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Epigenetic mutations of the imprinted IGF2-H19 domain in Silver-Russell Syndrome (SRS): Results from a large cohort of patients with SRS and SRS-like phenotypes.

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Key words:
Silver-Russell syndrome, imprinting, methylation defect, clinical scoring, 11p15

Word count: 3849

Abbreviations:
BWS; Beckwith-Wiedemann syndrome, DMR differentially methylated region. ICR1; imprinting control region 1, mUPD7; Maternal uniparental disomy for chromosome 7, MLPA; multiplex ligation dependant probe amplification, OFD; occipito frontal diameter, SD; standard deviation, SRS; Silver-Russell syndrome
Abstract

**Background:** Silver-Russell syndrome (SRS) is a clinically and genetically heterogeneous condition characterized by severe intrauterine and postnatal growth retardation. Loss of DNA methylation at the telomeric imprinting control region 1 (ICR1) on 11p15 is an important cause of SRS.

**Methods:** We studied the methylation pattern at the H19-IGF2 locus in 201 patients with suspected SRS. In an attempt to categorize the patients into different subgroups, we developed a simple clinical scoring system with respect to readily and unambiguously assessable clinical features. In a second step, the relationship between clinical score and epigenetic status was analyzed.

**Results and conclusions:** The scoring system emerged as a powerful tool for identifying those patients with both a definite SRS phenotype and carrying an epimutation at 11p15. 53% of the 201 patients initially enrolled fulfilled the criteria for SRS and about 40% of them exhibited an epimutation at the H19-IGF2 locus. Methylation defects were restricted to patients who fulfilled the diagnostic criteria for SRS. Patients carrying epimutations had a more severe phenotype than either the SRS patients with mUPD7 or the idiopathic SRS patients. The majority of patients with methylation abnormalities showed hypomethylation at both the H19 and IGF2 genes. However, we also identified SRS patients where hypomethylation was restricted to either the H19 or the IGF2 gene. Interestingly, we detected epimutations in siblings of normal parents, most likely reflecting germ cell mosaicism in the fathers. In one family, we identified an epimutation in an affected father and his likewise affected daughter.
Introduction

Silver-Russell syndrome (SRS, MIM 180860) is a clinically heterogeneous condition characterized by severe intrauterine growth retardation, poor postnatal catch up growth, craniofacial features such as a triangular shaped face and a broad forehead, body asymmetry and a variety of minor malformations. The phenotypic expression changes during childhood and adolescence and the facial features and asymmetry usually become much more subtle with age. Although different groups have established diagnostic criteria for SRS, diagnosis often remains doubtful and children with intrauterine growth retardation of various etiologies are spuriously diagnosed with SRS. [1,2] This has caused confusion with regard to the true incidence and natural history of SRS.

It has been recognized since a long time that SRS, which mostly occurs sporadically, is very likely a genetically heterogeneous condition with a complex genetic background. [3] However, until recently a genetic defect could only be identified in a small subset of patients with SRS: maternal UPD7 in about 5-10% of SRS cases and a chromosomal rearrangement such as maternal dup(11p15) and dup(7p11.3-p13) or segmental mUPD(7q31-qter) in less than 1%. [4,5,6,7,8,9] This situation changed in 2005 when hypomethylation of the differentially methylated IGF2/H19 imprinting center region (ICR1) at 11p15 was reported in a small number of SRS patients. [10] This finding was soon confirmed in larger cohorts of SRS patients and it turned out that partial or complete loss of DNA methylation at the telomeric ICR1 on 11p15 represents a major cause for SRS. [11,12,13,14] Interestingly, opposite epimutations to the ones observed in SRS namely hypermethylation of IGF2/H19 are observed in about 5-10% of patients with the Beckwith-Wiedemann syndrome (BWS), an overgrowth syndrome representing in many respects the countertype to SRS. [15]

The 11p15 region comprises two imprinted domains important for the control of fetal and postnatal growth, ICR1 and ICR2. ICR1 coordinates the expression of two oppositely imprinted genes, H19 and IGF2. H19 encodes a non translated RNA and is expressed exclusively from the maternal allele. [16] Hypomethylation of H19 in SRS is proposed to lead to biallelic expression of H19, down regulation of IGF2 and hence growth restriction. How the 11p15 epimutation causes the specific SRS phenotype is not completely understood at the moment. Up to now about 100 patients - mainly children - with 11p15 epimutations have been reported in the literature. However, the phenotypic spectrum of epimutation carriers and their long term perspective still remain vague.

The present study aimed at determining more precisely the clinical phenotype(s) associated with epimutations at the 11p15 region. We wanted to determine whether the patients with methylation defects might comprise a distinct group from idiopathic SRS patients and the ones with mUPD7. As a first step we developed a simple clinical scoring system with regard to the severity of the SRS phenotype. Then, in a second step, we analyzed the relationship between clinical score and epigenetic status.
Patients and Methods

Study population (Table 1)
The study population consisted of 201 individuals with suspected SRS. The patients were followed in one of the Genetic Clinics participating in this study (Zürich, Warsaw, Minsk, Istanbul) between 1990 and 2007, with the exception of 15 individuals whose blood samples were sent to Zürich for genetic analysis. Informed consent for analysis was obtained from all individuals included in the study: i.e. adults (patients and parents) and parents of underage patients. The study was performed in accordance with national ethics guidelines.

Table 1
Characteristics of study population

<table>
<thead>
<tr>
<th>Patients included</th>
<th>201</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio male/female</td>
<td>106/95</td>
</tr>
<tr>
<td>Patients excluded because of other diagnosis, e.g. chromosomal aberration, etc.</td>
<td>4</td>
</tr>
<tr>
<td>Patients excluded because clinical dataset incomplete</td>
<td>29</td>
</tr>
<tr>
<td>Sufficient clinical data available</td>
<td>168</td>
</tr>
<tr>
<td>Ratio male/female</td>
<td>87/81</td>
</tr>
<tr>
<td>Mean age at evaluation</td>
<td>6.5 y (1-42 y)</td>
</tr>
</tbody>
</table>

Clinical scoring system (Table 2)
Each patient was physically examined by a geneticist in one of the participating centers. The mean age at physical examination, usually coinciding with the blood sampling, was 6.5 years, with a range from 1 to 42 years of age. A form with information concerning birth measurements, perinatal adaptation, postnatal course, dysmorphic signs, feeding behavior, intellectual development and family history was completed by the examining physician. Facial features and body asymmetry were assessed from hospital charts or childhood photographs in those patients who had already reached adulthood (about 5% of the study sample). The clinical data of all the patients were entered into a database. Upon gathering the entire body of clinical information of the cohort we decided to omit information on bone age, target height, feeding difficulties, hypoglycemia, sweating and teeth abnormalities: although these symptoms represent important diagnostic features in SRS, they were often reported imprecisely or were missing. We therefore decided to retain in the database only those clinical features of the patients that were readily and unambiguously - and therefore reliably - assessable. Using the clinical criteria for SRS defined by Price et al. in 1999 as its basis, we established a refined “SRS severity” scoring system: Each patient was scored for the five domains, 1. Parameters at birth, 2. Postnatal course, 3. Asymmetry, 4. Facial features, 5. Other features (see Table 2). For four of the five domains 3 features were scored and 0-3 points were given with 0 depicting that none of the three features was present, 1 that one was present, etc.
Asymmetry was assessed in a different way: Since this is a very important feature in SRS but difficult to quantify we decided to score this feature simply as present (3 points) or missing (0 points). According to this procedure a minimum total score of 0 points and a maximum total score of 15 points was assigned.

Scoring of all the patients (on the basis of the information collected in the database) was performed by one of the authors who was not involved in examination of the patients and who was blind with respect to the findings of the molecular analysis. When scoring of the cohort was completed, we carefully compared the clinical presentation of those patients who displayed scores in the middle range (between 6 to 10 points). It was arbitrarily defined that patients with a score $\geq 8$ points were classified as SRS patients whereas those with lower scores were not (non-SRS patients).

### Table 2

**Clinical SRS score**

<table>
<thead>
<tr>
<th>Parameters at birth</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight $\leq 10^{th}$ percentile$^1$</td>
<td>1</td>
</tr>
<tr>
<td>Length $\leq 10^{th}$ percentile$^1$</td>
<td>1</td>
</tr>
<tr>
<td>Relative macrocephaly$^2$</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Postnatal course</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td>No catch up growth; length $\leq 3^{rd}$ percentile$^3$</td>
<td>1</td>
</tr>
<tr>
<td>Normal head circumference:</td>
<td></td>
</tr>
<tr>
<td>OFD $\geq 3^{rd}$ and $\leq 97^{th}$ percentile$^3$</td>
<td>1</td>
</tr>
<tr>
<td>Normal cognitive development$^4$</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Asymmetry</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Face/body/limbs</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Facial features</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Triangular shaped face</td>
<td>1</td>
</tr>
<tr>
<td>High/Bossing forehead</td>
<td>1</td>
</tr>
<tr>
<td><em>Other:</em> e.g. small chin, thin lips, down turned corners of the mouth, late closure of fontanelle</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other features</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinodactyly 5th finger</td>
<td>1</td>
</tr>
<tr>
<td>Genital abnormalities (e.g. cryptorchidism, hypospadias)</td>
<td>1</td>
</tr>
<tr>
<td><em>Other:</em> e.g. brachymesophalangy, syndactyly toes inguinal hernia, pigmentary changes</td>
<td>1</td>
</tr>
</tbody>
</table>

Maximal score 15$^*$

$^1$ according to [17]

$^2$ defined as OFD (occipito frontal diameter) at least 1.5 SD above birth weight or length according to [17]

$^3$ according to [18]

$^4$ defined as attending regular school

$^*$ Patients with scores $\geq 8$ were classified as SRS
**Statistical analysis**
The characteristics of the population were described as percentage for qualitative traits or as median, mean and SD for quantitative traits (clinical scoring). The relationship between the severity score and the epigenetic status ($H19/IGF2$ hypomethylation group, $H19$ hypomethylation group, mUPD7 group, idiopathic SRS group) was assessed by one-way analysis of variance (ANOVA). $P$ values $< 0.05$ were considered to indicate statistical significance.

**Methylation analysis for the 11p15 region**
DNA was extracted from peripheral blood samples of all individuals: control individuals, patients, and at times parents and siblings. One hundred age-matched healthy individuals served as controls.

The methylation status at $H19$ and $IGF2$ was investigated by MLPA (ME030, MRC, Holland, version 03, 3-1-2007) according to the protocol supplied by the manufacturer. Briefly, approximately 100 ng DNA of each individual were first denatured and hybridized overnight with the mixture of probes (supplied with the kit), and then, after splitting the sample in two, treated either with ligase alone or with ligase and $HhaI$. PCR reactions were then performed with the reagents and primers supplied in the kit. The PCR products were separated by capillary electrophoresis (ABI310). The relative ratio of methylation at $H19$ and $IGF2$ were calculated as follows: the areas of the peaks of interest were divided by the average peak area of the closest control peaks. For each peak, the relative area of the sample treated with $HhaI$ was divided by the value obtained for the undigested sample. These ratios were normalised by dividing by the average ratios obtained for control individuals. Mean values of the 100 control individuals are depicted in the legend of Figure 1. The results obtained for the probes 6266-L5772 H19DMR (301 bp) and 7175-L6784 IGF2 (AY375532.1, 9046-9047) DMR0 (211 bp) were in agreement with previous methylation analysis by MSP/DHPLC (data not shown) and were not subject to high variability due to the presence of polymorphisms in the target sequences.

**Analysis for mUPD7**
The possible occurrence of maternal UPD7 of the two critical regions on chromosome 7 (7p11.2-p13 and 7q32-34) was investigated prior to this study (data not shown). Analysis was performed as described in one of our earlier reports by genotyping DNA samples from the patient, mother, and father with 13 chromosome-7-specific polymorphic microsatellite markers. [19] At least one and preferably two informative markers per region were considered necessary to exclude UPD.

**Analysis of familial cases**
In the three families with either two affected siblings or vertical transmission (father and daughter affected) we sequenced the CTCF-binding sites in the DMR 5' of the $H19$ gene. Primer sequences were as reported previously [12]. To document the methylation status at another paternally imprinted locus the DMR of the $DLK1-GTL2$ locus on chromosome 14q32 was analysed, as described previously. [20]
Results

Clinical scoring
The characteristics of the study population are summarized in Table 1 and 3. According to the scoring system described above, 106 individuals had scores of ≥ 8 points and were classified as SRS, and this constituted 63% of all the patients scored. A diagnosis of SRS was accordingly excluded in 62 individuals (37%). These non-SRS individuals were primarily patients with prenatal growth retardation. No definitive diagnosis could be established in these patients.

Chromosomal analysis (data not shown)
One individual with a female phenotype turned out to have a male karyotype in blood lymphocytes (46,XY). This patient also carried an epimutation at 11p15 and will be reported on in detail in a separate publication. The remainder of the individuals had normal karyotypes.

Molecular evaluation
Epimutations at 11p15 are specific for SRS and are not a cause of nonspecific pre- or postnatal growth retardation (Table 3 and 4, Figure 1)
In 46 patients, hypomethylation at the ICR1 on 11p15 was detected. The clinical data set was incomplete in four of these and they were therefore excluded. From the 42 remaining patients, 41 had a score of ≥ 8 points and were therefore classified as SRS. From our cohort of 200 patients, we could therefore demonstrate that epimutations at 11p15 were specific to patients with classical SRS and were not present in patients, primarily children, either with isolated growth retardation, body asymmetry or minor anomalies similar to those occurring in SRS. There was only one exception to this, a female patient in mid-childhood who had a score of 6 and did therefore not fulfill the criteria for SRS. This girl actually exhibited a partial hypomethylation specific to \textit{H19} (not \textit{IGF2}). She showed borderline prenatal and pronounced postnatal growth retardation, no facial dysmorphisms, mild brachyphalangy, and no asymmetry. Unfortunately it was not possible to organize a follow up visit for this girl to verify her clinical profile.

Epimutations at 11p15 are restricted to the telomeric gene cluster and are not always congruent for the \textit{H19} and \textit{IGF2} gene (Table 4, Figure 1)
The MLPA technique applied allowed us to analyze the telomeric and the centromeric gene cluster on 11p15 simultaneously. In our cohort no methylation defects were detected at the \textit{centromeric} cluster where \textit{CDKN1C} and \textit{KCNQ1OT1} are located. Differential analysis of the \textit{H19} and \textit{IGF2} genes in the telomeric cluster revealed that the majority of patients showed hypomethylation of both \textit{H19} and \textit{IGF2}. Interestingly, 11 patients showed selective hypomethylation of \textit{H19} and three patients selective hypomethylation of \textit{IGF2}. The \textit{IGF2}-specific probe showed a broader variation in controls as compared to the \textit{H19} probe and these results should be interpreted with some reservation, therefore.

Epigenetic- phenotype correlation (Table 4 and Figure 1)
Patients carrying epimutations had higher scores then the ones with mUPD7 or the idiopathic SRS patients, indicating that hypomethylation at 11p15 was associated with a severe SRS phenotype. Median severity scores, means and SDs are depicted in Table 4. The differences of the severity scores were highly significant when comparing the group with combined hypomethylation of \textit{H19} and \textit{IGF2} with the mUPD7 patients (P<0.001) or the idiopathic SRS patients (P<0.001) and still significant when comparing the group with hypomethylation of \textit{H19} to the mUPD7 and idiopathic SRS group (P<0.01). The
scores of the mUPD7 group were not significantly different when compared to the idiopathic SRS group (P>0.05). Since we identified only 3 patients with hypomethylation restricted to IGF2, no statistical analysis could be performed for this group.

The methylation index, reflecting the degree of hypomethylation in a given individual, seemed to correlate with the score and therefore with clinical severity, at least for H19. That is, the relationship between hypomethylation of H19 and clinical severity might be quantitative. The small number of patients did not allow statistical analysis with regard to this point.

Our scoring system was not designed to establish a correlation between the presence of individual features and the epigenotype. However, it was obvious that asymmetry was more frequent in those SRS patients with a methylation abnormality when compared to the other groups: 65% (27 of 42) of SRS patients with a methylation defect exhibited body asymmetry, versus 40% (23 of 58) of idiopathic SRS patients, 20% (2 of 10) of patients with mUPD7 and 5% (3 of 62) of non-SRS patients.

**Familiar cases with epimutations at 11p15**

Two families, each with two children who displayed hypomethylation of H19 and IGF2, were identified. In both families neither of the parents showed clinical signs of SRS and methylation analysis in both fathers revealed normal results. In the first family a school-aged boy and a girl were affected, both of them showing a classical SRS phenotype with asymmetry of the limbs in the boy and an asymmetrical face in the girl. In the second family the affected siblings were both girls. A report on the second family has already been published before epimutations at 11p15 were demonstrated in SRS. [21] Both girls were scored at 14 points, reflecting the severe phenotype. In a third family we detected an epimutation at 11p15 in both a clinically affected father in his third decade (displaying a score of 10) and his affected daughter (score of 12 points). None of the familiar cases showed any clinical symptoms in addition to the SRS phenotype.

Sequence analysis of the CTCF-binding sites in the DMR between the IGF2 and the H19 genes did not reveal any mutations, and analysis of the paternally imprinted DLK1-GTL2 locus on chromosome 14q32 showed a normal methylation pattern in the three families.

**Maternal uniparental disomy (mUPD7)** (Table 4 and Figure 1)

Ten individuals displayed maternal uniparental disomy of chromosome 7. Interestingly, only 7 out of these 10 individuals fulfilled the diagnostic criteria for SRS according to our scoring system. The 7 patients with SRS phenotypes showed significantly lower scores when compared to the ones displaying hypomethylation of 11p15. Comparison with the idiopathic SRS patients did not show a statistically significant difference of the severity scores.
Table 3  
Frequency of methylation defects and mUPD7 in the patient cohort

<table>
<thead>
<tr>
<th></th>
<th>Patients of initial cohort</th>
<th>Patients clinically scored</th>
<th>Patients clinically scored as SRS*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong># of patients</strong></td>
<td>201</td>
<td>168</td>
<td>106</td>
</tr>
<tr>
<td>Methylation defect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H19 and IGF2</td>
<td>32</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>Methylation defect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H19</td>
<td>11</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Methylation defect</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF2</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>Total methylation defect</strong></td>
<td><strong>46 (23%)</strong></td>
<td><strong>42 (25%)</strong></td>
<td><strong>41 (38.6%)</strong></td>
</tr>
<tr>
<td>Telomeric cluster</td>
<td>none</td>
<td>none</td>
<td>none</td>
</tr>
<tr>
<td>Maternal UPD7</td>
<td>10</td>
<td>10</td>
<td>7 (6.6%)</td>
</tr>
<tr>
<td>No genetic abnormality identified</td>
<td>141</td>
<td>116</td>
<td>58 (54.7%)</td>
</tr>
</tbody>
</table>

*Patients with scores ≥ 8 were classified as SRS.
Table 4
Distribution of clinical severity scores in different groups of patients

<table>
<thead>
<tr>
<th>Clinical Score</th>
<th>H19 and IGF2 hypomethylation</th>
<th>H19 hypomethylation</th>
<th>IGF2 hypomethylation</th>
<th>Maternal UPD7</th>
<th>Idiopathic SRS patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;8</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>11</td>
<td>5</td>
<td>2</td>
<td>-</td>
<td>1</td>
<td>8</td>
</tr>
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<td>12</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>1</td>
<td>-</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>15</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Median score</td>
<td>13</td>
<td>11</td>
<td>-</td>
<td>8</td>
<td>9</td>
</tr>
</tbody>
</table>

# of patients
29 patients 11 patients 2 patients 10 patients 58 patients

Column 2
A  B  C  D  E

Clinical scores are ranging from 1 (minimal) to 15 (maximal). Patients with scores ≥ 8 were classified as SRS phenotypes, the ones with scores <8 as non SRS phenotypes. Columns: Means and SDs: Column A: 12.7±1.7, Column B: 11±2.4, Column C: no values due to low number of patients, Column D: 8.6±2.3, Column E: 9.7±1.6. P values: Column A vs D: P<0.001, A vs E: P<0.001, B vs D: P<0.01, B vs E: P<0.01, D vs E: P>0.05. Statistical significance was considered with a P value <0.05.
Figure 1
Average ratios of methylation at H19 and IGF2 in SRS patients compared to normal controls

Designation of patient groups (A-E) and number of patients in each group: see Table 4. Small symbols: relative methylation at H19 and IGF2 for single patients (one each with clinical scores of 6, 8 and 14, respectively). The relative ratios of methylation represent the results obtained with the probes: 6266-L5772: H19, and 7175-L6784: IGF2 (MRC-Holland, kit ME030 BWS/RSS). Analysis of 100 control individuals revealed a mean value of 1.048 (SD 0.106) for H19 and 0.99 (SD 0.121) for IGF2.
Discussion

This study of 201 individuals with SRS and SRS-like phenotypes confirms the high frequency of methylation abnormalities at the H19/IGF2 locus in SRS: epimutations occurred in about 40% of patients with SRS. In total, we identified more than 40 individuals with partial hypomethylation at the ICR1 on 11p15, which is the largest cohort of SRS patients with epimutations reported in the literature so far. Methylation defects were with one exception restricted to patients who fulfilled the diagnostic criteria for SRS.

Establishing the diagnosis of SRS is not always easy and there is no general consensus for diagnostic criteria. This has added confusion to the question of frequency of 11p15 epimutations in SRS: While the French group which was first to describe methylation abnormalities on 11p15 in SRS detected epimutations in 64% of a large series of SRS patients, other groups identified this abnormality in their cohorts at lower frequencies of 30-40%. Differences in detection rates might be explained to some extent by the molecular techniques used: it was recently shown that MLPA is more sensitive than Southern blot analysis for assessment of the methylation status at 11p15. Much more important, however, are the criteria used for establishing the SRS diagnosis in a given individual. The frequency of 40% epimutation carriers in our cohort therefore reflects the fact that we included patients with a broad clinical spectrum from borderline to classical SRS phenotypes.

To address the question of clinical heterogeneity and stringency of diagnostic criteria in SRS we developed a very simple scoring system which permits calculating a SRS severity score ranging from 0-15 points. The advantage of this scoring system is that it allows the determination of the phenotype on a quantitative basis. It emerged that patients with hypomethylation had significantly higher scores and therefore more severe phenotypes when compared to the idiopathic SRS patients where no genetic abnormality was identified or the patients with mUPD7: 95% of the patients with a methylation abnormality had scores between 10 and 15 points, whereas only 46% of the idiopathic SRS patient were within this range. This has important implication for the diagnostic strategy in an individual with SRS: The chance of detecting a methylation abnormality in a child with SRS and a low score of 8 or 9 points is very small and analysis for mUPD7 would be recommended first. On the contrary, methylation analysis should be the first diagnostic step in those patients with high scores between 10 and 15 points, especially in individuals with asymmetry. One might speculate that the milder phenotype of the SRS patients in whom we could not identify a genetic abnormality might carry a methylation abnormality on 11p15 which escaped analysis in blood samples. It is well known from BWS that methylation defects at the 11p15 locus mostly occur as somatic mosaics and that the detection rate depends on the tissue analyzed. It would be interesting, therefore, to test other tissues as for example fibroblasts for methylation abnormalities in those “blood negative” patients.

IGF2 is a paternally expressed fetal growth factor and H19 a maternally expressed non-coding RNA for which the function is still not very well understood. Imprinted expression of these two genes is regulated by the action of the H19 DMR. It largely remains unknown how biallelic silencing of IGF2 and activation of H19 translates into the specific SRS phenotype. It has been shown that hypomethylation of H19 in SRS patients is associated with decreased levels of IGF2 mRNA in fibroblasts, but IGF2 serum levels seem to be mostly normal in epimutation carriers. [10, 13, 24]. This observation is not surprising given the development- and tissue-dependent expression pattern of this growth factor, with monoallelic expression from the paternal allele in fetal life and biallelic expression in postnatal life. [25].
We observed selective hypomethylation for either *H19* or *IGF2* in 13 patients, indicating that hypomethylation of *IGF2* alone seems to be sufficient to cause classical SRS. The phenotype of those patients with selective hypomethylation at one locus was generally milder than in the patients displaying hypomethylation of *H19* and *IGF2*. However, given the small number of patients (11 for selective *H19* and 2 for *IGF2* demethylation) this observation needs to be confirmed. To date there is limited knowledge on the methylation status of *IGF2* in SRS and it is not known to what extent the proposed silencing contributes to the SRS phenotype. A recent paper on methylation changes at the *IGF2/H19* locus in SRS, BWS and Wilms’s tumor, reports on similar methylation abnormalities at *H19* and *IGF2* in the congenital growth disorders, but different methylation profiles in tumors. [26] The authors propose different mechanisms of imprinting loss in neoplastic cells and growth disorders. Along these lines it remains open whether loss of imprinting in those SRS patients with combined *H19/IGF2* hypomethylation is caused by a different mechanism than in the cases with selective hypomethylation of *H19* or *IGF2*.

In this study we describe for the first time familial cases of hypomethylation at the 11p15 locus. This finding has important implications for both genetic counseling and determination of the origin of the epimutations. The setting of epigenetic marks in germ cells has been studied in detail in mouse and to a lesser extend also in humans. [27] Methylation marks at imprinted loci are thought to be erased in primordial germ cells and then reacquired during spermatogenesis and oogenesis. In the majority of epimutation carriers identified in this study the loss of the paternal methylation mark may have resulted from a deficient acquisition of methylation during spermatogenesis or from lack of maintenance of the methylation mark on the paternal allele after fertilization. The two families for whom we identified epimutations in siblings most likely represent germ cell mosaicism of an incorrect methylation mark at the ICR1 during spermatogenesis in the fathers.

The third family where the epimutation was transmitted from an affected father to his daughter is the first case of vertical transmission through the male germ line described in the literature. The underlying mechanism is difficult to explain in this family. Obviously the affected father displays a missing methylation mark at the *H19/IGF2* DMD in somatic and germ cells. Reprogramming of the methylation pattern in his primordial germ cells did not lead to a correction (setting of a methylation mark) but the “mistake” was continued when resetting of the methylation pattern at imprinted loci occurred during spermatogenesis. How this mistake was “memorized” in the germ cells of the father remains to be explained. Father and daughter showed a classical SRS phenotype and did not have additional symptoms indicative of a different etiology compared to the sporadic cases. Both displayed partial hypomethylation at the *H19/IGF2* DMD and analysis of another paternally imprinted locus at 14q (*DLK1-GTL2*) showed a normal methylation pattern. Therefore, although the clinical picture and the molecular findings do not point into this direction it can not be excluded that father and daughter carry a mutation in one of the DNA methyl-transferase genes or an other gene involved in DNA-methylation.

It is interesting to note that the 4 children of the two families with suspected germ cell mosaicism in the fathers displayed hemihypoplasia. It has been hypothesized that asymmetry in BWS and SRS is caused by mosaic distribution of post-zygotically acquired epimutations. [28] However, the observation of asymmetry in the offspring of men with suspected germ cell mosaicism for epimutation at the *H19/IGF2* locus renders it rather unlikely that asymmetry can be explained by mosaic distribution of the epimutation: In those children the epimutation did most likely not emerge post-zygotically and the asymmetry has therefore to be explained by another still unknown mechanism.
In summary, our study has important implication for diagnosis and genetic counseling in SRS. First, the clinical scoring system we developed is a simple but powerful tool to direct the diagnostic efforts rapidly towards methylation analysis in those patients with a severity score in the upper range. Second, we demonstrate that hypomethylation of *H19* or *IGF2* alone appears to be sufficient to cause SRS. Third and most importantly, the observation of germ cell mosaicism and vertical transmission from an affected father to his child demonstrate that there is a recurrence risk for epimutations in SRS.
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