Malan syndrome (Sotos syndrome 2) in two patients with 19p13.2 deletion encompassing *NFIX* gene and novel *NFIX* sequence variant

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Background and Aim. Sotos syndrome 2 (MIM #614753), known also as Malan syndrome, is caused by heterozygous mutations/deletions of the *NFIX* gene located on chromosome 19p13.2. It manifests in developmental delay, intellectual impairment, macrocephaly, central nervous system anomalies, postnatal overgrowth, and craniofacial dysmorphism. Unusual behavior with/without autistic traits, ophthalmologic, gastrointestinal, musculo-skeletal, and hand/foot abnormalities are also frequent. Due to the limited number of such cases, no definitive conclusions about genotype-phenotype correlations have been possible. In the following paper, we discuss physical features consistent with Sotos syndrome 2 based on literature review and two new cases [a patient with *de novo* 19p13.2 deletion encompassing a part of the *NFIX* gene and a patient with *de novo* (not described so far) heterozygous missense mutation c.367C>T (p.Arg123Trp) in the *NFIX* gene].

Results. Apart from overgrowth and psychomotor developmental delay, the most consistent physical features of our two patients are dysmorphism including high forehead, downslanting palpebral fissures, pointed chin, and abnormalities of the pinna. Both show abnormal behavior and present with long, tapered fingers and toenail defect. No severe congenital malformations were noted.

Conclusions. We hope these data will serve as a material for further studies and provide an opportunity to make more reliable genotype-phenotype correlations.

Key words: Malan syndrome, Sotos syndrome 2, *NFIX* gene, 19p13.2 deletion, *NFIX* mutation

Received: September 14, 2015; Accepted with revision: February 3, 2016; Available online: February 29, 2016
<http://dx.doi.org/10.5507/bp.2016.006>

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INTRODUCTION

Aberrations in 19p13.2 (or 19p13.13 according to the hg18) result in diverse clinical phenotypes depending on the size of the affected fragment and involved genes (in large deletions/duplications) as well as type of point mutations. Descriptions of patients presented so far in the literature comprise several cases of chromosomal aberrations in 19p13.2/19p13.13 band reported by Lysy et al.¹, Dolan et al.², Malan et al.³, Bassuk et al.⁴, Wangenstein et al.⁵, Natiq et al.⁶, Schwemmle et al.⁷, Klaassens et al.⁸, Shimojima et al.⁹ and point mutations in *NFIX* gene (localized within 19p13.2) reported by Malan et al.³, Klaassens et al.⁸, Priolo et al.¹⁰, Yoneda et al.¹¹ in patients with Sotos-like and Marshall-Smith (MRSHS) syndromes. Unfortunately, due to the limited number of these cases, no definitive conclusions about the genotype-phenotype correlations can be made.

As patients with 19p13.2 and *NFIX* gene aberrations have been described in previous papers^{2,3,6,8,10}, we will not present another detailed comparison. Instead our goal is to discuss physical features consistent with Sotos syndrome 2 (Malan syndrome) based on literature review and

two new cases. We hope these data will serve as a material for further studies, involving larger group of patients, and hence will provide an opportunity to make more reliable genotype-phenotype correlations.

MATERIAL AND METHODS

After obtaining informed consent, the genomic DNA was extracted from probands' and their parents' peripheral blood samples using the automatic method (MagnaPure, Roche).

High-resolution chromosomal analyses were performed using the whole-genome oligonucleotide microarrays, i.e., PerkinElmer CGXTM HD v1.1 v1.1 4-plex (180 K array; hg19) and NimbleGen CGX-3 v1.0 3-plex (720 K array; hg18). Digestion, ligation, PCR, labeling, and hybridization of test and reference DNA were performed according to the manufacturer's recommendations. The slides were scanned into image files using the MS 200 Microarray Scanner (NimbleGen). Feature extraction and primary data analysis were performed using Agilent CytoGenomics Edition 2.7.22.0 software or NimbleGen

Table 1. Growth parameters of patient NM (with 19p13.2 deletion, including *NFIX* gene).

	Prenatal growth: 40 Hbd	Postnatal growth: 6 years
Weight	3020 g (15th-50th centile)	22 kg (50th centile)
Length / height	56 cm (>97th centile)	123.9 cm (75th-90th centile)
OFC	36 cm (85th-97th centile)	54 cm (97th centile)

DEVA software (depending on the type of microarray). Signature Genomics' Genoglyphix genome browser software was used for detailed analysis and data visualization.

NGS (next-generation sequencing) analysis was performed using TruSightOne Sequencing Panel (Illumina) according to the manufacturer's instructions. The samples were run on 1/12 of lane each on HiSeq 1500 using 2x75 bp paired-end reads. Bioinformatics analysis was performed as previously described¹². Briefly, after initial processing by the CASAVA, the generated reads were aligned to the hg19 reference genome with Burrows-Wheeler Alignment Tool¹³ and further processed by Genome Analysis Toolkit¹⁴. Base quality score recalibration, indel realignment, duplicate removal and the SNP/INDEL calling were done as described¹⁵. The detected variants were annotated using Annovar¹⁶ and converted to MS Access format for final manual analyses. In the analyzed probe 76.6% of target was covered minimum 20 times. Alignments were viewed with Integrative Genomics Viewer v.2.3.40 (ref.¹⁷).

PATIENT NM (DELETION OF 19p13.2)

The girl was referred for genetic counseling because of psychomotor delay. Her family history is unremarkable. She is a first child born at 40 weeks gestation after a pregnancy complicated by preterm contractions, by caesarean section (because of breech position) with 10 points in the Apgar scale, a weight of 3020 g (15th-50th centile), length of 56 cm (>97th centile) and occipitofrontal circumference (OFC) of 36 cm (85th-97th centile) (Table 1).

During further development, delay in language and motor skills as well as poor coordination were noted. She presents poor wound healing, bruising susceptibility, accelerated skeletal maturation (+2.5 years), dental malocclusion, strabismus, and constipation. The craniofacial examination revealed: triangular face with high forehead, hypertelorism, short and downslanting palpebral fissures, smooth philtrum, abnormality of the pinna, narrow mouth with open-mouth appearance, and pointed chin (Fig. 1). Moreover, long and tapered fingers, aplasia/hypoplasia of the palmar creases, clinodactyly of the 4th finger as well as overlapping toe and abnormality of the toenails were observed (all features are listed in Table 2).

She also presents behavioral anomalies (anxiety, tantrums, autoaggression), but in general, she is rather a cheerful child. Even though she can speak only a few words (mummy, daddy), she understands much more and executes commands. She also has a preference for water-related items.

Fig. 1. Facial features of patient NM (with deletion of 19p13.2). **Fig. 2.** PerkinElmer CGX™ HD v1.1 v1.1 4-plex array result – deletion within 19p13.2 region (with genomic coordinates chr19:13020206-13178390; hg 19).

The previous genetic diagnostics testing: GTG-banding karyotyping, MLPA (Multiplex Ligation-dependent Probe Amplification) for subtelomeric regions (SALSA MLPA P036 and P070 kits; MRC-Holland), MLPA for *MECP2*, *CDKL5*, *ARX*, *NTNG1* genes (SALSA MLPA P015 kit) and MS-MLPA toward Angelman syndrome (SALSA MS-MLPA ME028 kit) gave normal results.

PerkinElmer 180 K array revealed deletion within 19p13.2 (with genomic coordinates chr19:13020206-13178390; hg19) of about 158.2 kb, including 7 genes: *SYCE2*, *FARSA*, *CALR*, *RAD23A*, *GADD45GIP1*, *DAND5*, and *NFIX* (Fig. 2). NimbleGen 720 K array showed deletion of 19p13.13 region (with genomic coordinates

Table 2. Clinical features present in Sotos syndrome 2 described in the literature (symptoms observed in both our patients are marked in bold).

		NM Deletion of 19p13.2	AB <i>NFIX</i> mutation
Development	Motor retardation	+	+
	Hypotonia		+
	Speech delay	+	+
	Mental deficiency	+	+
	Behavioral anomalies	+	+
	Autistic traits		
Craniofacial features	Long / narrow face		+
	Downslanting palpebral fissures	+	+
	Hypertelorism	+	
	Proptosis		
	Epicanthal folds		
	Small mouth	+	
	Thin upper lip		+
	Everted lower lip		
	Prognathia		
	Small nose		
	Short nose		
	Anteverted nares		
	Low nasal bridge		
	High forehead	+	+
	Frontal bossing		
	Complex craniosynostosis		
	Flat occiput		
Eyes	Hypermetropia		
	Strabismus	+	+
	Nystagmus		
	Astigmatism		
	Optic nerve hypoplasia		
Musculo-skeletal abnormalities	Abdominal wall hypotonia		+
	Pectus excavatum		
	Coxa valga		
	Scoliosis		
	Advanced bone age	+	
Hand / foot abnormalities	Long fingers	+	+
	Clinodactyly of the 5th finger		
	Overlapping toes	+	
Brain MRI	Ventricular dilatation		+
	Hypoplasia of the corpus callosum		
	Mild atrophy		
	Chiari I malformation		
Seizures / EEG anomalies	Abnormal EEG	+	+
	Seizures	+	
Gastrointestinal abnormalities	Chronic diarrhea		
	Abdominal pain		
	Constipation	+	
	Vomiting		
	Poor feeding		
	Celiac disease		
	FTT (G-tube)		
Other abnormalities	Malformed nails	+	+
	Premature eruption of teeth		
	Generalized livedo		
	Hearing loss		

Table 2. continued

Other features of our cases			
General	Broad-based gait		+
	Poor coordination	+	+
	Generalized joint laxity	interphalangeal	+
	Myopia		+
	Poor wound healing	+	
	Bruising susceptibility	+	
	Dental malocclusion	+	
Craniofacial / Brain	Triangular face	+	
	Broad forehead		+
	Pointed chin	+	+
	Short columella		+
	Short philtrum		+
	Smooth philtrum	+	
	Short palpebral fissures	+	
	Open mouth	+	
	High, narrow palate	+	
	Abnormality of the pinna	+	+
	Cavum septum pellucidum	+	
	Pineal cyst	+	
Hand / Foot	Tapered fingers	+	+
	Aplasia/hypoplasia of the palmar creases	+	
	Clinodactyly of the 4th finger	+	

Table 3. Growth parameters of patient AB (with mutation in *NFIX* gene).

	Prenatal growth: 40 Hbd	Postnatal growth: 6 years
Weight	3350 g (50th-75th centile)	23.5 kg (50th-75th centile)
Length / height	56 cm (>97th centile)	123 cm (75th-90th centile)
OFC	33 cm (15th-50th centile)	52 cm (50th-75th centile)

chr19:12881047-13048119; hg18) of about 167.1 kb, encompassing the same genes (Fig. 3). No other potentially pathogenic losses or gains in other chromosome regions were identified except for known copy number variations. Parental chromosome investigations for the 19p13.2 deletion (array CGH by PerkinElmer) gave normal results, proving the *de novo* occurrence of this aberration in patient NM.

PATIENT AB (*NFIX* MUTATION)

The girl was diagnosed because of psychomotor delay. Her family history is unremarkable. She was born after infertility treatment (with intrauterine insemination) at 40 weeks gestation, after an uneventful pregnancy with 10 points in the Apgar scale, a weight of 3350 g (50th-75th centile), length of 56 cm (>97th centile) and OFC of 33 cm (15th-50th centile) (Table 3).

During further development, delay in language and motor skills, muscular hypotonia, broad-based gait with poor coordination, and generalized joint laxity were noted. The craniofacial examination revealed: long face with

Fig. 3. NimbleGen CGX-3 v1.0 3-plex array result – deletion of 19p13.13 region (with genomic coordinates chr19:12881047-13048119; hg 18).

high and broad forehead, downslanting palpebral fissures, short columella and short philtrum, thin vermillion border, abnormality of the pinna, and pointed chin (Fig. 4). Moreover, long, tapered fingers and abnormality of the toenails were observed. She presents behavioral abnormal-

Fig. 4. Facial features of patient AB (with *NFIX* mutation).

ity with aggressive and self-injurious behavior. Myopia, strabismus, and EEG abnormalities were also diagnosed (all features are listed in Table 2).

The previous genetic diagnostics testing which gave normal results included: GTG-banding karyotyping, FISH (fluorescence in situ hybridization) for 22q13, MLPA for subtelomeric regions (SALSA MLPA P036 and P070 kits) as well as for 5q35.3 region (SALSA MLPA P064 kit) and Cohen syndrome (SALSA MLPA P321 kit).

Finally, next-generation sequencing performed on HiSeq 1500 using TruSightOne Enrichment Kit revealed the presence of a molecular variant in the *NFIX* gene: a missense heterozygous substitution c.367C>T (p.Arg123Trp) in exon 2 (34/64 reads) which may be disease-causing (Fig. 5). At the same time, aberrations within coding sequences of *NDS1* and *VPS13B* (*COH1*) genes were excluded. The identified alteration is a novel molecular variant not yet described in the genetic databases or literature. In order to evaluate the pathogenicity of the substitution bioinformatic analysis was performed. Novel variant acquired the status of disease-causing change in seven predictive algorithms: CADD, RadialSVM, PolyPhen-2, LRT, LR, SIFT, and Mutation Taster. The variant in the patient was validated and the parental origin of this mutation was excluded by the Sanger sequencing. The nomenclature of mutation follows the guidelines of the Human Genome Variation Society (HGVS www.hgvs.org/mutnomen) using human *NFIX*-cDNA NM_001271043.2 as a reference sequence followed the Human Gene Mutation Database (HGMD www.hgmd.cf.ac.uk).

DISCUSSION

In 2010, 244 K oligonucleotide arrays were performed by Malan et al. in 18 patients from non-consanguineous parents with developmental delay, height >95th centile and/or occipitofrontal circumference >95th centile, and

Fig. 5. Integrative Genomics Viewer view of *NFIX* mutation in patient AB by next-generation sequencing – c.367C>T in exon 2 (34/64 reads).

at least two of the following features: 1. advanced bone age, 2. dysmorphic craniofacial features, 3. congenital malformations led to identification of first two cases with 19p13.1 monosomy³. Both patients had advanced bone age, long/narrow faces with high forehead, long fingers, slender habitus, and presented behavioral anomalies with autistic traits. Since the deleted region involved a single common gene, *NFIX* (Nuclear Factor I, X-type), it was considered to be a strong candidate in the cause of overgrowth in the investigated patients.

Subsequently, these authors screened for *NFIX* mutations 76 patients with unexplained syndromic overgrowth, confirming a heterozygous *de novo* nonsense mutation in a patient previously diagnosed with Sotos-like syndrome³. Moreover, based on the observation of *Nfix*-deficient mouse model presenting a phenotype similar to Marshall-Smith syndrome, the group screened 9 patients with MRSHSS for *NFIX* mutations that allowed for the identification of 7 independent frameshift mutations and 2 different mutations within the donor splice site of exon 6.

Links between *NFIX* gene disturbances and phenotype resembling Sotos syndrome were also verified shortly after by Yoneda et al. who identified different heterozygous missense mutations in 2 patients negative for *NSD1* mutation¹¹. In 2012, Priolo et al. found in frame deletion of *NFIX* gene in a patient with overgrowth and suspected a mild form of Marshall-Smith syndrome (i.a. blue sclerae, mild proptosis, slightly bullet-shaped phalanges, and sleep apnea that spontaneously resolved with age) (ref.¹⁰). As is now recognized, mutations in the DNA binding/dimerization domain of *NFIX* (leading to *NFIX* haploinsufficiency) are likely to cause Sotos-like syndrome, while Marshall-Smith syndrome is rather due to mutations with dominant negative effect [mutated RNAs escape nonsense-mediated decay (NMD)] (ref.^{3,10,11}). However, the NMD mechanism pertains exclusively to mutations leading to premature

stop codons, and hence is not expected in our patient AB. As noted by Klaassens et al., variants found in the Marshall-Smith syndrome are frameshift and splice site within 6-8 exons⁸. In patient AB, the novel heterozygous missense mutation c.367C>T (p.Arg123Trp) in the DNA-binding/dimerization domain of *NFIX* was identified. Arginine 123 is a very highly conserved residue across species (Fig. 6). This substitution is predicted to be disease-causing. Thus it might be assumed that the missense mutation affects DNA-binding/dimerization domain of *NFIX* gene leading to its haploinsufficiency.

As was just recently proposed by Klaassens et al., who reviewed cases with Sotos-like overgrowth, a phenotype caused by whole gene deletions, nonsense variants and missense variants affecting the DNA-binding domain may be referred to as Malan syndrome⁸. Referring to these authors' results, we can support the data on the high frequency of strabismus among cases with Sotos syndrome 2, while none of described herein patients present with nystagmus or optic disc pallor/hypoplasia [noted in 25% of cases review by Klaassens et al.⁸]. In none of our patients pectus excavatum or scoliosis were observed, noted by cited authors as other recurrent features (respectively in 40% and 25% of cases).

As shown in Tables 1 and 3, birth weight was within normal limits in both our patients, and no overweight in postnatal period is observed. On the other hand, in both girls, birth length was above 97th centile, and constantly tends to be within 75th-90th centile. Patient NM (diagnosed with 19p13.2 deletion) was born with larger OFC than patient AB (with point mutation in *NFIX* gene) – 36 cm (85th-97th centile) vs. 33 cm (15th-50th centile), what may result from the fact that the deletion also includes other than *NFIX* genes. This measurement increased later in patient AB to 50th-75th at the age of 6. The postnatal onset in weight and OFC gain are noteworthy features of Malan syndrome, in contrast to Sotos syndrome (caused by mutation in the nuclear receptor binding SET domain protein 1, *NSD1* gene), where the overgrowth is usually in prenatal period and is more significant.

Recognition of Malan syndrome on a clinical basis is however difficult. The differential diagnosis should take into account other overgrowth conditions that may be confused with the Sotos syndrome, such as: Weaver syndrome, Beckwith-Wiedemann syndrome, in males – Simpson-Golabi-Behmel syndrome as well as fragile X syndrome or benign familial macrocephaly in neurologically normal individuals.

Looking at Table 2, where clinical features described in patients with Sotos syndrome 2 are specified, it can be noted that, apart from overgrowth and psychomotor developmental delay, the most consistent physical features of our patients are dysmorphism including high forehead, downslanting palpebral fissures, pointed chin, and abnormalities of the pinna. Moreover, both present long, tapered fingers and abnormality of the toenails. No severe congenital malformations were noted. In general, they share a limited number of dysmorphic features.

On the contrary, they have a similar spectrum of motor abnormalities, such as poor coordination, which is

V L S N P D Q K G K I W R I D C L	<i>mutated</i>
V L S N P D Q K G K I R R I D C L	<i>Human</i>
V L S N P D Q K G K I R R I D C L	<i>Ptrogodytes</i>
V L S N P D Q K G K I R R I D C L	<i>Mmulatta</i>
V L S N P D Q K G K I R R I D C L	<i>Mmusculus</i>
V L S N P D Q K G K I R R I D C L	<i>Trubripes</i>
V L S N P D Q K G K I R R I D C L	<i>Drerio</i>
V L S N P D Q K G K M R R I D C L	<i>Dmelanogaster</i>
I I S V A D Q K G K M R R I D C L	<i>Celegans</i>

Fig. 6. Alignment of R123 amino acid in the *NFIX* protein showing conservation of the amino acid across species.

known to be characteristic for patients with Sotos syndrome and *NSD1* mutation. Likewise abnormal EEG was noted in both, however only patient with deletion 19p13.2 suffers from seizures. Also both our probands show abnormal behavior, such as aggression, self-injurious behavior and – noted in patient NM – tantrums. In contrast to the observation of Malan et al.³, no autistic traits were observed in our patient with *NFIX* mutation. This is consistent with the observation of Yoneda et al. that missense mutations in the DNA-binding/dimerization domain do not lead to autistic traits¹¹. Our patients show only some central nervous system anomalies – persistent cavum septum pellucidum and pineal cyst in patient NM and unilateral ventriculomegaly in patient AB.

When we compare the craniofacial appearance of our two probands, their gestalt is consistent with Sotos syndrome 2. However, it is quite clear that patient NM (diagnosed with 19p13.2 deletion) has more pronounced dysmorphism, which, moreover, more closely corresponds to the facial characteristics found in Sotos syndrome 2 (regarding the ocular region, philtrum, and chin).

The clinical status of patient NM is probably influenced by the absence of other than *NFIX* genes located within the 19p13.2 region – *SYCE2*, *FARSA*, *CALR*, *RAD23A*, *GADD45GIP1*, and *DAND5*. The *DAND5* gene (*609068) encodes a member of the bone morphogenic protein antagonist family and may play a role in regulating organogenesis, body patterning, and tissue differentiation¹⁸. The *CALR* gene (*109091) encodes a chaperone protein mediating the attenuating effect of glucocorticoids in Wnt signalling in osteoblastic cells and thus plays an important role in controlling osteoblastogenesis¹⁹. It is also located in neurons in the human small intestine and therefore might play a role in the gastrointestinal issues². The *NFIX* gene (*164005), which is known to be essential for normal brain development and is probably required for neural stem cell homeostasis, is also associated with the features observed in our presented patients²⁰. As for the rest of mentioned genes, based on current data on their function we found no correlation to the phenotype of patient NM.

The *de novo* heterozygous missense mutation c.367C>T (p.Arg123Trp) in *NFIX* gene in patient AB affects highly

conserved arginine and is suspected to be disease-causing by predictive algorithms. *NFIX* is a member of the nuclear factor I (NFI) family proteins implicated in site-specific DNA-binding proteins involved in viral DNA replication and gene expression regulation. Identified point mutation in the DNA-binding/dimerization domain of the *NFIX* protein might cause loss of dimerization, DNA-binding and replication activities but its exact role can only be verified in future studies.

CONCLUSIONS

This paper provides insight into the phenotype of Sotos syndrome 2, providing new clinical data important for distinguishing patients with *NFIX* mutations and deletions within 19p13.2 region. We hope that it will help in uncovering the molecular pathway leading to the Sotos-like phenotype and more precise diagnostics based on patients' clinical features.

Acknowledgement: This study was supported by grants from the Polish Ministry of Science and Higher Education (project no. 0193/IP1/2013/72) and from the National Science Centre (Harmony project no. UMO-2013/08/M/NZ5/00978).

Author contributions: AJS: study design, data interpretation, critically revised the manuscript; MKuc, DJ: data interpretation and analysis, critically revised the manuscript; KF, MM, MR: data analysis; DW, MKug, AC: clinical evaluation of the patients and their parents; EC, RP: data interpretation, critically revised the manuscript; MKW: final approval.

Conflict of interest statement: The authors state that there are no conflicts of interest regarding the publication of this article.

Study approval: This study was approved by the Bioethics Committee of the Children's Memorial Health Institute in Warsaw.

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