From NSD1 to Sotos syndrome : a genetic and functional analysis

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Chapter 7

RNF135 mutations are not present in patients with Sotos syndrome-like features

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Research letter in: Am J Med Genet A 2009; 149A: 806-808

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Abstract

Sotos syndrome is an overgrowth disorder which is characterized by an increased height and head circumference, typical facial features and mental retardation. It is caused by haploinsufficiency of *NSD1*, which is detected in approximately 60-90% of the patients. Consequently, a considerable number of patients with some characteristics of Sotos syndrome remain without a molecular diagnosis. Aberrations of the *RNF135* gene were recently identified in children with tall stature, macrocephaly, facial dysmorphism, mental retardation and variable other features. *RNF135* is located in the Neurofibromatosis type 1 microdeletion region and Neurofibromatosis type 1 patients harbouring such a deletion showed an increased height in comparison with patients with intragenic *NF1* mutations. Due to the phenotypic overlap, we hypothesized that *RNF135* aberrations might be the cause in patients with features of Sotos syndrome but without *NSD1* defects. However, in 160 patients no pathogenic abnormalities affecting solely *RNF135* were detected. Therefore routine screening of *RNF135* after *NSD1* analysis is not recommended. In one patient a classic Neurofibromatosis type 1 microdeletion was identified. Hence this phenotypic presentation should be considered in the differential diagnosis of young Sotos syndrome-like patients. To facilitate future research, an open access online database for *RNF135* mutations was created at www.lovd.nl/RNF135.

Introduction

Sotos syndrome (SoS; OMIM 117550) is characterized by three cardinal features i.e. overgrowth (height and/or head circumference > +2.0 SDS), facial dysmorphism and a certain degree of learning disability (1). The craniofacial features include dolichocephaly, a broad forehead with a receding hairline, a pointed chin and downslanting of the palpebral fissures. SoS is caused by haploinsufficiency of the *NSD1* gene and abnormalities of *NSD1* are found in approximately 60% - 90% of the patients (2). Although the detection rate is high in phenotypic classical patients, it is reported to be much lower (36%) in non-typical patients (3). Therefore there are a considerable number of patients with SoS-like features in whom no *NSD1* abnormalities are found.

Recently, haploinsufficiency of the Ring finger protein 135 gene (*RNF135*; OMIM 611358), located on 17q11.2, was reported as the cause of overgrowth in combination with dysmorphic features and a certain degree of mental retardation (4). In six unrelated patients, four heterozygous truncating mutations, one missense mutation and a microdeletion including four neighbouring genes of *RNF135* were described. All patients exhibited a postnatal overgrowth phenotype with macrocephaly (head circumference ≥ +2.0 SDS) and tall stature (height > +2.0 SDS), although the height of the patient with the missense mutation was within normal range (+1.1 SDS). The dysmorphic characteristics included a broad forehead, anti-mongoloid slant of the eyes, a broad nasal tip, a long philtrum, a thin upper lip and a full lower lip. In all patients a variety of additional features were described, such as advanced bone age (3/6), hearing problems (2/6) and eye abnormalities (2/6). The level of developmental delay varied from mild to severe. All mutations were also detected in either the father or the mother as well as in siblings of two patients. The dysmorphic features, except for macrocephaly, were reported to be less distinctive in these parents and three of them showed a normal intellectual development.

RNF135 was previously reported as one of the 14 genes deleted in the common 1.4Mb microdeletion, which causes neurofibromatosis type 1 (*NF1*; OMIM 162200) in approximately 5% of the cases (5-7). Because patients carrying this common deletion showed a tall stature in contrast to patients with intragenic *NF1* mutations (8), *RNF135* was considered to be one of the candidate genes involved in overgrowth (4). Furthermore, a phenotypic overlap of two familial patients carrying a deletion of the *NF1* region and Weaver syndrome was noted

(9). However, no *RNF135* mutations were found in classical Weaver syndrome patients (4).

The features described in patients with *RNF135* gene abnormalities show also an overlap with the characteristics found in SoS. We hypothesized that *RNF135* mutations might therefore be the culprit in patients referred to our laboratory on suspicion of SoS but without an *NSD1* defect and investigated whether analysis of *RNF135* should be performed as a standard secondary step after *NSD1* analysis.

Material and methods

Patients

The DNA samples of 160 patients referred for *NSD1* analysis on suspicion of SoS to our diagnostic laboratory were analyzed. They were mainly from the Netherlands and a few from western European countries. Twenty-two patients were previously described by de Boer et al. (3) and their detailed clinical information is presented in Table 1. Informed consent was obtained at the time of blood collection from parents, guardians or adult patients. *NSD1* mutations and microdeletions were previously excluded by sequencing and Multiplex Ligation Probe Amplification (MLPA; Salsa MLPA kit SoS, MRC Holland, Amsterdam, The Netherlands) and/or FISH-analysis following standard protocols in our laboratory.

Molecular studies

The reference sequence for the largest isoform of *RNF135* (NM_032322) was obtained from the Entrez Nucleotide database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=Nucleotide) and primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers were extended with an M13 tail. Primer sequences, product size and annealing temperature are shown in Table 2. PCR products were amplified using standard protocols and purified on the Biomek FX Laboratory Automation Workstation (Beckman Coulter, Mijdrecht, The Netherlands). Sequencing reactions were performed with uniform M13 primers, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Cheshire, UK) and purified with the Biomek Workstation. After electrophoresis on the ABI 3730 Genetic Analyzer (Applied Biosystems), results were analyzed with the SeqScape v2.5 software (Applied Biosystems). Splice-site predictions were performed using the NNSPLICE 0.9 version software at (http://www.fruitfly.org/seq_tools/splice.html) (10).

Table 1. Clinical information for 22 patients with SoS-like features but without *NSD1* alterations[1]

No.	Sex	Age (years)	Facial features[2]						Macro-cephaly	Tall stature	Developmental delay	Advanced bone age
			1	2	3	4	5	6				
7	M	5.8	+	+	+	+	+	+	+	+	+	+
15	M	15.3	-		+	+	-	-	+	+	+	+
24	M	3.8	+	-	+	-	+	+	+	-	+	+
30	M	5.6	-	+	-	+	+	-	+	-	+	+
31	F	7.2	-	+	+	-	+	+	+	-	+	+
32	F	9.2	-	+	-	+	-	-	+	-	+	+
33	M	20.6	+	+	-	-	+	+	+	-	+	+
37	M	1.8	-	-	-	+	+	+	-	-	+	+
39	F	4.8	-	+	-	+	+	+	+	+	+	-
40	F	6.3	-		-	-	-	-	+	+	+	+
42	F	14.0	-	+	+	-	+	+	+	-	+	
43	M	36.3	-	+	-	-	+	+	+	-	+	
45	M	6.2	-	+	-	-	+	+	-	+	+	-
49	M	48.4	-	+	+	-	-	-	-	-	+	
50	M	7.6	-	-	+	-	+	-	+	-	-	-
51	M	10.1	-	-	-	-	-	-	-	+	+	
53	M	12.8	-	+	-	-	-	-	+	+	-	-
54	F	5.7	-	+	-	-	-	-	-	+	-	-
56	M	8.6	-	-	-	-	-	-	+	-	+	-
57	M	8.8	-	-	-	-	-	-	+	-	+	-
58	M	7.3	-	-	-	-	-	-	-	-	+	-
59	M	9.6	-	-	-	-	-	-	-	-	+	-

[1] Adapted from de Boer et al. (3) with permission from S. Karger AG, Basel

[2] Facial features: 1 = down-slant palpebral fissures; 2 = high arched palate; 3 = prominent jaw; 4 = dolichocephaly; 5 = frontal bossing; 6 = high hairline

Table 2. Primer sequences, product sizes and annealing temperatures

Exon	Primer name	Forward primer (5'-3')	Reverse primer (5'- 3')	Product size (bp)[2]	Annealing Temp. (°C)
1	RNF135-Ex01	M13F-AGACTCGCCCGGCTCAA[1]	M13R-GCCACATGGGAAAGGGTCAG[1]	493	59
2	RNF135-Ex02	M13F-TGGTTCCTGGGTCCAGTTT	M13R-CAATCCCTAGCCCTATTCCCTTC	252	57
3	RNF135-Ex03	M13F-TAATAGTTGATAGACTGCATAGG	M13R-ATAGAGCACTCTGCTAC	293	57
4	RNF135-Ex04	M13F-TGGAGACCTTCAGTTTGCCTTT	M13R-GCTCTGCGGCAAGCCAA	200	57
5	RNF135-Ex05A	M13F-AAGATGACCGGCCATGT	M13R-CCATGCAGAGAGCTGGCTA	356	57
	RNF135-Ex05B	M13F-GGACTATGGACTCTTGTTG	M13R-TGAGATCCCATCTCTATCGTA	395	55
	RNF135-Ex05C	M13F-AATCAGGGTAGTAACTTGAC	M13R-AAGCTGATACTGCATGG	362	55
	RNF135-Ex05D	M13F-TTGCCTGATAAGAGTGTTT	M13R-AGTGATTCACTAGAAGGAC	400	55

[1] M13F (Forward tail): 5'-TGTAAAACGACGGCCAGT-3', M13R (Reverse tail): 5'-CAGGAAACAGCTATGACC-3'

[2] Product size in base pairs does not include the M13-tail.

Prediction of the functional effects of mutations on the protein were made with the SIFT (http://blocks.fhcrc.org/sift/SIFT.html) (11) and Polyphen (http://genetics.bwh.harvard.edu/pph/) (12) prediction software.

MLPA for deletion or duplication detection was performed using the SALSA MLPA Kit P122-B1 NF1 Area (MRC-Holland). This kit contains 25 probes covering *NF1* and 14 neighboring genes. *RNF135* is represented with a single probe. MLPA reactions and analysis were performed as described previously by White et al. (13).

Results

In 160 patients, the five exons of *RNF135* were fully sequenced and the results are shown in Table 3. However, no mutations resulting in protein abrogation were found. One mutation in intron 1 (c.372+14G>T) and one synonymous missense mutation in exon 3 (c.642C>T; pSer214Ser) were not reported previously. Splice site predictions for the mutations were performed but no changes in splice-site scores were observed in comparison with the wild

type (data not shown).

In three patients a missense mutation (c.1245G>T; p.Trp415Cys) was detected in exon 5. This nucleotide change was predicted to have a deleterious effect on the protein according to the SIFT and Polyphen software. In two of these patients parental DNA was available and both fathers were confirmed to carry this mutation.

Furthermore, MLPA was performed in 140 patients, since in 20 patients there was not enough DNA left. No copy-number abnormalities were detected affecting solely *RNF135*. However, in one patient a heterozygous deletion of 14 probes was detected extending from probe CRLF3 till LRRC37B, encompassing 14 Refseq genes including *RNF135* but also the *NF1* gene.

In order to facilitate future mutational research, an online open access database for *RNF135* mutations was created as part of the Leiden Open Source Variant Database (14). This database is freely accessible at http://www.LOVD.nl/RNF135.

Table 3. Mutations identified in *RNF135* in our patient cohort

Location	Nucleotide change	Predicted protein change	No. identified (n/160)	dbSNP	References
Exon 1	c.213C>G	p.His71Gln	8	rs7225888	Douglas et al. (4)
	c.322T>C	p.Ser108Pro	8	rs7211440	Douglas et al. (4)
	c.344G>A	p.Arg115Lys	5		Douglas et al. (4)
	c.360G>T	p.Pro120Pro	8	rs7224960	Douglas et al. (4)
Intron 1	c.372+14G>T		1		This study
Exon 3	c.642C>T	p.Ser214Ser	1		This study
Exon 5	c.1245G>T	p.Trp415Cys	3		Douglas et al. (4)
3'-UTR	c.1299+292C>T		6	rs10221201	This study
	c.1299+437G>A		1	rs13373	This study
	c.1299+526C>G		7	rs10221206	This study

Discussion

Abnormalities of *RNF135* were recently reported in 6 out of 245 patients (4). In contrast, our patient cohort of 160 patients referred for *NSD1* analysis on suspicion of SoS was negative for pathogenic alterations which can be attributed solely to *RNF135*. In three of our patients, the missense mutation c.1245G>T; p.Trp415Cys located in the B30.2/SPRY domain was detected and *in silico* analysis predicts a deleterious effect on the protein. Furthermore, changes in cysteine residues are well-known to have a pathogenic effect in diseases such as CADASIL and Marfan syndrome (15,16). However, this mutation was classified by Douglas et al. as a polymorphism since it was found in 1 patient and 4 (out of 510 analyzed) normal controls. The detection of the mutation in two non-affected, non-macrocephalic fathers renders phenotypic expression indeed unlikely. Although this can not be completely excluded since the parental phenotype of *RNF135* defects is also very mild (4).

With MLPA a heterozygous deletion was identified in one patient but the deleted region also included *NF1* and overlapped with the classic 1.4Mb *NF1* microdeletion (7). At the time of referral for *NSD1* analysis she was approximately 4 years of age, exhibiting a tall stature, developmental delay, a coarse facies with frontal bossing, long philtrum, epicanthus and a few café-au-lait spots. In the approximately two years' interval between referral and the start of our study, she had already been diagnosed with NF1 but this information had not been communicated to us. Diagnosis of NF1 in children younger than 8 years old can indeed be difficult, since major clinical features such as inguinal or axillary freckling, Lisch nodules or neurofibromas may not yet be present (17). In addition, a Weaver syndrome-like phenotype has been described in patients with a *NF1* microdeletion and the phenotypic overlap between Weaver and SoS is well-known (9,18). Thus it can be appreciated that NF1 caused by a microdeletion can indeed present with an overlapping phenotype in childhood and should be included in the differential diagnosis of young SoS-like patients. Because of the divergent phenotype in time and the low prevalence (<0.1%) in our population, standard molecular *NF1* analysis is however not warranted.

The issue remains why no *RNF135* abnormalities were detected in our patient cohort in contrast to the findings of Douglas et al. (4). Some possible explanations can be postulated. First of all, because of the setting of a diagnostic laboratory, our patients were referred by various physicians. Therefore our patient group might be more heterogeneous since

detailed phenotypic information is not available. However, in a subset of our study cohort, macrocephaly, tall stature, facial dysmorphism and developmental delay were frequent features (Table 1) and were probably prompting the physicians to suspect SoS. Secondly, the *RNF135* associated phenotype might alter when more patients are reported. The principal question indeed to be solved is to what extent *RNF135* defects are responsible for the clinical features, especially since parental carriers of the nonsense mutations exhibited an almost normal phenotype with sometimes macrocephaly as the only finding. In contrast, the children displayed a wide spectrum of additional features and were more severely affected. Additional patients carrying *RNF135* mutations and screening of patient cohorts with overlapping phenotypic characteristics are therefore necessary to further delineate the *RNF135* associated phenotype.

In conclusion, we analyzed *RNF135* in 160 patients referred for *NSD1* analysis on suspicion of SoS and did not detect any pathogenic abnormalities of this gene. Consequently, routine screening is not recommended after negative results of *NSD1* analysis. The detection of a NF1 patient carrying a microdeletion emphasizes that this phenotypic presentation should be considered in the differential diagnosis of childhood SoS-like patients. The phenotypic spectrum of *RNF135* defects remains to be elucidated.

Acknowledgements

R.Visser was supported by grant number 920-03-325 from The Netherlands Organisation for Health Research and Development and this research was supported by Stinafo (“Dutch fund for disabled children”).

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