THE DEGREE OF H19 HYPOMETHYLATION IN CHILDREN WITH SILVER-RUSSEL SYNDROME (SRS) IS NOT ASSOCIATED WITH THE SEVERITY OF PHENOTYPE AND THE CLINICAL SEVERITY SCORE (CSS)

Zoran S Gucev1, Ljiljana Saranac2, Aleksandra Jancevska1, Velibor Tasic1

1 Medical Faculty Skopje, 50 Divizija BB, 1000 Skopje, R. Macedonia
2 Medical Faculty Nish, Serbia

Corresponding Author: Zoran S. Gucev, Medical Faculty Skopje, 50 Divizija BB, 1000 Skopje, R. Macedonia, E-mail: gucevz@gmail.com

Abstract

Background: Hypomethylation of the imprinting control region 1 (ICR 1) at the IGF2/H19 locus on 11p15 is linked to Silver-Russel syndrome (SRS).

Methods and results: We tested the hypothesis that the severity of the phenotype in SRS patients is dependent on the clinical severity score (CSS) (1). Three SRS patients were clinically scored and their scores ranged between 12, 13 and 13. Two of the three SRS patients (66%) had hypomethylation of one allele.

Conclusion: All three patients had high CSS. Nevertheless, only two of them had hypomethylation of one H19 allele. Interestingly, two of them had ventricular septal defects, but only one had H19 hypomethylation. All children had low birth length and weight, a classic facial phenotype, haemihypertrophy (> 2.5 cm thinner left arm/leg in comparison to the right one), shorter leg, and striking thinness (BMI of > 16.0). One child was operated for cryptorchidimus, and the same child had elbow contracture. Two children had scoliosis. All three children were short (–3 to 5.5 SD), and treatment with GH resulted in growth on the third percentile. Since one child had no hypomethylation and two had a lower degree of hypomethylation, the higher CSS (12, 13 and 13) was not followed by a higher degree of hypomethylation of the IGF2/H19 locus.

Key words: Silver-Russell Syndrome, methylation index, IGF2/H19 locus.

Introduction

The diagnosis of SRS is notoriously difficult. Clinical diagnosis is mostly based on the criteria proposed by Price et al. [2]. This system requires at least four of the following criteria for the diagnosis of SRS: intrauterine growth retardation (IUGR), poor postnatal growth, relatively normal head circumference, classic facial phenotype (triangular-shaped face, broad forehead, pointed, small chin and a wide, thin mouth) [2]. The reliability of this criteria system was confirmed by the analysis by Wollmann et al. [3] of 386 SRS patients. Nevertheless, a number of additional symptoms and signs were added to the description of SRS: generalized camptodactyly (many with distal arthrogryphosis), hypospadias, inguinal hernia, severe feeding problems. Sweating and pallor were described in 52% of parents in the early weeks of life. A third of the patients are considered for special education. Anderson et al. [4] 2002 found high incidence of gastrointestinal symptoms (77%): gastroesophageal reflux disease (34%), oesophagitis (25%), food aversion (32%), and failure to thrive (63%).

The proposals of Rossignol et al. [5] and Netchine et al. [6] emphasised the definition of small for gestational age, requiring low birth weight and/or length as mandatory features for the diagnosis of SRS. Given the complexities
of SRS diagnosis a simplified clinical score
system proposed by Bartholdi et al. [2] 2009
seemed to be of much value.

A number of publications claim that the
severity of the SRS phenotype is dependent on
the level of hypomethylation [6–8]. We looked
at the clinical data and the methylation indexes
(MI) in the three SRS patients in order to test
the hypothesis that the severity of the pheno-
type in SRS is linked to a higher MI.

Methods, patients and results

Methylation Analysis of the H19 DMD,
H19 promoter and KCNQ1OT1 region

2 µg of genomic DNA was converted by
bisulphite treatment using the EZ Methylation
Kit™ (Zymo Research) and 4 µl of the 20 µl
eluted converted genomic DNA was used for
each PCR reaction. The following primers were
used: H19 promoter F-ggtatggttttttgaggggagat; H19
promoter R-catcccaccccctccctcacccta; H19
DMD F-gtagggtttttggtaggtatagag; H19 DMD
R-cttaaataacccraaacrtttccac. KCNQ1OT1 F-tgt-
tgaggagtttyggggaggatta and KCNQ1OT1 R-ca-
cctcacacccaaccaatacctcat.

HotStarTaq® (Qiagen) DNA polymerase
in a total volume of 25 µl was treated as fol-
lows: activation at 96ºC for 16 minutes, for 1
cycle; 44 cycles of denaturation at 96ºC for 30
seconds, ATºC for 30 seconds, extension at
72ºC for 30 seconds; 72ºC final extension for 5
minutes, where the annealing temperatures for
the amplicons H19 promoter, H19 DMD and
KCNQ1OT1 were 58ºC, 53ºC and 50ºC respec-

tively. 5 µl of PCR product was chec-ked on a
1% agarose gel (Bioline) to verify that the cor-
rect size product was generated (H19 promoter,
H19 DMD and KCNQ1OT1 were 329bp, 212
bp and 373 bp respectively).

The PCR product (12 µl) was radiolabel-
led by incorporating 32P dATP (Amersham) in
one additional cycle of PCR to a final volume
of 20 µl of the appropriate Methylation sensi-
tive restriction enzyme (5 units) was added to
the PCR product and digested for 3 hours at the
recommended temperatures. For H19 promoter,
PCR products were digested with Taq I (Fer-
mentas) for 3 hours at 65ºC digesting the 329
bp product into 167 bp and 162 bp fragments.
For H19 DMD, PCR products were digested
with BstU I (New England Biolabs) for 3 hours
at 60ºC digesting the 212 bp product into 163
bp and 49 bp fragments and for the KCNQ1OT1
product, PCR products were digested with Taq
I (New England Biolabs) for 3 hours at 65ºC
digesting the 373 bp product into 152 bp, 116
bp, 86 bp and 19 bp fragments.

Radiolabelled digests were electrophore-
sed on 6% polyacrylamide gels in TBE buffer,
gels were dried and exposed to Phosphor screens
and radioactive incorporation was measured
volumetrically. MI was determined by calculat-
ing the ratio of methylated volume divided by
the sum of methylated and non-methylated
volume.

In healthy Caucasians, the MI range is
0.41 to 0.83, with the majority of cases at 0.5–0.6.

Patients and results

Patient 1: The patient was the third child
of healthy parents. He was born after a preg-

nancy of 40 weeks and 2 days, with a birth
weight of 2,450 g (−2.1 SD), birth length 45
cm (−3.0 SD), and skull circumference of 33.5

cm (−0.4 SD).

In the first months, there was a
failure to thrive. At age 5 months, a ventricular
septal defect was detected, and surgically cor-
rected at the age of five. He has the classic
facial phenotype of triangular-shaped face, broad
forehead, pointed, small chin and a wide, thin
mouth. There is asymmetry of the arms and
legs (left side shorter by 1.5 cm), his BMI is
14.9. Clinodactyly and elbow contractures are
present. Mental development is retarded;
he
studies at a school for the mentally challenged.
Tests of GH pituitary reserve, T4, TSH were
uneventful. After 8 years of growth hormone
treatment his final height at the age of 17.5
years is 154.7 cm. His MI is: H19 Promoter
0.51 (normal), H19 DMD 0.45 (normal).

Patient 2: The patient was born after a
pregnancy of 39 wk and 3 d, with a birth weight
of 2,450 g (−2.1 SD), birth length 45
cm (−3.0 SD), and skull circumference of 33.5

cm (−0.4 SD). Referred at the age of 2 years she
was slender with a height of 77 cm (−2.5 SD),
head circumference of 47.5 cm (+0.01 SD),
and a weight of 8,050 g (−3.15 SD for her
length). She has the classic facial phenotype of
triangular-shaped face, broad forehead, pointed,
small chin and a wide, thin mouth. The left leg
is 2 cm shorter than the right one. Her intelli-
gence is normal (IQ 100). Tests of GH pituitary reserve, T4, TSH were uneventful. She has been treated with growth hormone for eight years and grows on the third percentile of the growth curve. At the age of 14 years her height is 141.7 cm (−2.5 SD). She is an excellent student in a regular primary school. Her MI is: H19 Promoter 0.26 (low), H19 DMD 0.65 (normal).

Patient 3: The patient was born after a pregnancy of 39 wk, with a birth weight of 1,880 g (−3.55 SD), a birth length of 41 cm (−4.5 SD). Triangular-shaped face, broad forehead, pointed, small chin and a wide, thin mouth are prominent. The left leg is 2 centimetres shorter than the right. Mental development is retarded, she visits a special school. BMI was 14.2. Pituitary reserve testing was uneventful, T4, TSH was within the normal range. Karyotype was uneventful, microdeletion 22q11.2 was not found. Treated with GH she grows on the third percentile and has a height of 100.6, at age 5.5 years. Midparental height is not known, since the child is adopted. Clinodactyly was also present, the BMI was 13.2. She has been treated with growth hormone, but as she has moved out of the country the response to the GH treatment is not evaluated. Her MI is: H19 Promoter 0.50 (n), H19 DMD 0.23 (hypomethylated).

<table>
<thead>
<tr>
<th>Parameters at birth</th>
<th>Points</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight &lt; 10\text{th} percentile</td>
<td>1</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Length &lt; 10\text{th} percentile</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Relative macrocephaly</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Postnatal Course</th>
<th>Points</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No catch up growth; lengths 3\text{rd} percentile</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Normal head circumference</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OFD &gt; 3\text{rd} and &lt; 97 percentile</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Normal cognitive development</td>
<td>1</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Asymmetry</th>
<th>Points</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Face/body/limbs</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Facial features</th>
<th>Points</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triangular shaped face</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>High/bossing forehead</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Other: e.g. small chin, thin lips, down turned corners of the mouth, late closure of fontanelle</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Other features</th>
<th>Points</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinodactily 5\text{th} finger</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Genital Abnormalities (e.g. cryptorchidism, hypospadias)</td>
<td>1</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Other: e.g. Brachymesos-Phalangy, yndactyly toes, inguinal hernia, pigmentary changes</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total</th>
<th>Points</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>13</td>
<td>13</td>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Methylation index</th>
<th>Patients</th>
<th>H19 Promoter</th>
<th>H19 DMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>0.51 (n)</td>
<td>0.45 (n)</td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>0.26 (low)</td>
<td>0.65 (n)</td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td>0.50 (n)</td>
<td>0.23 (low)</td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

Several reports found that the severity of the phenotype or some SRS features is dependent on the degree of the H19 methylation [7]. Significant correlations were identified between the H19-DMR methylation index and the body and placental size, and between the placental weight and the body size in the epimutation-positive patients [9].

Interestingly, head circumference and facial features were found to be associated in patients with hypomethylation [9]. Kotzot [10] reported a higher frequency of relative macrocephaly and high fore-head/frontal bossing in patients with epimutations of the ICR1 on 11p15 than in SRS patients of unexplained etiology or maternal UPD 7. Others reported that a prominent forehead, relative macrocephaly, body asymmetry and low BMI were sig-
nificantly associated with ICR1 [6]. However, some reports did not confirm the association of the head circumference to hypomethylation [9].

The patients with SRS-like features were found to have lower methylation indexes compared with the patients with asymmetry and growth retardation [11].

Netchine et al. [6] found that the birth weight, birth length, and postnatal body mass index (BMI) were lower in the abnormal 11p15 SRS group than in the normal 11p15 SRS group. In the same study, no molecular abnormalities were found in the non-SRS SGA group. In addition, IGF-I and IGFBP-3 serum levels were lower in patients with 11p15-SRS, compared with the low levels in UPD7-SRS and in the cohort of 58 nonsyndromic SGA children [12]. It was also reported that children with SRS and an 11p15 epimutation have IGFBP-3 excess and show endocrine characteristics suggesting IGF-I insensitivity [13]. In addition, children with SRS and UPD7 were not different from nonsyndromic short children born SGA [13].

The height gain was also affected: more height gain in children with UPD7 than in those with 11p15 epimutation under GH therapy. Binder et al. 2008 [12] concluded that children with SRS and an 11p15 epimutation show endocrine characteristics of IGF-I insensitivity. We now describe a patient without hypomethylation and multiple pituitary deficiencies that grew better than the SRS patients without growth hormone deficiency. He attained a favourable adult height of 166 cm.

Further, some specific anomalies of the spine, elbows, hands and feet, and genital defects were found in patients with severe H19 hypomethylation, while milder symptoms were reported in SRS patients with matUPD7 [8]. The contracture of the elbows was found in one of our patients without hypomethylation.

Some explanation in the genotype–phenotype relations was given by the fact that densely methylated regions of DNA show low crossover rates therefore influencing the complex traits [14].

In our patients the degree of hypomethylation was not linked to the severity of the phenotype. Namely, all three of them had a severe SRS phenotype despite the fact that only one of them was H19 hypomethylated.

Acknowledgement

We are very grateful to Prof. Goodrun Moore and Dr Sayeda Abu-Amaro for all their excellent assistance in the laboratory analysis.

REFERENCES


Резиме

СТЕПЕНОТ НА Х19 ХИПОМЕТИЛАЦИЈАТА КАЈ ДЕЦАТА СО СИЛВЕР-РАСЕЛ-ОВ СИНДРОМ (СРС) НЕ Е АСОЦИРАН СО ТЕЖИНАТА НА ФЕНОТИПОТ И ВКУПНИОТ ЗБИР НА КЛИНИЧКАТА ЕКСПРЕСИЈА (ВЗКЕ)

Зоран С. Гучев С1, Љиљана Шараанц2, Александра Јанчевска1, Велибор Тасик1

1 Медицински факултет Скопје, 50 Дивизија 6б, 1000 Скопје, Р. Македонија
2 Медицински факултет, Ниш, Србија

Вовед: Хипометилација на импринтинг контролниот регион 1 (ИЦП 1) на локусот ИГФ2/Х19 на 11p15 е поврзана со Силвер-Раселовиот синдром (СРС).

Методи и резултати: Ние ја тестирахме хипотезата дека тежината на фенотипот кај па-циентите со РСС зависи од вкупниот збир на клиничката експресија (ВЗКЕ) (1). Три пациенти со РСС беа клинички рангиран и нивните резултати беа 12, 13 и 13. Две од тие СРС пациенти имаа хипометилација на едниот алел.

Заклучок: Сите три пациенти имаа висок ВЗКЕ. Сепак, само двајца од нив имаа хипометилација на едниот Х19 алел. Интересно, двата од нив имаа вентрикуларни септални дефекти, а од нив само едниот Х19 хипометилација. Сите деца имаа мала родилна должина и тежина, класичен лицев фенотип, хемихипертрофија (> 2,5 цм потенка лева рака/нога во споредба со десните), пократка нога и впечатлива грацилност (БМИ > 16,0). Едно од децата беше оперирано од крипторхизам и истото имало и контрактура на рамето. Две деца имаа сколиоза. Сите три деца беа ниски (–3,5 до 5,5 СД), а третманот со HR резултираше пораст во височина до третиот перцентил. Бидејќи едното дете немаше воопшто хипометилација, а кај другите две таа беше со низок степен изразена, високот ВЗКЕ (12, 13 и 13) не беше проследен со висок степен на хипометилација на ИГФ2/Х19 локусот.

Ключни зборови: Силвер-Расел синдром, метилиационен индекс, ИГФ2/Х19 локус.