From NSD1 to Sotos syndrome : a genetic and functional analysis

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Chapter 6

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

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Abstract

Sotos syndrome (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 *n*uclear receptor binding *S*ET *d*omain protein *1* (*NSD1),* due to either intragenic mutations or whole-gene microdeletions is found in the majority of patients with Sotos syndrome. However in approximately 10-40 % of patients with a typical Sotos syndrome 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 Sotos syndrome 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 Sotos syndrome. Additional methods are necessary for detection of other genetic or epigenetic causes of Sotos syndrome.

Introduction

Sotos syndrome (SoS; OMIM 117550) is a congenital overgrowth syndrome with characteristic craniofacial features and variable degrees of developmental delay (1). Aberrations of the *n*uclear receptor binding *S*ET *d*omain protein *1* (*NSD1*) gene at 5q35 include intragenic mutations and submicroscopic whole-gene deletions (2-9). 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 (10)]. 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) (11). 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* (12).

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 (13). Recently, in two individuals with multiple colorectal tumors, germline hypermethylation of the DNA mismatch repair gene *MLH1* was identified (14). 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.

Material and methods

Patients

The study population comprised of 18 patients with characteristic SoS features in whom NSD1 abnormalities were excluded. Seventeen patients were reported before (2,5,15) and 1 was newly added. The clinical inclusion criteria and the methods for *NSD1* analysis, consisting of gene sequencing and FISH analysis, were reported elsewhere (5,15). 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/) (16), FirstEF (http://rulai.cshl.org/tools/FirstEF/) (17), and CpGProD (http://pbil.univ-lyon1.fr/software/cpgprod_query.html) (18). If masking of repeats was deemed necessary (19), 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 0.8µM of each primer, 1 unit of JumpStart™ REDTaq™ DNA polymerase (Sigma, St. Louis, MO, USA), 0.2 mM each dNTP and 1X PCR buffer. Conditions included initial denaturation at 94 °C for 2 min, 45 cycles of 94 °C for 30 sec, 60 °C for 30 sec, 72 °C for 40 sec 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) (20) and Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)(21). Degenerate primers were designed if primers contained a CpG nucleotide. Primers are *forward*: 5' GAGTTGTTGTTTTTATTTTGTTTTTTGT 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 (22).

Genomic analysis of the *NSD1* promoter region

Primers were designed with the online version of Primer3 (21). 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 x 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 Figure 1. The region for methylation analysis was selected based upon overlapping predictions and proximity to the starting site of transcript variant 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 proximal located CpGs. For each patient at least 18 clones were analyzed

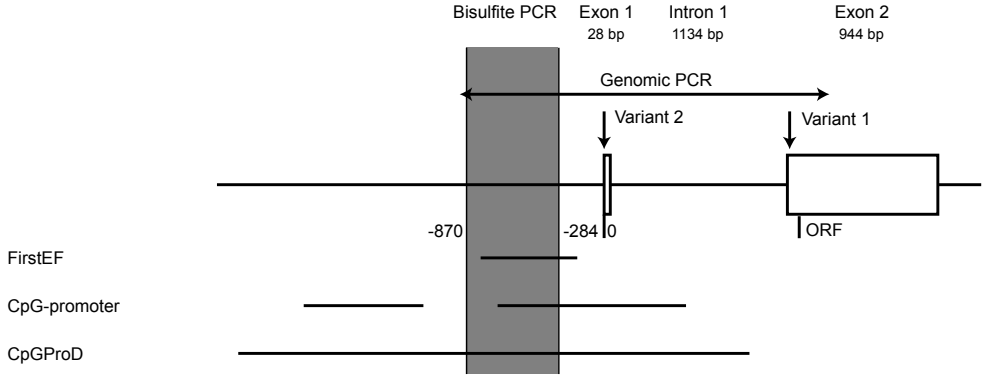

Figure 1. Computational analysis of the promoter region of *NSD1*

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 prediction programs are shown on the left and their predicted promoter regions 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 the ~2.3 kb PCR product used for genomic sequencing. *bp:* base-pair, *ORF:* open reading frame

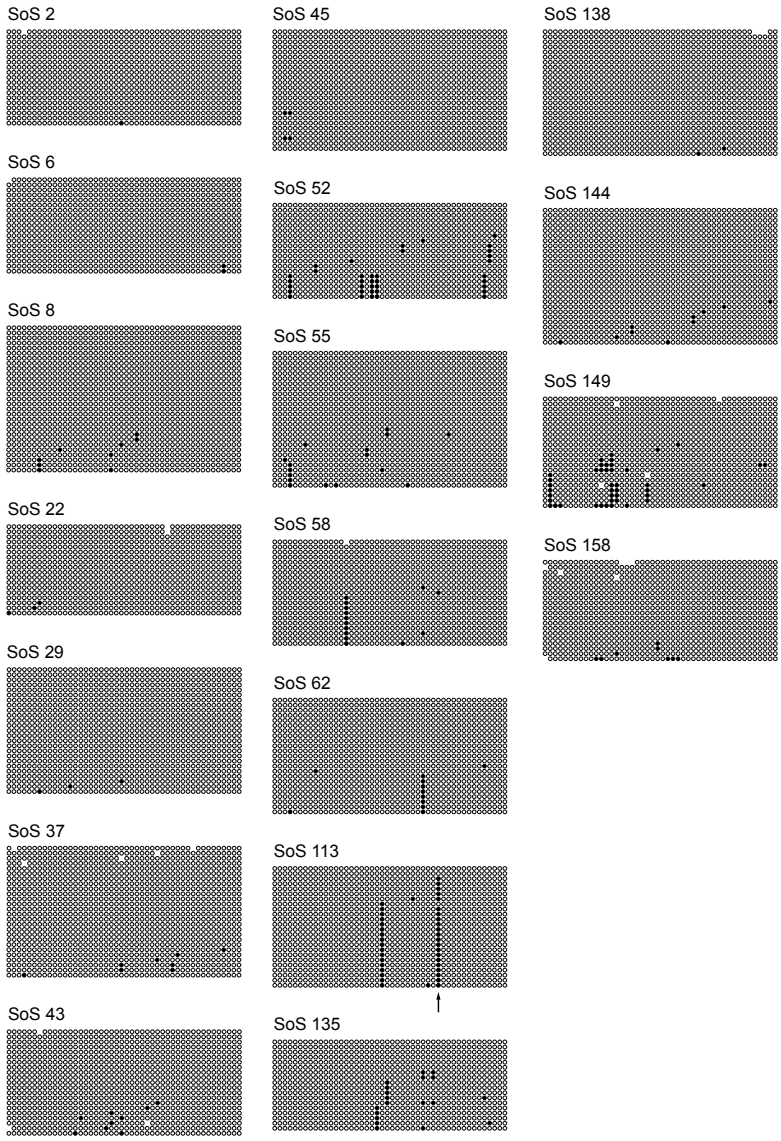

Figure 2. Methylation status of CpGs per individual SoS patient

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.

to ensure an accurate distribution of possible methylated and non-methylated clones. A total number of 18 SoS patients was analyzed, including one patient with a confirmed well-differentiated hepatocellular carcinoma. In all patients with SoS, no hypermethylation of the analyzed region was detected (Figure 2). In some patients (SoS 58, 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 (Figure 2).

A ~ 2.3 product was amplified containing the genomic region of ~ 0.9 kb proximal of exon 1 till within exon 2 (Figure 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, 5 homozygous T/T and 3 patients were heterozygous for this SNP.

Discussion

Mutations and deletions of *NSD1* account for the majority of patients with SoS (2-9). 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 (23). 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 can't 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 signalling 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 (19). 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 syndrome is estimated to be ~2 - 3.9% (24,25), however a direct involvement of *NSD1* in tumor growth in Sotos syndrome is not confirmed (10,25). Since the identification of *NSD1*, to our knowledge, only a few SoS patients with neoplasia were confirmed to harbor a *NSD1* alteration. This included three neuroblastomas (6,8,26), a ganglioglioma (27), a presacral ganglioneuroma, three sacrococcygeal teratomas, a small cell lung carcinoma, T-cell lymphoma and acute lymphocytic leukaemia (25,26). 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 (28,29). 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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