Targeted Next Generation Sequencing Approach in Patients Referred for Silver-Russell Syndrome Testing Increases the Mutation Detection Rate and Provides Decisive Information for Clinical Management

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Objective To investigate the contribution of differential diagnoses to the mutation spectrum of patients referred for Silver-Russell syndrome (SRS) testing.

Study design Forty-seven patients referred for molecular testing for SRS were examined after exclusion of one of the SRS-associated alterations. After clinical classification, a targeted next generation sequencing approach comprising 25 genes associated with other diagnoses or postulated as SRS candidate genes was performed.

Results By applying the Netchine-Harbinson clinical scoring system, indication for molecular testing for SRS was confirmed in 15 out of 47 patients. In 4 out of these 15 patients, disease-causing variants were found in genes associated with other diagnoses. These patients carried mutations associated with Bloom syndrome, Mulibrey nanism, KBG syndrome, or IGF1R-associated short stature. We could not detect any pathogenic mutation in patients with a negative clinical score.

Conclusions Some of the differential diagnoses detected in the cohort presented here have a major impact on clinical management. Therefore, we emphasize that the molecular defects associated with these clinical pictures should be excluded before the clinical diagnosis "SRS" is made. Finally, we could show that a broad molecular approach including the differential diagnoses of SRS increases the detection rate. *(J Pediatr 2017;187:206-12).*

Silver-Russell syndrome (SRS, OMIM 180860) has a heterogeneous phenotype and is caused by epigenetic and genetic molecular alterations.[1,2] The clinical diagnosis is difficult, and several scoring systems have been suggested.[3] By comparing these scores, it has been shown that the Netchine-Harbison clinical scoring system (NH-CSS) is the most sensitive and, therefore, is in the recently published SRS consensus statement.[2,3] The NH-CSS is based on 6 key features: severe intrauterine growth retardation (IUGR) as well as postnatal growth retardation (≤–2 SDS), relative macrocephaly at birth (≥1.5 SDS above birth weight/length SDS), body asymmetry (leg length discrepancy ≥0.5 cm or <0.5 cm with at least 2 other asymmetrical body parts), feeding difficulties (use of feeding tube or cyproheptadine, or body mass index ≤–2 SDS at 24 months), and a protruding forehead (age 1-3 years). The score is positive, and the clinical diagnosis is considered when at least 4 out of 6 criteria are present.[2,3]

The majority of patients with a positive Netchine-Harbison clinical score (NH-CS) carries disturbances affecting imprinted loci.[4] The most frequent alteration, in 50% of patients, is a (mosaic) hypomethylation of the distal imprinting control region (ICR1, *H19/IGF2* IG-DMR) in 11p15.5.[2,3] In up to 10% maternal uniparental disomy of chromosome 7 (upd(7)mat) can be detected. A further subgroup comprises carriers of chromosome 14 changes, molecularly corresponding to Temple syndrome.[2,5] In single patients, maternal uniparental disomy of chromosomes 16 or 20, as well as chromosomal copy number variations and single point mutations in imprinted genes in 11p15 have been reported (for review Wakeling et al[2]) However, nearly 30% of all patients with a positive NH-CS remain without a molecular diagnosis, though some of them escape routine detection of ICR1 hypomethylation because of its mosaic distribution.[3,6,7]

Broad molecular testing allows the identification of additional mutations and epimutations in clinically typical patients with SRS (eg, deletions or duplications)[8] rather associated with other congenital syndromes (eg, *NSD1* duplication) as well as epimutations on other chromosomes than 7 and 11. Thereby, the significant contribution of disturbances of the imprinted 14q32 region and pathogenic copy number variations could be confirmed, and are now included in the molecular

bp	Base pair	NH-CSS	Netchine-Harbison clinical scoring system
GH	Growth hormone		
IUGR	Intrauterine growth retardation	SRS	Silver-Russell syndrome
NGS	Next generation sequencing		
NH-CS	Netchine-Harbison clinical score		

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diagnostic workup of SRS.[2,3,9] Furthermore, single case reports have proven significant clinical overlap between various differential diagnoses and SRS (for review Wakeling et al[2]) The most important differential diagnoses for SRS include cancer predisposition syndromes as Bloom syndrome, Nijmegen breakage syndrome, and Mulibrey nanism, growth retardation because of mutations in *IGF1* or *IGF1R*, as well as Microcephalic Osteodysplastic Primordial Dwarfism II, Meier-Gorlin syndrome, 3M syndrome, Short stature, hyperextensibility, hernia, ocular depression, Rieger anomaly, and teething delay syndrome, Floating Harbor syndrome, and Intrauterine growth restriction (IUGR), Metaphyseal dysplasia, Adrenal hypoplasia congenita, and GEnitourinary abnormalities syndrome.[2]

With next generation sequencing (NGS), a further step toward a comprehensive analysis of patients with growth retardation and NH-CSS features has been reached. As we assumed that a significant number of patients referred for molecular testing for SRS without a molecular diagnosis after standard testing is actually affected by one of the many differential diagnoses, we set up a NGS-based approach to analyze the currently known genes for differential diagnosis of SRS.

Methods

The cohort consisted of 47 patients with IUGR and/or postnatal growth retardation, referred for SRS routine diagnostic testing. Whenever possible, clinical scoring for SRS was carried out with the NH-CSS.[3] Postnatal growth was examined at varying ages because biometric data at the same ages were not available for all. Therefore, the criterion "protruding forehead" was scored using childhood pictures in patients 1 and 2 who were examined above the age of 3 years. SDS were calculated by using Ped(z) according to German and Swiss epidemiologic data.[10-13]

In the routine diagnostic workup, upd(7)mat and hypomethylation *of H19/IGF2* IG-DMR had been excluded for all patients. Aberrant methylation/uniparental disomy of the imprinted loci *PLAGL1:alt-TSS-DMR* in 6q24, *IGF2R:Int2-DMR* in 6q25, *MEG3:TSS-DMR* and *MEG3/DLK1*:IG-DMR in 14q32, *SNURF:TSS-DMR* in 15q11, *GNAS-AS1:TSS-DMR* and *GNAS-XL:Ex1-DMR* in 20q13 as well as upd(16)mat and pathogenic submicroscopic copy number variations (by SNP6.0 or Cytoscan Array; Affymetrix, Wycombe, United Kingdom) were excluded in nearly all patients.[8,14] The study was approved by the Ethical Review Board of the University Hospital Aachen (Germany).

DNA from peripheral blood samples was isolated either by a conventional salting out method or with the Qiagen Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations.

A NGS panel was designed covering 25 genes, which either have been reported to be associated with differential diagnostic disorders of SRS or which have been suggested as SRS candidate genes (**Table I**; available at www.jpeds.com).

The library enrichment was performed by using a custom enrichment kit designed with the Illumina DesignStudio (Illumina, Inc, San Diego, California) in 46 patients or, in 1 case, the Nextera Rapid Capture Exome (FC-140-1083; Illumina, Inc) according to the manufacturer's protocols.

Sequencing of the custom enrichment library was performed on a MiSeq platform with 2 × 151 paired end reads and v2 chemistry. Sequencing data were aligned to the hg19 reference genome and variant call format files and binary sequence alignment/map files were generated by the Illumina MiSeqReporter with standard software settings.

The whole exome enrichment library was sequenced on a NextSeq500 platform with 2 × 151 paired end reads and NextSeq high output v2 chemistry. Fastq files were locally generated from sequencing raw data (BCL2fastq2). Using Illumina BWA Enrichment workflow (v 2.1.0, reference genome: hg19) binary sequence alignment/map files and variant call format files were generated. On average, a coverage of ×10 was reached in 97.8% of regions of interest in case of the targeted NGS assay. For the whole exome sequencing approach ×10 coverage was obtained in 98.9% of regions of interest. Variants located in regions that had a low coverage as well as variants that did not pass the defaults filter variables provided by Illumina were not considered.

Variant annotation was performed using the Illumina Variant Studio (v 2.2.1). For variant filtering, the following variables were chosen: variants with a minor allele frequency >1% in the 1000 genomes project database (April 2012 phase 1 call set), in the Exome Variant Server (NHLBI GO Exome Sequencing Project, Seattle, Washington (evs.gs.washington.edu/EVS, accessed November 2012), or the Exome Aggregation Consortium database (Cambridge, Massachusetts (http://exac.broadinstitute.org, accessed August 2016), and synonymous variants were excluded. All remaining insertion, deletion, start loss, stop loss, stop gain, splicing as well as missense variants scored as "damaging" by PolyPhen and "deleterious" by SIFT in Variant Studio were investigated and rated with the American College of Medical Genetics guidelines.[30] For the further estimation of pathogenicity of missense variants Alamut 2.7.2, SIFT, PolyPhen-2, and MutationTaster (all accessed August 2016) were applied.[31-33] However, it must be considered that the accuracy of these in-silico prediction programs is less than 80%.[30]

Confirmation and segregation of pathogenic and likely pathogenic variants detected in the NGS approaches were performed by Sanger sequencing on an ABI3130 Sequencer (Applied Biosystems, Waltham, Massachusetts) in the patients and their families.

In patients carrying homozygous mutations, CytoScan HD Array data (Affymetrix, Santa Clara, California) were reanalyzed for the detection of regions with loss of heterozygosity to exclude a compound heterozygosity for a point mutation and a larger deletion.

Results

Samples from 47 patients referred for molecular genetic testing for SRS were analyzed with a targeted NGS-based approach. Clinical data for at least 4 NH-CSS criteria were available for all but 2 patients. Thus, the indication for molecular testing