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Development of a Method To Measure DNA Methylation Levels by Using Methyl CpG-Binding Protein and Luciferase-Fused Zinc Finger Protein

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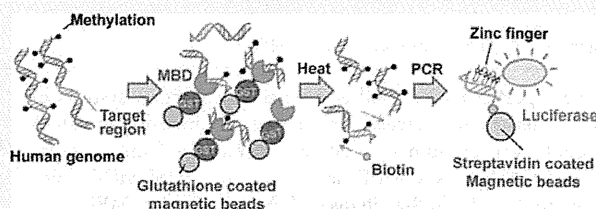
Supporting Information

ABSTRACT: DNA methylation, which is an important epigenetic event for transcriptional regulation, is regarded as a biomarker for cancer. A rapid and sensitive method for measuring DNA methylation levels in target genomic regions may enable early diagnosis of cancer. To detect DNA methylation levels conveniently, we developed a detection system for DNA methylation, designated as methylated DNA precipitation combined luciferase-fused zinc finger assay (MELZA), which uses methyl CpG-binding domain (MBD) and luciferase-fused zinc finger protein. This system comprises the following 3 steps: (1) MBD-based methylated DNA precipitation, (2) PCR amplification of the target genomic region, and (3) detection of the PCR product quantity by using luciferase-fused zinc finger protein. Using this system, we have accurately measured methylation levels of the androgen receptor gene promoter region in LNCaP, PC3, Du145, and whole blood cells. This system does not require bisulfite treatment, and all the steps can be automated. Therefore, it might be useful for measuring DNA methylation levels in clinical cancer diagnoses.



DNA methylation is an epigenetic event that is catalyzed by methyltransferases, and it occurs in mammals at the 5′-position of cytosine in CpG dinucleotides.[1] DNA methylation is important for regulation of gene expression, genomic stability, and cell development.[2,3] Aberrant methylation is detected at the promoter regions of tumor suppressor genes in tumors, and this aberrant methylation pattern is dependent on the type of tumor.[4] Aberrant DNA methylation is regarded as a biomarker in the early diagnosis of cancer, because aberrant methylation occurs only in the early stage of carcinogenesis,[5] and is tissue-specific; furthermore, DNA methylation is more stable than marker proteins or mRNAs.[6] In addition, DNA based-analyses have increased sensitivity when combined with PCR. Therefore, a method to measure the DNA methylation levels in target genomic regions is required for the early diagnosis of cancer.

The sodium-bisulfite sequencing assay has been used widely in DNA methylation analysis;[7] however, this assay requires several hours to convert all the unmethylated cytosine residues in the genome to uracil.[8] To detect DNA methylation levels rapidly, an assay that does not need bisulfite treatment is required. Methylation-sensitive restriction enzyme-based assays can detect DNA methylation without bisulfite treatment;[9] however, this assay cannot target all genomic regions because of limitations in the recognition sequence.[10]

In order to develop a convenient DNA methylation detection system, we focused on MBD, which recognizes methylated CpG in double-stranded DNA (dsDNA) with nanomolar K_d.[11] MBD has been used for methylated DNA precipitation (MeDP)[12,13] or direct detection of methylated DNA.[14] The MBD-based assay does not require bisulfite treatment and can target all genomic regions. Recently, a methyl-CpG (mCpG)-sequence enabled reassembly (mCpG-SEER) assay, which employs a zinc finger protein and MBD, was reported.[15,16] Zinc finger protein is the most popular DNA-binding protein in mammals and is able to bind dsDNA with high affinity and sequence specificity.[17] The assay detects DNA methylation by means of luciferase or GFP complementation; thus, DNA methylation can be detected in a homogeneous assay. On the other hand, in order to detect DNA methylation with high sensitivity, a bound/free separation step is required for signal amplification using PCR.

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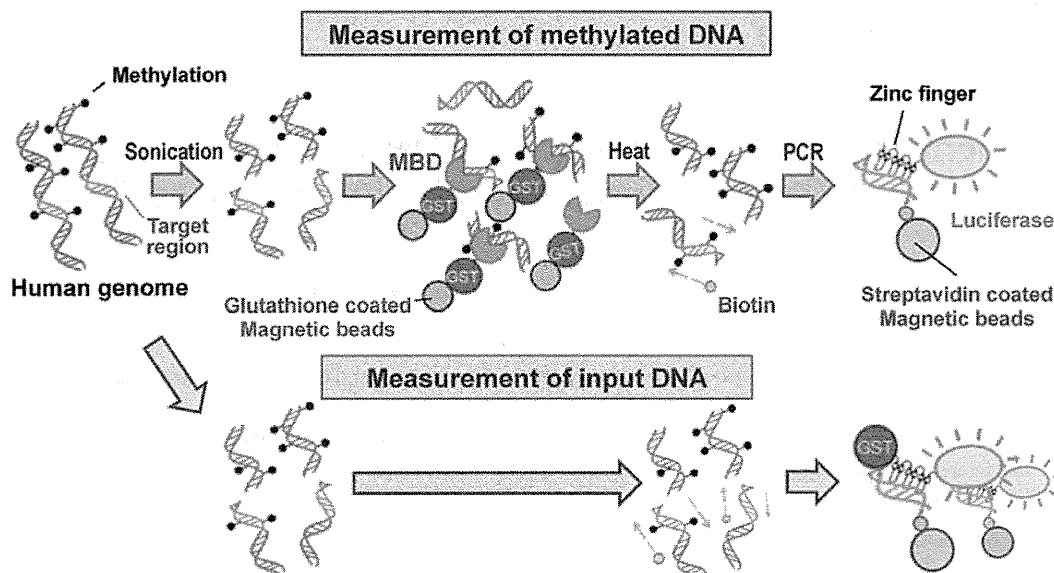

Figure 1. Schematic representation of MELZA.

We report herein a rapid and sensitive detection system for DNA methylation levels, designated as methylated DNA precipitation combined luciferase-fused zinc finger assay (MELZA), that uses MBD and luciferase fused zif268 zinc finger protein (zif268-luciferase). The scheme for MELZA is shown in Figure 1. First, genomic DNA from cells or tissues is sonicated. Next, methylated DNA is captured by MBD immobilized on glutathione-coated magnetic beads via GST. MBD-captured DNAs are then eluted by heating, and a target region, which includes a zinc-finger recognition site, is amplified via PCR using biotinylated primers. Finally, PCR products are immobilized on streptavidin-coated magnetic beads via biotin, and they are then detected using luciferase-fused zinc finger protein. In this system, PCR is used to increase assay sensitivity, and zinc finger recognition is used for more specific selection of the target region. In this study, we have used MELZA to measure DNA methylation levels of the androgen receptor (AR) promoter region in several cell lines and human blood cells.

Methylated DNAs are captured using MBD immobilized on glutathione-coated magnetic beads via GST. The captured DNAs are eluted by heating, and the target region is amplified using PCR with biotinylated primers. PCR products are immobilized on streptavidin-coated magnetic beads. The quantities of subsequent PCR products are detected using luciferase-fused zinc finger protein. In parallel, the same procedure without MBD-based MeDP was carried out. The MeDP sample and input sample were amplified by PCR, and then the amount of PCR product was determined by luciferase-fused zinc finger. The signal ratio of MeDP sample to input sample is defined as the normalized signal, which reflects the DNA methylation levels.

■ EXPERIMENTAL SECTION

Preparation of DNA Binding Proteins. A plasmid expressing GST-tagged MBD, comprising 1−82 amino acids of MBD1 protein (pGEX-2TH-MBD), was constructed as reported in a previous study.[18] We also constructed a plasmid that expresses GST-tagged zif268-luciferase.[19] Briefly, we constructed a plasmid encoding the mouse fusion protein zif268, which is a C_2H_2-type zinc finger protein that recognizes 5′-GCG TGG GCG-3′ and firefly luciferase. The proteins were expressed in *E. coli* BL21 (*DE3*) cells by incubating in an overnight express autoinduction system (Novagen, WI) at 20 °C for 24 h. The expressed proteins were then purified using a GSTrap HP column (GE Healthcare Bio-Sciences, NJ).

Preparation of Genomic DNA. The prostate cancer cell lines LNCaP, PC3, and Du145 were cultured in RPMI 1640 medium (GIBCO, Uxbridge, U.K.) containing 10% fetal bovine serum (FBS, GIBCO, Uxbridge, U.K.), 100 μg/mL streptomycin, and 100 U/mL penicillin (Omega Scientific, CA) at 37 °C in 5% CO_2. Genomic DNA from each cultured cell was extracted using a FastPure DNA Kit (Takara, Shiga, Japan). Whole blood from healthy women and men was collected using heparin-coated syringes, and the genomic DNAs were extracted using an Agencourt Genfind v2 DNA isolation kit (Beckman Coulter, FL).

Sodium Bisulfite Sequencing. Bisulfite treatment of genomic DNAs was carried out using EpiTect Bisulfite Kits (Quiagen, Hilden, Germany). The AR promoter region was amplified via PCR by using 20 ng of bisulfite-treated DNA, EX taq HS (Takara, Shiga, Japan), and AR primer set (Table S1) in a 20-μL reaction volume under the following PCR conditions: 95 °C for 5 min; 35 cycles of 98 °C for 10 s, 56 °C for 30 s, and 72 °C for 30 s. The PCR product was cloned using a pGEM-T Easy vector system (Promega, WI) and transformed into *E. coli* DH5α strain. White colonies were collected, and the plasmid was then amplified using a TempliPhi DNA amplification kit (GE Healthcare, NJ) and sequenced using a BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, CA) with an ABI3100 genetic analyzer (Applied Biosystems, CA). The sequenced data were analyzed using the QUMA web tool (http://quma.cdb.riken.jp/).[20]

MELZA for Synthesized dsDNA. Unmethylated or fully methylated oligonucleotide complementary to 85 bp in the promoter region of the AR gene was synthesized (Takara).

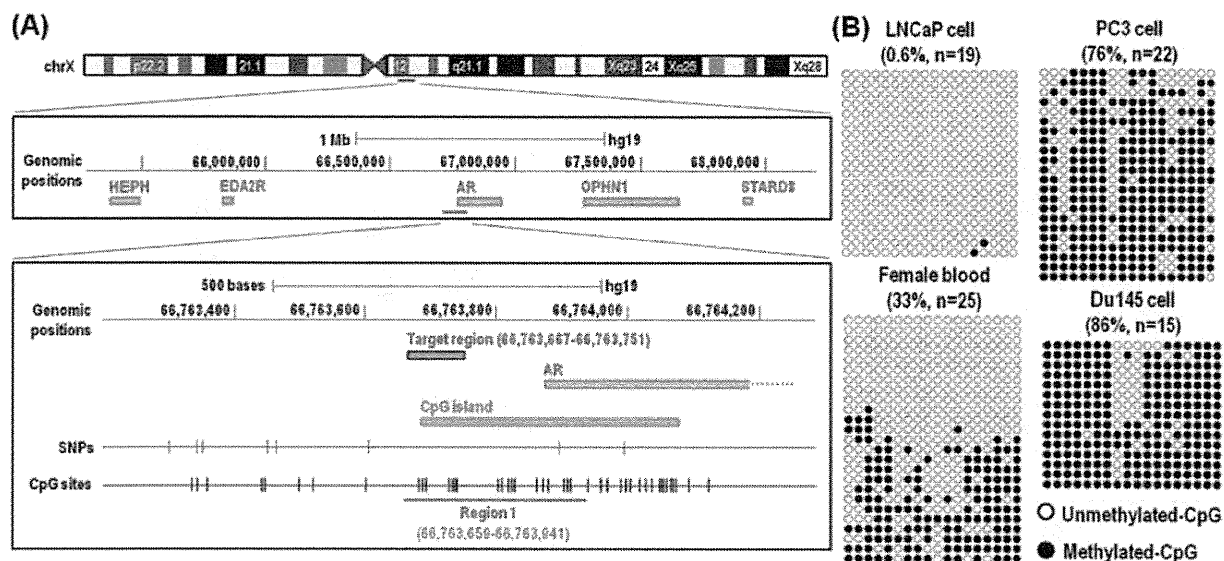

Figure 2. Target genomic region for methylation analysis by MELZA. (A) Map of the proximal AR gene in chromosome X of the human genome. Information with regard to each gene was obtained from the USCS genome browser on human February 2009 (GRCh37/hg19) assembly. The 85-bp target region, which has a zif268 recognition sequence, is amplified using PCR. The methylation level in region 1 was analyzed by bisulfite sequencing. The numbers in the parentheses show the genomic positions of each region. (B) The methylation status of each genomic DNA in region 1 was determined by bisulfite sequencing. The percentages in the parentheses indicate methylation levels. Dots correspond to each of the CpG sites in region 1. The black dots indicate methylated CpG sites, and the white dots indicate unmethylated CpG sites. A set of horizontally aligned dots represents an individual clone. The "*n*" refers to the number of sequenced genomes.

Blocking buffer (200 μL, 20 mM Tris-HCl, 170 mM NaCl, 5 mM EDTA, 2% skim milk, 1 mg/mL tRNA, pH 7.5), including 500 nM GST-MBD, was added to 15 μL of 25% of glutathione-coated magnetic beads (Pierce, IL) to immobilize GST-MBD on the beads. After incubation for 1 h at room temperature (RT), the beads were washed twice in washing buffer (20 mM Tris-HCl, 170 mM NaCl, pH 7.5). To capture methylated DNA, 200 μL of the DNA with various concentrations in binding buffer (20 mM Tris-HCl, 2 mM $MgCl_2$, 0.05% (v/v) tween-20, 400 mM NaCl, pH 7.5) was added to the beads, and the mixture was incubated for 1 h at RT. After washing twice in binding buffer and once in 10 mM Tris-HCl (pH 8.0), 40 μL of DNase free water was added to the beads, and captured DNA was eluted by heating at 95 °C for 20 min. The eluted DNA was used directly as the PCR template, and the PCR amplification reaction was performed using AmpliTaq gold (Applied Biosystems, CA) with an AR primer set (Table S-1) in 80 μL solution under the following conditions: 95 °C for 5 min; 27 cycles at 95 °C for 30 s, 60 °C for 30 s, and 75 °C for 30 s. PCR products (75 μL) were added to 15 μg of streptavidin-coated magnetic beads (JSR, Tokyo, Japan) in 85 μL of zinc buffer (PBS with 90 μM $ZnCl_2$, pH 7.3), and the mixture was then incubated for 15 min at RT. The beads were washed twice in zinc-washing buffer (zinc buffer containing 0.05% (v/v) Tween20), followed by the addition of 160 μL of zinc-blocking buffer (wash buffer, 2% (w/v) skim milk). Next, 1.0×10^7 count per second (CPS) of zif268-luciferase was added, and the mixture was incubated for 45 min at RT. Finally, the beads were washed thrice in zinc-washing buffer and twice in zinc buffer, and then 100 μL of PicaGene (TOYO INK, Tokyo, Japan) was added to the beads in 50 μL of zinc buffer. Luciferase chemiluminescence was measured using a plate reader (PerkinElmer, Kanagawa, Japan) after 1-min incubation at RT.

MELZA for Genomic DNA. To produce random fragments ranging in size from 100 to 500 bp, 1.0×10^6 copies of genomic DNA from LNCaP, PC3, Du145 cells, and whole blood cells of the women were sonicated. The sizes of the fragmented DNAs were confirmed via 3% agarose gel electrophoresis. MBD-based MeDP was carried out with 7.5×10^5 copies of the fragmented genomic DNA, as described above. Luciferase chemiluminescence obtained in this procedure was defined as methylated DNA signal. In parallel, to measure total quantities of genomic DNA, the same procedure without MBD-based MeDP was carried out with 2.5×10^5 copies of fragmented genomic DNA. Luciferase chemiluminescence obtained in this procedure was defined as total-DNA signal. The ratio of methylated DNA signal to total DNA signal is defined as normalized signal, which reflects the DNA methylation levels. To generate a calibration curve, fragmented genomic DNA from LNCaP cells and Du145 cells was mixed in different ratios to prepare samples containing various levels of methylation (ranging from 0.6% to 86%).

RESULTS AND DISCUSSION

Characterization of the GST-Tagged zif268-Luciferase and MBD. GST-tagged zif268-luciferase and GST-tagged MBD were expressed in *E. coli* BL21 (DE3) cells, and they were then purified using a GSTrap HP column. We confirmed that the eluted fraction contained a main band of GST tagged-zif268-luciferase at 98 kDa (Figure S-1A) and a single band of GST tagged-MBD at 37 kDa (Figure S-1B). The luciferase enzymatic activity of zif268-luciferase was 1.8×10^9 CPS/mg. We selected an 85-bp site in the promoter region of the androgen receptor gene (AR) as a target genomic region because it contains a zif268 recognition site and its aberrant methylation has been correlated with hormone-independent prostate cancer[21] (Figure 2A). We determined the DNA

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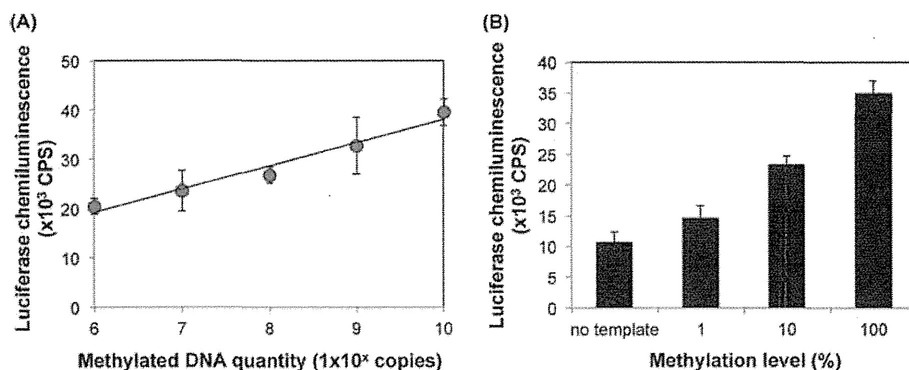

Figure 3. Characterization of MELZA by using synthetic methylated dsDNA. (A) Several copies of methylated dsDNA (1.0×10^6–1.0×10^{10} copies) were captured using MBD, and the target was then amplified using PCR with zif268-luciferase. Each sample was prepared via serial dilution. (B) Several methylation levels of target dsDNAs (1.0×10^{11} copies) were captured by MBD, and then the PCR-amplified target was detected using zif268-luciferase.

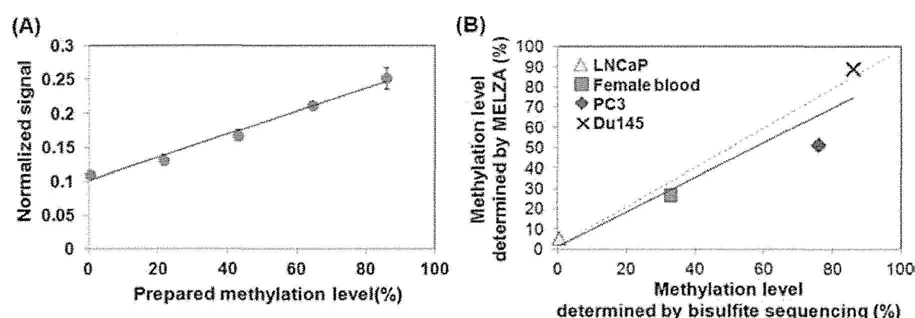

Figure 4. Measurement of the methylation levels at the AR promoter region in the human genome via MELZA. (A) Calibration curve for MELZA showing DNA methylation measurements at the AR promoter. Genomic DNA from LNCaP cells (with a methylation level at the AR promoter of 0.6%) and Du145 cells (methylation level at the AR promoter of 86%) were mixed to prepare several samples having various methylation levels (ranging from 0.6% to 86%). (B) Methylation levels of each genome as determined using MELZA. Methylation levels determined using MELZA or bisulfite sequencing are shown on the *y*-axis and *x*-axis, respectively. The dotted line corresponds to the ideal 1:1 correlation between the two methods.

methylation levels in the AR promoter region of DNA from LNCaP, PC3, Du145, and whole blood cells of the women via bisulfite sequencing. The DNA methylation levels were as follows: LNCaP, 0.6%; PC3, 76%; Du145, 86%; and whole blood cells of women, 33% (Figure 2B). We carried out qPCR to identify the best PCR cycle number for MELZA (Figure S-2). The qPCR results indicated that 26 or 27 PCR cycles showed significant correlation between quantity of template DNA and fluorescence intensity. Thus, we chose 27 PCR cycles to amplify the MeDP sample. To investigate whether the target DNA concentration could be quantified using zif268-luciferase, various concentrations of synthetic target DNA were amplified by 27 cycles of PCR with biotinylated primers, and the resultant PCR product was then immobilized on streptavidin magnetic beads. The beads were then mixed with zif268-luciferase, and the associated luciferase activity was then measured. We confirmed that luciferase activity increased with an increase in the template DNA concentration (Figure S-3). These results suggest that zif268-luciferase has the ability to bind the target PCR product and that the luciferase activity is proportional to the amount of PCR-amplified template DNA. The binding ability of GST tagged-MBD to methylated-dsDNA was confirmed using ELISA (Figure S-4), and the binding signal was obtained when the dsDNA was methylated. These results indicate that GST tagged-MBD recognized methylated DNA specifically.

DNA Methylation Analysis for Synthetic dsDNA by MELZA. To investigate whether the luciferase detection signal for MELZA is dependent on the quantity of methylated DNA, we prepared various quantities of synthetic, fully methylated dsDNA containing the target 85-bp DNA sequence, including 4 CpG sites. We carried out MBD-based MeDP, PCR-amplification, and then quantification of the PCR product by using zif268-luciferase. The results show that luciferase chemiluminescence increased in methylated dsDNA in a quantity-dependent manner, ranging from 1.0×10^6 copies to 1.0×10^{10} copies (Figure 3A). Furthermore, we prepared 1.0×10^{11} copies of samples with different methylation levels and investigated whether the luciferase signal is dependent on the methylation levels. The results show that luciferase chemiluminescence increased in a DNA methylation level-dependent manner (Figure 3B). Overall, these results collectively indicate that the methylation levels of synthetic DNA could be determined using MELZA.

DNA Methylation Analysis of the Human Genome via MELZA. We next tried to measure the DNA methylation levels in the AR promoter region of the human genome by using MELZA. To calibrate the assay, we mixed LNCaP genome and Du145 genome to prepare genomic DNA samples with serial

methylation levels of the AR promoter region. MBD-based MeDP was carried out with the genomic DNAs, and quantities of the PCR-amplified captured DNA or input DNA were detected using zif268-luciferase. The obtained luciferase signal from captured DNA was normalized to the luciferase signal from input DNA, and the calibration curve was obtained using LNCaP and Du145 genomic DNA (Figure 4A). Additionally, we analyzed the methylation levels of AR promoter regions in genomic DNA samples from PC3 cells and whole blood cells from women by using MELZA. We obtained normalized signals for MELZA against each genomic DNA, and the methylation level was calculated using a calibration curve. These methylation levels were then correlated with bisulfite sequencing results (Figure 4B). Although many factors such as MeDP or the PCR process will affect the results of MELZA, there was significant correlation between MeDP efficiency calculated by qPCR and the normalized signal (Figure S-5), indicating that the normalized signal correctly reflects the DNA quantity collected by MeDP. The results demonstrate that the methylation levels of target genomic regions can be correctly determined using MELZA.

Although 1.0×10^{11} copies of synthetic DNA were required for MBD-based MeDP, we were able to determine the methylation levels in 1.0×10^{6} copies of genomic DNA. The differences could be attributed to avidity effects of MBD on methylated CpG. The 85-bp synthetic DNA contains only 4 CpG sites. On the other hand, sonicated genomic DNA contains 8−13 CpG sites in the target AR region because the average size of sonicated DNA was 200 bp. It has been reported that increases in the total number of methylated CpG results in an increase in the MBD-binding affinity.[22] Moreover, there is evidence indicating that multivalent interactions are 1000 times stronger than monovalent interactions.[23] Therefore, differences in sensitivity between synthetic DNA and genomic DNA seem to be attributed to the avidity effects of MBD on multi-methylated CpG sites.

The detection limit of MELZA depends on a methylated DNA precipitation process because the precipitated DNA can be easily amplified using PCR. In this study, PCR conditions were determined using qPCR to amplify 1.0×10^{6} copies of genomic DNA, which is conveniently prepared from 1-mm^2 tumors[24] or 0.2 mL of human whole blood. The dissociation constant of MBD to methylated CpG is several tens in the nanomolar order, and to unmethylated CpG from several micromolar to tens of micromolar.[22] Therefore, under equilibrium conditions, when 500 nM of MBD and 1 copy to 1.0×10^{12} copies of DNA were used, about 90% of methylated dsDNA will form DNA-MBD complexes, and <0.3% of unmethylated dsDNA will form DNA-MBD complex. Therefore, when the elution efficiency is assumed to be 100%, 90% of the methylated DNA will be collected via DNA precipitation by using MBD. However, the actual precipitation efficiency is lower than the ideal value. We determined MBD-based MeDP efficiency at the AR promoter region in 1.0×10^{6} copies of genomic DNA by using qPCR. When the AR promoter was fully methylated, the precipitation efficiency was about 30% (Figure S-6). We calculated that 3.3 copies of genomic DNA would be required to collect 1 copy of the target DNA when the target methylation level is 100%. We thus assumed that 3.3×10^{2} copies of genomic DNA would be required to collect 1 copy of template DNA from a sample with a methylation level of 1%. This suggests that MELZA could detect DNA methylation levels from approximately 3.3×10^{2} copies of genomic DNA. The detection limit for mCpG-SEER is 1.2×10^{11} copies.[15] Therefore, MELZA seems to be a sensitive method for detecting DNA methylation.

The sodium bisulfite method takes at least half a day to measure the DNA methylation level because bisulfite treatment takes several hours. MELZA takes 3.5 h to measure the DNA methylation level, indicating that MELZA is a rapid DNA methylation sensing system. In both methods, PCR is needed to amplify the target genomic region. In the sodium bisulfite method, PCR primers have to comprise only 3 nucleotides (A, T, G) because cytosine is converted to uracil, except for methylated cytosine. Therefore, specific PCR amplification often fails in the sodium bisulfite method. On the other hand, normal PCR primers can be used in MELZA, suggesting that MELZA can measure DNA methylation with high specificity compared to the sodium bisulfite method. The MeDP sample can be amplified with biotin-labeled primer, and the biotinylated PCR product can be detected by streptavidin-fused enzyme. However, the biotin-streptavidin-based sensing system also detects nonspecific PCR products such as primer dimer products. Since the luciferase-fused zinc finger protein can recognize a particular DNA sequence within the PCR product, we used it to specifically detect the PCR product as opposed to using the biotin-streptavidin-based methods.

In MELZA, the target region has to contain a zinc finger recognition site as we reported earlier with regard to specific bacterial detection.[25] Approximately 110 genes contain zif268 recognition sites in the region from 1 kb upstream to 1 kb downstream of the transcriptional start site (TSS) in the human genome. On the other hand, we have developed luciferase-fused sp1 zinc finger protein that recognizes a 5′-GGG GCG GGG-3′ sequence.[19] About 1100 genes contain sp1 recognition sites in the region described above. For example, the zif268 recognition site is found in the same regions as the telomerase reverse transcriptase (TERT) gene, whose methylation in mucous is reported to be a predictive marker for Barrett's esophagus,[26] and the thrombospondin-1 (THBS1) gene, which is a tumor suppressor gene whose down regulation is associated with digestive cancer and displays aberrant methylation in colon cancer cell lines.[27] The sp1 recognition site is found in the same region as the Cadherin-4 (CDH4) gene, whose aberrant methylation in circulating blood cells is associated with gastrointestinal tumors.[28] Therefore, MELZA is useful for the diagnoses of such diseases. We used zif268 to construct a luciferase-fused zinc finger protein because zif268 has been well-characterized. On the other hand, various methods have been developed for the efficient design of artificial zinc finger proteins that recognize a variety of sequences. In particular, modular assembly is a simple method for engineering zinc finger, which regards 1 finger that can recognize a specific 3-bp DNA sequence as a modular unit and designs any zinc finger by combining several modular units.[29−32] A web tool for designing artificial zinc finger proteins by using modular assembly is available[33] so that any target region can be analyzed using MELZA by means of an artificial zinc finger protein.

CONCLUSIONS

We developed MELZA to measure DNA methylation levels without bisulfite treatment by using MBD and luciferase fused zinc finger. We demonstrated that the methylation levels in the AR promoter region can be determined accurately with MELZA. In MELZA, MBD captured methylated DNA can be amplified using PCR, and the PCR product is then detected on

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the basis of the amount of luciferase activity; thus, a highly sensitive assay was achieved. Moreover, the assay can be automated easily because the MELZA assay is based on magnetic beads. Therefore, we believe that MELZA is a suitable method for detecting aberrant DNA methylation in the clinical diagnosis of early stage cancer.

ASSOCIATED CONTENT

Supporting Information

Additional table and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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RESEARCH ARTICLE

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Complex Genomic Rearrangement in the *SOX9* 5′ Region in a Patient With Pierre Robin Sequence and Hypoplastic Left Scapula

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Pierre Robin sequence (PRS) can occur as a component of campomelic dysplasia (CD) and acampomelic CD (ACD) caused by dysfunction or dysregulation of SOX9, although it can also take place as an isolated form. Recently, genomic alterations in the far upstream and the far downstream region of *SOX9* have been identified in patients with isolated PRS. Here, we report on a male patient with PRS and a heterozygous genomic rearrangement in the 5′ region of *SOX9*. Clinical analysis revealed PRS-compatible craniofacial anomalies, mild hypoplasia of the left scapula, and normal male external genitalia. Molecular analysis identified a paracentric inversion on the long arm of chromosome 17 with breakpoints at 17q21.31 and 17q24.3, and a microdeletion spanning from −4.15 to −1.16 Mb relative to *SOX9*. These findings indicate that the chromosomal region more than 1.16 Mb apart from *SOX9* contains at least one developmental enhancer(s) for *SOX9* that plays a critical role in the development of the mandible and a relatively small role in the development of the scapula. Moreover, the concept of exclusion mapping argues that putative CD/ACD loci are located within the 1.16 Mb region closest to *SOX9* coding exons, which remain intact in this Non-CD/ACD patient. This study provides a novel example for long-range *cis*-regulatory mutations of *SOX9*. © 2012 Wiley Periodicals, Inc.

Key words: campomelic dysplasia; deletion; inversion; enhancer; noncoding element

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INTRODUCTION

Pierre Robin sequence (PRS) (OMIM 261800) is a congenital malformation sequence characterized by micrognathia, glossoptosis, and posterior U-shaped cleft palate [Robin, 1934]. The primary defect of PRS is assumed to be mandibular hypoplasia caused by impaired chondrogenesis or aberrant proliferation of neural crest cells [Gordon et al., 2009]. PRS frequently occurs as a component of known syndromes such as campomelic dysplasia (CD) (OMIM 114290), acampomelic CD (ACD), and Stickler syndrome (OMIM 108300), although PRS can also take place as an isolated (nonsyndromic) form [Holder-Espinasse et al., 2001].

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CD and ACD are caused by dysfunction or dysregulation of SOX9; multiple intragenic mutations of *SOX9* as well as various types of chromosomal rearrangements around the coding exons have been identified in patients with CD and ACD [Meyer et al., 1997; Gordon et al., 2009]. In addition to PRS, patients with CD manifest bowing of the long bones (campomelia), hypoplastic scapulae, pelvic malformations, a missing pair of ribs, clubfeet, and 46,XY gonadal dysgenesis. ACD represents a mild variant of CD lacking campomelia. Since PRS is present in most patients with CD and ACD [Gordon et al., 2009], SOX9 likely plays a particularly important role in the development of the mandible.

Recently, molecular defects in the far upstream and the far downstream region of *SOX9* have been identified in patients with isolated PRS. Jamshidi et al. [2004] and Jakobsen et al. [2007] identified balanced translocations of t(2;17) in familial and sporadic PRS cases, respectively, and found that the 17q breakpoints are located more than 1.0 Mb upstream of *SOX9*. Subsequently, Benko et al. [2009] identified variable genomic abnormalities (translocations, deletions, and a nucleotide substitution) at a position more than 1.0 Mb apart from *SOX9* in two sporadic and five familial cases with PRS. Furthermore, Benko et al. [2009] showed that the deletions and translocations included several highly conserved noncoding elements (HCNE) and the nucleotide substitution abolished the tissue-specific enhancer activity of one of these HCNEs (HCNE-F2). These data provide the first evidence that dysfunction of the 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 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.

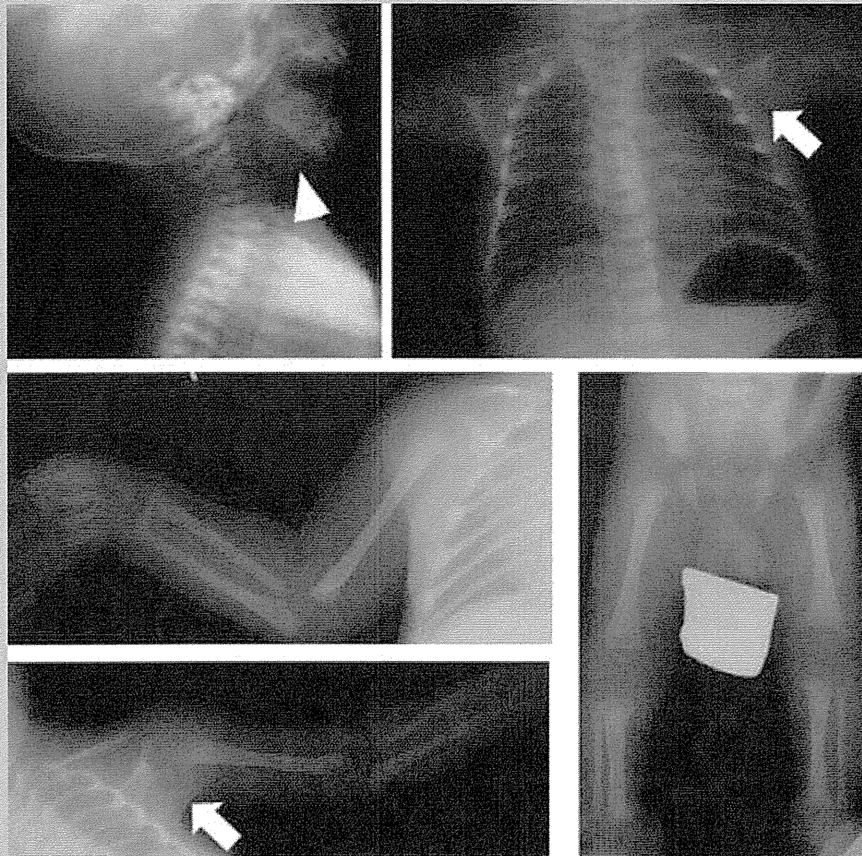

FIG. 1. Roentgenograms of the patient at 2 weeks of age. Mild hypoplasia of left scapula (white arrows) and micrognathia (a white arrowhead) are indicated.

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 CD, 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 C). Signals for *SOX9* were detected on two chromosome 17. Comparative genomic hybridization (CGH) analysis using a human genome oligoarray (1 × 1 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 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 boy with PRS. The genomic lesion 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

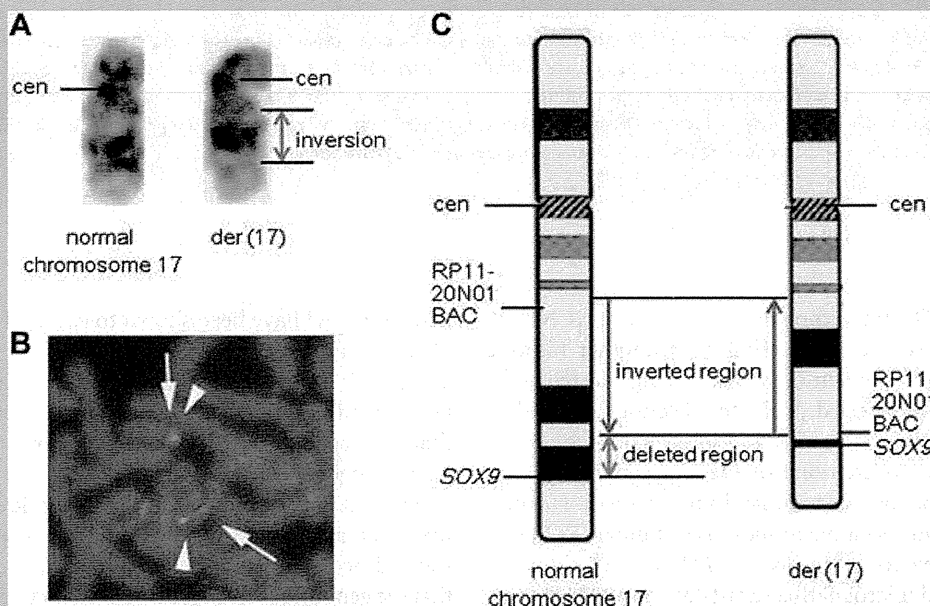

FIG. 2. Chromosomal banding and FISH analysis. A: High-resolution chromosomal banding indicating the presence of a deletion and an inversion on the long arm of chromosome 17. cen, centromere. B: Representative results of FISH analysis. The arrowheads denote RP11-84E24-BAC containing *SOX9* (green signals); 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.

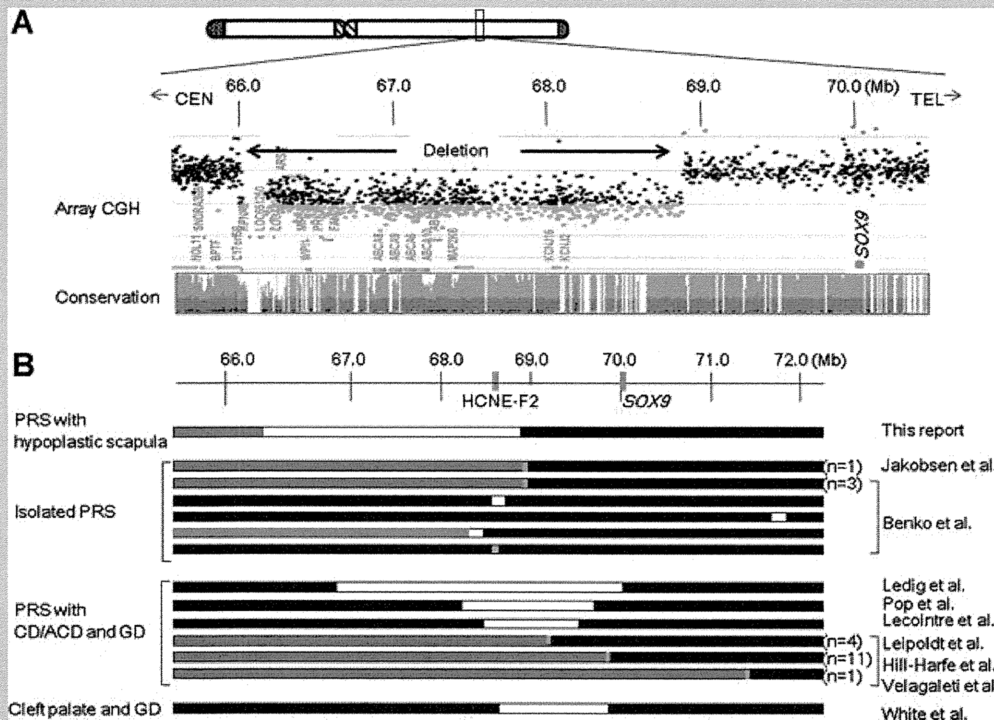

FIG. 3. Genomic abnormalities around *SOX9*. A: Oligoarray CGH analysis in the patient. The black, the red, and the green dots denote signals indicative of the normal, the increased (>+0.5), and the decreased (<−1.0) copy numbers, respectively. The deletion is 2.99 Mb in length and encompasses 18 Refseq genes and several highly conserved noncoding 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; Hill-Harfe et al., 2005; Velagaleti et al., 2005; Jakobsen et al., 2007; Leipoldt et al., 2007; Benko et al., 2009; Lecointre et al., 2009; Ledig et al., 2010; 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 noncoding element with enhancer activity reported by Benko et al. [2009]; PRS, Pierre Robin sequence; ACD, acampomelic campomelic dysplasia; GD, gonadal dysgenesis; CD, campomelic dysplasia.

would be ascribed to *SOX9* misexpression due to loss of HCNE-F2, although we cannot exclude the possibility of another hitherto unidentified *cis*-regulatory element(s) of *SOX9* being affected by the deletion/inversion. In this regard, while the deletion 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 [Zaritsky et al., 2000; Andelfinger et al., 2002], 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* have been shown to cause a PRS as a component of 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 and Lovell-Badge, 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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