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1 Deletions and rearrangements of the *H19/IGF2* enhancer region in patients with Silver-
2 Russell syndrome and growth retardation

3

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3

4 **KEYWORDS**

5 Silver-Russell syndrome, H19 enhancer, deletion, methylation

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9

1 **ABSTRACT**

2 Silver-Russell syndrome is characterized by pre- and postnatal growth retardation,
3 dysmorphic facial features and body asymmetry. In 35-60% of SRS cases the paternally-
4 methylated imprinting control region (ICR) upstream of the *H19* gene (H19-ICR) is
5 hypomethylated, leading to down regulation of *IGF2* and biallelic expression of *H19*. *H19*
6 and *IGF2* are reciprocally-imprinted genes on chromosome 11p15. The expression is
7 regulated by the imprinted methylation of the ICR, which modulates the transcription of *H19*
8 and *IGF2* facilitated by enhancers downstream of *H19*. A promoter element of *IGF2*,
9 IGF2P0, is differentially-methylated equivalently to the H19-ICR, though in a small number
10 of SRS cases this association is disrupted, i.e. hypomethylation affects either H19-ICR or
11 IGF2P0.

12 Here we present three pedigrees associated with hypomethylation of IGF2P0 in the probands,
13 two with paternally-derived deletions, and one with a balanced translocation of inferred
14 paternal origin. They all have a breakpoint within the *H19/IGF2* enhancer region. One
15 proband has severe growth retardation, the others have SRS. This is the first report of
16 paternally derived structural chromosomal mutations in 11p15 causing SRS. We propose that
17 these cases define a novel etiology of the growth retardation in SRS, namely, dissociation of
18 *IGF2* from its enhancers.

19

20

1 INTRODUCTION

2 Silver-Russell syndrome (SRS, MIM 180860) is a genetically and clinically heterogeneous
3 condition characterized by intrauterine and postnatal growth retardation, dysmorphic facial
4 features and body asymmetry [1]. 35–60% of SRS cases show hypomethylation of the *H19*
5 imprinting control region (H19-ICR; Fig. 1a) at 11p15, which is believed to result in reduced
6 expression of *IGF2* (MIM 147470) [2-4]. The underlying causes of hypomethylation include
7 mosaic maternal uniparental disomy (UPD) of chromosome 11 [5], maternal duplication of
8 11p15 [6, 7], and possibly gene mutations of H19-ICR [8], but is unknown in most cases. In
9 most SRS patients hypomethylation occurs at both H19-ICR and IGF2P0, but is occasionally
10 restricted to H19-ICR or IGF2P0 [9].

11 The H19-ICR is located within the *H19* promoter and is normally methylated on the
12 paternally-derived allele. *H19* (MIM 103280) encodes a maternally-expressed non-coding
13 RNA while *IGF2* encodes the paternally-expressed growth factor IGF-II. In mice the
14 endodermal enhancers of *IGF2* are located +10 kb from *H19* transcription start site; skeletal
15 muscle and mesodermal enhancers are located at +35 kb and other mesodermal enhancers at
16 +120 kb [10]. The interaction of these enhancer sequences with the promoters of *H19* or
17 *IGF2* is modulated by CTCF for which the H19-ICR contains seven potential binding sites
18 [11]. CTCF binding to unmethylated H19-ICR prevents interaction between the *IGF2*
19 promoter and enhancers, while methylation of the H19-ICR prevents CTCF binding, enabling
20 the enhancers to interact with the *IGF2* promoter. Naturally occurring structural anomalies in
21 the enhancer region of *H19/IGF2* in patients are very informative in testing the hypothesis of
22 complex imprinting and competing enhancer regulation of *H19* and *IGF2* and ultimately help
23 to more clearly define the roles of these genes in humans. We report here for the first time
24 SRS patients with chromosomal structural mutations in the *H19/IGF2* enhancer region.

25

1 **METHODS**

2 **Patients**

3 Patient 1 was referred for SRS testing at the age of 2 years due to pre- and postnatal growth
4 retardation, characteristic dysmorphic features, congenital anomalies and failure to thrive.

5 Patient 2 was referred at age 14 weeks with pre- and postnatal growth retardation, typical
6 facial features and severe feeding difficulties. Subsequently his sister (patient 3) presented
7 neonatally with the same clinical features. Patient 4 was identified after karyotyping due to
8 recurrent miscarriages. She had pre- and postnatal growth retardation and atypical diabetes,
9 but there was no clinical suspicion of SRS. Informed consent for the investigations performed
10 and publication was obtained for all patients. Patients 1 and 4 were recruited via the initial
11 diagnostic testing, while patients 2 and 3 were recruited into the research study "Imprinting
12 Disorders Finding Out Why?" (IDFOW), approved by Southampton and South West
13 Hampshire Research Ethics committee 07/H0502/85.

14

15 **Molecular genetic investigations**

16 Karyotyping was performed on cultured lymphocytes using standard techniques.
17 Fluorescence-in-situ-hybridization (FISH) analysis was performed on metaphases by standard
18 techniques using BAC and fosmids clones. Genomic DNA was extracted from blood
19 leukocytes by standard procedures.

20

21 **MLPA**

22 MLPA analysis was performed by methylation-specific MLPA kit ME030 version B1 from
23 MRC-Holland (Amsterdam, The Netherlands) following the manufacturer's instructions. 250
24 ng of DNA was used for analysis.

25

1 Pyrosequencing

2 Genomic DNA (2µg) was bisulphite treated using the EZ-DNA Methylation™ kit, according
3 to manufacturer's instructions (Zymo Research, Orange, CA USA), except that DNA was
4 eluted in 50µl of elution buffer and 50µl of TE pH 8.0.

5 Pyrosequencing assays of bisulphite- induced C/T polymorphisms [12] for H19 and IGF2P0
6 were designed using Biotage Assay Design Software™ v1.0.6 (Biotage, Uppsala, Sweden)
7 and performed essentially as previously described [13]. Briefly, primary amplification
8 reactions (50µl, performed in duplicate) contained forward and reverse primers, one of which
9 was biotinylated, Titanium Taq and buffer (Clontech, Mountain View, Ca. USA), and ~20ng
10 bisulphite-treated DNA. After 45 amplification cycles, 20µl single-stranded biotinylated PCR
11 products were pyrosequenced using Pyrosequencing Gold Reagents (Biotage) according to
12 the manufacturer's instructions. The percentage methylation at each CpG site was determined
13 by AQ software. AQ levels >5% were indistinguishable from background fluorescence. In
14 each pyrosequencing assay, a bisulphite treatment control was included in the form of a
15 cytosine not in a CpG dinucleotide; this would be expected to be converted entirely to uracil.

16 Primer sequences were: H19 pyrosequencing: forward
17 GTATAGTATATGGGTATTTTTGGAGG, reverse
18 CCATAAATATCCTATCCCAAATAACC (bio); sequencing
19 GTTYGGGTTATTTAAGTT. This amplifies the region chr11: 1977625-1977877.

20 IGF2(P0) pyrosequencing: forward TGAGGATGGGTTTTTGTGGTAT; reverse
21 TCCTCAATCCACCCAAAATAATAT (bio); sequencing
22 AAAAGTTATTGGATATATAGT.

23

24 Array CGH analysis

1 Array CGH analysis (aCGH) used a custom 8 X 60K Agilent array (Agilent Technologies,
2 South Queensferry, UK) designed to densely cover known human imprinted loci. Probes
3 were designed using Agilent Technologies' e-Array design tool with the following filters
4 applied: T_m filter, similarity filter and catalog probe preference. Across the region
5 chr11:1,674,000-2,979,000 (hg18) probes were tightly tiled to provide maximum resolution
6 with an average spacing of 50nt. The remainder of chr11 was represented by Agilent aCGH
7 control probes, which provide a genome-wide backbone to enable efficient normalization.
8 aCGH was performed according to the manufacturer's instructions. Briefly, 500ng of blood
9 genomic DNA was labeled using the genomic DNA enzymatic labeling kit, hybridized to the
10 array for 24 hours and subsequently washed. Arrays were scanned using an Agilent
11 Technologies' DNA microarray scanner G2539A and data extracted using Agilent
12 Technologies' Feature Extraction software version 10.5.1. Duplications and deletions were
13 mapped and end points determined by Agilent Technologies' DNA Analytics software
14 version 4.0 with the following settings: data was analyzed using the ADM-2 Algorithm with
15 a threshold of 6.0 using the DLR error model, with fuzzy zero turned on and the continuous
16 moving average applied.

17

18 Web resources

19 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>

20 UCSC Human Genome Database Build hg18, March 2006, <http://www.genome.ucsc.edu>

21 Database of Genomic Variants (DGV): <http://projects.tcag.ca/variation/?source=hg18>

22

23 **RESULTS**

24 The clinical features of the patients are summarized in Table 1. Cytogenetic analysis of
25 patient 1 showed a normal karyotype, 46,XY while MLPA analysis showed deletion of *H19*

1 and part of H19-ICR (Fig. 1a); the peak height of the deleted probes suggested mosaicism,
2 while MLPA analysis of parental DNA was normal, suggesting that the deletion had arisen *de*
3 *novo*. Microsatellite analysis showed reduced dosage ratios of D11S4046 (paternal:maternal
4 ratio 1:2.3) consistent with mosaicism (Fig. 1c). Cytogenetic analysis of patients 2 and 3
5 revealed a pericentric inversion of chromosome 11 (karyotypes 46,XY,inv(11)(p15.5q21)pat
6 and 46,XX,inv(11)(p15.5q21)pat, respectively), inherited in both children from their
7 phenotypically normal father. FISH analysis showed a split signal using BAC clone RP11-
8 534I22, while fosmids W12-1875E5 and W12-1660M14 hybridized to 11p and 11q,
9 respectively (Fig. 1a and Fig. 1d) mapping the 11p15.5 breakpoint to chr11:1,862,216-
10 1,921,555 (hg18).

11 Patient 4 had a balanced translocation (karyotype 46,XX,t(1;11)(p36.22;p15.5)). FISH
12 analysis mapped the 11p15.5 breakpoint between W12-1875E5 and W12-1660M14 [14].
13 Paternal origin of the translocation was inferred through karyotyping of the patient's mother,
14 who had a normal karyotype, and the patient's daughter, who had the same translocation as
15 the patient but normal growth.

16 DNA methylation analysis showed partial hypomethylation of both H19-ICR and IGF2P0 in
17 patient 1. In patients 2, 3 and 4 IGF2P0 was partially hypomethylated while the methylation
18 of H19-ICR was in the normal range (Fig. 1b).

19 All four patients were analyzed by array CGH (aCGH). In patient 1 aCGH showed a 58.8 kb
20 deletion (chr11:1,918,222-1,977,026 (hg18)) encompassing the endodermal, skeletal muscle
21 specific and part of the mesodermal enhancers, as well as *H19* and part of H19-ICR including
22 the seventh CTCF binding site, but not the two repeat blocks (Fig. 1a), though the log
23 intensity difference suggested a mosaic deletion. In patients 2 and 3 aCGH showed an 8.8 kb
24 deletion (chr11:1,918,312-1,927,132 (hg18)) overlapping with the mesodermal enhancer.
25 Deletions partially overlapping with the above deletions have been registered as rare

1 variations in apparently normal individuals (<http://projects.tcag.ca/variation/?source=hg18>)
2 but the parental origin of these deletions are unknown. aCGH did not reveal any deletion in
3 patient 4.

4

5 **DISCUSSION**

6 We present three pedigrees with disruption of the shared *H19/IGF2* enhancer region and
7 separation of the mesodermal enhancers from *IGF2*, one by deletion, one by an inversion
8 breakpoint accompanied by deletion, and one by breakpoint of an apparently balanced
9 translocation. *IGF2* expression was reduced in mesodermal tissue in patient 4 [14].
10 Previously, the small stature of patient 4 was unaccounted for, but we now hypothesize that
11 hypomethylation of IGF2-P0 due to the separation of IGF2 from its enhancers causes growth
12 retardation. The deletion in patient 1 may truncate these enhancers and moreover reduces
13 their distance from *IGF2* by 50%, thus potentially affecting the local three-dimensional
14 structure of the DNA and hence its function. We therefore suppose that the mesodermal
15 enhancers are non-functional in all four patients. It is interesting that all four patients are
16 growth-retarded, but only patients 1, 2 and 3, who harbor deletions including the sequence
17 chr11:1,918,312-1,927,132 (hg18), presented with the clinical diagnosis of SRS. One
18 interpretation of this observation is that reduced *IGF2* expression from mesodermal
19 enhancers is responsible for only the growth retardation seen in SRS, while other genetic or
20 epigenetic defects cause the other phenotypic traits of SRS. Alternatively, given that SRS is a
21 disorder with marked clinical heterogeneity it may be that patient 4 showed an atypical
22 presentation.

23 It was striking that all patients showed hypomethylation of IGF2P0, while hypomethylation
24 of the H19-ICR was observed only in patient. This observation suggests that IGF2P0
25 methylation may not directly depend on H19-ICR methylation in *cis*, but may instead reflect

1 the promoter activity of IGF2P0 *per se*, and be established as a consequence of its interaction
2 with distant *IGF2* enhancers. Disruption of the enhancer region represents a possible
3 explanation of rare SRS cases with hypomethylation restricted to IGF2P0 [9].
4 The effects of deletions and mutations in the *H19* region depend not only on their parent of
5 origin, but also on their localization. Deletions or mutations of H19-ICR described to date are
6 apparently without phenotypic effect when on the paternal allele, whereas in maternal
7 inheritance they are associated with the overgrowth disorder Beckwith-Wiedemann syndrome
8 (MIM 130650) [15, 16]. Thus, we hypothesize that a paternal deletion of *H19*, such as seen in
9 patient 1, may be of less clinical significance than the separation of *IGF2* from its enhancers.
10 For the enhancer region, no data have hitherto been presented in humans; however, mice with
11 paternally inherited deletions were 80% of normal size whereas mice with maternal deletions
12 were of normal size [17]. We predict that detailed characterization of the *IGF2* promoter and
13 enhancer elements in humans will uncover further genetic and epigenetic changes that
14 predispose to growth retardation and/or SRS. Moreover, we confirm that IGF2P0
15 hypomethylation is found in a proportion of SRS cases with normal H19-ICR methylation.
16 Therefore, in agreement with Bartholdi et al. [9] we suggest that IGF2P0 methylation
17 analysis should be included in standard molecular testing for SRS.

18

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20 MLPA was performed by Judy Boe Rasmussen, The Kennedy Center; FISH was performed
21 by Viv Maloney, Wessex Genetics Service. Jette Bune Rasmussen performed the graphic
22 work.

23

24 **COMPETING INTERESTS**

25 The authors declare no competing interests

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1 Table 1: clinical data for patients 1-4

	1	2	3	4
Gender	Male	Male	Female	Female
Age	14 y	14 y	7 m	31 y
<i>Pregnancy</i>				
History of infertility/assisted reproduction	NK	No	No	NK
Placental abnormality	NK	"Poor condition"	No	NK
<i>Growth</i>				
Birth weight (weeks gestation)	2.1kg (0.4 th -2 nd) (38/40)	1.95kg (0.4 th) (38/40)	1.48kg (0.4 th -2 nd) (35/40)	1.25kg (<<0.4 th) (40/40)
Height (age)	84 cm (<0.4 th) (3y) 163 cm (50 th -75 th) (14y)	60cm (<<0.4 th) (7.5m) 102cm (2 nd -9 th) (4y10m)	41.2cm (<<0.4 th) (7wk) 52cm (<<0.4 th) (6m)	145cm (<<0.4 th) (31y)
Relative macrocephaly (age)	33.5cm (25 th -50 th) (birth)	47.5cm (91 st -98 th) (7.5m)	36cm (50 th -75 th) (7wk)	NK
<i>Congenital abnormalities</i>				
Male genital anomaly	Cryptorchidism, inguinal	No	n/a	n/a
Skeletal abnormality	hernia Delayed bone age at 14y	No	No	No
<i>Other problems</i>				
Psychomotor / speech delay	No	No	No	No

Excessive sweating	No	Yes	No	No
Hypoglycaemia	No	In infancy	No	No
Feeding difficulties	Yes	Yes	Yes	No
Gastrointestinal reflux	No	Yes	No	No
<i>Craniofacial features</i>				
Triangular face	Yes	Yes	Yes	No
Frontal bossing	Yes	Yes	Yes	No
Micro/retrognathia	Yes	No	No	No
Irregular/crowded teeth	NK	No	n/a	No
Small teeth	NK	Yes	n/a	No
Downturned corners of mouth	Yes	Yes	Yes	No
Thin upper lip	Yes	No	No	No
Low set/posteriorly-rotated ears	Yes	low set	Yes	No
Epicanthus	Yes	NK	NK	No
<i>Other clinical signs</i>				
5th finger clinodactyly	Yes	Yes	Yes	No
Camptodactyly	No	No	No	No
Joint contractures	No	No	No	No
Café au lait patches	No	No	No	No

Other	Multiple haemangiomas			Atypical diabetes Lactation failure
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1

2 NK, not known; n/a, not applicable; y, years; m, months; wk, weeks

1 FIGURE LEGENDS

2 **Figure 1:** genetic organization of the human *H19-IGF2* region, and cytogenetic, genetic and
3 epigenetic analysis of patients 1-4. **a.** Sequence numbering according to human genome
4 release 18 of March 2006. Genes are represented by solid blue lines; putative enhancer
5 elements by dashed lines (meso: mesodermal enhancer, skm: skeletal muscle and
6 mesodermal enhancers, endo: endodermal enhancer); H19-ICR and IGF2P0 regions are
7 marked in solid blue with a "lollipop". FISH probes are marked in solid black. The triangular
8 arrowheads mark MLPA probes deleted in patient 1. MLPA probes written in blue contain a
9 HhaI recognition site. Solid red lines indicate the location of deletions in patients 1-3 mapped
10 by aCGH and dashed red line indicate translocation breakpoint in patient 4 mapped by FISH.
11 **b.** Pyrograms for patients 1, 3 and 4 illustrating allele quantification of bisulfite-induced C/T
12 polymorphisms within *H19* and IGF2P0. NC: normal control. Each table cell contains a
13 representative pyrogram of the first C/T polymorphism. Figures above each pyrogram
14 indicate the proportions of methylated and unmethylated product. **c.** Electropherogram of
15 microsatellite D11S4046. Middle panel: patient 1; upper panel: father; lower panel: mother.
16 **d.** FISH analysis of patient 3. Upper panel: W12-1875E5 (red) retained on 11p; lower panel:
17 W12-1660M14 (red) which is transferred to 11q. The centromere probe D11Z2 (green)
18 hybridizes to both normal and derivative chromosomes 11.

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