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ORIGINAL ARTICLE

Remco Visser · Tomonobu Hasegawa · Norio Niikawa
Naomichi Matsumoto

Analysis of the *NSD1* promoter region in patients with a Sotos syndrome phenotype

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Abstract Sotos syndrome (SoS, OMIM#117550) is an overgrowth disorder characterized by excessive growth—especially in the first years of childhood—distinctive craniofacial features, and various degrees of mental retardation. Haploinsufficiency of the nuclear receptor binding SET domain containing protein 1 (*NSD1*) gene, due to either intragenic mutations or whole-gene microdeletions, is found in the majority of patients with SoS. However, in approximately 10–40% of patients with a typical SoS phenotype, no abnormalities are detected. In this study, hemizygous hypermethylation or genomic sequence abnormalities of the promoter region of *NSD1* were hypothesized to be the underlying cause in patients with a SoS phenotype, but without confirmed *NSD1* alterations. In 18 patients, including one patient with a reported hepatocellular carcinoma, the promoter region of *NSD1* was analyzed. However, no hypermethylation or sequence abnormalities in the promoter region could be detected. It therefore seems unlikely that such abnormalities of *NSD1* are a major culprit in patients with phenotypical SoS. Additional methods are necessary for detection of other genetic or epigenetic causes of SoS.

Keywords Epimutation · Methylation · *NSD1* · Promoter · Sotos syndrome

Abbreviations BWS: Beckwith–Wiedemann syndrome · *NSD1*: Nuclear receptor binding SET domain containing protein 1 · *NSD2*: Nuclear receptor binding SET domain containing protein 2 · *NSD3*: Nuclear receptor binding SET domain containing protein 3 · SNP: Single nucleotide polymorphism · SoS: Sotos syndrome

R. Visser · N. Niikawa
Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

R. Visser
Department of Pediatrics,
Leiden University Medical Center, Leiden, The Netherlands

R. Visser · N. Matsumoto (✉)
Department of Human Genetics, Yokohama City University Graduate School of Medicine, 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004, Japan
E-mail: naomat@yokohama-cu.ac.jp
Tel.: +81-45-7872606
Fax: +81-45-7865219

R. Visser · N. Niikawa · N. Matsumoto
CREST, Japan Science and Technology Agency, Kawaguchi, Japan

R. Visser · N. Niikawa
International Consortium for Medical Care of Hibakusha and Radiation Life Science, The 21st Century Center of Excellence, Nagasaki, Japan

T. Hasegawa
Department of Pediatrics, Keio University School of Medicine, Tokyo, Japan

Introduction

Sotos syndrome (SoS, OMIM#117550) is a congenital overgrowth syndrome with characteristic craniofacial features and variable degrees of developmental delay (Cole and Hughes 1994). Aberrations of the nuclear receptor binding SET domain containing protein 1 (*NSD1*) gene at 5q35 include intragenic mutations and submicroscopic whole-gene deletions (Kurotaki et al. 2002, 2003; Douglas et al. 2003; Nagai et al. 2003; Rio et al. 2003; Turkmen et al. 2003; de Boer et al. 2004; Tatton-Brown et al. 2005). In approximately 10–40% of typical SoS patients without a detected *NSD1* abnormality, different aberrations of *NSD1* or locus heterogeneity should be considered [see review by Visser and Matsumoto (2003)]. In two SoS patients, abnormalities were detected in the imprinted region of 11p15, which is a common cause of Beckwith–Wiedemann syndrome (BWS, OMIM#130850) (Baujat et al. 2004). However, to date, no new cases are reported. Furthermore, a

screening of the *NSD*-gene family in patients with a SoS phenotype, but without *NSD1* aberrations, excluded involvement of *NSD2* and *NSD3* (Douglas et al. 2005).

In cancer genetics, epigenetic changes in tumors, such as promoter methylation of tumor repressor genes, are well known to result in transcriptional silencing of genes (Baylin and Herman 2000). Recently, in two individuals with multiple colorectal tumors, germline hypermethylation of the DNA mismatch repair gene *MLH1* was identified (Suter et al. 2004). Similar epimutations of the promoter region of *NSD1* were hypothesized to be responsible for transcriptional silencing of *NSD1* and would subsequently lead to SoS. In this study, 18 patients with a typical SoS phenotype but without aberrations of *NSD1* were screened for epimutations. Furthermore, the promoter regions were sequenced in all patients to exclude possible genomic mutations.

Materials and methods

Patients

The study population comprised of 18 patients with characteristic SoS features, in whom *NSD1* abnormalities were excluded. Seventeen patients were reported previously (Kurotaki et al. 2002, 2003; Kamimura et al. 2003) and one was newly added. The clinical inclusion criteria and the methods for *NSD1* analysis, consisting of gene sequencing and FISH analysis, have been reported elsewhere (Kamimura et al. 2003; Kurotaki et al. 2003). After informed consent, genomic DNA was obtained from peripheral blood cells. Experimental protocols were approved by the Committee for Ethical Issues at Yokohama City University School of Medicine, and by the Committee for Ethical Issues on Human Genome and Gene Analysis at Nagasaki University.

NSD1 promoter region

A 7.2-kb sequence was downloaded from the National Center for Biotechnology Information (NCBI) build 35 database (May 2004) available on the UCSC Genome Bioinformatics web site (http://genome.ucsc.edu/). This sequence includes the starting sites of the two known transcripts of *NSD1*: the shorter variant 1 (GenBank accession number NM_172349) and the longer variant 2 (GenBank accession number NM_022455). Furthermore, it extends 5 kb upstream from the most proximal transcript (variant 2). The following programs were used for predictions of promoter locations and CpG-islands: CpG-promoter (http://rulai.cshl.org/tools/CpG_promoter/) (Ioshikhes and Zhang 2000), FirstEF (http://rulai.cshl.org/tools/FirstEF/) (Davuluri et al. 2001), and CpGProD (http://pbil.univ-lyon1.fr/software/cpgprod_query.html) (Ponger and Mouchiroud 2002). If masking of repeats was deemed necessary (Bajic et al. 2004), the RepeatMasker webserver was used (http://www.repeatmasker.org/). Only promoter predictions coinciding with a correct prediction of the first exon (according to transcript variants 1 and 2) were kept in analysis. Transcription factor-binding sites were identified using the DNASIS Pro software (Hitachi Software Engineering Co., Tokyo, Japan).

Evaluation of the methylation status of the *NSD1* promoter region

The DNA was treated with sodium bisulfite according to the manufacturer's guidelines (CpGenome™ DNA Modification Kit, Chemicon International, Temecula, CA, USA). Polymerase chain reaction (PCR) was performed in a 25 µl mixture containing 0.8 µM of each primer, 1 unit of JumpStart™ REDTaq™ DNA polymerase (Sigma, St. Louis, MO, USA), 0.2 mM of each dNTP and 1× PCR buffer. Conditions included initial denaturation at 94°C for 2 min, 45 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 40 s, and a final extension of 72°C for 7 min. Primers for bisulfite PCR (degenerate and non-degenerate primers) were designed with Methprimer (http://www.urogene.org/methprimer/index1.html) (Li and Dahiya 2002) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen and Skaletsky 2000). Degenerate primers were designed if primers contained a CpG nucleotide. Primers are *forward* 5′-GAGTTGTTGTTTTATTTTGTTTTTTGT-3′ and *reverse* 5′-CCCTTCTCTCACTCTTC*R*AAATTC-3′. This PCR product was subsequently subjected to nested PCR with the following primers: *forward* 5′-GGTGGTGGTGTGGGTTTG-3′ and *reverse* 5′-CTCTCACTCTTC*R*AAATTCAAAAC-3′. The product was cloned with the Topo-TA kit (Invitrogen, Carlsbad, CA, USA). DNA was obtained after overnight cultures, and sequencing was performed as described previously (Visser et al. 2005).

Genomic analysis of the *NSD1* promoter region

Primers were designed with the online version of Primer3 (Rozen and Skaletsky 2000). A ~2.3 kb product was amplified with primers *forward* 5′-TGCCTCCATTTTGTTTCCTG-3′ and *reverse* 5′-CATGGAGGCCAAATCCTGTA-3′ using LaTaq (Takara Bio, Otsu, Shiga, Japan) with the provided 2× GC buffer. Nested primers were used for sequencing. All primers and conditions are available upon request.

Results

The identified CpG-islands by CpGProD and CpG-promoter and the prediction of promoter locations by FirstEF are shown in Fig. 1. The region for methylation analysis was selected based on overlapping predictions and on proximity to the starting site of transcript variant

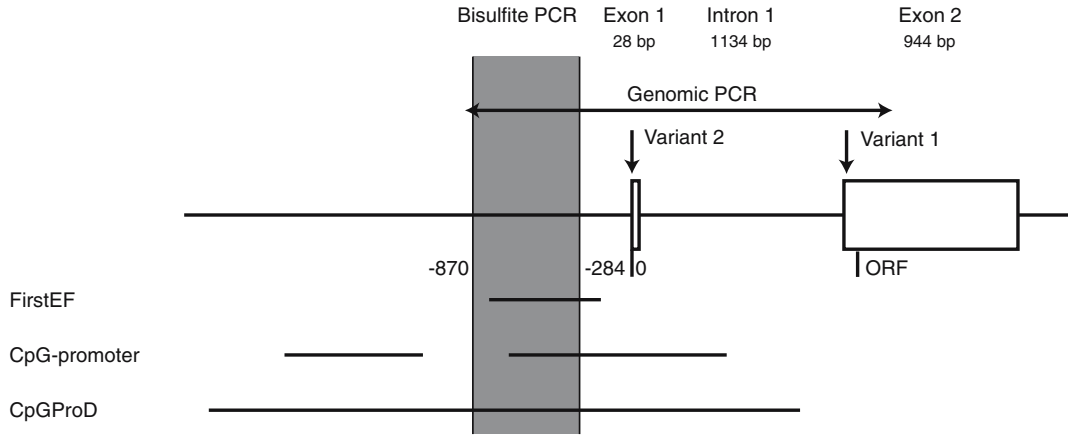

Fig. 1 Computational analysis of the promoter region of *NSD1*. *NSD1* and its 5′ region (5 kb) are shown schematically. The starting sites of transcript variant 1 and variant 2 are depicted by *vertical arrows*. The first nucleotide of variant 2 (position) is used as a starting point for numbering of the nucleotides. The names of the promoter and CpG-islands prediction programs are shown on the left and their predicted promoter regions and CpG-islands are shown under the 5′-region of *NSD1*. The region amplified after bisulfite conversion is the *gray-shaded* area between the vertical lines. A *horizontal bidirectional arrow* depicts ~2.3 kb PCR product used for genomic sequencing. *bp* base-pair, *ORF* open reading frame

2. Bisulfite PCR and subsequent nested PCR produced a product of 587 bp containing a total of 60 CpGs. Sequence information was obtained with a single reverse primer for a total of 46 most proximally located CpGs. For each patient, at least 18 clones were analyzed to ensure an accurate distribution of possible methylated and non-methylated clones. A total number of 18 SoS patients were analyzed, including one patient with a confirmed well-differentiated hepatocellular carcinoma. In all patients with SoS, no hypermethylation of the analyzed region was detected (Fig. 2). In some patients (SoS 58, SoS 62, and SoS 113), single hypermethylated cytosine-nucleotides were found. In SoS 113, a putative AP-2 transcription factor-binding site was identified to be co-localizing with such a hypermethylated CpG nucleotide (Fig. 2).

A ~2.3 kb product was amplified containing the genomic region of ~0.9 kb proximal of exon 1 until within exon 2 (Fig. 1). None of the 18 patients showed any mutations within this region. In the NCBI SNP database build 124 (http://www.ncbi.nlm.nih.gov/SNP/), only a C/T polymorphism was deposited for this region (refSNP ID, rs3733873). Ten patients were homozygous C/C, five homozygous T/T, and three patients were heterozygous for this SNP.

Discussion

Mutations and deletions of *NSD1* account for the majority of patients with SoS (Kurotaki et al. 2002, 2003; Douglas et al. 2003; Nagai et al. 2003; Rio et al. 2003; Turkmen et al. 2003; de Boer et al. 2004; Tatton-Brown et al. 2005). However, in a considerable group of patients with characteristic SoS features, no abnormalities of *NSD1* can be detected. In this study, we hypothesized that heterozygous hypermethylation or sequence abnormalities of the promoter region of *NSD1* would lead to impairment of the gene expression. However, the 18 patients analyzed did not show methylation changes of this region, nor did sequence analysis of the promoter region reveal any mutations. In SoS 113, a hypermethylated CpG nucleotide was found to co-localize with a putative AP-2 transcription factor-binding site. Site-specific methylation of the AP-2 transcription factor-binding site was detected in tumors in neurofibromatosis type 1, but was also found in 4/20 controls (Harder et al. 2004). However, repeated analysis of a different DNA sample of SoS 113 could not confirm this hypermethylation, favoring possible bias due to incomplete conversion during the bisulfite reaction (data not shown). Although we cannot completely exclude the influence of site-specific methylation in the repression of *NSD1*, it seems unlikely that this plays a major role in SoS patients. Intragenic microdeletions, altered splicing due to mutations in introns, aberrations affecting the yet unknown expression regulatory mechanisms of *NSD1*, or abnormalities in one of the components in the *NSD1*-related signaling pathway could be responsible for the SoS patients without confirmed *NSD1* haploinsufficiency.

In this study, *in silico* analysis was used for promoter prediction. It is known, however, that the individual programs do not always achieve a good correlation of the sensitivity and positive predictive value (Bajic et al. 2004). Although we used different programs in combination with knowledge of the starting sites of transcription, it would therefore be possible that the actual promoter region is located outside the analyzed regions and/or not related to a CpG-island. Enhancement of the computational programs is necessary for the correct promoter location.

The frequency of neoplasia in SoS is estimated to be ~2–3.9% (Cohen 1999; Rahman 2005); however, a direct involvement of *NSD1* in tumor growth in SoS is

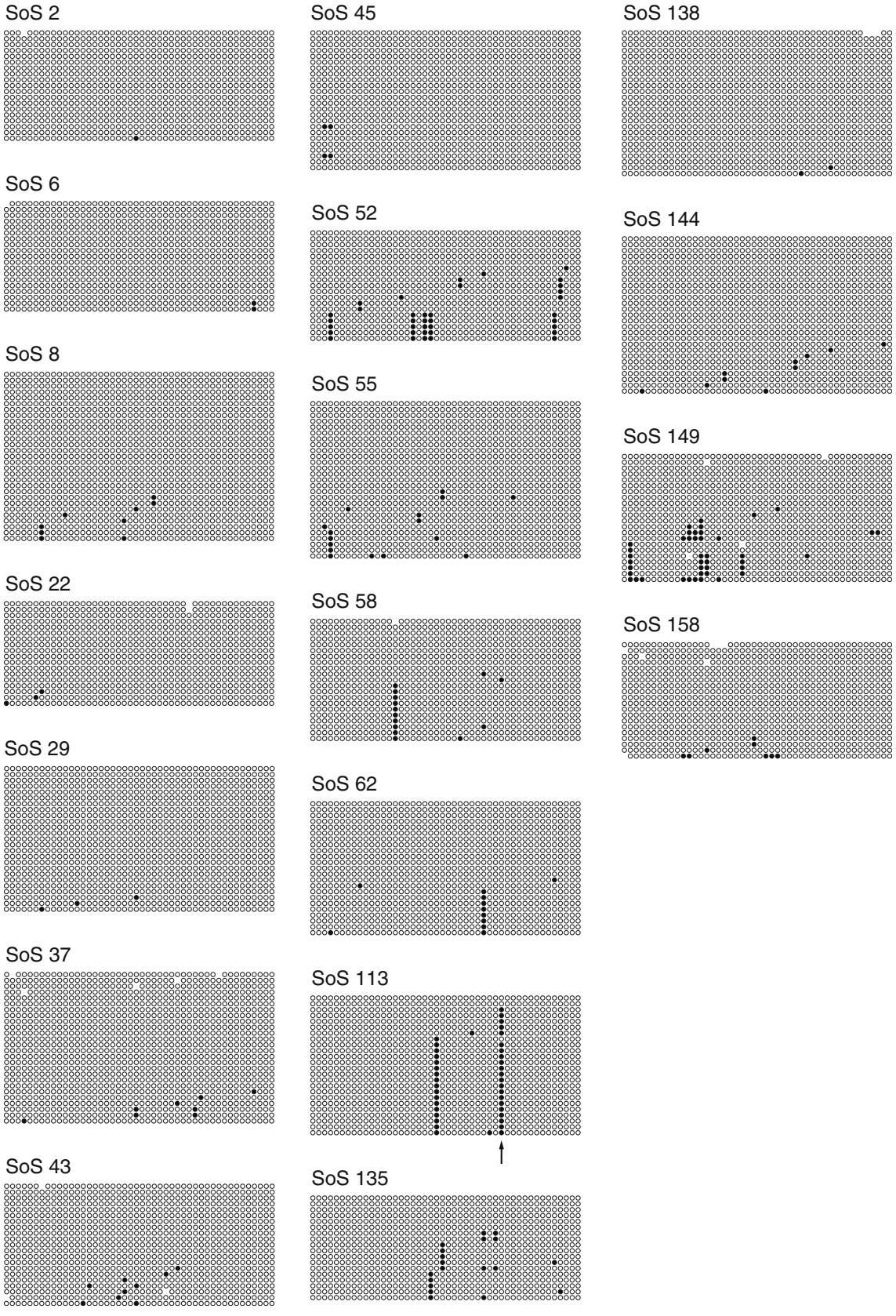

Fig. 2 Methylation status of CpGs per individual SoS patient. Each CpG is depicted by a *circle*. The CpGs (a total of 46) per single clone are shown horizontally and the clones are ordered vertically. *Open* and *closed circles* indicate non-methylated and methylated CpGs, respectively. *Missing circles* indicate nucleotides were the sequence could not be analyzed. A *vertical arrow* in SoS 113 indicates the position of the CpG nucleotide which co-localizes with a AP-2 transcription factor-binding site

not confirmed (Visser and Matsumoto 2003; Rahman 2005). Since the identification of *NSD1*, to our knowledge only a few SoS patients with neoplasia have been confirmed to harbor a *NSD1* alteration. This included three neuroblastomas (Nagai et al. 2003; Turkmen et al. 2003; Tatton-Brown and Rahman 2004), a ganglioglioma (Deardorff et al. 2004), a presacral ganglioneuroma, three sacrococcygeal teratomas, a small cell lung carcinoma, T-cell lymphoma, and acute lymphocytic leukaemia (Tatton-Brown and Rahman 2004; Rahman 2005). Our analysis of the methylation status of the *NSD1* promoter region could easily be applied to tumor tissues in Sotos patients with a *NSD1* alteration. Subsequently, differentiation would be possible between a primary NSD1 aberration (loss or mutation) or a combination with secondary hypermethylation, considering the Jones's newly-revised Knudson two-hit hypothesis (Knudson 1971; Jones and Laird 1999). Our patient with a hepatocellular carcinoma exhibited a specific SoS phenotype, but no alterations of *NSD1* coding regions were identified. Unfortunately, tumor tissue was not available for analysis. Further investigations in other SoS patients, with and without *NSD1* alterations, who developed neoplasia are necessary for elucidation of the possible relation between *NSD1* abnormality and neoplasia development.

In conclusion, it is unlikely that epimutations or genetic abnormalities of the *NSD1* promoter region are the main culprit for phenotypical SoS patients without yet-detected *NSD1* alterations. Future research might shed light on other genetic or epigenetic causes leading to SoS.

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