

Hypomethylation of the *H19* Gene Causes Not Only Silver-Russell Syndrome (SRS) but Also Isolated Asymmetry or an SRS-Like Phenotype

Jet Blik,^{1,*} Paulien Terhal,^{2,*} Marie-José van den Bogaard,² Saskia Maas,¹ Ben Hamel,³ Georgette Salieb-Beugelaar,¹ Marleen Simon,⁴ Tom Letteboer,² Jasper van der Smagt,² Hester Kroes,² and Marcel Mannens¹

¹Department of Clinical Genetics, Academic Medical Center, Amsterdam; ²Department of Medical Genetics, University Medical Center Utrecht, Utrecht; ³Department of Human Genetics, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; and ⁴Department of Clinical Genetics, Erasmus Medical Center, Rotterdam

The *H19* differentially methylated region (DMR) controls the allele-specific expression of both the imprinted *H19* tumor-suppressor gene and the *IGF2* growth factor. Hypermethylation of this DMR—and subsequently of the *H19* promoter region—is a major cause of the clinical features of gigantism and/or asymmetry seen in Beckwith-Wiedemann syndrome or in isolated hemihypertrophy. Here, we report a series of patients with hypomethylation of the *H19* locus. Their main clinical features of asymmetry and growth retardation are the opposite of those seen in patients with hypermethylation of this region. In addition, we show that complete hypomethylation of the *H19* promoter is found in two of three patients with the full clinical spectrum of Silver-Russell syndrome. This syndrome is also characterized by growth retardation and asymmetry, among other clinical features. We conclude that patients with these clinical features should be analyzed for *H19* hypomethylation.

Silver-Russell syndrome (SRS [MIM 180860]) is a clinically heterogeneous syndrome, first described by Silver et al. (1953) and Russell (1954). Diagnosis can be difficult, and at least four of the following criteria should be present: intrauterine growth retardation (IUGR), poor postnatal growth, relatively normal head circumference, classic facial phenotype, and asymmetry (Price et al. 1999). At the genetic level, the syndrome is heterogeneous: although mostly sporadic, in familial cases, the syndrome can be transmitted in an autosomal dominant, autosomal recessive, and/or X-linked dominant way (Duncan et al. 1990; Teebi 1992; Al-Fifi et al. 1996; Ounap et al. 2004). In most patients, the molecular pathology of SRS is unknown; however, abnormalities have been described for chromosome 7 (especially maternal uniparental disomy [mUPD], in ~10% of cases) (Monk et al. 2002). Although, in general, complete mUPD 7 is found in these cases, a single case was reported with a partial mUPD 7q31-qter (Hannula et al. 2001). Analysis of genes in this region did not lead to the identification of a candidate gene involved in the syndrome. Various other chromosomal abnormalities have been described, including trisomy 1q32.1-q42.1 (van Haelst et al. 2002), deletion of chromosome 15q or ring chromosome 15 (Rogan et al. 1996), 18p- (Christensen and Nielsen 1978), translocations involving breakpoints 17q25 (Ramirez-Duenas et al. 1992) and 17q23-24 (Dorr et al. 2001), and, finally, a paternally inherited deletion of the

CSH1 gene at chromosome 17q22-24 (Eggermann et al. 1998).

In 2002, Fisher et al. (2002) reported three patients with a phenotype that includes growth retardation. These patients presented with maternal duplications of chromosome 11p15. In 2005, Eggermann et al. (2005) described two patients with maternal duplications of 11p15 and SRS. Recently, Gicquel and coworkers (2005) published a series of patients with SRS who had hypomethylation of the *H19* region at 11p15 (MIM 103280).

The 11p15 region is also associated with the Beckwith-Wiedemann syndrome (BWS [MIM 130650]), characterized by macroglossia, omphalocele, fetal gigantism, and other abnormalities, including various childhood tumors (DeBaun et al. 2002). In many cases, the overgrowth is asymmetric, producing hemihypertrophy, which is probably more accurately termed “hemihyperplasia.” The syndrome can be caused by various molecular defects, which lead to altered expression of imprinted genes on chromosome 11p15, including *H19* and *IGF2* (MIM 147470) (Blik et al. 2001) (fig. 1a). Given the opposite phenotypes of SRS and BWS, Chitayat et al. hypothesized, as long ago as 1988, that the growth abnormality and asymmetry in both disorders might be caused by dysregulation of the same gene (Chitayat et al. 1988). Here, we report support for that hypothesis from a series of patients with *H19* hypomethylation and clinical fea-

Received October 26, 2005; accepted for publication January 20, 2006; electronically published March 1, 2006.

Address for correspondence and reprints: Dr. Marcel Mannens, Department of Clinical Genetics, Academic Medical Center, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands. E-mail: m.a.mannens@amc.uva.nl

* These two authors contributed equally to this work.

Am. J. Hum. Genet. 2006;78:604–614. © 2006 by The American Society of Human Genetics. All rights reserved. 0002-9297/2006/7804-0008\$15.00

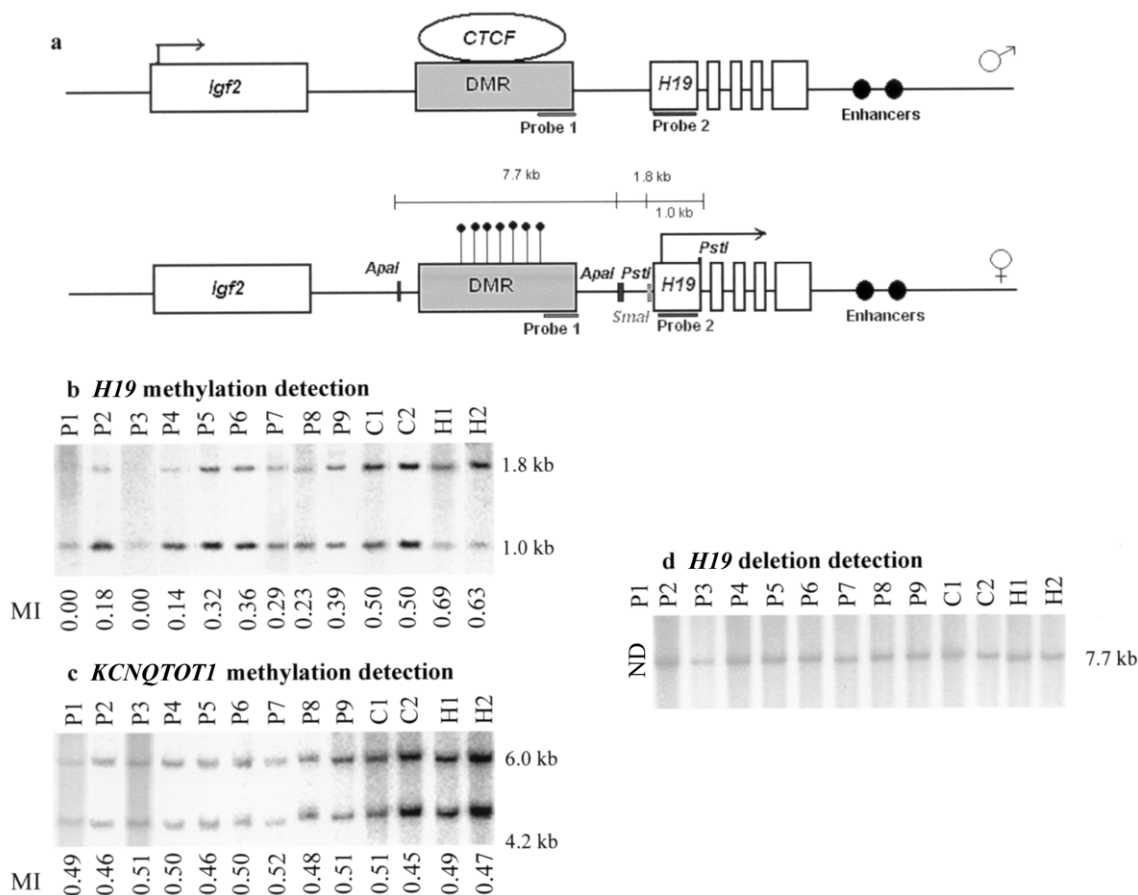


Figure 1 *a*, Schematic representation of the *H19/IGF2* region of chromosome 11p15. Probe 1 is the deletion detection probe, probe 2, the promoter methylation detection probe. CTCF-binding sites within the DMR are indicated by blackened circles. *b*, Results of the *H19* probe 2 hybridization. MI = methylation index. *c*, Results of the *KCNQ1OT1* hybridization. *d*, Results of the *H19* probe 1 hybridization. P1–P9 are the patients described in this study; C1 and C2 are normal controls; H1 and H2 are patients with hemihypertrophy and hypermethylation of *H19*. ND = not determined.

tures ranging from isolated asymmetry to the full clinical spectrum of SRS.

Material and Methods

Methylation and Deletion Studies

Methylation indices of both *H19* and *KCNQ1OT1* (MIM 604115) were measured in blood lymphocytes as described elsewhere (Blik et al. 2001). In brief, DNA was digested overnight with a methylation-sensitive restriction enzyme (*NotI* for *KCNQ1OT1* and *SmaI* for the *H19* promoter), was precipitated, and was digested overnight with a second restriction enzyme (*BamHI* for *KCNQ1OT1* and *PstI* for *H19*). Completion of digestion of the methylation-sensitive enzyme was monitored by the use of control probes that recognize the non-methylated restriction site. After Southern blotting, the filters were hybridized with the probes listed below. Hybridization was measured in a phosphorimager (Amersham). The Imagequant program (Amersham) was used to measure the intensity of the radioactive bands. The mean methylation index (inten-

sity of the measured band divided by the intensity of both bands) (\pm SD) for control individuals for *H19* is $0.5 (\pm 0.03)$ and for *KCNQ1OT1* is $0.51 (\pm 0.025)$. Normal methylation was defined as the mean ± 2 SD.

Deletion studies were performed on patient DNA digested with *ApaI*. After Southern blotting, the filter was hybridized with the following probes and primer sets: deletion detection probe (probe 1 in fig. 1*a*), forward 5'-ATTCCTGAGTCTCCCTTGG-3' and reverse 5'-TCGGCAAACCCTCTGTCC-3'; methylation detection probe (in first exon of *H19*) (probe 2 in fig. 1*a*), forward 5'-GTGGGAGCCAAGGAGCACCTTGACATCTG-3' and reverse 5'-TCCTGGTGACGTCCTGCTGCAACTCCCCGA-3'; and methylation detection probe (*KCNQ1OT1*), forward 5'-CCAGGTGAGAGGTAGTGGTAGAAGTC-3' and reverse 5'-TCTTTGCATTTCCTAGAGCAATCC-3'.

UPD Analyses

DNA containing CA repeats was amplified using standard PCR methods. Markers were taken from the ABI PRISM Link-

age Mapping Sets version 2.5 kit or were retrieved from the National Center for Biotechnology Information Web site. The PCR was done with Cy5-dCTP, and the fragments were analyzed by polyacrylamide gel electrophoresis with ALFexpress (Amersham Pharmacia). The PCR products generated with the ABI PRISM Linkage Mapping Sets version 2.5 kit were analyzed by capillary electrophoresis by use of Genetic Analyzer ABI310 (Applied Biosystems).

Mutation Analyses

Three primer sets were used to amplify all seven CTCF-binding sites, described elsewhere (Bell and Felsenfeld 2000), in the differentially methylated region (DMR) between the *IGF2* and the *H19* genes. The first three sites were amplified with the primers CTCFS1-3F, 5'-GCCCATCTTGCTGACCTCAC-3', and CTCFS1-3R, 5'-AGAAGACCTCCGAGAACCCTG-3'. For CTCF site 4-6, the following primers were used: CTCFS4-6F, 5'-GGTAGGACCCTTGACGAGCC-3', and CTCFS4-6R, 5'-GACCTGAAGATCTGGTGCGG-3'. For CTCF site 4, we used the following primer set: CTCFS7E, 5'-ATTTCCTGAGTCTCCCCTTG-3', and CTCFS7R, 5'-TCGGCAAACCCTCTGTTCC-3'. Two additional sequence primers were used: CTCFS site 2R, 5'-AATGTGGCTCCCATGAGTG-3', for CTCF site 2, and CTCF site 5R, 5'-AGAAGGGTTTCACACTAGGGCCG-3', for CTCF site 5.

PCR reactions were done in a total volume of 25 μ l with MgCl₂ (1.5 μ M), dNTP (0.24 mM), Betaine (50 mM), primers (1.2 μ M), and 0.3 U Amplitaq Gold (Applied Biosystems), with an annealing temperature of 60°C. After purifying the PCR product with QIAquick PCR Purification Kit 250 (QIAGEN), a sequence reaction was performed using the BigDye Terminator v1.1. Cycle Sequencing Kit (Applied Biosystems), with an annealing temperature of 60°C. Samples were analyzed on an ABI 310 genetic analyzer after ethanol precipitation.

Patients

Patient 1.—The patient was the third child of healthy parents. She was born after a pregnancy of 40 wk and 2 d. Birth weight was 1,870 g (−3.6 SD), birth length was 41 cm (−4.6 SD), and skull circumference was 33.5 cm (−1 SD). In the first weeks, nasogastric tube feeding was necessary, and, in the first months, there was a failure to thrive. At age 7 mo, a ventricular septal defect was detected. At age 8 mo, she developed circulatory and respiratory insufficiency after gastroenteritis, followed by multiorgan failure and postanoxic encephalopathy. Because of severe feeding problems, she was fed by percutaneous gastrostomy from age 2.5 years onward. Mental development is retarded because of encephalopathy.

Physical examination at age 2 years and 9 mo showed a height of 87 cm (−2 SD). Unfortunately, skull circumference was not measured. She had dysmorphic features suggestive of SRS. An iris coloboma was noted in the left eye. There was asymmetry of the arms and legs (left side larger) and bilateral mild ulnar deviation of the third, fourth, and fifth fingers (fig. 2a–2c).

Patient 2.—The patient was born after a spontaneous gemelli pregnancy. The twin sister is healthy. Because of IUGR of the patient, a cesarean section was performed at 35 wk and 6 d of pregnancy. The proband had a birth weight of 1,310 g (−2.4

SD) and a skull circumference of 32 cm (−0.8 SD). Length at age 2 wk was 38 cm (−6.5 SD). Pathologic examination showed dichorionic, diamniotic placentas. The placenta of the patient was smaller than the placenta of her healthy twin sister (1:2). DNA analysis confirmed dizygosity. Postpartum, features compatible with arthrogryposis multiplex congenita were present. The proband had ulnar deviation of the hands; flexion deformity of the fingers, especially on her right hand; and camptodactyly of the index finger. A luxation of the left hip, bilateral knee luxation, and bilateral pes equinovarus deformity were noted. In the first years, she had a severe failure to thrive. Partial nasogastric tube feeding was necessary until age 1 year and 9 mo. Mental development was normal.

Physical examination at age 4 years and 1 mo showed a height of 84.5 cm (−5 SD), a head circumference of 49.5 cm (−0.3 SD), and a weight of 9.6 kg (−2.4 SD for her length). She had facial features that suggested SRS. Two café-au-lait spots were present. Total hand length, leg length, and leg circumference were smaller on the right side than on the left side (fig. 2d–2f).

Cardiac evaluation showed two small ventricular septal defects. Metabolic studies were normal except for a temporary, isolated elevation of pipecolic acid. An electromyography showed no abnormalities compatible with neuropathy.

Patient 3.—The patient was born after a pregnancy of 39 wk and 3 d, with a birth weight of 1,780 g (−3.6 SD), a birth length of 40 cm (−5.5 SD), and a skull circumference of 33 cm (−2 SD). After birth, she underwent surgery because of ambiguous genitalia with a large clitoris. In the first weeks, she was fed partially by nasogastric tube. At age 14 years, her development is completely normal. Because of absence of breast development and hypergonadotropic hypogonadism, magnetic resonance imaging of the abdomen was performed and showed absence of ovaries and a hypoplastic uterus. She has been treated with growth hormone; her height is currently 1.60 m.

Physical examination at age 2 years showed a slender girl with a height of 77 cm (−3.3 SD), head circumference of 47.5 cm (−0.3 SD), and a weight of 8,050 g (−2.6 SD for her length). She had facial characteristics of SRS. There was asymmetry of the body (left side larger). Physical examination at age 14 years was not allowed.

Patient 4.—Patient 4 was born after a pregnancy of 41 wk, with a birth weight of 1,800 g (−4.2 SD), a birth length of 42 cm (−5 SD), and a skull circumference of 34 cm (−1 SD). He had ambiguous genitalia consisting of severe periscrotal hypospadias and a small introitus. An atrial septal defect was detected. Hepatosplenomegaly and a conjugated hyperbilirubinemia with progressive liver function disturbance was present. Liver biopsy at age 3.5 mo showed nonspecific fibrosis and ductular proliferation. The patient had a severe failure to thrive. Nasogastric tube feeding was necessary. He died at age 7 mo, probably as a result of an influenza pneumoniae.

Physical examination shortly after birth showed several dysmorphic features suggestive of SRS (fig. 2g). Metabolic and endocrine investigations showed no abnormalities. Obduction showed mild asymmetry of the arms and legs (left side larger), severe liver cirrhosis without biliary atresia, and fibrous material in the pericardiac sac.

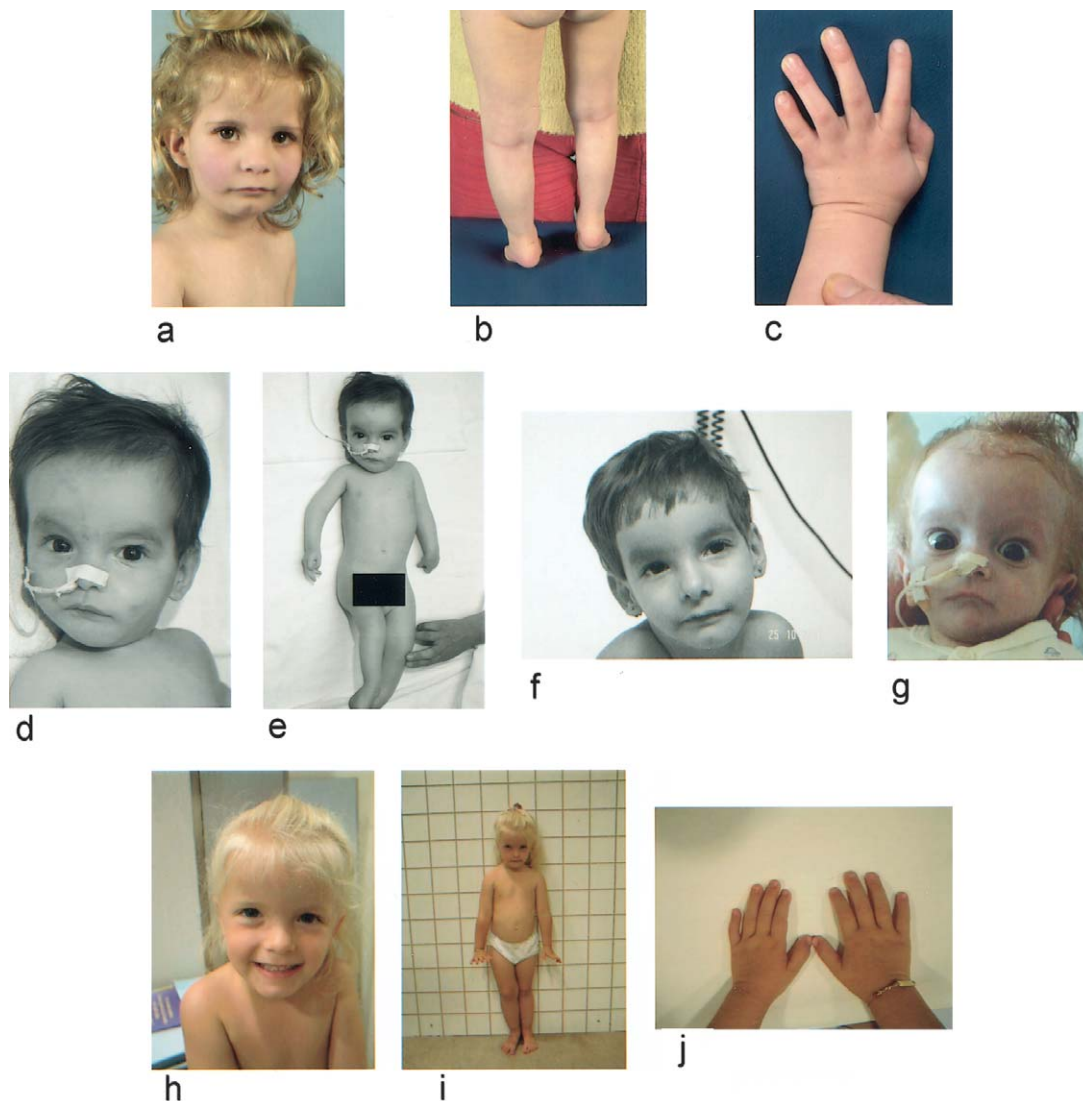


Figure 2 *a*, Patient 1, aged 2 years and 9 mo. Note triangular, asymmetric face, long eyelashes, thin lips, and mild retrognathia. A coloboma is present in the left eye. *b*, Asymmetry of the legs (left leg larger). *c*, Mild ulnar deviation of digits 3, 4, and 5, with shortening of the index finger and clinodactyly of the little finger. *d*, Patient 2, aged 9 mo. Broad forehead; small viscerocranium; triangular, asymmetric face; long eyelashes; thin lips with downturned corners of the mouth; and mild retrognathia. *e*, Asymmetry of the legs (left leg larger), internal rotation of the left arm, flexion deformity of the left wrist, and abnormal position of the feet (status after multiple surgical corrections). *f*, Patient 2, aged 4 years and 1 mo. Facial features are less characteristic of SRS. *g*, Patient 4, aged 5 mo. Note small viscerocranium, triangular face, large eyes with sunset phenomena, and thin lips with downturned corners of the mouth. *h*, Patient 9, aged 3 years and 3 mo. Note mild asymmetry of the face (right side larger), broad forehead, flat philtrum, and thin upper lip. *i*, Asymmetry (right side larger) of the body. *j*, Asymmetry of the hands (right hand larger), mild clinodactyly of little finger.

Patient 5.—Patient 5 was born after a pregnancy of 39 wk and 2 d, with a birth weight of 2,260 g (−2.5 SD) and a birth length of 47.5 cm (−1.75 SD). Skull circumference was 32.2 cm (−2.2 SD). No feeding problems were present in the neonatal period. Psychomotor development was normal. At age 10 years, she was referred to the orthopedic surgeon because of leg length discrepancy and asymmetry of the arms (right side larger). No clinical features of SRS were present.

Patient 6.—The parents of patient 6 are of Turkish descent. The patient was born after a pregnancy of 41 wk and 3 d,

with a birth weight of 2,800 g (−1.8 SD) and a birth length of 50 cm (−1 SD). Skull circumference was 35.5 cm (+0.4 SD). Feeding problems started at age ~4 mo, when she often refused fluids. At age 8 years, she was referred to the Department of Medical Genetics because of growth retardation in combination with discrepancy of leg length and circumference (left side larger). Mental development was normal.

Physical examination at age 8 years showed a height of 119.2 cm (−2 SD according to the growth curve for Turkish children). Head circumference was 51 cm (−0.5 SD). No facial

abnormalities were present except a slightly narrow face with thin lips. She had three small café-au-lait spots.

Patient 7.—This patient was born after a pregnancy of 41 wk and 3 d, complicated with a beginning HELLP syndrome (hemolysis, elevated liver enzyme levels, and a low platelet count), which prompted inducing labor. She had a birth weight of 2,560 g (−2 SD). Birth length was 46 cm (−3 SD). The neonatal period was uneventful. At age 7 wk, her occipital-frontal circumference was 38.3 cm (0 SD). Asymmetry was noticed from birth and involved limbs and face, which clinically looked more like a rightsided hemihypotrophy than hemihypertrophy. At age 1 year, her height was 72 cm (−1.5 SD), and, at age 4 years and 9 mo, it was 109 cm (0 to −1 SD). Apart from bilateral fifth finger clinodactyly, no SRS-like features were present. The right hand showed a variant simian crease. Otherwise, she is a healthy young girl of mixed Dutch-Angolese descent. Her mental and motor development has been completely normal. Methylation studies were performed for this patient because of body asymmetry.

Patient 8.—This patient was born after a pregnancy of 38 wk, with a birth weight of 1,500 g (−3.4 SD). Information about his birth length and skull circumference was not available. An asymmetry (left side larger) of the body was present. Since age 23 years, he has suffered from progressive muscle weakness in his right arm. At age 38 years, he developed insulin-dependent diabetes. Recently, it was discovered that his creatine kinase (CK) is elevated (1,264 U); the explanation of the elevated levels is still unknown. Intelligence is normal. Height currently is 1.70 m. He has no dysmorphic features suggestive of SRS.

Patient 9.—This pregnancy was achieved after intracytoplasmic sperm injection. The patient was born after a pregnancy of 40 wk and 3 d, with a birth weight of 2,500 g (−2 SD) and a birth length of 49 cm (−1 SD). No feeding problems were present in the neonatal period. Shortly after birth, a leg length discrepancy of 3 cm was noted (right leg larger). Height was 90.5 cm at age 3 years and 3 mo (−2.2 SD). Physical examination showed mild dysmorphic features (fig. 2*b–2j*). Her mental development has been normal.

For patients 1 and 3, a diagnosis of SRS was made. For patients 2 and 4, the clinical features were suggestive of SRS; however, the additional features of severe arthrogryposis multiplex congenital and liver cirrhosis have not been described before in patients with SRS. In the remaining patients, the diagnosis of SRS could not be made.

Chromosome analysis was performed for patients 1, 2, 3, 4, 6, 8, and 9 and showed a normal karyotype. Comparative genome hybridization was performed for patient 2 and patient 4, and no abnormalities were detected.

Results

Methylation Studies

Methylation studies of the *H19* gene on chromosome 11p15 are routinely performed in our laboratory as part of a diagnostic test for BWS (Blik et al. 2001). We noticed that, in seven patients with asymmetry, the *H19* promoter was hypomethylated rather than hypermethy-

lated (fig. 1*b*, patients 2, 4, 5, 6, 7, 8, and 9). All seven patients also exhibited IUGR or postnatal growth retardation (table 1 and fig. 2). This suggested to us that the asymmetry in the patients could be due to hemihypotrophy rather than hemihypertrophy. In addition, patients 2 and 4 had an SRS-like phenotype (table 1). Therefore, we examined patients 1 and 3 and one additional patient, whose diagnoses were clearly SRS, according to the criteria of Price et al. (1999). Among these patients, patients 1 and 3 had a complete hypomethylation of the *H19* gene (fig. 1). The third patient with SRS had no methylation defect.

UPD Analyses

Hypermethylation of *H19* is often seen in patients with BWS in conjunction with hypomethylation of the imprinted *KCNQ1OT1* gene, as a result of a UPD, mostly in a mosaic form (Blik et al. 2001). All *H19* hypomethylated patients had a normal methylation pattern at the *KCNQ1OT1* locus (fig. 1), so we exclude mUPD of a large part of chromosome 11p15 as an explanation for the methylation defects seen in these patients. To further exclude a small mUPD around the *H19* gene, we analyzed a series of polymorphic markers in this region (fig. 3 and table 2). In all patients, biallelic signals with normal intensity were found, excluding uniparental isodisomy in all cases. For patients 1, 2, 3, 6, and 7, parents were available and uniparental heterodisomy could be excluded. Additionally, we found no evidence of UPD-7 in any of our patients (table 2). We conclude that UPD around *H19* is not the mechanism that leads to the *H19* hypomethylation in our patient group.

Deletion Studies

Sparago et al. (2004) and Prawitt et al. (2005) demonstrated that maternal inheritance of small deletions (2–3 kb) at the *H19* DMR could cause hypermethylation of *H19* in patients with BWS. In our patient group, we could exclude the existence of such deletions (fig. 1); therefore, it is unlikely that paternal inheritance of an *H19* DMR deletion would prevent methylation of this locus during spermatogenesis and so cause the hypomethylation defects observed in our patients.

Sequence Analyses of the CTCF-Binding Sites

Imprinted expression of the *H19/IGF2* locus depends on a DMR that acts both as a maternal-specific, methylation-sensitive insulator and as a paternal-specific site of hypermethylation (Bell and Felsenfeld 2000). In humans, seven repeats in DMR bind the CTCF-binding factor on the hypomethylated maternal allele and have been proposed as attracting methylation on the paternal allele (Bell and Felsenfeld 2000). Binding of CTCF to the DMR mediates the insulator function of this region

Table 1

Clinical Features of Patients with Hypomethylation of H19

Clinical Features	P1	P2	P3	P4	P5	P6	P7	P8	P9
Neonatal period:									
Pregnancy	40 wk 2 d	35 wk 6 d (twins)	39 wk 3 d	41 wk	39 wk 2 d	41 wk 3 d	41 wk d	38 wk	40 wk 3 d
≤ -2 SD, birth weight (g)	+1,870	+1,310	+1,780	+1,800	+2,260	-2,800	+2,560	+1,500	+2,500
≤ -2 SD, birth length (cm)	+41	+38 (at age 2 wk)	+40	+42	-47.5	-50	+46	Unknown	-49
Skull circumference (cm)	33.5	32	33	34	32.2	35.5	38.3 (at age 7 wk)	Unknown	Unknown
Hypoglycemia	-	-	-	-	-	Unknown	-	Unknown	-
Facial features:									
Triangular face	+	+	+	+	-	-	-	-	+
Frontal bossing	+	+	+	+	-	-	-	-	+
Micro- or retrognathia	+	+	+	+	-	-	+	-	-
Downturned corners of the mouth	+	+	+	+	-	-	-	-	+
Thin lips	+	+	+	+	-	+	-	-	+
Crowded teeth/enamel defects	-	-	-	Unknown	-	-	-	-	-
Delayed closure or large fontanel anterior	+	-	+	+	-	-	+	Unknown	-
Delayed development:									
Motor	+	+	+	+	-	-	-	-	-
Mental	+	-	+	Unknown	-	-	-	-	-
Skeletal:									
Postnatal growth retardation	+	+	+	+	-	+	-	+	+
Asymmetry	+	+	+	+	+	+	+	+	+
Clinodactyly digit 5	+	+	+	+	-	-	+	-	+
Joint luxation	+	+	-	-	-	-	-	-	-
Camptodactyly	-	+	-	-	-	-	-	-	-
Gastrointestinal:									
Feeding problems	+	+	+	+	-	+	-	-	-
Liver anomalies	+	+	-	Cirrhosis	-	-	-	-	-
Urogenital:									
Abnormal genitalia	- ^b	-	+	+	-	-	-	-	
Renal abnormalities	+	-	-	+	-	-	-	-	
Congenital cardiovascular anomalies ^g	+	+	-	+	-	-	-	-	-
Skin findings:									
Café-au-lait spots	-	+	+	-	-	+	-	-	-
Hyperhidrosis	+	-	+	+	-	Unknown	-	-	-
Ophthalmological	Coloboma	-	-	-	-	-	-	-	-
Cancer	-	-	-	-	-	-	-	-	-
Other	-	-	-	-	-	-	-	+	-

NOTE.—A plus sign (+) = present; a minus sign (-) = absent.

^a Temporary increased pipecolic acid.

^b Neonatal vaginal prolapse.

^c Ambiguous genitalia with clitoral hypertrophy, absent ovaries, and hypoplastic uterus.

^d Ambiguous genitalia and periscrotal hypospadias with small introitus.

^e Small kidneys and postnaxotic acute tubular necrosis.

^f Subcortical renal cysts.

^g VSD = ventricular septal defect; ASD = atrial septal defect.

^h And fibrinous layer pericard.

ⁱ Elevated CK, diabetes mellitus, and muscle weakness.

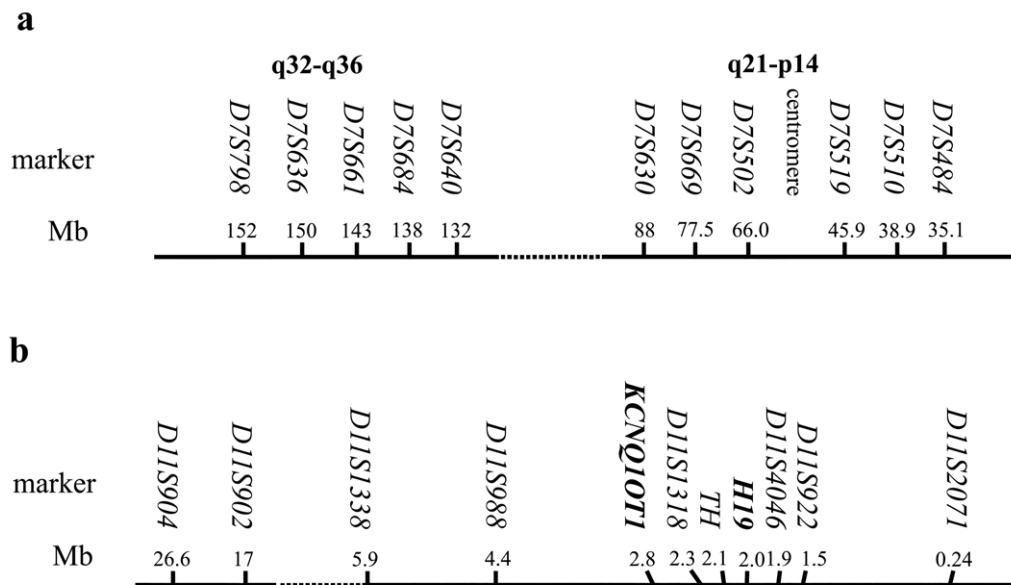


Figure 3 *a*, Chromosomal order (given in megabases, from the telomere of the short arm) of chromosome 7 markers used for UPD screening. *b*, Chromosomal order (given in megabases, from the telomere of the short arm) of chromosome 11 markers used for UPD screening.

and controls the reciprocal expression of the *H19* and *IGF2* genes in both mouse and human. Engel and co-workers (2004) introduced point mutations into the mouse DMR to deplete the repeats of CpGs while retaining ctf binding and enhancer blocking activity. Maternal inheritance of the mutations left *h19* and *Igf2* expression intact, consistent with the idea that the DMR acts as an insulator. Conversely, paternal inheritance of these mutations disrupted maintenance of DMR methylation, resulting in biallelic *h19* expression. Furthermore, an insulator was established on the paternally inherited mutated allele in vivo, which reduced *Igf2* expression and resulted in a 40% reduction in size of newborn offspring. Additionally, Sparago et al. (2004) and Gicquel et al. (2005) demonstrated aberrant methylation of CTCF binding sites in patients with BWS or SRS, suggesting that mutations in the DMR repeats could account for the loss of methylation in the *H19* region in our patients. We therefore sequenced the DMR repeat in our patients and found all were normal (table 3).

Discussion

It is striking that two patients with SRS have complete hypomethylation of the *H19* region and that the two patients with SRS-like features have lower methylation indexes in blood tissue compared with the patients with asymmetry and growth retardation without additional SRS features. In the study by Gicquel et al. (2005), a correlation between the degree of hypomethylation in

patients with SRS and the severity of the phenotype is also present, but no case of complete demethylation was reported. The complete demethylation of the patients with SRS in our studies suggests lack of somatic mosaicism, at least in major parts of the fetus, and suggests that it may have occurred prefertilization. We suggest that the severity of the phenotype may be a result of the tissues that are affected and how that tissue is influenced by a change in methylation. In addition, the possibility exists that normal cells would have a growth advantage relative to *H19* hypomethylated cells in a time- and tissue-dependent manner and generate tissues with apparent varying proportions of *H19* hypomethylation. Under this hypothesis, placental hypomethylation of *H19* might be responsible for decreased placenta size and hence decreased body size of the fetus, as seen in the twin described in this study. In placentas of sporadic IUGR, downregulation of *IGF2* and other paternally imprinted genes, in conjunction with upregulation of maternally imprinted genes, has been described by McMinn et al. (2005). Interestingly, one of these upregulated imprinted genes was the *MEST* gene (MIM 601029), localized to the SRS region of chromosome 7.

In contrast to patients with SRS, there seems to be no correlation between the degree of mosaicism and the severity of the clinical features of patients with BWS and hemihypertrophy (J.B., unpublished material).

In the present study, every patient with hypomethylation of the *H19* locus had prenatal and/or postnatal growth retardation and asymmetry, an observation also

Table 2

UPD Screening Results

CHROMOSOME AND MARKER (LOCATION)	ALLELES BY PATIENT (METHYLATION INDEX OF <i>H19</i>)								
	P1 (.00)	P2 (.18)	P3 (.00)	P4 (.14)	P5 (.32)	P6 (.36)	P7 (.29)	P8 (.23)	P9 (.39)
7p14-q21:									
D7S484 (35.1 Mb)	ND	NI	<u>3/7</u>	3/6	3/5	<u>3/7</u>	<u>3/4</u>	3/4	NI
D7S510 (38.9 Mb)	ND	<u>3/5</u>	<u>3/4</u>	3/5	3/6	<u>3/6</u>	<u>4/5</u>	1/2	NI
D7S519 (45.9 Mb)	ND	<u>3/4</u>	<u>4/5</u>	3/5	4/6	<u>4/9</u>	<u>4/5</u>	2/8	5/7
D7S502 (66.0 Mb)	ND	<u>3/8</u>	<u>2/5</u>	NI	4/4	<u>5/10</u>	ND	2/3	5/7
D7S669 (77.5 Mb)	ND	<u>2/7</u>	<u>1/3</u>	5/9	3/7	<u>5/6</u>	ND	7/10	7/8
D7S630 (88.0 Mb)	ND	<u>1/5</u>	7/8	1/7	NI	<u>3/7</u>	<u>6/7</u>	4/7	6/7
7q32-36:									
D7S640 (132 Mb)	ND	<u>2/7</u>	<u>2/4</u>	4/6	NI	<u>4/5</u>	<u>1/2</u>	5/8	2/6
D7S684 (138 Mb)	ND	<u>6/7</u>	NI	NI	2/3	1/6	<u>4/7</u>	NI	1/7
D7S661 (143 Mb)	ND	<u>6/8</u>	<u>5/6</u>	2/8	4/6	<u>6/7</u>	<u>2/8</u>	3/5	NI
D7S636 (150 Mb)	ND	<u>7/12</u>	<u>4/11</u>	4/6	8/9	<u>5/8</u>	<u>3/8</u>	6/11	3/9
D7S798 (152 Mb)	<u>5/6</u>	NI	<u>5/6</u>	4/6	3/6	<u>4/6</u>	NI	5/6	3/6
11p15:									
D11S2071 (0.24 Mb)	<u>1/3</u>	NI	3/8	2/4	ND	<u>1/5</u>	<u>3/7</u>	NI	ND
D11S922 (1.5 Mb)	NI	<u>5/6</u>	<u>7/12</u>	1/4	ND	<u>4/7</u>	<u>5/9</u>	6/9	ND
D11S4046 (1.9 Mb)	ND	NI	<u>7/9</u>	3/8	6/8	<u>3/8</u>	<u>2/5</u>	1/10	7/8
TH (2.1 Mb)	ND	NI	NI	1/5	ND	<u>2/3</u>	<u>1/2</u>	ND	ND
D11S1318 (2.3 Mb)	<u>6/8</u>	<u>2/8</u>	<u>1/7</u>	ND	ND	<u>3/7</u>	NI	ND	ND
D11S988 (4.4 Mb)	<u>6/9</u>	<u>4/10</u>	<u>7/9</u>	6/9	ND	<u>1/9</u>	<u>2/6</u>	6/7	ND
D11S1338 (5.9 Mb)	<u>1/4</u>	NI	<u>3/4</u>	1/4	ND	NI	NI	2/4	ND
D11S902 (17 Mb)	ND	<u>7/8</u>	ND	5/6	3/7	<u>1/3</u>	NI	7/8	NI
D11S904 (27 Mb)	ND	<u>2/6</u>	<u>2/5</u>	6/7	1/3	<u>4/6</u>	<u>2/3</u>	2/5	6/7

NOTE.—When parents are available, the paternal allele is underlined and the maternal allele is in boldface italics. ND = not done; NI = not informative.

made, with the exception of one individual who lacked asymmetry, by Gicquel et al. (2005). Therefore, hypo- or hypermethylation of the *H19* region is not necessarily associated with asymmetry in SRS or BWS. In patients of both studies, feeding problems were often present. Besides features that can be present in SRS, additional features were seen (table 1) that have not been previously described in the syndrome, including iris coloboma, structural heart malformations, arthrogryposis multiplex congenita, absent ovaries, hypoplastic uterus, and hepatomegaly (cirrhosis). The birth prevalence of iris coloboma is ~1 in 10,000 births (EUROCAT), and it is possible that this is a chance occurrence.

The birth prevalences of ventricular septal defect and atrial septal defect are 26 in 10,000 and 11 in 10,000, respectively (EUROCAT). Two of our patients have a ventricular septal defect, and one had an atrial septal defect, and it is possible that, compared with the general population, patients with *H19* hypomethylation have a higher risk for these heart defects.

Over 20% of patients with SRS have generalized campodactyly, many with signs of distal arthrogryposis (Price et al. 1999). Physical examination clearly showed bilateral ulnar deviation of the third, fourth, and fifth fingers in patient 1 and arthrogryposis in patient 2, and it is possible that these features also belong to the anomalies caused by *H19* hypomethylation, with arthrogryposis

multiplex congenita as the most severe end of the spectrum. Genital anomalies are common in male patients with SRS, often cryptorchism, sometimes hypospadias or ambiguous genitalia (Price et al. 1999); Mayer-Rokitansky-Kuster-Hauser syndrome (MIM 277000) has been reported in a female with SRS with ectopic localization of the ovaries and primary amenorrhea of adrenal origin (Bellver-Pradas et al. 2001) and in another female with a bicornuate uterus (Price et al. 1999). Elevated urinary gonadotropins were described in the first reported patients with SRS and also in later reports (Silver et al. 1953; Curi et al. 1967). We suggest that at least some females with hypomethylation of the *H19* locus do have uterus anomalies and/or ovarian insufficiency. The cause of hepatomegaly and cirrhosis in patient 4 remains unknown. Cholestasis and cirrhosis are sometimes seen in infants with total parenteral feeding; however, patient 4 was largely fed by nasogastric tube. It seems logical that the generalized growth defects seen in the patients described in this study are due to the methylation defect at 11p15, but no data are available from the literature

Table 3

Sequence of the CTCF-Binding Sites in Patients 1–9

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

that hypomethylation of *H19* also accounts for other SRS or non-SRS features. Some of these features might very well occur by chance.

Unlike the present study, Gicquel et al. (2005) selected their patients on the basis of the SRS diagnosis. This explains the more frequent occurrence of facial abnormalities in their study. In the study reported here, patients were selected on the basis of asymmetry and growth retardation, and we have shown that the *H19* hypomethylation found in SRS can also be found in patients with asymmetry and pre- and/or postnatal growth retardation only. In seven of the nine patients, asymmetry was with the left side larger; whether this is a chance occurrence remains unknown. In previous reports (Price et al. 1999; Gicquel et al. 2005), the sidedness of the asymmetry was not mentioned.

SRS has been described elsewhere in MZ twins, all of them discordant for the SRS phenotype (Samn et al. 1990; Bailey et al. 1995). A preponderance of female MZ twins among patients with BWS has been described (Weksberg et al. 2002), with the twins being discordant for the imprinting defect at the *KCNQ1OT1* locus. Weksberg et al. (2002) further suggested that it is possible that unequal splitting of the inner cell mass during twinning leads to differential maintenance of imprinting of the *H19* locus, and we suggest that a similar mechanism might apply in SRS. However, data on the methylation status of *H19* in discordant MZ SRS twins are not yet available.

DeBaun et al. (2003) found increased prevalence of assisted reproductive technology (ART) (4.6%) in mothers of patients with BWS compared with the control population (0.8%). It has been suggested that different factors—for example ART embryo culture conditions or the treatment for infertility—could theoretically have an effect on epigenetic alterations (Maher 2005), and there is an increased incidence of IUGR in children conceived by ART (Schieve et al. 2002). Recently, two cases of SRS after ART were reported (Källén et al. 2005; Svensson et al. 2005). Patient 9 in our series is the third reported patient. Since *H19* hypomethylation is seen not only in patients with classic SRS but also in patients with growth retardation and asymmetry, it seems wise to perform methylation studies of the *H19* locus in patients conceived by ART who have growth retardation of unknown cause.

Mulibrey nanism (muscle-liver-brain-eye [MIM 253250]) has several features that overlap with SRS—for example, pre- and postnatal growth retardation, triangular face, frontal bossing, and asymmetry. Some features—like pericarditis, yellow dots in the fundi, fibrous dysplasia of long bones, and hepatomegaly—do not occur in SRS. About 4% of patients with Mulibrey nanism develop Wilms tumor (Karlberg et al. 2004a). Recently, hypergonadotropic premature ovarian failure with in-

complete breast development was described in Mulibrey nanism (Karlberg et al. 2004b). Homozygous mutations in the *TRIM37* gene (MIM 605073) that are localized close to an SRS translocation on chromosome 17q23-q24 give rise to Mulibrey nanism (Avela et al. 2000). Because patient 4 has some severe features of SRS in combination with hepatomegaly, which can be present in Mulibrey nanism, one can hypothesize that both disorders are part of the same genetic pathway.

A remaining question is whether patients with hypomethylation of the *H19* locus should be screened for intra-abdominal embryonal tumors by ultrasound screening. Although it is not to be expected that overexpression of *H19*, a tumor-suppressor gene, and subsequent reduction of *IGF2* expression would lead to tumor development, hypomethylation of *H19* has been found in human bladder cancer (Takai et al. 2001). In addition, tumors have been described in association with SRS. Testicular cancer, hepatocellular carcinoma, testicular seminoma, craniopharyngoma, and Wilms tumor have been reported in patients with SRS (Chitayat et al. 1988; Dang et al. 2004) but are rare. However, neither our patients nor those reported by Gicquel et al. (2005) developed tumors.

In conclusion, we believe it is appropriate to perform methylation studies of the *H19* locus in all patients with short stature and asymmetry, full SRS, or SR-like syndrome, especially when these are accompanied by feeding problems. Ultrasound screening has a great impact on the patients and their parents. In our opinion, it is not justified to continue ultrasound screening for embryonal tumors conform the BWS protocol in SRS or SRS-like patients with hypomethylation of the *H19* locus.

Acknowledgments

We thank Karin van der Lip and Sebastiaan de Leng, for excellent technical assistance, and the physicians who referred their patients to our clinic. We are grateful for the willingness of the patients and their parents to contribute to this study. We are most grateful to Prof. Dr. P. F. R. Little for critically reading the manuscript. This work was supported by Netherlands Organization of Scientific Research grant 901-04-208.

Web Resources

The URLs for data presented herein are as follows:

EUROCAT, <http://www.eurocat.ulst.ac.uk/>

National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for SRS, BWS, *H19*, *IGF2*, *KCNQ1OT1*, *MEST*, Mayer-Rokitansky-Kuster-Hauser syndrome, Mulibrey nanism, and *TRIM37*)

References

- Al-Fifi S, Teebi AS, Shevell M (1996) Autosomal dominant Russell-Silver syndrome. *Am J Med Genet* 61:96–97
- Avela K, Lipsanen-Nyman M, Idanheimo N, Seemanova E, Rosengren S, Makela TP, Perheentupa J, Chapelle AD, Lehesjoki AE (2000) Gene encoding a new RING-B-box-coiled-coil protein is mutated in Mulibrey nanism. *Nat Genet* 25:298–301
- Bailey W, Popovich B, Jones KL (1995) Monozygotic twins discordant for the Russell-Silver syndrome. *Am J Med Genet* 58:101–105
- Bell AC, Felsenfeld G (2000) Methylation of a CTCF-dependent boundary controls imprinted expression of the *Igf2* gene. *Nature* 405:482–485
- Bellver-Pradas J, Cervera-Sanchez J, Boldo-Roda A, Martin-Cortes A, Ferreres-Gomez L, Serra-Serra V, Romeu-Sarrio A (2001) Silver-Russell syndrome associated to Mayer-Rokitansky-Kuster-Hausler syndrome, diabetes and hirsutism. *Arch Gynecol Obstet* 265:155–157
- Bliet J, Maas SM, Ruijter JM, Hennekam RC, Alders M, Westerveld A, Mannens MM (2001) Increased tumour risk for BWS patients correlates with aberrant *H19* and not *KCNQ1OT1* methylation: occurrence of *KCNQ1OT1* hypomethylation in familial cases of BWS. *Hum Mol Genet* 10:467–476
- Chitayat D, Friedman JM, Anderson L, Dimmick JE (1988) Hepatocellular carcinoma in a child with familial Russell-Silver syndrome. *Am J Med Genet* 31:909–914
- Christensen ME, Nielsen J (1978) Deletion short arm 18 and Silver-Russell syndrome. *Acta Paediatr Scand* 67:101–103
- Curi JF, Vanucci RC, Grossman H, New M (1967) Elevated serum gonadotrophins in Silver's syndrome. *Am J Dis Child* 114:658–661
- Dang YN, Shin IC, Gordon RA, Karcioğlu ZA (2004) Bilateral reactive lymphoid hyperplasia of the orbit in a child with Russell-Silver syndrome. *J AAPOS* 8:588–591
- DeBaun MR, Niemitz EL, Feinberg AP (2003) Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of *LIT1* and *H19*. *Am J Hum Genet* 72:156–160
- DeBaun MR, Niemitz EL, McNeil DE, Brandenburg SA, Lee MP, Feinberg AP (2002) Epigenetic alterations of *H19* and *LIT1* distinguish patients with Beckwith-Wiedemann syndrome with cancer and birth defects. *Am J Hum Genet* 70:604–611
- Dorr S, Midro AT, Farber C, Giannakudis J, Hansmann I (2001) Construction of a detailed physical and transcript map of the candidate region for Russell-Silver syndrome on chromosome 17q23-q24. *Genomics* 71:174–181
- Duncan PA, Hall JG, Shapiro LR, Vibert BK (1990) Three-generation dominant transmission of the Silver-Russell syndrome. *Am J Med Genet* 35:245–250
- Eggermann T, Eggermann K, Mergenthaler S, Kuner R, Kaiser P, Ranke MB, Wollmann HA (1998) Paternally inherited deletion of *CSH1* in a patient with Silver-Russell syndrome. *J Med Genet* 35:784–786
- Eggermann T, Meyer E, Obermann C, Heil I, Schuler H, Ranke MB, Eggermann K, Wollmann HA (2005) Is maternal duplication of 11p15 associated with Silver-Russell syndrome? *J Med Genet* 42:e26
- Engel N, West AG, Felsenfeld G, Bartolomei MS (2004) Antagonism between DNA hypermethylation and enhancer-blocking activity at the *H19* DMD is uncovered by CpG mutations. *Nat Genet* 36:883–888
- Fisher AM, Thomas NS, Cockwell A, Stecko O, Kerr B, Temple IK, Clayton P (2002) Duplications of chromosome 11p15 of maternal origin result in a phenotype that includes growth retardation. *Hum Genet* 111:290–296
- Gicquel C, Rossignol S, Cabrol S, Houang M, Steunou V, Barbu V, Danton F, Thibaud N, Le MM, Burglen L, Bertrand AM, Netchine I, Le BY (2005) Epimutation of the telomeric imprinting center region on chromosome 11p15 in Silver-Russell syndrome. *Nat Genet* 37:1003–1007
- Hannula K, Lipsanen-Nyman M, Kontiokari T, Kere J (2001) A narrow segment of maternal uniparental disomy of chromosome 7q31-qtter in Silver-Russell syndrome delimits a candidate gene region. *Am J Hum Genet* 68:247–253
- Källén B, Finnström O, Nygren KG, Olausson PO (2005) In vitro fertilization (IVF) in Sweden: risk for congenital malformations after different IVF methods. *Birth Defects Res A Clin Mol Teratol* 73:162–169
- Karlberg N, Jalanko H, Perheentupa J, Lipsanen-Nyman M (2004a) Mulibrey nanism: clinical features and diagnostic criteria. *J Med Genet* 41:92–98
- Karlberg S, Tiitinen A, Lipsanen-Nyman M (2004b) Failure of sexual maturation in Mulibrey nanism. *N Engl J Med* 351:2559–2560
- Maher ER (2005) Imprinting and assisted reproductive technology. *Hum Mol Genet Spec* 14:R133–R138
- McMinn J, Wei M, Schupf N, Cusmai J, Johnson EB, Smith AC, Weksberg R, Thaker HM, Tycko B (2005) Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. *Placenta* (http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WPD-4GY893H-2&_coverDate=08%2F24%2F2005&_alid=367534473&_rdoc=1&_fmt=&_orig=search&_qd=1&_cdi=6988&_sort=d&view=c&_acct=C00001358&_version=1&_urlVersion=0&_userid=5745&md5=cb441b9679541ac3b7fc7232f578d2c3) (electronically published August 24, 2005; accessed February 17, 2006)
- Monk D, Bentley L, Hitchins M, Myler RA, Clayton-Smith J, Ismail S, Price SM, Preece MA, Stanier P, Moore GE (2002) Chromosome 7p disruptions in Silver Russell syndrome: delineating an imprinted candidate gene region. *Hum Genet* 111:376–387
- Ounap K, Reimand T, Magi ML, Bartsch O (2004) Two sisters with Silver-Russell phenotype. *Am J Med Genet A* 131:301–306
- Prawitt D, Enklaar T, Gartner-Rupprecht B, Spangenberg C, Oswald M, Lausch E, Schmidtke P, Reutzel D, Fees S, Lucito R, Korzon M, Brozek I, Limon J, Housman DE, Pelletier J, Zabel B (2005) Microdeletion of target sites for insulator protein CTCF in a chromosome 11p15 imprinting center in Beckwith-Wiedemann syndrome and Wilms' tumor. *Proc Natl Acad Sci USA* 102:4085–4090
- Price SM, Stanhope R, Garrett C, Preece MA, Trembath RC (1999) The spectrum of Silver-Russell syndrome: a clinical and molecular genetic study and new diagnostic criteria. *J Med Genet* 36:837–842
- Ramirez-Duenas ML, Medina C, Ocampo-Campos R, Rivera H (1992) Severe Silver-Russell syndrome and translocation (17;20)(q25;q13). *Clin Genet* 41:51–53
- Rogan PK, Seip JR, Driscoll DJ, Papenhausen PR, Johnson VP, Raskin S, Woodward AL, Butler MG (1996) Distinct 15q genotypes in Russell-Silver and ring 15 syndromes. *Am J Med Genet* 62:10–15
- Russell A (1954) A syndrome of intra-uterine dwarfism recognizable at birth with cranio-facial dysostosis, disproportionately short arms, and other anomalies (5 examples). *Proc R Soc Med* 47:1040–1044
- Samn M, Lewis K, Blumberg B (1990) Monozygotic twins discordant for the Russell-Silver syndrome. *Am J Med Genet* 37:543–545
- Silver HK, Kiyasu W, George J, Beamer WC (1953) Syndrome of congenital hemihypertrophy, shortness of stature, and elevated urinary gonadotropins. *Pediatrics* 12:368–376
- Schieve LA, Meikle SF, Ferre C, Peterson HB, Jeng G, Wilcox LS (2002) Low and very low birth weight in infants conceived with use of assisted reproductive technology. *N Engl J Med* 346:731–737
- Sparago A, Cerrato F, Vernucci M, Ferrero GB, Silengo MC, Riccio A (2004) Microdeletions in the human *H19* DMR result in loss of *IGF2* imprinting and Beckwith-Wiedemann syndrome. *Nat Genet* 36:958–960
- Svensson J, Björnståhl A, Ivarsson SA (2005) Increased risk of Silver-Russell syndrome after in vitro fertilization? *Acta Paediatr* 94:1163–

- Takai D, Gonzales FA, Tsai YC, Thayer MJ, Jones PA (2001) Large scale mapping of methylcytosines in CTCF-binding sites in the human *H19* promoter and aberrant hypomethylation in human bladder cancer. *Hum Mol Genet* 10:2619–2626
- Teebi AS (1992) Autosomal recessive Silver-Russell syndrome. *Clin Dysmorphol* 1:151–156
- van Haelst MM, Eussen HJ, Visscher F, de Ruijter JL, Lindhout D, Wouters CH, Govaerts LC (2002) Silver-Russell phenotype in a patient with pure trisomy 1q32.1-q42.1: further delineation of the pure 1q trisomy syndrome. *J Med Genet* 39:582–585
- Weksberg R, Shuman C, Caluseriu O, Smith AC, Fei YL, Nishikawa J, Stockley TL, Best L, Chitayat D, Olney A, Ives E, Schneider A, Bestor TH, Li M, Sadowski P, Squire J (2002) Discordant *KCNQ1OT1* imprinting in sets of monozygotic twins discordant for Beckwith-Wiedemann syndrome. *Hum Mol Genet* 11:1317–1325