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ARTICLE

Methylation analysis of 79 patients with growth restriction reveals novel patterns of methylation change at imprinted loci

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This study was an investigation of 79 patients referred to the Wessex Regional Genetics Laboratory with suspected Russell–Silver Syndrome or unexplained short stature/intra uterine growth restriction, warranting genetic investigation. Methylation status was analysed at target sequences within eleven imprinted loci (*PLAGL1, IGF2R, PEG10, MEST1, GRB10, KCNQ1OT1, H19, IGF2P0, DLK1, PEG3, NESPAS*). Thirty seven percent (37%) (29 of 79) of samples were shown to have a methylation abnormality. The commonest finding was a loss of methylation at *H19* (23 of 29), as previously reported in Russell–Silver Syndrome. In addition, four of these patients had methylation anomalies at other loci, of whom two showed hypomethylation of multiple imprinted loci, and two showed a complete gain of methylation at *IGF2R*. This latter finding was also present in five other patients who did not have demonstrable changes at *H19.* In total, 7 of 79 patients showed a gain of methylation at IGF2R and this was significantly different from a normal control population of 267 individuals (*P*=0.002). This study in patients with growth restriction shows the importance of widening the epigenetic investigation to include multiple imprinted loci and highlights potential involvement of the *IGF2R* locus.
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INTRODUCTION

Imprinted genes are of fundamental importance in normal human growth and development. Their expression is restricted in a parent-of-origin specific manner by epigenetic modifications, including DNA methylation and histone modification.[1]

A number of imprinting syndromes due to aberrant expression of imprinted genes have been described. These include transient neonatal diabetes (TND), Russell–Silver syndrome (RSS), Beckwith–Wiedemann syndrome (BWS), Prader–Willi syndrome, Angelman syndrome, Pseudohypoparathyroidism type 1b, maternal (also called Temple syndrome[2]) and paternal UPD 14 related disorder. Though this list represents diverse phenotypes, there are common features: phenotypically, many are associated with disordered growth; molecularly, each may be caused by aberrant methylation at a differentially methylated region (DMR). In some of these conditions in addition to the site-specific loss of methylation (LOM) associated with the disorder, LOM also occurs at diverse additional imprinted loci. This has been termed hypomethylation of multiple imprinted loci (HIL)[3] and has been observed in BWS and TND.[3–5]

RSS is characterised by pre- and post-natal growth restriction; other salient features include relative head sparing, a triangular face with prominence of the forehead, a low body mass index, asymmetry, and feeding difficulties in infancy. RSS remains a difficult diagnosis to make due to clinical and (epi)genetic heterogeneity. Various clinical criteria have been proposed[6,7] but many of the features are relatively non-specific, and there is considerable phenotypic overlap between RSS, and low birth weight or short stature due to other causes. Furthermore, some of the features become less apparent with increasing age. Molecularly, two well described causes exist: LOM at *H19*, which is found in 30–60%,[7–9] and maternal uniparental disomy of chromosome 7 (mUPD7), which is found in around 5% of well-defined cases.[7] The critical region on chromosome 7 contributing to the RSS phenotype has not yet been established, and there are currently three candidate imprinted regions (*MEST/PEG1, GRB10* and *PEG10*) at which an isolated epigenetic change may conceivably lead to an RSS phenotype.

Maternal duplications of the ICR2 region on 11p15 are also implicated in a small number of RSS cases.[10–12] Thus, within RSS there are clear subgroups, which may have discrete clinical

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implications. Furthermore, at least 30% of clinically diagnosed cases have no recognised cause.

In patients with RSS due to LOM at *H19*, HIL has not yet been observed; in mice LOM at *H19* can be accompanied by LOM at *DLK1*[13] but a recent study examining methylation at four loci (*PLAGL1, DLK1, MEST, H19*) in RSS patients, found no additional LOM.[14]

The possibility that LOM at *H19*, or mUPD7 might not be specific to RSS and might be found in a proportion of patients with isolated pre- and postnatal growth retardation, has also been considered by others.[7,8,15,16] Until recently, the conclusion in each of these studies was that mUPD7 and LOM at *H19* were restricted to those with typical RSS features. More recently, less typical patients have been reported.[17] Comprehensive methylation studies at other known DMRs have not yet been published in the cohort with atypical RSS.

We hypothesized that altered methylation patterns at the DMRs of imprinted genes on chromosome 7, or elsewhere, would be found in patients with growth restriction. Furthermore, given the observed HIL in some imprinting syndromes, we postulated that additional LOM would be seen in patients with LOM *H19*, defining a new subgroup of patients.

MATERIALS AND METHODS

DNA samples from 79 patients referred to the Wessex Regional Genetics Laboratory with growth restriction were included in the study. There were two recruitment groups: the first consisted of 19 patients recruited into the Imprinting Disorders Finding Out Why? (IDFOW) study. This group was referred with growth restriction, where the referring geneticist felt there was a potential imprinting disorder. Detailed phenotypic information accompanied these referrals. The second group comprised 60 samples sent to the NHS laboratory for the routine genetic investigation of unexplained short stature/intrauterine growth retardation, or possible SRS, as the major reason for referral. This latter group was identified by a single observer searching the laboratory database of referrals for the period 2000–2008. An appropriate referral reason and sufficient DNA were prerequisite selection criteria. These samples were anonymised and linked to clinical data. Twenty-seven of the 60 were referred by NHS consultants in Clinical Genetics from the United Kingdom. The remaining 33 samples originated from local paediatricians.

Each recruitment group had its own ethics approval: the first group was adopted by the UK comprehensive local research network, and approved by Southampton and South West Hampshire Research Ethics committee 07/H0502/85; the second group were approved by the Salisbury Research Ethics Committee REC05/Q2008/52, but the approval did not allow us to go back to the patients or referring clinicians for more detailed phenotypic information.

The two recruitment arms generated four main categories of referral groups: RSS; short stature (SS) sufficient to warrant genetic investigation; intrauterine growth retardation (IUGR); IUGR and unexplained short stature.

DNA was extracted from peripheral blood lymphocytes by standard procedures. No additional tissue samples were available for this study.

Methylation status was analysed at target sequences within 11 imprinted loci (*PLAGL1, IGF2R, PEG10, MEST1, GRB10, KCNQ1OT1, H19, IGF2P0, DLK1, PEG3, NESPAS*). Genomic DNA was modified by bisulphite treatment (EZ DNA Methylation Kit; Zymo Research). Methylation-specific PCR (MS-PCR) was performed at these loci as previously described.[3] Previously undescribed MS-PCR primer sets are given in Table 1. Each experiment was performed in duplicate.

DNA methylation was calculated as the peak height ratio of unmethylated and methylated amplicons and normalised against normal controls (>4 per experiment). The methylation ratio is therefore the normalised unmethylated/methylated peak height ratio as exemplified in Figure 1. A complete gain of methylation is represented as '0'. Hypomethylation, that is, greater peak intensity for the unmethylated than the methylated amplicon, is represented by numbers >1. Hypermethylation, that is, greater peak intensity for the methylated than the unmethylated amplicon, is represented by numbers <1.

A patient result was considered abnormal when it fell outside three SD from the control mean normalised ratio.

Table 1 Previously undescribed primer sets, for loci *IGF2R, PEG10*, and *IGF2P0*

	MS-PCR primers: genomic sequence (NCBI build 36.1)	*'Methylated' primer amplicon size (bp)*	*'Unmethylated' primer*	*Common (fam-labelled) primer*
IGF2R	Chr6:160 346 341–160 346 523	GAGGAGG**CG**AGGGG**CGCGCG**AGG**CG**GC	G**tGtG**GAGGGTATGAGGAGG**tG**AGGGG**tGtG**t**G**	CCATTCTCTCCCCTCCCCAACTACAACCTTCC
6q27		256	270	
PEG10	Chr7:94 123 695–94 123 917	**C**GAGTTTGG**C**GAAAGGTT**C**GTTGAG**C**GGGTTGT**C**	GTGAAAGGTT**T**GTTGA**GT**GGGTTGTT**G**TTT**G**GAG	CTAAAATACTACTCCATCTCCCRCAACTCCC
7q21		224	216	
IGF2P0	Chr11:2 126 061–2 126 227	GtttGA**C**GAGGTTAGTGAGGGA**C**GGCG	ATAGTTTTGTTTG**AT**GAGGTTAGTGAGGG**AT**GGT**G**	CCAAAACAATTTCCCTAAAAATACTCATTCATAC
11p15		155	164	

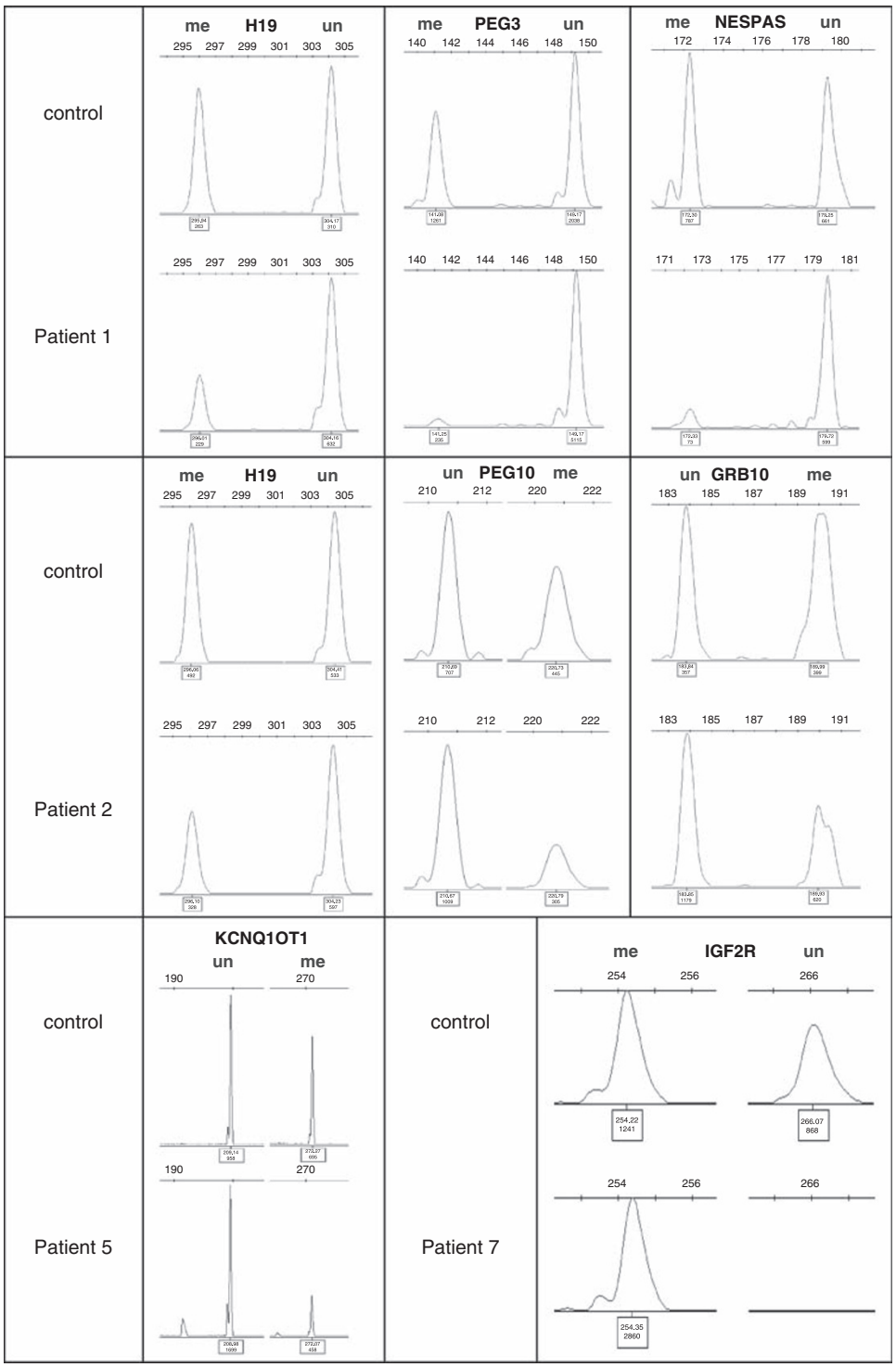

Figure 1 Electropherograms of methylation-specific PCR amplicons. Top row, patient 1; second row, patient 2; bottom row left, patient 5, bottom row right, patient 7. In each case an upper electropherogram represents the result from a control within the same experiment, and the lower electropherogram the result from the patient. The amplicon is identified above each panel, as are the methylated and unmethylated peaks (marked in red or blue for maternal or paternal origin, respectively). Presence of both peaks at equivalent abundance to the normal control is consistent with a normal methylation profile; reduction of the methylated peak height indicates relative hypomethylation at this site in a patient.

This method has been published elsewhere[3,18,19] and has been used to detect methylation changes at several loci. To further substantiate the methods for each loci analysed within this paper, separate experiments were performed using control DNA derived from 126 normal controls (for *IGF2R* we used 267 controls). These experiments generated the SD of the ratios, providing further evidence of reproducibility (Table 3).

Abnormal results were confirmed using a separate bisulphited sample and also, in most cases, utilising a second methodology: for *H19, IGF2R, GRB10,*

Table 2 Summary of results, recruitment method, and referral category for all patients

Ascertainment cohort	*Clinical referral category*	*Number of patients with loss of methylation at H19 DMR*		*Number of patients who in addition to H19 result showed* *Methylation aberrations in at least one other locus (see Table 3)*	*Normal methylation at other loci (data not shown)*
IDFOW (*n*=19)	RSS (*n*=6)	LOM	5	2	3
		Normal	1	0	1
	IUGR and short stature (*n*=9)	LOM	0	NA	NA
		Normal	9	4	5
	Short stature (*n*=4)	LOM	0	NA	NA
		Normal	4	1	3
Anonymised (*n*=60)	RSS[a] (*n*=28)	LOM	16	2	14
		Normal	12	0	12
	IUGR and short stature[a] (*n*=3)	LOM	1	0	1
		Normal	2	1	1
	Short stature[a] (*n*=7)	LOM	0	NA	NA
		Normal	7	0	7
	IUGR[a] (*n*=22)	LOM	1	0	1
		Normal	21	0	21

IDFOW, patients recruited through the Imprinting Disorders Finding Out Why study in which detailed phenotypic information accompanied samples; RSS, Russell-Silver syndrome; IUGR, intra-uterine growth retardation; LOM, loss of methylation; NA, not applicable.
[a]The clinical referral categories for those individuals recruited as the anonymised cohort were derived from details given on referral cards, accompanying samples. Due to the potential inaccuracy in this phenotypic information, there is the possibility that an individual may have been inaccurately assigned to a given clinical category.

PEG3 and *NESPAS,* pyrosequencing was performed, using standard methodology[3] and with primers designed to be non overlapping with MS–PCR primer sequences; for *KCNQ1OT1* (and *H19*) an MLPA kit was used (obtained from MRC Holland http://www.mlpa.com).

RESULTS

The results of all 79 patients, their recruitment method and referral category are tabulated in Table 2. The MS–PCR results of patients with novel imprinting signatures are given in Table 3, and depicted in Figure 1. Examples of corroboratory pyrosequencing assays are depicted in Figure 2.

Of the 79 patients included in this study, 23 patients had LOM at *H19,* and 21 of these were referred with a clinical suspicion of RSS. The remaining two patients were referred with variable phenotypes and were recruited from the anonymised arm: one had IUGR and suffered from numerous chest infections; the other was a twin with IUGR, exomphalos, hypospadias, a cleft palate and a cardiac defect.

Of the 23 patients with LOM at *H19* there were two with additional widespread LOM (Table 3). Of these, patient 1 had LOM involving *NESPAS/GNAS* and *PEG3*. This patient was born with a cleft lip and palate, and had a birth weight below the 0.4th centile. By 7 years of age, without the use of growth hormone, she had shown catch up growth with her height on the 25th centile. She was reported to have speech and language delay. This patient had a half sibling with BWS, and there was a family history of recurrent pregnancy losses (manuscript in preparation). The other patient (patient 2) had LOM at *IGF2R*, *KCNQ1OT1*, *PEG10, GRB10* and *NESPAS*. This patient was one of monochorionic monoamniotic female twins with discordant growth. She was born at 31 weeks, with her birth weight on the 9th centile. She developed renal failure in the neonatal period due to renal dysplasia. Her height and weight at age 4 years were on the 2–9th centiles, with her head circumference on the 50th centile. Asymmetry was noted, and motor and speech milestones were met later than in her twin. DNA was not available from her twin.

Patients 3 and 4 had, in addition to LOM at *H19*, a complete gain of methylation (GOM) at *IGF2R*. Their clinical features are summarised in Table 4.

Of the 56 patients with no LOM at *H19*, one patient (patient 5) showed LOM at *KCNQ1OT1* (chr11p15, DMR2), ordinarily associated with BWS. MLPA analysis of the 11p15 region did not reveal any copy number changes to account for the LOM at *KCNQ1OT1.* This female patient was born at 38 weeks gestation and weighed 2.03 Kg (0.4th centile). Her mother had been diagnosed with polycystic ovarian syndrome, and the pregnancy was complicated by pre-eclampsia (from 34 weeks). In the neonatal period she developed hypoglycaemia and feeding problems with a poor suck. At 7 years of age she had height and head circumference $<$0.4th centile, with her weight on the 3rd centile. She showed developmental delay, particularly affecting her speech and language.

In five patients (patients 6–10) the only epigenetic anomaly found was complete GOM at *IGF2R.* The known clinical features within this group are shown in Table 4. The incidence of GOM in our study population (7 of 79) was significantly higher than in an anonymised control population of unknown anthropometry (3 of 267: *P=0.002* Fisher Exact).

DISCUSSION

In this cohort of patients referred for investigation of RSS/unexplained growth restriction, 37% (29 of 79) of DNA samples showed LOM at one or more imprinted loci. LOM at *H19* was the most common methylation anomaly in such patients. Most other studies have concluded that LOM at *H19* is very unlikely in those patients without features of RSS;[7,8,15,16] however, in this series two patients with LOM at *H19* did not have a diagnosis of RSS clearly suggested on the referral details. They were both from the anonymised recruitment arm and therefore detailed phenotypic information has not been confirmed, but both were referred by clinical geneticists. In one of these patients there was a complex clinical picture and multiple congenital

Table 3 Summary of results in the 10 patients with unusual methylation studies

						Imprinted loci						
Patient no.	*PLAGL1 6q24*	*IGF2R 6q27*	*MEST1 7q32*	*GRB10 7p12*	*PEG10 7p21*	*KCNQ1OT1 11p15*	*IGF2PO 11p15*	*H19 11p15*	*DLK1 14q32*	*PEG3 19q13*	*NESPAS 20q13*
Patients with LOM at H19											
1	0.79	1.35	1.26	0.84	1.27	1.10	**1.65**	**2.01**	1.05	**12.14**	**25.2**
2	0.99	**1.80**	1.21	**2.20**	**1.90**	**1.90**	0.99	**1.60**	0.85	1.10	**1.67**
3	0.98	**0.00**	0.83	1.09	1.23	0.77	**2.75**	**4.12**	1.06	1.09	0.89
4	0.92	**0.00**	0.69	1.10	1.21	0.68	**3.01**	**3.41**	1.20	1.12	0.84
Patients with no LOM at H19											
5	0.95	1.13	0.99	1.06	1.20	**2.28**	0.66	1.08	0.90	1.09	0.97
6	1.03	**0.00**	0.80	0.72	1.07	0.66	0.81	0.74	1.08	0.97	1.04
7	1.09	**0.00**	0.99	0.95	1.02	1.12	1.04	1.12	0.89	1.02	0.74
8	0.92	**0.00**	0.97	0.80	1.19	0.85	0.82	1.01	0.78	1.45	0.94
9	0.80	**0.00**	0.87	0.56	0.68	1.20	0.90	0.82	0.67	0.95	0.76
10	0.89	**0.00**	1.04	0.66	0.94	1.19	0.81	1.03	1.05	0.95	1.21
Control experiments											
SD of mean ratio	0.16	0.23	0.2	0.17	0.22	0.17	0.14	0.18	0.3	0.22	0.22
(−3 SD to +3 SD)	(0.52–1.48)	(0.31–1.69)	(0.4–1.6)	(0.49–1.51)	(0.34–1.66)	(0.49–1.51)	(0.58–1.42)	(0.46–1.54)	(0.1–1.9)	(0.34–1.66)	(0.34–1.66)
n=126		*n*=267									

The average methylation ratio for each patient is shown corrected to two decimal places. Complete gain of methylation is '0'. Hypomethylation, that is, greater peak intensity for the unmethylated than the methylated amplicon, is represented by numbers >1. Atypical ratios for *IGF2R, GRB10, KCNQ1OT1, H19, PEG3* and *NESPAS* were confirmed using at least two bisulphite treatments as well as independent assays: pyrosequencing, MLPA, or a second MS-PCR interrogating different CpG dinucleotides in the same differentially methylated region.

anomalies. It is possible that RSS had not been suspected clinically, because of the severity of comorbid features, or his young age.

We found HIL in 2 of 23 patients with LOM at *H19*, a phenomenon which was not observed in a previous study that tested a smaller number of DMRs.[14] These two patients are remarkable. Patient 1 had a half-sibling with BWS. Siblings with RSS and BWS due to underlying HIL have not been previously described, and this family probably represents a new genetic disorder. Patient 2 was one of discordant monozygous twins, which might be aetiologically relevant: the incidence of monozygotic twinning in BWS is increased[20,21] and some of these show HIL.[22] Monozygous twins discordant for RSS have also been reported ;[23–25] although, to our knowledge this latter group has not been investigated for HIL. We believe the discovery of HIL in patient 2 with growth restriction may represent a new aetiological group, with HIL arising as part of the twinning process.

With the exception of patient 2, who had LOM at *GRB10* and *PEG10* on chromosome 7 as part of a wider HIL, we did not detect epigenetic changes on chromosome 7 to account for the restricted growth in this cohort. These results corroborate those of another study that identified no methylation change at *MEST/PEG1* in 54 RSS patients.[26] Taken with our own results this suggests that isolated imprinting abnormalities of *MEST1/PEG1, GRB10* and *PEG10* on chromosome 7 are not a common cause of growth retardation or RSS.

Surprisingly, this study has also revealed that LOM at *KCNQ1OT1,* previously associated with BWS, can be found in patients with growth restriction (patients 2 and 5). Recently there has been another report of LOM at *KCNQ1OT1* in three children without clinical BWS.[27] In our study, although MLPA did not reveal a copy number change in the 11p15 region, we cannot exclude an atypical paternal duplication, or a maternal deletion; however, it is difficult to explain the phenotype of growth restriction in the context of the epigenetic result in these two patients. In patient 2, the HIL involving multiple loci, including *H19,* may have prevented a net overgrowth; yet, a similar pattern, with hypomethylation at both maternally and paternally methylated loci, was also seen in discordant twins with BWS.[22] The modest changes in methylation at multiple loci in patient 2 may reflect a dilutional effect resulting from circulation sharing with her twin. For patient 5, we did not show HIL, though we cannot exclude hypomethylation of DMRs not analysed in this study.

The finding of a complete gain of methylation at *IGF2R* in 7 of 79 patients is also intriguing. This occurred significantly more commonly in the patients (cases) than in our control group (*P=0.002* using Fisher's Exact test).

Although the proliferative effects of IGF2 are mediated through the IGF1 receptor (IGF1R), the main function of the IGF2 receptor is anti-proliferative, achieved by clearing IGF2 from the circulation.[28] Not surprisingly therefore, *Igf2r* knockout mice are up to 30% larger than wild-type mice.[29–31] The expression of human and mouse *IGF2R* differs: in mice, *Igf2r* imprinted expression is observed in all fetal and adult tissues; in humans, *IGF2R* imprinted expression has not been found in adult tissues, but has been found in fetal tissues and Wilms' tumours, in a proportion of samples tested.[32] There is also a difference in the DMRs between these two species: DMR1, in the promoter of the *Igf2r* gene, occurs only in mice; DMR2 occurs in both species and is located in intron 2, methylated on the maternal allele. In mice the DMR2 acts as a promoter for *Air*, a non-coding RNA, which is antisense to *Igf2r*. Maternal methylation of DMR2 silences maternal *Air*, allowing monoallelic maternal expression of *IGF2R*. There is now some evidence of a human Air homologue.[33] Given this control and expression pattern, we hypothesised that a gain of methylation at DMR2 in humans might increase the expression of *IGF2R* (at a critical

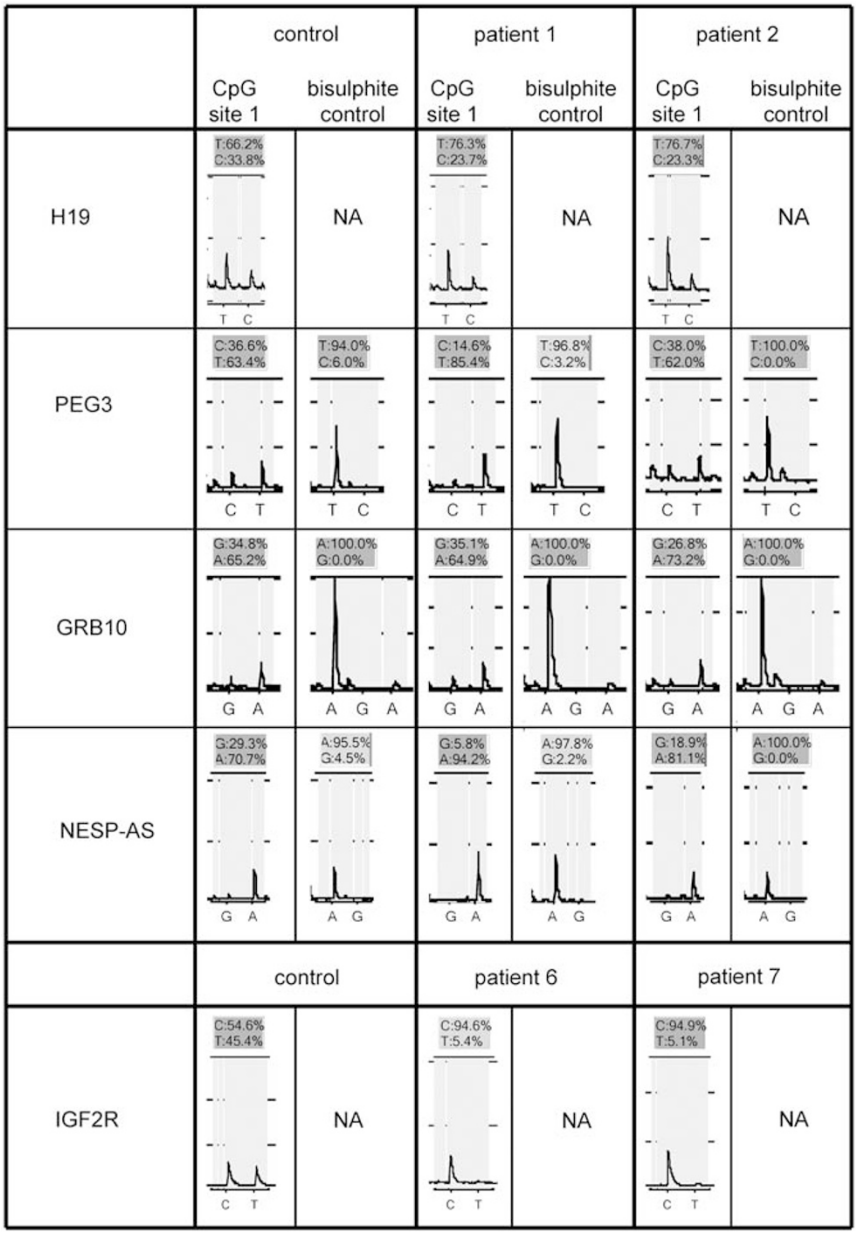

Figure 2 Results of allele quantification of bisulfite-induced C/T polymorphisms by pyrosequencing; examples of pyrograms. Successive columns illustrate a normal control, and patients. Successive rows illustrate the H19, PEG3, GRB10, NESPAS and IGF2R DMRs. Each table cell contains two images: on the left, the first C/T polymorphism of the pyrosequencing reaction, and on the right, a bisulfite conversion control from the same pyrosequencing reaction (a C nucleotide not within a CpG dinucleotide, which would be expected to undergo 100% C>T conversion). NA: Not available; for some assays no suitable cytosine was available to act as a conversion control. Figures above each image indicate the proportions of methylated and unmethylated product present at that position; alleles measured at 5% or less of total are indistinguishable from zero, owing to imprecise quantification at this level.

stage of embryonic development), which could lead to a decrease in circulating IGF2, and so decreased growth. The opposite epigenetic modification, in the form of demethylation of the DMR2 of *Igf2r*, leads to overgrowth of sheep.[34] Furthermore, in humans a partial demethylation of DMR2 was detected in one of 55 patients with idiopathic overgrowth, and was associated with a decreased serum IGF2R level and decreased IGF2 binding, at age 3 years.[35] We are not able to go back to the patients in this study to compare clinical findings in this subgroup with the rest of the cohort, or test IGF2 levels. The available clinical details reveal failure to thrive and post natal growth retardation, with or without prenatal growth retardation (Table 4). The findings at *IGF2R* warrant further investigation and may have highlighted a novel subset of growth retarded individuals.

The main limitation of this study is the minimal phenotypic information available on the 60 individuals within the anonymised cohort. It is recognised that referral cards can be difficult to interpret and this may limit our epigenotype–phenotype correlations; however, almost half of this group originated from Clinical Geneticists who may provide more reliable referral details. Furthermore, this group does reflect routine clinical practice, being drawn from unselected referrals to a service laboratory of patients with growth restriction. Thus, this study mirrors the real difficulty of diagnosing RSS in the clinic: in

Table 4 The phenotypic features of patients with GOM at *IGF2R*

Patient	*Methylation status at H19*	*Birth weight centile*	*Other features*
3	LOM	Not known	Not known.
4	LOM	Not known	Asymmetry, mild developmental delay, ventricular septal defect.
6	Normal	2nd	Growth failure from 3 months. Age 19/12 <0.4th centile for height; OFC 2nd centile. Normal development, mild concern regarding speech.
7	Normal	<0.4th	Feeding difficulties, micrognathia. Delayed dental eruption, vitiligo and anterior chamber dysgenesis. Age 10 years, height remains <0.4th centile.
8	Normal	Not known but referred with IUGR	0.4th centile at time of referral.
9	Normal	50th	Developed unexplained short stature.
10	Normal	<0.4th	Assisted conception; <0.4th centile for weight at term and <0.4th centile at 3.5 years; developmental delay but minimal dysmorphic features.

many instances, although doctors know there is definite intrauterine growth retardation and or short stature, there maybe uncertainty about the presence of other RSS features, some of which evolve with time.

We believe this is the most extensive molecular study of its kind, analysing 11 DMRs in individuals with suspected RSS or growth retardation. We report new cases of HIL in patients with LOM at *H19*. We have ascertained further patients with LOM at *KCNQ1OT1* who do not have clinical BWS. Although we did not identify the loci on chromosome 7 that may account for the growth restriction observed in maternal UPD7, we did identify a potentially important change involving *IGF2R*. We believe this study warrants confirmation prospectively alongside accurate clinical phenotyping.

ADDENDUM

During the review process, Azzi *et al*[36] also published evidence that some patients with RSS have hypomethylation involving multiple imprinted loci. Interestingly, they reported the involvement of different loci, which may indicate that more than one mechanism may be involved.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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