a substrate in 21 µl of 50 mM Tris (pH 7.9), 150 mM NaCl, 10 mM $MgCl_2$, 2.5 mM $MnCl_2$, 25 µM ATP and 5 Ci ^{32}P-γ-ATP (Amersham Pharmacia) for 30 min at 30°C. The reactions were subjected to polyacrylamide gel electrophoresis using 10% gels followed by autoradiography.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/InoueCC8-8-Sup.pdf

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

Screening of DNA Methylation at the H19 Promoter or the Distal Region of its ICR1 Ensures Efficient Detection of Chromosome 11p15 Epimutations in Russell–Silver Syndrome

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Over a 10-year period blood samples were collected from 57 individuals with growth restriction and RSS-like features. Our goal was to identify epigenetic abnormalities in this cohort, including uniparental disomy of chromosome 7 (UPD7), methylation changes at chromosome11p15, as well as new epigenomic alterations. We evaluated the methylation status of 7 imprinting control regions on chromosomes 7, 11, 14, and 15. UPD7 and chromosome 7 structural abnormalities had been previously identified in five patients. Epigenetic alterations on chromosome 11p15 were identified in 11 patients. Of interest, in 3 of these 11 patients, the epigenetic alterations were limited to the H19 promoter and the distal region of its associated imprinting center, ICR1. In addition, in one patient, we detected methylation changes consistent with maternal UPD at all tested imprinted regions. This patient series suggests that epimutations on chromosome 11p15 can be most efficiently detected in RSS patients by screening for DNA methylation defects at the H19 promoter or the distal region of ICR.

Key words: Russell–Silver syndrome; H19; DNA methylation; genomic imprinting; maternal UPD

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INTRODUCTION

Russell–Silver syndrome (RSS) (OMIM 180860), is a clinically and genetically heterogeneous disorder usually diagnosed in children with prenatal and postnatal growth restriction, a relatively large head circumference, dysmorphic facial features and body asymmetry [Silver et al., 1953; Russell, 1954]. The majority of RSS patients

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are sporadic but a few familial cases have been described [Robichaux et al., 1981; Duncan et al., 1990; Teebi, 1992; Al-Fifi et al., 1996].

Two different epigenetic defects have been associated with RSS. These are maternal uniparental disomy (mUPD) for chromosome 7 and loss of paternal methylation at the differentially methylated region on chromosome 11p15.5 upstream of the H19 gene.

Maternal uniparental disomy of chromosome 7 (mUPD7) was first described in a girl with short stature [Langlois et al., 1995] and later was reported in 7–10% of RSS and RSS-like individuals [Eggermann et al., 1997; Preece et al., 1997; Nakabayashi et al., 2002].

A link between RSS and chromosome 11p15.5 was initially established by identifying RSS patients with structural chromosomal anomalies disrupting this region [Fisher et al., 2002; Eggermann et al., 2005; Schonherr et al., 2007b]. Later, loss of methylation at the H19 differentially methylated region (ICR1) was reported to occur in patients with RSS, including one of a pair of monozygotic twins with phenotypic discordance [Gicquel et al., 2005]. Since then several reports have been published describing similar findings in a number of RSS, RSS-like and even isolated asymmetry patients [Binder et al., 2006, 2008; Bliek et al., 2006; Eggermann et al., 2006, 2008a,b; Schonherr et al., 2006; Netchine et al., 2007; Zeschnigk et al., 2008; Bartholdi et al., 2009]. The frequency of 11p15.5 epigenetic anomalies in RSS is estimated to be in the range of 35%. Thus, the two most commonly found molecular anomalies could explain approximately 50% of the RSS and RSS-like patients.

Human chromosome 11p15.5 harbors an imprinted gene cluster of 1 Mb with two imprinting control regions (ICR1 and ICR2) (Fig. 1a). In addition to its role in RSS, this imprinted domain is implicated in a wide variety of malignancies and the overgrowth disorder Beckwith–Wiedemann syndrome (BWS, OMIM 130650) [Weksberg et al., 2003]. RSS and BWS display a number of opposite clinical features, and have, in fact, opposite epigenetic errors on chromosome 11p15.5. While epimutations and genetic structural anomalies have been found in both ICRs associated with BWS, no primary ICR2 epimutation has been reported to date in RSS patients. However, a single RSS patient with a maternally inherited cryptic duplication involving ICR2 has been reported [Schonherr et al., 2007b].

In spite of all the advances in delineating the causes of RSS, the etiology in a significant proportion of RSS (~50%) and RSS-like patients remains unclear. Given the known functional relationship between imprinted regions and growth, it is possible that individuals diagnosed with growth restriction phenotypes, including but not limited to RSS, may have a broader range of epigenetic abnormalities than is currently appreciated. A comprehensive analysis of such phenotypes could identify new epigenetic abnormalities beyond mUPD7 or 11p15.5 methylation anomalies. Therefore, we studied a cohort of patients with a broad clinical spectrum of RSS features for epigenetic abnormalities in several differentially methylated regions, including the *H19* promoter, ICR1, *IGF2* DMR2, and the ICR2 on chromosome 11p15.5, *PEG1/MEST* on chromosome 7q32, *MEG3/GTL2* on chromosome 14q32 and *SNRPN* on chromosome 15p11–13. Chromosome 7 anomalies, such as UPD7 and structural chromosome 7 anomalies had been excluded in this cohort.

Our comprehensive DNA methylation analysis of chromosome 11p15.5 identified epigenetic alterations in 11 RSS patients. Interestingly we found loss of methylation at the *H19* promoter to be the most consistent epimutation in the 11 RSS patients. Furthermore, in one of the patients we detected methylation changes consistent with mUPD across all four chromosomes tested, prompting consideration of more complex diagnoses. Analysis of the phenotype and epigenotype data allowed us to develop a set of clinical criteria that will enable clinicians to better predict the likelihood of chromosome 11 epimutations or mUPD7 in patients with RSS features.

MATERIALS AND METHODS

Clinical and Biological Samples

We used DNA obtained from peripheral blood of patients for whom RSS was in the differential diagnosis as per the referring physician. The most consistent clinical feature in the cohort was either fetal growth restriction (birthweight equal or less than 3rd centile) or short stature at observation (height equal or less than 3rd centile). Some patients had some RSS facial dysmorphic features (triangular face, down-turned corners of the mouth, micrognathia/retrognathia, frontal bossing/high forehead, blue sclera, prominent nasal bridge, low set ears). All patients had a normal karyotype. Five other patients, that were originally part of this cohort, had been found to have mUPD7 or chromosome 7 structural anomalies [Nakabayashi et al., 2002] and were therefore excluded from the present analysis. The study was approved by the Research Ethics Board at the Hospital for Sick Children, Toronto, Canada.

Methylation Studies

Southern blot. To analyze the methylation status of imprinted loci on chromosome 11p15.5, 7q32, and 14q32, 5 μg of DNA was digested with *Rsa*I and methylation-sensitive *Sma*I restriction enzymes. For the imprinted locus in chromosome 15q11–13, the same amount of DNA was digested with *Xba*I and methylation-sensitive *Not*I restriction enzymes. The resulting fragments were fractionated on a 1.0% agarose gel and transferred by Southern blotting onto Amersham Hybond™-N+ (GE Healthcare UK, Buckinghamshire, England). The blots were hybridized with ^{32}P-labeled probes that were generated by PCR of genomic DNAs. Primer sequences used for the probes are presented in supporting information Table I (supporting information Table I may be found in the online version of this article). X-ray films were exposed to the Southern blots and they were analyzed qualitatively. The films of *H19* promoter and ICR1 were scanned using a HP digital scanner and the bands of the good quality images were quantitated using AlphaEaseFc Software, version 4.0.0, 2003 from Alpha Innotech, San Leandro, CA.

Pyrosequencing. For patients with abnormal DNA methylation results at ICR1 or *H19* promoter, if DNA was still available the epimutations were validated using pyrosequencing of bisulfite converted DNA. The pyrosequencing assay targets a more distal region of ICR1 than the position probed by southern blot (Fig. 1b). This technique involves bisulfite conversion of the DNA and quantitative sequencing of a PCR fragment including several CG dinucleotides. The technique allows for quantitation of each

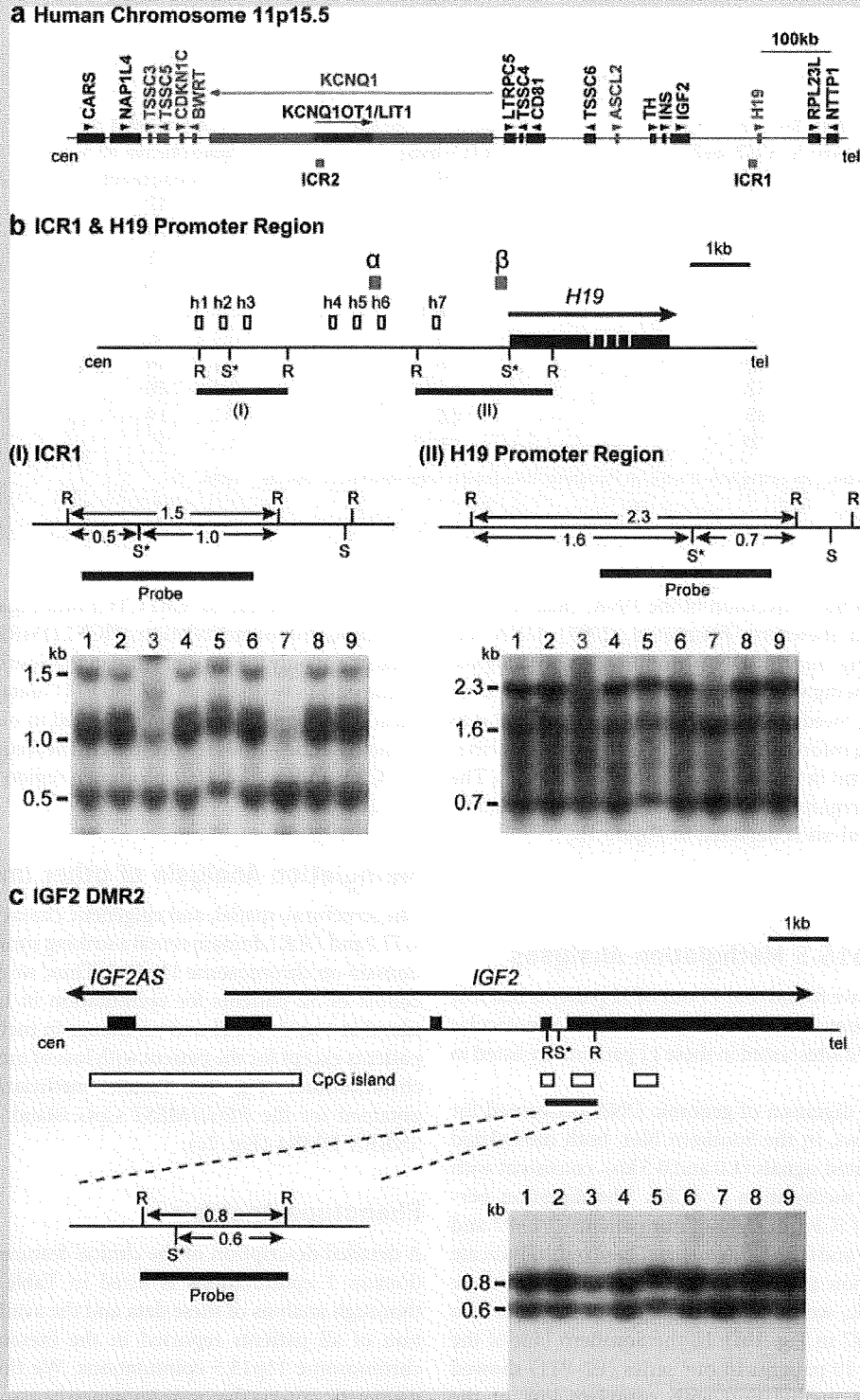

FIG. 1. Methylation analysis of chromosome 11p15.5 locus in individuals with RSS. a: Physical map of the imprinted gene cluster in the diagram of human chromosome 11p15.5. Previously identified genes or transcripts (boxes) are drawn approximately to scale. The transcriptional orientation is indicated by arrows. Blue, paternally expressed genes; Red, maternally expressed genes; Black, biallelically expressed genes. b: Southern blot methylation analysis of ICR1 (I) and *H19* promoter region (II). The filled boxes indicate the exons of *H19*. The open box (h1–h7) indicates the CTCF binding sites. The probes used for Southern blot analysis are shown below the restriction maps. R and S indicate the *RsaI* and *SmaI* restriction sites. α, β (green boxes) indicates the pyrosequencing target regions. It is shown that Southern blot analysis of ICR1 targets a dinucleotide in the area of CTCF binding site 2 whereas the pyrosequencing analysis targets a more distal region corresponding roughly to CTCF binding site 6. c: Methylation analysis of *IGF2* DMR2. The open boxes indicate the CpG islands. The transcriptional orientation is indicated by arrows. In the Southern blot examples shown, individual 3 and 7 showed loss of methylation of ICR1 and *H19* promoter but normal methylation of *IGF2* DMR2. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Molecular Data for Cases With Domain 1 Epimutations

Patient #	ICR1 SB quantitative MI % (CTCF-bs2)	ICR1 pyrosequencing (CTCF-bs6)	H19 promoter SB quantitative MI %	H19 promoter pyrosequencing
P1	38	29	Inconclusive	28
P2	27	na	12	na
P3	25	na	14	na
P4	22	19	25	17
P5	23	9	26	10
P6	32	na	9	na
P7	14	na	6	na
P8	65	26	14	22
P9	53	na	26	na
P10	65	22	14	18
P11	18	na	7	na

SB, Southern blot; CTCF-bs2 and bs6, CTCF binding sites 2 and 6; MI, methylation index (control range 45–65%); na, DNA not available.

SNPcreated by the bisulfite conversion of the DNA. Details of this technique are provided elsewhere [Byun et al., 2007]. DNA was bisulfite converted using Imprint DNA Modification Kit (Sigma, St. Louis, MO). Sequencing was done on a PyroMar™Q24 system from Biotage, Uppsala, Sweden. The primers used in the PCR step are listed in supporting information Table I (supporting information Table I may be found in the online version of this article). The relative location of the regions probed by the Southern blot and by the Pyrosequencing analysis is depicted in Figure 1b.

RESULTS

Chromosome 11p15.5 Methylation Analyses

In the present analysis, we found that 11 out of 52 patients had loss of methylation on chromosome 11. Details about the molecular findings at the imprinted sites tested in these 11 patients are listed in Table I.

In normal controls, digestion of genomic DNA performed for the study of ICR1 shows, in the Southern blot, both methylated (1.5 kb) and unmethylated signals (1.0 and 0.5 kb), consistent with paternal-specific CpG methylation at ICR1. The digestion performed for the study of ICR1 in eight of our patients (P1–P7 and P11) produced only 1.0 and 0.5 kb fragments. These results indicate that paternal methylation at the ICR1 locus was altered in these patients. As an example, we present the analysis of ICR1 for two patients (lane #3 and #7 in Fig. 1bI). In the Southern blot of the *H19* promoter region, 10 patients in our series (P2-P11) showed loss of *H19* promoter methylation with complete loss of the methylated signal (2.3 kb) (examples shown in lane #3 and #7 in Fig. 1bII). The Southern blot data of ICR1 and *H19* promoter was further validated using pyrosequencing of bisulfite converted DNA for five patients with available DNA. We were able to confirm the epimutation at the *H19* promoter in P4, P5, P8, and P10. Further, we verified that P1 (inconclusive by Southern blot) also had loss of methylation at the *H19* promoter. Interestingly, for ICR1, targeting of CTCF binding site 6 by pyrosequencing showed loss of methylation in all five patients tested, including P8 and P10 who showed normal methylation for ICR1 CTCF binding site 2 by Southern Blot. In contrast loss of methylation of IGF2 DMR2, demonstrated by the presence of only the smaller band in the Southern blot (Fig. 1c), was found only in two patients (P7 and P11) (data not shown). Thus, the most consistent epimutation identified in our RSS cohort was loss of methylation at the *H19* promoter. The frequency of epimutations of ICR1 and/or the *H19* promoter region in our study cohort is ~21% (11/52).

Methylation Analysis of other Imprinted Loci

The structural, spatial, and epigenetic characteristics of the *MEG3/GTL2* and *DLK1* domain reveal a striking similarity to the *IGF2/H19* domain on chromosome 11p15.5. Thus, we also screened the same cohort of 52 patients for epimutation in the *MEG3/GTL2* CpG island at 14q32. We found no change in methylation in any of the patients except for the patient with loss of methylation for multiple chromosomes (Fig. 2a). Parallel methylation results were also obtained for the *PEG1/MEST* CpG island (Fig. 2b) and for the SNRPN DMR1 (Fig. 2c).

Phenotype Analysis

A detailed description of the clinical features of each patient with domain 1 epimutations is listed in Table II. We performed a thorough analysis of these data and the available clinical information of all patients reported in the literature with mUPD7 or chromosome 11p15.5 epimutations. We then developed a set of diagnostic criteria that were known to be associated with epigenetic alterations of chromosome 7 or 11, excluding P11 (Table III). We applied these criteria to our patients who were negative for chromosome 11p15.5 epimutations and mUPD7. Only 19 of these 41 patients would meet such criteria. Further, of those 19 patients, five had features not previously described in the phenotypic spectrum currently accepted for RSS such as hypogammaglobulinemia, hypothyroidism, sensorineural hearing loss, cerebral cortical dysplasia, branchial arch cyst, coarctation of the aorta with bicuspid aortic valve. If we also exclude these patients, ICR1 epimutations

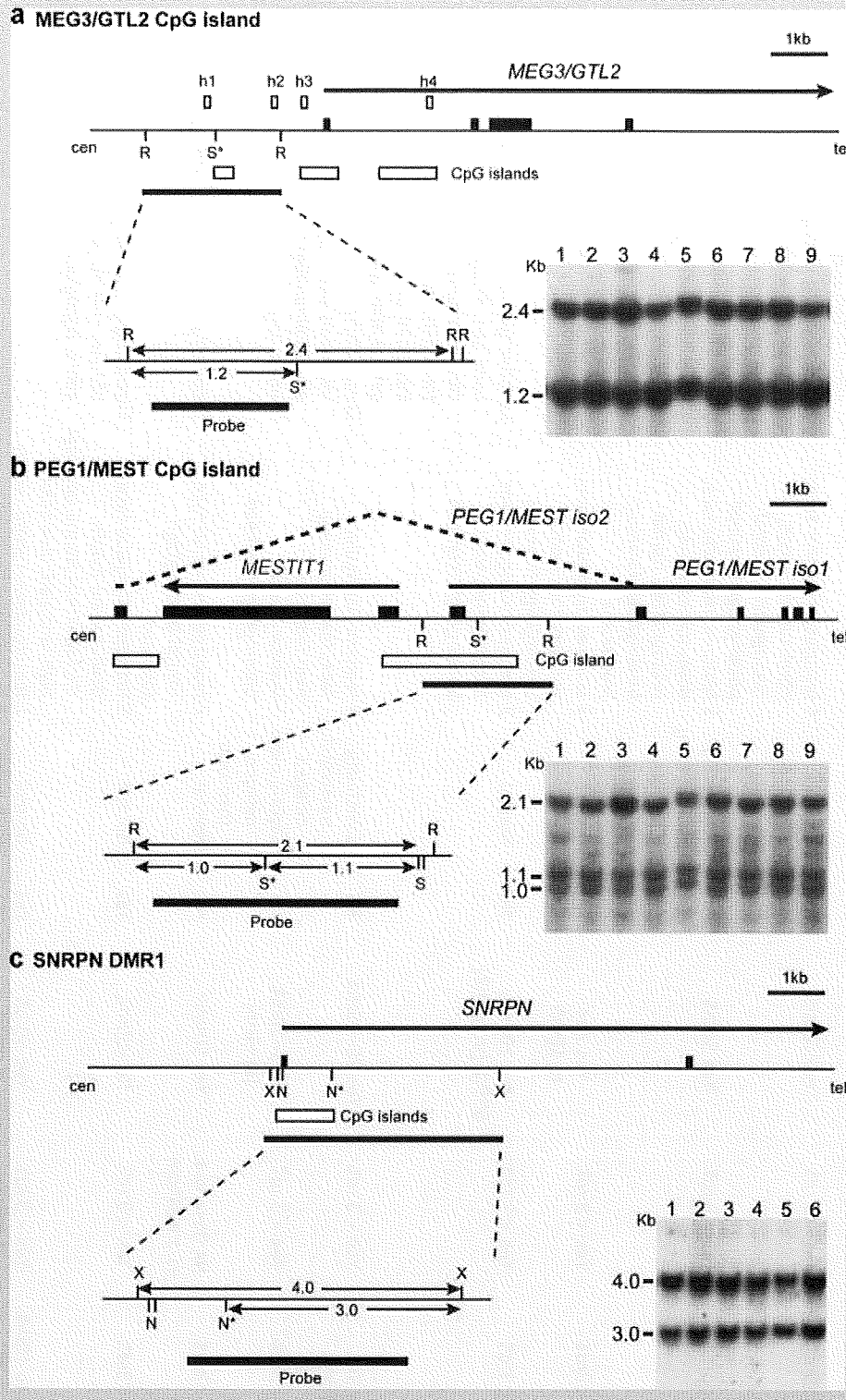

FIG. 2. Methylation analyses of the *MEG3/GTL2* and *PEG1/MEST* CpG islands and of the *SNRPN* DMR1. a: Methylation analysis of the *MEG3/GTL2* CpG island. b: Methylation analysis of the *PEG1/MEST* CpG island. c: Methylation analysis of the *SNRPN* DMR1. The filled boxes indicate the exons. The open boxes indicate the CpG islands. The open box (h1–h4) indicates the CTCF binding sites. The probes used for Southern blot analysis are shown below the restriction maps. R, S, X, and N indicate the *RsaI*, *SmaI*, XbaI, and NotI restriction sites respectively.

TABLE II. Summary of Clinical Phenotype of all Cases Found With Chromosome 11 Epimutations

Patient #	Sex	BW <10th% or ~1.3 SD	Postnatal height < 3rd% or ~1.6 SD	HC >3rd% or ~1.6 SD	Body asymmetry	Mental development	Facial RSS features	Additional RSS features	Additional non-RSS features
P1	F	Yes <3rd%	Yes <3 rd%	Yes ~25th%	No	Normal	1 feature: frontal bossing/ high forehead	Clinodactyly, delayed bone age, syndactyly of toes, delayed closure of anterior fontanel	Shoulder and elbow dimples
P2	M	Yes <3rd%	Yes <3 rd%	Yes ~25th%	Yes	Normal	4 features: triangular face, down-turned mouth, micrognathia, frontal bossing/high forehead	Clinodactyly, syndactyly of toes, thin skin	
P3	F	Yes <3rd%	Yes <3 rd%	Yes 50th%	Yes	Moderately to severely delayed	5 features: triangular face, down-turned mouth, micrognathia, frontal bossing/high forehead, blue sclera	Clinodactyly, increased sweating	
P4	M	Yes <3rd%	Yes <3 rd%	Yes 2nd to 10th%	Yes	Normal	3 features: triangular face, micrognathia, frontal bossing/high forehead	Clinodactyly, thin skin	
P5	M	Yes <3rd%	Yes <3 rd%	Yes 10th to 50th% (at birth?)	No	Normal	3 features: triangular face, micrognathia, frontal bossing/high forehead	Clinodactyly, delayed bone age, delayed closure of anterior fontanelle, thin skin, increased sweating, genital anomalies	Increased levels of T3 and T4
P6	M	Yes 3rd to 10th%	Yes <3 rd%	Yes 25–75th%	No	Selectively moderately delayed	2 features: triangular face, frontal bossing/ high forehead	Clinodactyly, increased sweating, genital anomalies	
P7	F	Yes <3rd%	Yes <3 rd%	Yes 3–10th% (at birth)	No	Slightly more delayed then twin	1 feature: frontal bossing/high forehead	Clinodactyly	
P8	M	Yes <3rd%	NA Observed as a newborn	Yes 50th%?	Yes	Normal	2 features: triangular face, frontal bossing/high forehead	Clinodactyly	Metopic ridge
P9	M	Yes <3rd%	Yes <3 rd%	No <2nd%	Yes	Normal	5 features: triangular face, down-turned mouth, micrognathia, frontal bossing/ high forehead, blue sclera	Clinodactyly, syndactyly of toes, café au lait spots, hypoglycemia	
P10	M	Yes 3rd to 10th%	Yes <3 rd%	Yes 50th%	Yes	Normal	1 feature: down-turned mouth		
P11	**M**	**Y <3rd%**	**N 25th%**	**Y 50th%**	**Yes**	**Moderately delayed**	**1 feature: down-turned mouth**	**Clinodactyly, syndactyly of toes, delayed closure of anterior fontanelle, hypoglycemia, genital anomalies**	**Dizygotic twin with growth discrepancy**

BW, birthweight; HC, head circumference; SD, standard deviation; RSS, Russell–Silver syndrome; M, male; F, female; %, centile.
The last patient, in bold, is the child with multiple methylation defects suggesting UPD for multiple chromosomes in blood DNA.

TABLE III. Criteria Developed Based on the Clinical Findings of RSS Cases With ICR1 Epimutation or mUPD7 in Our Cohort (Excluding P11) and From Cases Reported in the Literature

BIRTHWEIGHT < 10%	And, at least, 2 of:	POSTNATAL HEIGHT < 3% HEAD CIRCUMFERENCE > 3%	
		ONE OR MORE FACIAL FEATURES:	triangular face, down-turned mouth, micrognathia / retrognathia, frontal bossing/high forehead, blue sclera, low set ears
and/or			
ASYMMETRY (with any of the above or isolated)			

Other anomalies previously described in RSS include: increased sweating, 5th finger clinodactyly, simian creases, joint contractures, delayed bone age, 2–3 syndactyly of toes, delayed closure of anterior fontanel, café-au-lait spots, neonatal hypoglycemia, renal anomalies, heart anomalies, thin skin, genital abnormality in males, uterine or ovarian anomalies in females, sacral abnormalities, vertebral abnormalities, muscular hypoplasia, early puberty, feeding difficulties, mental retardation, irregularities of teeth, ear anomalies, simian crease.
The presence of features other than those listed above decreases the likelihood of finding H19 promoter/ICR1 hypomethylation or mUPD7.

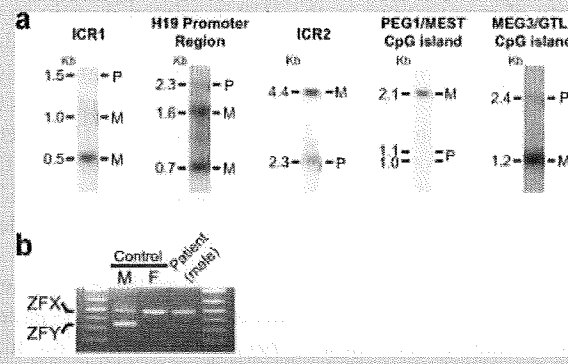

FIG. 3. Blood DNA molecular tests of patient found to have epimutations in all tested sites. a: Methylation analysis of ICR1, *H19* promoter region, ICR2, *PEG1/MEST* CpG island, *MEG3/GTL2* CpG island showing that all loci (11p15.5, 7q32, 14q32) display maternal methylation pattern. b: Molecular analysis of *ZFX* and *ZFY*; the PCR co-amplifies distinguishable products from X and Y chromosomes. PCR products are resolved in 1.5% agarose gels. Lane M: Male control showing 2 bands, Lane F: female control showing only one band. The patient, third lane, failed to yield a Y-specific product, despite its male phenotype, contributing to the hypothesis of maternal UPD for all chromosomes in blood DNA.

would then be found in 40% (10/25) of patients selected by these stricter criteria. If we include the five patients with chromosome seven anomalies previously identified in our cohort [Nakabayashi et al., 2002], we find ICR1 epimutations in 10 out of 30 patients (33%, 95th CI 18–53%), and mUPD7 in 5 out of 30 patients (16%, 95th CI 5–35%). These data are consistent with the 50% rate of mUPD7 and chromosome 11 epimutations reported in the literature for patients with a clinical diagnosis of RSS.

Complex Mosaic Patient

In our screening of this series of RSS patients, we identified a male patient (P11) in whom we found changes in methylation at all sites surveyed (ICR1 and *H19* promoter region, ICR2, *MEG3/GTL2*, *SNRPN*, and the *PEG1/MEST* locus) (Fig. 3a). These data were consistent with mUPD for all chromosomes tested. Although this patient is phenotypically male, no Y specific PCR product was detected in blood (Fig. 3b). Follow-up clinical information included a chromosome analysis done on skin fibroblasts which showed a 46, XY karyotype (1 cell out of 100 with 69, XXY). Unfortunately biological material for further molecular characterization was not available. Therefore, we were unable to test our hypothesis that this individual is a mosaic for multiple cell lines: a 46,XY normal male cell line, a 69,XXY cell line and, in blood, a 46,XX cell line with mUPD for all chromosome pairs.

DISCUSSION

We screened a series of patients with growth restriction syndromic phenotypes and tested for abnormalities at several imprinted regions. Our comprehensive screen of chromosome 11p15.5 identified 11 patients with epimutations in the telomeric imprinted domain, which included both the primary imprinting control region, ICR1, and the imprinted *H19* promoter as well as *IGF2* DMR2. *IGF2* is known to be involved in regulation of fetal growth and development [Guo et al., 2008]. Loss of methylation of ICR1 can result in reduced transcription of *IGF2* [Bell and Felsenfeld, 2000; Hark et al., 2000].

Usually, the *H19* genomic region, which includes both the imprinting control region ICR1 and the *H19* promoter, is expected to reflect a consistent level of methylation. Our data suggest a consistent concordance of *H19* promoter methylation only in the distal (CTCF binding site 6) region of ICR1. In contrast, the methylation in the proximal (CTCF binding site 1) region is variably correlated with methylation at the H19 promoter. This type of subregional loss of methylation for the *H19* region has not been reported before, although data from Eggermann et al. [2008b] and Zeschnigk et al. [2008], showing that loss of methylation at ICR1 may occur only at some CTCF binding sites, gives support to our findings. This is important when evaluating tests for epigenetic errors in this region, since these data suggest that some subregions of the telomeric 11p15.5 differentially methylated regions are more likely than others to demonstrate loss of methylation in RSS patients. In retrospect, it is important to note that several of the first studies of this region in RSS likely underestimated the frequency of loss of methylation at the chromosome 11p15.5 telomeric imprinted region, as their assays were limited to the proximal ICR1 subregion of the *H19* genomic region (see supporting information Table II which may be found in the online version of this article). Indeed, when some of the patients with normal methylation at ICR1 were retested, they demonstrated epimutations in other regions of ICR1 [Zeschnigk et al., 2008; Eggermann et al., 2008b].

Based on our comprehensive analyses of the chromosome 11p15.5 imprinted cluster, we propose that testing for epimutations

at the *H19* promoter or the distal region of ICR1 will detect the majority of epimutations in the *H19* imprinted domain in RSS patients. One additional important factor that must be considered when testing for chromosome 11p15 domain 1 epimutations is the tissue being tested. Of note, in the normal placenta, the H19 promoter is unmethylated on both parental alleles [Jinno et al., 1995; Guo et al., 2008]. Therefore, in placenta, testing for DNA methylation at the H19 genomic region should focus on the CTCF binding site 6 region of ICR1. Larger cohort studies will be needed to validate these recommendations.

Loss of methylation at the DMR2 region of the IGF2 gene has been reported previously by Gicquel et al. [2005] in RSS patients. Indeed a study in mice showed that loss of methylation of Igf2 DMR2 can disrupt of *IGF2* expression [Murrell et al., 2004]. We found loss of CpG methylation of IGF2 DMR2 in only one of our patients in addition to the one with mUPD for several chromosomes.

Recently Kagami et al. [2007] reported a RSS patient with CG dinucleotide changes in the *PEG1/MEST* DMR [Kagami et al., 2007]. However, previous studies showed that CpG methylation patterns of the 5' region of *PEG1/MEST* were normal in a total of 127 RSS or RSS-like patients [Riesewijk et al., 1998; Kobayashi et al., 2001; Schöherr et al., 2008]. In our cohort, changes in methylation at the *PEG1/MEST* locus were detected only in the patient with multiple methylation defects at several imprinted loci. This contradiction between the previous reports is likely explained by the relative rarity of this type of molecular anomaly or due to the different methylation assays used by various investigators to analyze methylation at one or more CG dinucleotide sites.

SNRPN and MEG3 DMRs were not previously assayed in RSS patients. Our study did not identify any epimutations at these DMRs suggesting a low likelihood of their involvement in RSS.

Molecular heterogeneity in an unusual growth restriction phenotype was identified in one of our patients (P11). This patient had clinical features beyond those typically seen in RSS. In this patient the only RSS features present were prenatal growth restriction, relative macrocephaly and asymmetry. Molecular testing demonstrated multiple methylation defects that were best interpreted as maternal UPD for all chromosomes tested. Since mUPD for all chromosomes is expected to be lethal, it is likely that there is a more complex genetic constitution. This was supported by the finding of low level mosaicism with a normal male cell line in a follow-up karyotype (46,XY/69,XXY) of cultured fibroblasts. Although we did not have access to further biological specimens to confirm this finding we suspect, based on our limited data, that this patient represents an example of mosaicism in which one diploid cell line has UPD for all chromosomes and another cell line has biparental contributions. This type of molecular anomaly in humans has been previously reported [Strain et al., 1995]. Our patient demonstrates that an epigenetic alteration at an ICR in a patient with atypical phenotypic features should prompt consideration of other diagnoses. In this regard, testing of other imprinted loci may be informative in the detection of complex genetic alterations.

The consideration of more complex epigenetic alterations for RSS patients is further supported by the recent report of individuals with loss of methylation at multiple genomic sites [Mackay et al., 2006a,b]. In contrast, our results did not reveal any epimutations outside the two RSS candidate regions on chromosomes 7 and 11 supporting what has been previously reported [Schonherr et al., 2007a]. This discrepancy may be explained by the absence of such multiple methylation defects in RSS. Alternatively, it can be explained either by the rarity of these epimutations in RSS patients and/or by the fact that we and others [Schonherr et al., 2007a] have tested only a subset of the known genomic imprinted regions.

In conclusion, our data and the data from others [Bliek et al., 2006; Mackay et al., 2006a,b; Kagami et al., 2007], lend support to the idea that there are likely to be growth restriction syndromic phenotypes caused by as yet unidentified imprinting defects involving other genomic regions, for example, GRB10 or PEG10 on chromosome 7. As high throughput and more powerful methods of methylation analysis become available, it may be useful to revisit the patients in which no etiology has been established and to perform an analysis of multiple imprinted regions. Identification of the heterogeneous molecular causes of RSS and RSS-like phenotypes is important, not only from a mechanistic viewpoint, but also for prognostic considerations. In this regard, it is important to note that RSS patients with mUPD7 have a better response to growth hormone therapy than do patients with epigenetic anomalies on chromosome 11 [Binder et al., 2008].

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