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Less Frequent *NSD1*-Intragenic Deletions in Japanese Sotos Syndrome: Analysis of 30 Patients by *NSD1*-Exon Array CGH, Quantitative Fluorescent Duplex PCR, and Fluorescence In Situ Hybridization

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Sotos syndrome (SoS, OMIM #117550) is an autosomal dominant overgrowth syndrome with pre- and postnatal excessive growth, characteristic craniofacial features, and variable degrees of developmental delay. Haploinsufficiency of the nuclear receptor binding SET domain containing protein 1 (*NSD1*) gene causes SoS, as two thirds of SoS patients had either a whole-gene microdeletion or an intragenic point mutation. However, the etiology of other patients remains undetermined. In the present study, we analyzed 30 Japanese SoS patients on whether they have *NSD1* intragenic deletions by *NSD1*-specific exon microarray comparative genomic hybridization (array CGH). Although the analysis suggested a deletion at the 5' region of *NSD1* in 16 of the 30 patients, no such abnormalities were confirmed by subsequent quantitative fluorescent duplex PCR and fluorescence *in situ* hybridization. As no intragenic deletions have been identified in our series of SoS patients, other genetic aberrations need to be identified.

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Introduction

Sotos syndrome (SoS, OMIM #117550) is an overgrowth disorder characterized by excessive growth in childhood, distinctive craniofacial features, including macrocephaly, and mental retardation. SoS is caused by haploinsufficiency of the nuclear receptor binding SET domain containing protein 1 gene (*NSD1*) located at 5q35.2-q35.3.[1] Microdeletions encompassing the entire *NSD1* and intragenic point mutations account for about 50% and 10% of Japanese patients, respectively.[1-3] On the other hand, the point mutations are the main disease-causing abnormalities in non-Japanese patients (50% or more), and microdeletions occur in about 10% of them.[4-10] The reason for the difference between Japanese and non-Japanese patient groups remains unexplained. Partial, intragenic deletions of *NSD1*, comprising a single or multiple exons, were recently found by multiplex ligation-dependent probe amplification (MLPA) method in eight (6 %) of 124 SoS patients from UK.[11] As the etiology of about one third of Japanese SoS patients is undetermined, we hypothesized that intragenic deletions could frequently be associated with Japanese SoS patients. For analysis of such deletions, we developed an *NSD1*-specific exon microarray comparative genome hybridization (array CGH) system.

Here we report the results of analysis of 30 Japanese patients with SoS.

Materials and Methods

Subjects

The research was approved by the Committee for Ethical Issues

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on Human Genome and Gene Analysis at Nagasaki University. The subjects studied included 30 patients (seven females and 23 males) with typical SoS features (including macrocephaly, overgrowth, and mental retardation), in whom a whole-*NSD1* gene microdeletion and *NSD1* point mutation were excluded. Clinical manifestations of these cases were reported elsewhere.[2,3] After obtaining informed consent, DNA was isolated from patients' peripheral blood leukocytes. We also used DNA samples from six other patients with SoS who had been previously diagnosed to have a whole-gene deletion, as positive controls in microarray conperative genomic hybridization (CGH) and quantitative fluorescent duplex PCR experiments.

NSD1-specific exon array CGH

A total of 34 genomic regions were selected from the *NSD1* locus, chromosome X, and from chromosome 13 as elements for *NSD1*-specific exon array CGH: Five from an 18-kb segment upstream of *NSD1*, 23 exons of *NSD1*, and three regions from each of chromosomes X and 13 (Figure 2). Sequences of all these regions are unique according to RepeatMasker program (http://www.repeatmasker.org/), and range from 355 bp to 2765 bp (primer sequences and conditions for elements amplification available on request). PCR products of the regions were cloned into pCR™2.1-TOPO™ vector (Invitrogen, Carlsbad, CA), and used for transformation of DH5α-T1™ (Invitrogen). Insert sequences were confirmed on ABI Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA), and they were then amplified from corresponding plasmids using universal 5'-amino-linked primers (forward: TACCGAGCTCGGATCCACTAGTA; reverse: CAGTGTGATGGATATCTGDCA) by PCR in a volume of 100 μL. PCR products were purified with a membrane filter, Microcon YM-30 (Millipore, Badford, MA), dissolved in 0.25 M phosphate buffer/0.00025% N-lauroyl sacrosine, and spotted in quadruplicate on CodeLink™ aminosilane coated slides (Amersham Bioscience, Piscataway, NJ) with a single pin spotter, Stampman (Nippon Laser and Electronics Laboratory, Nagoya, Japan). The same set of probes was printed twice on one glass slide, forming Block-1 and Block-2. The slides were finally treated as described previously,[12] and stored at room temperature and low humidity until use.

After complete digestion with *Eco*RI, subject DNA (800 ng) was labeled with Cy-3 dCTP (GE Healthcare Bio-Sciences, Piscataway, NJ) and the same amount of reference DNA (with an opposite sex) was labeled with Cy-5 dCTP using Bioprime DNA Labeling Kit (Invitrogen) (Block-1). Dyes were swapped in Block-2 (subject DNA labeled with Cy5 and reference DNA labeled with Cy3) to confirm that signal patterns were opposite. Prehybridization and hybridization procedures were carried out as described previously.[13] Processed arrays were scanned with GenePix 4000B (Axon Instruments, Union City, CA) and analyzed with GenePix Pro 4.0 software (Axon Instruments). Signal intensity ratios between subject and reference DNA were calculated from the data of the single-slide experiment, using the ratio of means formula according to GenePix Pro. 4.0:

$$\text{Ratio Cy5/Cy3} = \frac{\text{mean Cy5 intensity of feature-median Cy5 intensity of the background}}{\text{mean Cy3 intensity of feature-median Cy3 intensity of the background}}$$

Ratios of all array elements were normalized to the average of elements from chromosome 13 as an internal normal control. The signal ratio was regarded as "abnormal," when it was out of ±3 SD (standard deviation) range which was determined from three elements of chromosome 13.

Quantitative fluorescent duplex PCR (QFD-PCR)

With a QFD-PCR method described previously,[14,15] we designed test primers to amplify a 302-bp region "A" that is corresponding to the 5'-5 region (Figure 1). Reverse primer was labeled at its 5' end

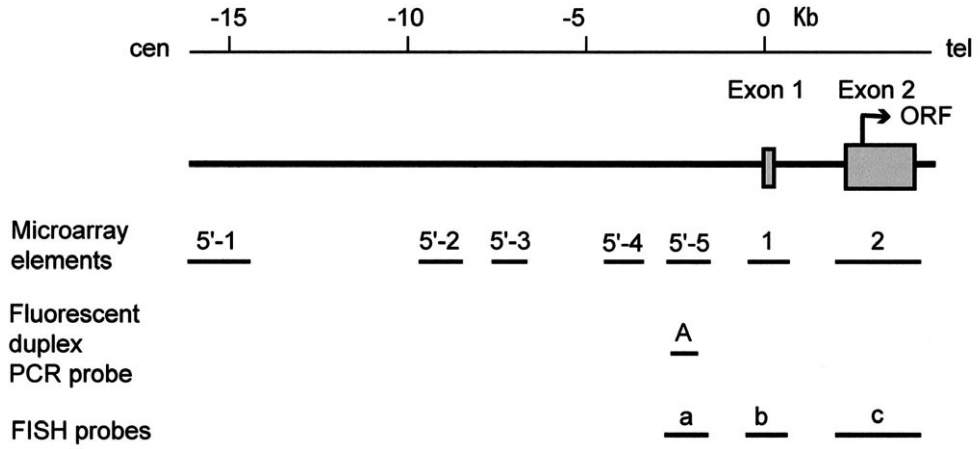

Figure 1. Schematic representation of a 20-kb segment covered by microarray. Exons 1 and 2 of the *NSD1* gene are presented as grey boxes, open reading frame (ORF) of *NSD1* starts from an arrow. Seven elements of the exon array are shown: five (5'-1, 5'-2, 5'-3, 5'-4, and 5'-5) covering the *NSD1* upstream region; two (1, 2) covering exons 1 and 2, and adjacent areas. Region A is a probe for QFD-PCR, and a, b and c are probes for FISH analysis.

with 6-FAM fluorophore (Applied Biosystems). Non-polymorphic STS marker G06854 (330 bp) mapped at 10q25.3 was chosen as an internal control, and control primers were designed according UCSC Genome Bioinformatics web site (http://genome.ucsc.edu). Reverse primer was labeled at its 5' end with HEX fluorophore (Applied Biosystems). Test and control regions were amplified simultaneously in a single tube. Reaction was carried out in a volume of 20 μL, containing 100 ng of template DNA, 1 X ExTaq buffer, 200 μM of each dNTP, 1 U of ExTaq DNA polymerase (Takara, Otsu, Japan), and 0.2 μM of test primers (forward: GTTGAGTCGAATT GCCCAGAT; reverse: ACAGGCCCTTAGCACATGTCT), and 0.3 μM of control primers (forward: AGACAGGGTTGGGAAGG ACT; reverse: CAGGAGAGCCTTGGTGAAAG). After an initial step of denaturation at 95 °C for 3 min, PCR was cycled 27 times at 95 °C for 30 sec, at 62 °C for 30 sec, and at 72 °C for 25 sec, followed by the final extension at 72 °C for 4 min. An aliquot (2 μL) of PCR products was mixed with 16 μL formamide, and the mixture (2 μL) was further combined with 2.5 μL loading buffer containing 0.3 μL of GENESCAN™-500XL size standard (Applied Biosystems). Samples were separated with ABI 377 automated sequencer (Applied Biosystems), and results were analyzed using GeneScan 3.1.2 and Genotyper 2.5 (Applied Biosystems).

We analyzed all but two SoS patients (SoS 151 and SoS 149) whose DNA was used up, six patients confirmed to have a 5q35 whole-gene deletion as positive controls, and six normal controls. To determine an *NSD1* intragenic deletion, we adopted a method described by Yau et al.,[15] and calculated 6 dosage quotients (DQ) for each SoS patient to be examined and each positive control patient using peak heights, according to the following formula:

$$DQ = \frac{\text{test region (patient sample)/control region (patient sample)}}{\text{test region (normal control sample)/control region (normal control sample)}}$$

Average DQ was determined for each subject patient and each positive control patient. Patients were considered as having an intragenic deletion if their average DQ was within the DQ range for deletion that was determined from average DQ of six positive controls ±2 SD.

Fluorescent in situ hybridization (FISH)

FISH was performed on metaphase chromosomes of a SoS patient (SoS 13). Plasmid DNA containing probe a, b, or c was labeled with biotin-16-dUTP (Roche Diagnostics, Mannheim, Germany) by nick translation at 37 °C for two hrs. A BAC clone, RP11-465I17, labeled with SpectrumOrange™-11-dUTP (Vysis, Downers Grove, IL) was used as a control. Probes a, b and c, and the control probe were combined with human Cot-1 and salmon sperm DNAs in a hybridization mixture, denatured at 70 °C for 10 min, applied on the chromosomes, and incubated at 37 °C for 72 hrs. Slides were washed, and solutions containing avidin D FITC, biotinilated anti-avidin D antibody (Vector Laboratories, Burlingame, CA), and again avidin D FITC were serially mounted on slides at 37 °C each for 15 min, and washed again. Finally, slides were mounted with an antifade solution (Vector Laboratories, Burlingame, CA) containing DAPI. Fluorescence photomicroscopy was performed as described previously.[13]

Results

NSD1-specific exon array CGH

We hybridized Cy3-labeled male and Cy5-labeled female control DNAs to the *NSD1*-specific microarray DNA. The Cy5/Cy3 signal ratios of all array elements of the *NSD1* locus as well as of three chromosome-13 elements were within the normal range, indicating no difference in the copy-number between the two controls. The Cy5/Cy3 ratios for three X-chromosome elements were higher than the +3 SD value, clearly reflecting copy-number difference in X chromosome (Figure 2 A). When hybridized with Cy3-labeled DNA from a male patient (SoS 3) who has a confirmed 5q35 whole-gene microdeletion, and with Cy5-labeled female control DNA, the Cy5/Cy3 ratios for the *NSD1* elements and X-chromosome elements were higher than the +3 SD value, the result indicating a deletion of the entire *NSD1* region (Figure 2 B). The Cy5/Cy3 ratios for chromosome-13 elements remained within the normal range, the result corresponding to normal choromosome-13 karyotype in both DNA samples.

Array CGH for DNA from the 30 patients without a whole-*NSD1* gene microdeletion or an *NSD1* intragenic mutation showed two patterns: A pattern of the normal ratio for all elements at the entire *NSD1* locus in 14 patients (Figure 2 C), and a possible pattern of deletion involving the 5' upstream region (elements 5'-1, 5'-2, 5'-3, 5'-4, and 5'-5) and exons 1 and 2 of *NSD1* in 16 patients (Figure 2 D).

QFD-PCR and FISH

To confirm the suspected copy-number changes of the 5' region of *NSD1* in 16 patients, QFD-PCR was performed (Figure 1). The region "A" (about 1.8 kb upstream of exon 1) was chosen for the analysis, because it showed high ratios in the microarray CGH analysis in all the 16 patients. Dosage ratios of the region "A" to the control region were evaluated in 28 patients (two patients were not evaluated as DNA was used up), six normal controls, and six other patients with confirmed 5q35 whole-gene microdeletions as positive controls. The DQ range for deletion determined from DQ values among the six positive controls was 0.16- 0.50 with the mean (SD) of 0.33 (0.08). In all but one patient (SoS13) examined, DQ was higher than 0.50 (Figure 3). These findings indicated that 15 of the 16 patients are unlikely to possess a deletion. FISH using combined probes a, b and c (Figure 1) was performed on metaphase chromosomes of SoS 13. Signal was observed on the long arm of both chromosomes 5 (Figure 4), indicating that SoS 13 did not have a deletion.

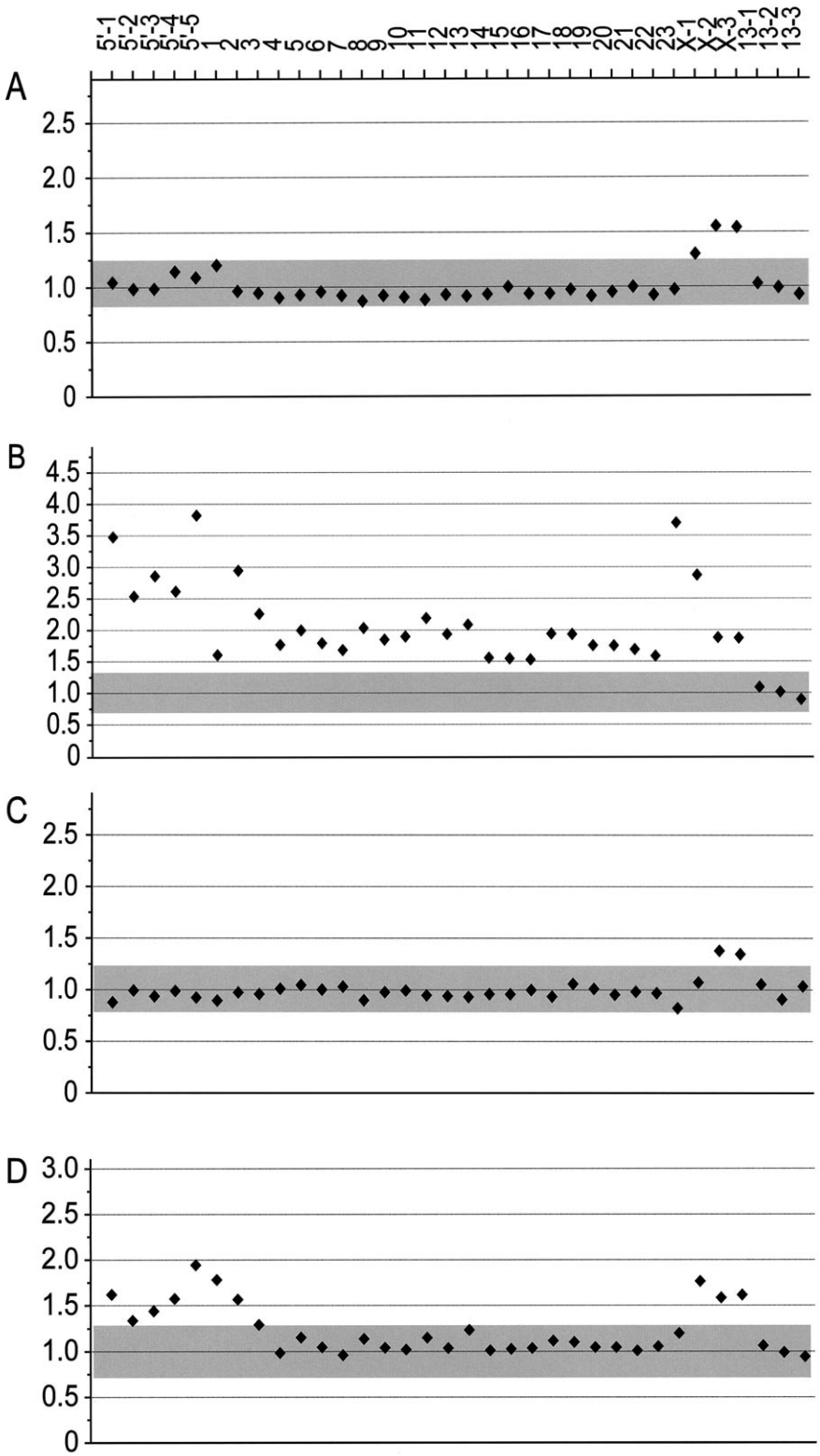

Figure 2. *NSD1* exon array CGH. The Cy5/Cy3 ratios are indicated on the y-axis. Range of ±3 SD is represented as gray area. **A.** Hybridization of normal male control DNA (Cy3) and normal female control DNA (Cy5). Note that the Cy5/Cy3 ratios of three elements of chromosome X are higher than the +3 SD value. **B.** Hybridization of SoS3 (with confirmed *NSD1* deletion) DNA (Cy3) and normal female control DNA (Cy5). Note that the Cy5/Cy3 ratios for all array elements except those of chromosome 13 are higher than the +3 SD value. **C.** Hybridization of SoS30 DNA (Cy5) and normal male DNA (Cy3). This is an example of a "normal" pattern seen in 14 out of 30 SoS patients. **D.** Hybridization of SoS13 DNA (Cy3) and normal female control DNA (Cy5). This is an example of a possible deletion pattern observed in 16 out of 30 SoS patients. Note that the Cy5/Cy3 ratios of 5'-1, 5'-2, 5'-3, 5'-4, 5'-5, 1, and 2 elements are higher than the +3 SD value, suggesting an abnormality at the 5' region of *NSD1*.

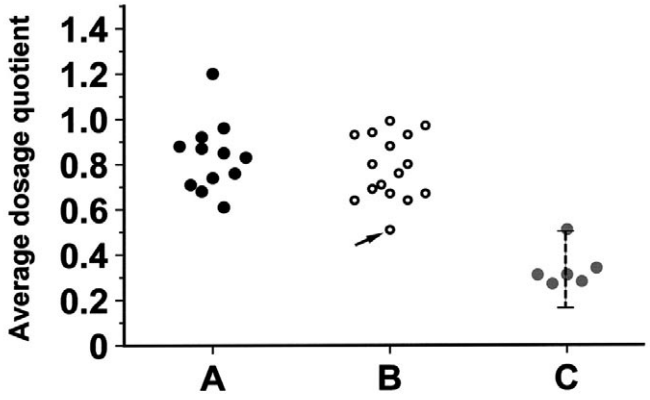

Figure 3. Confirmatory quantitative fluorescent duplex PCR. Average dosage quotients (DQs) for three groups of patients are shown: those with normal array CGH results (group A); those with suspected deletion (group B); and those with confirmed whole-gene microdeletion by FISH (group C). Possible deletion range (0.16-0.50) is shown by vertical bar. Note that average DQ in SoS13 (shown by arrow) is at the border of the deletion range.

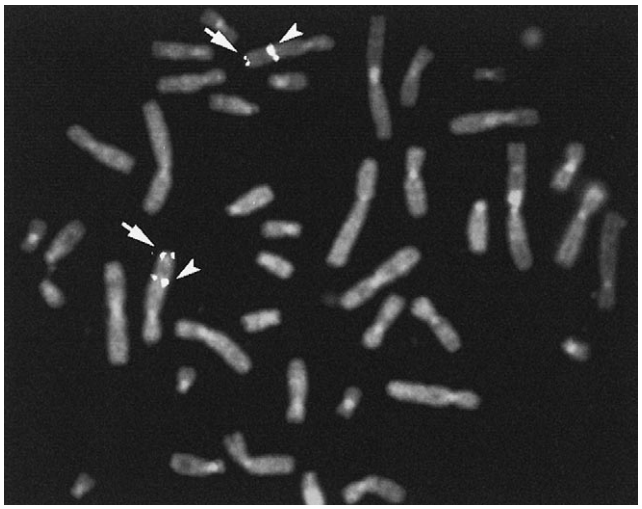

Figure 4. FISH on metaphase chromosomes of SoS 13. Arrowheads indicate signals from the control probe (RP11-465I17). Arrows show signals of combined probes a, b and c, which are visible on both chromosomes 5.

Discussion

Results of *NSD1*-specific exon array CGH suggested that the 5' region of *NSD1* might be deleted in 16 of 30 SoS patients analyzed. However, in none of them, the subsequent QFD-PCR confirmed such an abnormality. Moreover, FISH analysis never proved a deletion in SoS 13. Therefore, intragenic deletions were ruled out as a possible genetic cause in our series of 30 Japanese SoS patients without any *NSD1* point mutations or whole-gene microdeletions. Douglas et al. suggested that Alu mediated recombination leading to partial deletions would be the likely cause in some of European patients,[11] but we could not find such partial deletions in Japanese patients.

Exon-based array CGH has been successful in detecting copy-number changes.[16,17] Our *NSD1*-specific microarray covered all 23 exons as well as five regions in an 18-kb segment upstream of exon 1, where two CpG islands are located (according to UCSC genome browser). We cloned all probes necessary for PCR for the regions, and the use of a single set of 5'-amino-linked primers simplified further amplification of inserts. Although our system worked well in hybridization of normal and positive controls, seemingly positive results recognized in 16 of 30 patients on probes of 5'-1, 5'-2, 5'-3, 5'-4, 5'-5 and exons 1-2, were later denied by QFD-PCR and FISH analyses, though either QFD-PCR or FISH system did not cover all the areas with positive results in exon arrays. These false positive data may be explained by different nucleotide contents within the microarray elements used. The average CG content in the elements of a proximal 20-kb region (5'-1 to 2) of *NSD1* (Figure 1) is 54 % with the maximum of 74 % within exon 1, while it is only 39 % in elements from exon 3 to exon 23. High CG contents could affect efficiency of hybridization and washing of labeled probes. Also, as elements of the exon microarray are much smaller in size than those of BAC-clone microarray and have much less DNA complexity, they are likely to be more affected by sequence contents.

Since *NSD1* intragenic deletions are less frequent in Japanese patients with SoS, other disease-causing mechanisms must be considered. A mutation screening of *NSD2* and *NSD3* that belong to the same family as *NSD1* failed to identify any aberrations in 78 patients with overgrowth syndromes, including 12 typical SoS patients without *NSD1* mutations,[18] therefore excluding *NSD2* and *NSD3* as candidacy. In addition, epigenetic changes may be important. Two SoS patients without *NSD1* aberrations have been reported to have abnormities in 11p15, the region which contains two imprinting domains related to Beckwith-Wiedemann Syndrome (OMIM #130850).[19] One case had paternal isodisomy for chromosome 11 and showed demethylation of *KCNQ1OT*, and the other exhibited partial *KCNQ1OT* demethylation. However, additional patients with such imprinting defects have not been reported. Hypermethylation or a mutation in the promoter region of *NSD1* as another possible cause was ruled out in 18 Japanese patients.[20]

In conclusion, intragenic deletion was not identified in our series of 30 Japanese patients with SoS. Therefore, the cause in approximately one third of Japanese SoS patients still remains unexplained. Other genetic aberrations that impair function of the components of *NSD1*-related pathway could be associated with a subset of SoS patients. Finally, exon array CGH is potentially useful, but shorter array elements may be easily affected by sequence contents, therefore data should carefully be evaluated.

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