**Targeted Methylation Testing of a Patient Cohort Broadens the Epigenetic and Clinical Description of Imprinting Disorders.**

Rebecca L Poole1,2, Louise E Docherty1,2, Abeer Al Sayegh1,2,3, Almuth Caliebe1,2,4, Claire Turner5, Emma Baple6, Emma Wakeling6, Lucy Harrison1,2,8, Anna Lehmann1,2,9, I Karen Temple1,2\*, Deborah JG Mackay1,2 On behalf of the International Clinical Imprinting Consortium‡.

1 Faculty of Medicine, University of Southampton, UK

2 Wessex Genetics Service, Southampton University Hospitals Trust, Southampton, and Salisbury Hospital NHS Foundation Trust, Salisbury, UK

3 Current address: Genetic and Developmental Medicine Clinic, Sultan Qaboos University Hospital, P.O Box 38, Alkoudh, 123 Sultanate of Oman

4 Current address: Institut für Humangenetik des Universitätsklinikum Schleswig-Holstein, Campus Kiel, Kiel, Germany

5 Royal Devon and Exeter Hospital, Exeter, UK

6 Current address: Centre for Human Genetics, St George’s University of London, London, UK

7 North West Thames Regional Genetics Service, North West London Hospitals NHS Trust, Harrow, UK

8 Comprehensive Local Research Network (Hampshire & Isle of Wight), UK

9 Current address: South West Thames Regional Genetics Service, St George's University of London, UK

\* To whom correspondence should be addressed: Wessex Clinical Genetics Service, Princess Anne Hospital, Coxford Road, Southampton SO16 5YA, UK

‡ Consortium members are listed as an Appendix in the Supplementary Information

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# ABSTRACT

Imprinting disorders are associated with mutations and epimutations affecting imprinted genes, ie those whose expression is restricted by parent of origin. Their diagnosis is challenging for two reasons: firstly, their clinical features, particularly prenatal and postnatal growth disturbance, are heterogeneous and partially overlapping; secondly, their underlying molecular defects include mutation, epimutation, copy number and chromosomal errors, and can be further complicated by somatic mosaicism and multi-locus methylation defects. It is currently unclear to what extent the observed phenotypic heterogeneity reflects the underlying molecular pathophysiology; in particular, the molecular and clinical diversity of multilocus methylation defects remains uncertain. To address these issues we performed comprehensive methylation analysis of imprinted genes in a research cohort of 285 patients with clinical features of imprinting disorders, with or without a positive molecular diagnosis. 20 of 91 patients (22%) with diagnosed epimutations had methylation defects of additional imprinted loci, and the frequency of developmental delay and congenital anomalies was higher among these patients than those with isolated epimutations, indicating that hypomethylation of multiple imprinted loci (HIL) is associated with increased diversity of clinical presentation. Among 194 patients with clinical features of an imprinting disorder but no molecular diagnosis, we found 15 (8%) methylation anomalies, including missed and unexpected molecular diagnoses. These observations broaden the phenotypic and epigenetic definitions of imprinting disorders, and show the importance of comprehensive molecular testing for patient diagnosis and management.

**KEY WORDS**

Imprinting, Imprinting disorders, Silver-Russell-syndrome, Beckwith-Wiedermann-syndrome.

# INTRODUCTION

Congenital imprinting disorders (IDs) result from genetic or epigenetic mutations of imprinted genes, ie. genes whose expression is restricted by parent of origin [Walter and Paulsen, 2003; Robertson, 2005; Horsthemke, 2010]. Genomic imprinting affects groups of genes whose fine regulation is required for normal pre- and post-natal growth and development. Approximately 60 imprinted genes are currently known in humans, though more may remain to be discovered [Nakabayashi *et al*., 2011; Smallwood *et al*., 2011; Choufani *et al*., 2011].

Currently eight ‘classic’ IDs are recognised: the Beckwith-Wiedemann (BWS, OMIM 130650), Silver-Russell (SRS, OMIM 180860), Prader-Willi (PWS, OMIM 176270) Angelman (AS, OMIM 105830), and Wang syndromes (WS, OMIM 608149]), a, transient neonatal diabetes (TND, OMIM 601410), maternal uniparental disomy 14, and pseudohypoparathyroidism type 1b (PHP1b, OMIM 603233) [Choufani *et al*., 2010; Eggermann, 2010; Buiting, 2010; Kelsey, 2010; Ogata *et al*., 2008]. IDs are clinically heterogeneous, most likely reflecting their molecular heterogeneity with a variety of mutational causes: epigenetic (or primary) mutations; genetic (or secondary) mutations in both *cis* and *trans*, including copy number variations, chromosomal rearrangements and uniparental disomy. Additionally, IDs can present mosaically, ie not uniformly present in all somatic tissues. Recently, patients have been described with epimutations at more than one locus, and in many cases these loci are associated with more than one “classic” ID: hypomethylation of multiple (additional) imprinted loci (HIL: also known as multi-locus methylation disorder or MLMD[Azzi *et al*., 2010]) has been described among patients with TND, BWS, SRS, PHP1b, growth restriction, and idiopathic clinical features [Mackay *et al*., 2006; Rossignol *et al*,. 2006; Bliek *et al*., 2009a; Azzi *et al*., 2009b; Begemann *et al*., 2010; Turner *et al*., 2010; Baple *et al*., 2011; Perez-Nanclares *et al*., 2012]. Although the phenotype of the presenting ID is normally recognisable (possibly reflecting ascertainment bias), it may be modified, or additional features present; in extreme cases an individual with HIL may lack the cardinal clinical features of any single ID [Baple *et al*., 2011]. While HIL has been associated with mutations of *ZFP57* and *NLRP2* [Mackay *et al*., 2008; Meyer *et al*., 2009] for the majority of HIL patients the underlying genetic mutation, if any, remains unknown.

Although the shared molecular mechanisms of IDs may suggest shared principles of clinical and molecular diagnosis, in practice the clinical and (epi)genetic heterogeneity of IDs challenge both the sensitivity and cost of molecular diagnosis.

In order to investigate the clinical and epigenetic diversity of imprinting disorders we systematically analysed imprinting status in a large cohort of patients with either a known ID (i.e. with a molecular diagnosis of an ID), or a suspected ID (i.e. referral, but no positive diagnosis, for the closest syndromic presentation of ID).

# MATERIALS AND METHODS

## Patient recruitment via the IDFOW study cohort

Patients were recruited to the study Imprinting Disorders – finding out why (IDFOW) (supplementary material 1) and placed into 2 groups: 1. The molecular group; patients known to have an imprinting aberration in at least one imprinted locus confirmed in an accredited NHS genetics laboratory (molecular group) . 2. The clinical group; including patients seeking or having sought diagnosis for unexplained growth or developmental disorder attending paediatric or genetic clinics in NHS hospitals and patients referred to the Wessex Genetics Laboratory for investigation of an imprinting abnormality (in all cases routine molecular diagnosis has revealed no known cause for the clinical features). Inclusion criteria for the clinical group are unexplained short stature <10th centile or 3 or more of the following features: **Growth disturbance** - overgrowth at birth for gestational age – weight or length>90th centile; small for gestational age weight or length <10th centile; asymmetric growth obvious clinically; unexplained postnatal excessive weight or height (>98th centile); central obesity; **Developmental delay** - learning difficulties; verbal dyspraxia, hypotonia; **Glycaemic control** - unexplained hyper or hypoglycaemia; **Congenital abnormalities** - umbilical developmental defect, macroglossia, micrognathia, genital abnormality, scoliosis, deafness, other congenital abnormality; **Conception** - conception by assisted reproductive technology, monozygous twinning.

Written consent was obtained from patients or guardians as appropriate, including consent to access medical records and to obtain further samples if necessary from the patient and immediate family to support further investigation of molecular findings. Participating physicians completed a concise clinical information questionnaire (<http://www.southampton.ac.uk/geneticimprinting/informationclinicians/imprintingfindingoutwhy.page>? Acessed 8th January 2013). and forwarded DNA for molecular analysis. Selected patients also had a more detailed examination by a clinical geneticist (IKT/AC/AA).

**Molecular studies**

Targeted imprinting testing performed at the following loci: *PLAGL1* (6q24); *IGF2R* (6q25); *GRB10* (7p12); *PEG10* (7q21); *MEST* (7q32); *KCNQ1OT1* (ICR2:11p15), *H19* (ICR1: 11p15), *IGF2* DMR0 (11p15); *MEG3* (14q32); *SNRPN* (15q11); *PEG3* (19q32); *GNASAS*, and *GNAS* exon 1a (20q13), as detailed in Supplementary Material 1. All cases with an identified methylation error consistent with a recognised ID were investigated to exclude UPD using microsatellite markers. In some cases Array comparative Genomic Hybridisation (aCGH) was performed, using the design and analysis described [Docherty *et al*., 2010 and supplementary material 1].

The study was approved by the Southampton and South West Hampshire Research Ethics committee 07/H0502/85.**RESULTS**

## Recruitment

The recruited patients were in two categories (Table I): those with a confirmed molecular diagnosis of an ID (N=91 or 32%; molecular group), and those with clinical features of an ID but negative on routine molecular testing (N=194 or 68%; clinical group). In the clinical group, most had a suspected clinical diagnosis of a specific syndrome, but 25 (13%) were referred without a specific clinical diagnosis but fulfilling three or more clinical criteria for inclusion (“unknown” category).

The major referral in both (molecular and clinical) groups was SRS, with 38% and 66%, followed by BWS with 32% and 12% of the two groups respectively.

**Molecular group**

Epigenetic studies

20 of 91 (22%) patients with a prior molecular diagnosis of an ID had additional imprinting anomalies at loci independent from their primary clinical presentation (Table I), including six of 35 SRS patients (17%), eight of 29 BWS patients (28%), four out of 8 (50%) TND patients and 1/11 (9%) PHP1b patients.

*SRS*: Among 35 SRS referrals were 29 (83%) with ICR1 hypomethylation, five (14%) UPD7mat, and one with mosaic segmental maternal UPD11. Six cases with ICR1 hypomethylation (17%) showed HIL, in line with findings of other groups (Table I; Azzi *et al*., 2009; Begemann *et al*., 2010; Turner *at al*., 2010). Strikingly, 3 SRS-HIL cases showed hypomethylation of ICR2, an epimutation normally associated with BWS. Two individuals had partial hypomethylation of *PEG3* and two of *GNAS*. One case showed complete hypomethylation of the *MEG3* DMR, which has been described in >50% SRS-HIL [Azzi *et al*., 2009], and one had complete hypomethylation of *IGF2R*, a finding described previously in an individual with BWS [Gicquel *et al*., 2004].

*BWS*: The 29 referrals with a molecular diagnosis of BWS comprised 21 (72%) with ICR2 hypomethylation, four (14%) with ICR1 hypermethylation, and four (14%) with mosaic segmental paternal UPD11, a distribution broadly reflecting the known prevalence of different molecular causes of BWS [Choufani *et al*., 2010].

BWS-HIL was found only among patients with ICR2 hypomethylation, in line with other studies [Rossignol *et al*., 2006; Bliek *et al*., 2009a, Azzi *et al*., 2009]. Among 8 BWS-HIL patients, five had hypomethylation of *PLAGL1*, four of *MEST* and four of *GNAS*; but no two patients had an entirely conserved hypomethylation pattern across loci (Figure 1).

*Other IDs*: Of two cases referred with a molecular diagnosis of AS, one was found to have additional imprinting anomalies, affecting ICR2, *PEG3* and the *GNAS* locus, as reported elsewhere [Baple *et al*., 2011]. Of eight cases referred with TND to the study, six had hypomethylation of *PLAGL1*, and four of these had TND-HIL, with three having a mutation in *ZFP57*. Two have been reported previously (patients 2 and 3 [Mackay et al., 2006; Mackay *et al*., 2008]).

Eleven referrals with PHP1b had hypomethylation of the *GNAS* and *GNASAS* DMRs, two were members of monozygotic twin pairs. One was a discordant monozygotic twin; significant hypomethylation of *GNAS* and *GNASAS* DMRs was seen in fibroblast DNA of the affected twin only, but both twins showed modest hypomethylation in blood-derived DNA, suggesting sharing of haematopoietic stem cells. In the other twin pair, DNA from the second twin was not available for study. Interestingly, the affected twin of this second twin pair is our only case of HIL within the PHP1b group.

## Clinical comparison of patients with and without HIL.

SRS: There were no statistically significant differences between the SRS-HIL and SRS groups (Table II), although the frequency of developmental delay was considerably higher (67%) in SRS-HIL compared to 26% in ‘isolated’ SRS. 5/6 SRS-HIL cases had atypical clinical features, compared with 23% of isolated SRS: these included renal and radial anomalies, and one patient with cleft lip and palate (supplementary material 2).

BWS: The clinical presentation of BWS-HIL differed from BWS with isolated hypomethylation at ICR2 (Table II, Supplementary material 3). The most significant finding was the presence of mild to moderate developmental delay in 8/8 of the BWS-HIL group compared to 3/11 of ‘isolated’ BWS (P = 0.004). Additionally, abnormal glycaemic control was seen at an increased frequency amongst the HIL cases, although significance failed to meet the P≤0.05 treshhold after the application of multiple testing correction (Table II). 6/8 BWS- HIL had additional congenital anomalies atypical for BWS (2 cleft palate, 3 congenital heart disease, 1 duplex kidney, 1 cerebral ventriculomegaly, and 2 genital abnormalities: supplementary material 3) in comparison to 4/13 non HIL cases.

Other: No difference in glycaemic control was described between TND-HIL and TND (data not shown). Two TND-HIL patients had birth weights above the 10th centile, remarkable in a syndrome where the mean birth weight centile is 1.35. No additional features were described in the PHP1b-HIL patient compared to those with an isolated epigenetic mutation. By contrast, the AS-HIL case differed significantly from typical AS, having initially been referred for BWS and PWS testing (for a case report see [Baple *et al*., 2011]).

**Clinical group**

Among 194 patients with clinical suspicion of an ID but without molecular diagnosis, DNA methylation anomalies were identified in 15 (8%); none had HIL, 12 of these were referred with clinical features of SRS.

*SRS*: Of 127 individuals with clinical features suggestive of SRS, DNA methylation anomalies were found in 12 (9%), 9 of which have clinical relevance. These findings included one patient with UPD7mat (after a false negative diagnosis from a referring laboratory) one patient with UPD14mat, one patient with UPD15mat (all confirmed by microsatellite analysis – data not shown) and one patient with suspected UPD20mat (as parental samples were not available methylation analysis was performed at imprinted loci in addition to *GNAS* and *GNASAS* on chromosome 20 (*NNAT1* and *L3MBTL1*) complete maternalisation was seen for all loci). A sibling pair had hypomethylation of the *IGF2* DMR0 but not ICR1, resulting from an inversion separating *IGF2* from its enhancers, as reported elsewhere [Grønskov *et al*., 2011].

Three patients had hypomethylation of ICR2 (in the absense of CNVs, tested by arrayCGH, and in-cis mutations) in leukocyte but not buccal DNA (confirmed by pyrosequencing and bisulphite sequencing, Supplementary material 4). This is the commonest molecular cause of BWS, but has not been reported in growth-restricted individuals except as an additional epimutation in SRS-HIL [Azzi *et al*., 2009]. Other findings of unknown significance include individuals with partial hypomethylation of *PEG3, GNAS* exon 1a, or *MEG3* in the absence of CNVs.

Features of these individuals are summarised in Supplementary Material 5, which shows the significant clinical overlap among patients with variable molecular abnormalities.

*Other IDs*: Of 23 individuals referred with clinical features consistent with BWS, two had low-level mosaic hypermethylation of ICR1 and a third had mosaic UPD11pat (both consistent with BWS) that had escaped detection by standard diagnostic methods. No epimutations were observed in the reaminder of the clinical group.

# DISCUSSION

The clinical heterogeneity, diverse molecular aetiology and somatic mosaicism of IDs present challenges for clinical diagnosis and counselling [Choufani et al., 2010; Eggermann 2010; Buiting 2010; Kelsey 2010]. Some are caused by in-*cis* mutation [Bastepe et al., 2005; Linglart et al., 2005; Cerrato et al., 2008; Demars et al., 2010; Kagami et al., 2010], some are associated with in-*trans* mutations impacting control of multiple loci, [Mackay et al., 2008; Meyer et al., 2009] and for others the cause remains unknown. The continued testing that results from lack of a diagnosis indicates a significant unmet health need for the individuals concerned – especially since individuals without a positive molecular diagnosis remain without the important management interventions consequent upon that diagnosis. In 2007 we began accrual of a cohort with clinical and/or molecular features of IDs, to explore their epigenetic and phenotypic diversity in order to improve patient diagnosis and management.

## Increased diversity of clinical phenotype in patients with HIL defects:

Until recently it was thought that imprinting mutations in various human syndromes were events affecting only a given locus involved in a specific syndrome. Our group and others have reported patients with SRS, BWS and TNDM with multilocus imprinting defects [Azzi *et al*., 2009; Azzi *et al*., 2010; Mackay *et al*., 2006; Mackay *et al*., 2008; Rossignol *et al*,. 2006; Bliek *et al*., 2009a; Begemann *et al*., 2010; Turner *et al*., 2010; Baple *et al*., 2011; Perez-Nanclares *et al*., 2012]. Previous studies of HIL patients did not show phenotype:epigenotype correlations[Azzi *et al*., 2009]. In our cohort, the most significant finding was that all BWS-HIL patients are reported to have moderate developmental delay, mainly affecting speech. Detailed review of medical records showed congenital anomalies not classically associated with BWS, indicating potentially a more complex phenotype. Similarly, SRS-HIL patients were reported to have developmental delay and other congenital anomalies. Also recruited to this study is a patient with HIL in the molecular background of AS [Baple *et al*., 2011]. At this point it is important to re-emphasise the distinction between a clinical and molecular diagnosis: this patient was referred for diagnostic genetic testing for both PWS and BWS, as suggested by their clinical presentation. Diagnostic testing revealed methylation anomalies consistent with a diagnosis of AS (the opposite imprinting defect to PWS) and ICR2 BWS. Recruitment to this study identified the involvement of further additional loci. This case illustrates how HIL can blur the clinical boundaries of these disorders and further supports the need for wider genetic investigations of patients with features of IDs.

**Molecular diversity of HIL patients**

Our observed rates of HIL are broadly in line with those seen in other studies, with HIL most common among TND patients, followed by BWS and SRS, with PHP1b having the lowest occurrence rate; this may reflect a higher rate of in-cis mutations in the latter group of patients (it is not possible to comment on the other IDs due to low recruitment levels) [Chaofani *et al*., 2010; Rossignol *et al*., 2006; Bliek *et al*., 2009a; Azzi *et al*., 2009; Begemann *et al*., 2010; Turner *et al*., 2010; Perez-Nanclares *et al*., 2012].

In our cases of TND-HIL and BWS-HIL hypomethylation is restricted to maternally-methylated loci; however in SRS-HIL and AS-HIL both maternally- and paternally- methylated loci are affected. Although in many cases the epimutations appear restricted to maternally methylated loci, this may reflect the relatively larger number of maternally methylated loci, and it remains likely that maternally and paternally imprinted loci are equally affected.

If genetic in origin, it is predicted that HIL disorders result from mutations in trans-acting factors, for example: maternal-effect mutations of *NLRP7* and *C6ORF221* cause biparental complete hydatidiform mole, while *NLRP2* is mutated in atypical BWS and *ZFP57* in TND-HIL [Mackay *et al*., 2008; meyer *et al*., 2009; Murdoch *et al*., 2006; Parry *et al*., 2011]. Although among the HIL patients within this study, epimutation groups are emerging (such as the SRS-HIL cases with ICR1 and ICR2 involvment, and the BWS-HIL cases with involvement of TND, *IGF2R* and *MEST* ICRs as well as ICR2), the majority of the HIL cases reported here do not fall into obvious groupings. Whilst groups may become clearer as more patients are described, it remains possible that few ‘common’ causes exist and that many factors including trans-acting genes remain to be discovered.

Non-genetic causes of epimutation may include environmental insult, reproductive error and stochastic error. There is clear evidence of an association between assisted reproductive technology (ART) and IDs, with increased frequencies of IDs among individuals concieved by ART [Dupont and Sifer 2012; Talaulikar and Arulkumaran 2012]. However, evidence of a causal relationship, i.e. that the ID is a direct result of the ART and not that epimutations result in reduced fertility requiring the use of ART, remains unclear [Voet *et al*., 2011; Rancourt *et al*., 2012; Mertzanidou *et al*., 2013; Zheng *et al*., 2013]. More recent evidence suggests a more specific association between ART and HIL disorders, with 5/6 BWS and SRS patients [Hiura *et al*., 2012] and 3 out of 4 TND patients [Docherty *et al.*, *in press*] conceived by ART exhibiting aberrant methylation at multiple loci. As ART was one the inclusion criteria it is difficult to comment about its prevalence among this cohort; however, it is notable that none of our HIL patients were conceived via ART.

A number of our cases were discordant monozygotic (MZ) twins. MZ twinning was noted in 3/8 BWS-HIL cases, compared with 1/13 cases with isolated ICR2 hypomethylation, and in 1/6 SRS-HIL cases compared with 0/23 isolated ICR1 hypomethylation. Moreover, two of 11 PHP1b cases were discordant MZ twins; the second twin pair interestingly is our only case of PHP1b-HIL. The frequency of monozygotic twinning is ~0.1 among BWS cases with ICR2 hypomethylation, compared with ~0.01 in the normal population, with the vast majority being discordant for disease [Weksberg *et al*., 2002; Bliek *et al*., 2009b]. It has been suggested that stochastic methylation errors affecting a small cell population within the early zygote, cause subsequent unequal development and predisposition to splitting of the zygote to form discordant twins [Bestor *et al*., 2003]. It remains possible that twinning is not always a primary reproductive error, but may have an underlying epigenetic cause, or even a genetic cause leading to mosaic epimutation and subsequent twinning. Further (epi)genetic studies of twins will help resolve this question.

Further studies of HIL patients is required to clarify whether their atypical presentation represents (a) an ascertainment bias reflecting detailed study of specific patients, (b) the effect on primary presentation of additional known epimutations, or (c) epigenetic dysregulation of genes either not currently known to be imprinted, or not imprinted but controlled by the same epigenetic factors as imprinted genes.

**Epimutations in patients with no prior molecular diagnosis**

The IDFOW study was designed not to restrict recruitment to patients with a molecular diagnosis of an ID, or a classic clinical phenotype, but to capture referrals that did not necessarily align with a specific ID. By far the commonest referral category – threefold higher than any other – was clinical diagnosis of SRS without positive molecular diagnosis. This reflects the clinical concern for children with growth restriction, failure to thrive and severe feeding difficulties. The lower representation of other IDs reflected several factors such as a clear clinical definition as in the case of AS and rarity of disease such as PHP and TND.

Among patients referred with clinical features of SRS were one patient each with UPD14mat, UPD15mat (PWS), and UPD7mat (SRS); this illustrates the clinical overlap between these conditions in very young children, which can in some cases obscure diagnosis. In addition we identified one patient with apparent UPD20mat. Few patients have been reported with UPD20mat, and although most are growth restricted, no clear clinical definition is apparent at present [Velissariou *et al*., 2002; Chudoba *et al*., 1999; Powis *et al*., 2009].

Additionally, we describe six patients with epimutations of unknown significance. Three showed hypomethylation of ICR2, which although normally associated with BWS, has been described in phenotypically normal individuals conceived by ART [Gomes *et al*., 2009]; notably, the epimutations were apparently mosaic, in that they were absent from buccal DNA. We encountered one patient with isolated hypomethylation of the *PEG3* DMR, one with isolated partial hypomethylation at GNAS and a final patient with partial hypomethylation at DLK. Array-CGH and Sanger sequencing were performed where possible, but detected no sequence variants that might account for the epimutations, or might have given rise to artefactual results. Further work is required to determine the cause and significance of these findings.

It is interesting to note that all of the patients with a new diagnosis (excluding mis-diagnoses due to low level mosaicism) or novel epimutation were initially referred with growth restriction. This likely reflects the difficulty in the clinical diagnosis of SRS and that SRS is the most commonly requested ID test when growth restriction is observed. Perhaps more importantly these findings highlight the need to consider all imprinting disorders when performing molecular diagnoses on such patients and in doing so may help shed more light on the significance on the novel epimutations described here

In summary, we have explored the epigenetic and phenotypic features of 285 individuals in the IDFOW cohort. The prevalence of HIL described here is broadly in line with other studies, but HIL patients appear to have greater range and/or severity of clinical features than those with ‘classical’ IDs. We suggest that comprehensive clinical characterisation in IDs will improve diagnosis, counselling and clinical management of affected individuals, especially those with clinical features of BWS and SRS, as they may have additional health problems including developmental delay and congenital anomalies. Moreover, further genetic and epigenetic analysis of these patients is likely to reveal novel causes of congenital imprinting disorders. Importantly, this study highlights the need for the implementation of a broader diagnostic testing regime for patients with clinical features of imprinting disorders, and in particular for those with restricted growth.

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Zheng HY, Tang Y, Niu J, Li P, Ye DS, Chen X, Shi XY, Li L, Chen SL. 2013. Aberrant DNA methylation of imprinted loci in human spontaneous abortions after assisted reproduction techniques and natural conception. Hum Reprod 28: 265-273.Table I: Cohort summary

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Referred Cases** | **Molecular group** | | | **Clinical group** | |
|  | **Isolated** | **HIL** | **Total** | **isolated** | **Total** |
| **SRS** | 23 | 6 | 352 | 127 | 127 |
| **BWS** | 13 | 8 | 293 | 38 | 23 |
| **PHP** | 9 | 1 | 114 | 0 | 3 |
| **TND** | 2 | 4 | 85 | 0 | 1 |
| **AS** | 5 | 1 | 66 | 0 | 6 |
| **PWS** | 0 | 0 | 1 | 0 | 0 |
| **WS** | 0 | 0 | 1 | 0 | 1 |
| **UPD14mat** | 0 | 0 | 0 | 0 | 8 |
| **Unknown1** | 0 | 0 | 0 | 0 | 25 |
| **Total** | **52** | **20** | **91** | **14** | **194** |

The Molecular group represent patients recruited with a previous molecular diagnosis; the Clinical group represents patients recruited with a clinical diagnosis but negative by routine diagnostic molecular testing. Isolated: methylation abnormality detected at one imprinted locus

HIL: hypomethylation of multiple imprinted loci. 1. unknown: fulfilling clinical criteria of the study, ie three or more features of an ID, but no primary clinical diagnosis. 2: includes 5 UPD7mat and one mosaic UPD11mat; 3: includes 4 mosaic UPD11pat and 4 ICR1 hypermethylation; 4: includes 1 UPD20pat; 5: includes one UPD6pat and one segmental duplication of paternal origin; 6: includes one UPD15pat; 7: includes UPD7mat (SRS), UPD14mat, UPD15mat (PWS), UPD20mat, 2 isolated IGF2 DMR0 hypomehtylation cases (SRS), 3 cases of mosaic hypomethylation of ICR2 (BWS), 3 cases with isolated hypomethylation of PEG3, GNAS and MEG3 all of unknown significance; 8: includes mosaic UPD11pat and two with mosaic hypomethylation of ICR1, both missed detection by routine diagnostic analysis.

Table II: Clinical Features of BWS and SRS patients with isolated and HIL defects:

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | ***BWS*** | | | ***SRS*** | | |
| **Clinical Feature** | **Isolated (N=11)1** | **HIL**  **(N=8)2** | ***P*-value3** | **Isolated**  **(N=23) 1** | **HIL**  **(N=6)** | ***P*-value** |
| Assisted reproduction | 2 | 0 | 0.49 | 3 | 0 | 1.0 |
| Monozygous Twins | 1 | 3 | 0.26 | 0 | 1 | 0.21 |
| LGA (>98th centile)4 | 7 | 3 | 0.37 | - | - |  |
| SGA (<2nd centile)4 | - | - |  | 20 | 5 | 1.0 |
| Short Stature (<10th centile) | - | - |  | 14 | 4 | 1.0 |
| Macroglossia | 8 | 8 | 0.23 | - | - | - |
| Umbilical Hernia | 2 | 3 | 0.60 | - | - | - |
| Asymmetry | 4 | 7 | 0.06 | 10 | 4 | 0.39 |
| Feeding Difficulties | 1 | 3 | 0.25 | 17 | 4 | 1.0 |
| Developmental Delay | 3 | 8 | **0.004\*\*** | 6 | 4 | 0.14 |
| Abnormal Glycaemic control | 2 | 6 | **0.02\*** | 2 | 0 | 1.0 |
| Additional congenital abnormalities (cases affected)5  Total | 4 | 6 | 0.17 | 17 | 5 | 1.0 |

1 Isolated: methylation abnormality at ICR2 (BWS) 11/13 isolated cases, 2 had insufficient clinical data for inclusion here or ICR1 (SRS).

2 HIL: hypomethylation of multiple imprinted loci

3 P values calculated using χ-squared (Fisher test) and t-test.

4 twins excluded. Centile calculations after www.healthforallchildren.co.uk/pro.epl

5 Congenital anomalies affecting HIL cases are detailed in Supplementary Materials 3 and 4

\* Statistically significant (P≤ 0.05) but not after multiple testing correction (Holm-Bonferoni method).

\*\*Statistically significant (P≤ 0.05) after multiple testing correction (Holm-Bonferoni method).

**FIGURE and TABLE LEGENDS**

Figure 1: Results of allele quantification of bisulfite-induced C/T polymorphisms by methylation-specific PCR and pyrosequencing.

Successive columns contain representative data images of assays for the imprinting control regions of ICR2, *PLAGL1*, *IGF2R*, *MEST* and *NESP-AS*. In each column, the first row contains data from a control sample; subsequent rows contain data from the positive cases. For methylation-specific PCR (ICR2, *IGF2R*, *MEST*), red and blue bars mark the positions of “methylated” and “unmethylated” peaks, 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.

In the case of pyrograms, the relative proportions of the two pyrogram peaks 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.

Supplementary material 4: Molecular analysis of ICR2 in patients with clinical features of SRS.

Panel A: schematic representation of KCNQ1 and the imprinting control region ICR2 in the KCNQ1OT1 promoter. Top panel: Location of KCNQ1OT1 (blue line) and maternally-methylated ICR2 (red lollipops) within KCNQ1 (red dashed line with exons indicated as vertical bars). Scale (in Mb) is according to HG18. Second panel: ICR 2 region showing location of amplicons for molecular analysis. The transcription origin of KCNQ1OT1 is represented by a blue arrow, ICR2 by a red bar. Locations of amplicons for genomic sequencing, MS-PCR, bisulphite sequencing and pyrosequencing are indicated by black bars.

Panel B: Pyrograms of ICR2 amplicon 4 in patients and control. Each panel represents a representative pyrogram of the first bisulphite-induced C/T polymorphism in pyrosequencing reaction D. The percentage cytosine (ie percentage methylation) is marked over each pyrogram.

Panel C: Pyrosequencing analysis of ICR2 in the three patients and a normal control. For each patient the methylation index is represented as the percentage cytosine at the first bisulphite-induced polymorphism in the pyrosequence, as an average of duplicate tests. The control value is the mean and standard deviation of duplicate determinations of four normal controls at the same position.

Panel D: Summary of methylation index as determined by methylation-specific MLPA. For each patient the methylation index is stated for each of the four probes within ICR2, and the mean and standard deviation of the five probes within ICR1. In a normal individual the methylation indices at both ICR2 and ICR1 would be expected to approximate to 1.0, with abnormal calls being made outside the range 0.75-1.3.

Panel E: Bisulphite sequence analysis of ICR2 in the three patients, a normal control, and a control with BWS caused by ICR2 hypomethylation. Filled and empty circles represent methylated and unmethylated cytosines, respectively, within CG dinucleotides in the amplicon. Numbers to the right of each sequence represent the number of clones, out of a total of 20, in which that sequence was present.

