edu/; hg 19; NCBI Build 37) showed that the deletion was 2.99 Mb in physical length and flanked by the proximal and the distal breakpoints residing at -4.15 Mb and -1.16 Mb to *SOX9*, respectively. A total of 18 known genes were located within the deleted region, as assessed using the Refseq database (Fig. 3A).

DISCUSSION

A complex genomic rearrangement in the 5′ region of *SOX9* was identified in a male patient with PRS. The genomic lesion was started at a point 1.16 Mb upstream of *SOX9* and affected several HCNEs. In particular, HCNE-F2, previously shown to act as a developmental enhancer for the craniofacial region [Benko et al., 2009], was deleted in this patient (Fig. 3B). Thus, the PRS phenotype of this patient would be ascribed to *SOX9* misexpression due to loss of HCNE-F2, although we cannot rule out the possibility of another hitherto unidentified cis-regulatory element(s) of *SOX9* being affected by the deletion/inversion. In this regard, while

the deletion has removed 18 genes, clinical features of the patient can be explained by *SOX9* dysfunction alone. Moreover, none of the 18 genes, except for *KCNJ2,* are known to be involved in mandibular growth. Furthermore, whereas dominant negative mutations of human *KCNJ2* as well as homozygous deletion of mouse *KcnJ2* have been shown to result in cleft palate and micrognathia [Andelfinger et al., 2002; Zaritsky et al., 2000], haploinsufficiency of *KCNJ2/KcnJ2* has not been shown to cause such abnormalities. Hence, the patient represents a novel case with PRS caused by a *SOX9* cis-regulatory mutation. Such submicroscopic genomic rearrangements may also be present in other patients with isolated PRS. Indeed, only a few genes have been identified as causative genes for isolated PRS. In this regard, it is noteworthy that mutations of collagen genes including *COL11A2* and *COL11A1* were shown to cause a PRS-like phenotype, i.e., Stickler syndrome without apparent ocular involvement [Vikkula et al., 1995; Annunen et al., 1999]. Since collagen genes are known to be direct targets of SOX9 [Gordon et al., 2009], these data suggest that transactivation of collagen genes by SOX9 is critical for the development of the mandible.

This patient manifested PRS-compatible craniofacial abnormalities and mild hypoplasia of the left scapula. Nevertheless, he showed no typical CD/ACD skeletal features. These data indicate that the genomic rearrangement of the patient disrupted at least one enhancer for *SOX9* that plays a critical role in the development of the mandible and a small role in the development of the scapula. In addition, the concept of exclusion mapping implies that tissue-specific enhancers for long bones, pelvic bones and ribs are located within the 1.16 Mb region closest to *SOX9*, because CD/ACD is known to be a fully penetrant phenotype in patients with intragenic mutations of *SOX9* [Meyer et al., 1997]. Consistent with this, previous studies have suggested that putative loci for CD/ACD are located within the 1.0 Mb region from *SOX9* [Gordon et al., 2009]. Nevertheless, the phenotype of this patient could also be explained by assuming that there is a global developmental enhancer(s) of *SOX9* in the region more than 1.16 Mb apart from *SOX9* and that the mandible and the scapula are more sensitive to reduced transcriptional levels of *SOX9* than other skeletal tissues [Gordon et al., 2009]. Indeed, various

skeletal changes of the patient such as clubfeet, borderline broad ischia and relatively wide ischiopubic synchondroses, may be related to mildly impaired *SOX9* expression. In this context, it is noteworthy that CD, ACD and isolated PRS are currently regarded as a continuum of a disorder caused by *SOX9* abnormalities [Gordon et al., 2009]. Thus, this patient may represent an intermediate phenotype between ACD and isolated PRS.

This patient had normal male external genitalia, indicating that the testis-specific enhancer(s) of *SOX9* is preserved in this patient. Consistent with this, previous studies on translocation-positive patients suggested that a testis-specific enhancer(s) is located within the 789 kb region closest to *SOX9* [Gordon et al., 2009]. Moreover, animal studies have identified a testis-specific enhancer immediately upstream of *Sox9* [Sekido et al., 2008]. However, fairly well preserved masculinization of this patient may be ascribed to incomplete penetrance of gonadal dysgenesis in *SOX9* abnormalities, because normal testicular development has been observed in about 25% of 46,XY individuals with a *SOX9* intragenic mutation [Mansour et al., 1995].

To date, various types of cryptic deletions have been identified in patients with PRS (Fig. 3B). Notably, there is no overlapping region of deletion that is shared by all PRS cases, although the deletions of sporadic case 4 and familial case 1 reported by Benko et al. [2009] are located within the deleted region of the patient described herein. These results imply that multiple cis-acting elements around *SOX9* are required for the appropriate development of the mandible. Further analysis in a large cohort of PRS patients would enable us to clarify the precise locations of *SOX9* tissue-specific enhancers. In this regard, array CGH would serve as a powerful tool for screening of such patients, because it can detect various copy number alterations in a chromosomal region of several megabases.

In summary, the present study provides a novel example for long-range cis-regulatory mutations of *SOX9*. Our findings suggest that the genomic region more than 1.16 Mb upstream of *SOX9* includes at least one cis-acting element that regulates *SOX9* expression in the developing mandible, and, to a lesser extent, in the developing scapula. Further studies will

permit the full characterization of the genomic environment involved in tissue-specific regulation of *SOX9*.

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FIGURE LEGENDS

Fig. 1. Roentgenograms of the patient at 2 weeks of age. Mild hypoplasia of left scapula (white arrows) and micrognathia (a white arrowhead) are indicated.

Fig. 2. Chromosomal banding and FISH analysis. **A**: High-resolution chromosomal banding indicating the presence of a deletion and an inversion on the long arm of chromosome 17. cen, centromere. **B**: Representative results of FISH analysis. The arrowheads denote RP11-84E24-BAC containing *SOX9* (green signals); the arrows indicate RP11-20N01-BAC on 17q21.31 (red signals). Two signals of an apparently different distance are present on two chromosome 17, indicating an inversion on one of two chromosome 17. Signals for *SOX9* are normally present on both chromosome 17. **C**: Schematic representation of the genomic rearrangement of the patient.

Fig. 3. Genomic abnormalities around *SOX9*. **A**: Oligoarray CGH analysis in the patient. The black, the red, and the green dots denote signals indicative of the normal, the increased (>+0.5), and the decreased (<–1.0) copy numbers, respectively. The deletion is 2.99 Mb in length and encompasses18 Refseq genes and several highly conserved non-coding elements. The proximal border of the deletion is located at a point 1.16 Mb upstream of *SOX9*. Genomic positions are referred to the Human Genome (February 2009, hg 19; NCBI Build 37). **B**: Schematic representation of genomic lesions and clinical features of present case and previously reported patients [Pop et al., 2004; Velagaleti et al., 2005; Hill-Harfe et al., 2005; Leipoldt et al., 2007; Jakobsen et al., 2007; Lecointre et al. 2009; Ledig et al., 2010; Benko et al., 2010 and White et al., 2011]. The white areas denote monosomic regions and the black areas, the disomic regions. The purple area indicates the inverted region. The blue regions in translocation-positive patients indicate DNA sequences derived from other chromosomes; the approximate location of translocation breakpoint clusters are shown in green, and the number of breakpoints within each cluster is shown in parenthesis. The gray region depicts a dosage-unknown region. The orange

dot denotes a nucleotide substitution. HCNE-F2, the highly conserved non-coding element with enhancer activity reported by Benko et al. [2009]; PRS, Pierre Robin sequence; ACD, acampomelic campomelic dysplasia; GD, gonadal dysgenesis; CD, campomelic dysplasia.