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Clinical and molecular genetic features of Beckwith–Wiedemann syndrome associated with assisted reproductive technologies

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BACKGROUND: Beckwith–Wiedemann syndrome (BWS) is a model imprinting disorder resulting from mutations or epigenetic events affecting imprinted genes at 11p15.5. Most BWS cases are sporadic and result from imprinting errors (epimutations) involving either of the two 11p15.5 imprinting control regions (IC1 and IC2). Previously, we and other reported an association between sporadic BWS and assisted reproductive technologies (ARTs).

METHODS: In this study, we compared the clinical phenotype and molecular features of ART (IVF and ICSI) and non-ART children with sporadic BWS. A total of 25 patients with post-ART BWS were ascertained (12 after IVF and 13 after ICSI).

RESULTS: Molecular genetic analysis revealed an IC2 epimutations (KvDMR1 loss of methylation) in 24 of the 25 children tested. Comparison of clinical features of children with post-ART BWS to those with non-ART BWS and IC2 defects revealed a lower frequency of exomphalos (43 versus 69%, $P = 0.029$) and a higher risk of neoplasia (two cases, $P = 0.0014$). As loss of methylation at imprinting control regions other than 11p15.5 might modify the phenotype of BWS patients with IC2 epimutations, we investigated differentially methylated regions (DMRs) at 6q24, 7q32 and 15q13 in post-ART and non-ART BWS IC2 cases ($n = 55$). Loss of maternal allele methylation at these DMRs occurred in 37.5% of ART and 6.4% of non-ART BWS IC2 defect cases. Thus, more generalized DMR hypomethylation is more frequent, but not exclusive to post-ART BWS.

CONCLUSIONS: These findings provide further evidence that ART may be associated with disturbed normal genomic imprinting in a subset of children.

Key words: Beckwith–Wiedemann syndrome / imprinting disorder / assisted reproductive technologies / epimutations / loss of methylation

Introduction

Beckwith–Wiedemann syndrome (BWS) is a congenital overgrowth disorder resulting from altered expression or function of genes within the 11p15.5 imprinted gene cluster. In particular, reduced expression (or less frequently inactivation) of the maternally expressed growth suppressor *CDKN1C* and/or increased expression of the paternally expressed growth promoter *IGF2* appear to have a major role in the pathogenesis of BWS. Multiple genetic and epigenetic mechanisms including paternal uniparental disomy of chromosome 11p15 may lead to alterations in *CDKN1C* and *IGF2* function resulting in BWS (see Cooper *et al.*, 2005 and references within).

However, the most common mechanism observed in up to 50% of patients is loss of maternal allele methylation (LOM) at a differentially methylated region (KvDMR1) between *CDKN1C* and *IGF2*. KvDMR1 marks an imprinting control centre [imprinting centre 2 (IC2)] and KvDMR1 LOM is associated with loss of maternal allele *CDKN1C* expression, biallelic expression of the untranslated RNA *KCNQ1OT* (usually only expressed from the paternal allele) and, in some cases, biallelic expression (loss of imprinting) of *IGF2* (Lee *et al*., 1999; Smilinich *et al*., 1999; Diaz-Meyer *et al*., 2003). Rarely KvDMR1 LOM may result from a germline maternal allele deletion, but in most KvDMR1 LOM results from an IC2 epimutation (Niemitz *et al*., 2004).

Although IC2 epimutations represent the most common cause of BWS, little information is available regarding the aetiology of IC2 epimutations. However, we and others have reported an association between BWS and assisted reproductive technologies (ARTs) such as IVF and ICSI (DeBaun *et al*., 2003; Gicquel *et al*., 2003; Maher *et al*., 2003; Halliday *et al*., 2004). To date, most post-ART BWS children have been found to have KvDMR1 LOM but detailed comparison of the clinical and molecular features of post-ART and non-ART BWS children with IC2 defects has not been undertaken.

Materials and Methods

Patients

ART group

Twenty-five BWS children born after IVF or ICSI were referred to the West Midlands Regional Genetics Service/University of Birmingham for molecular testing and/or research studies.

Non-ART group

Eighty-seven BWS children without a history of ART with KvDMR1 LOM tested at the same laboratory were identified.

The clinical and molecular features of the ART group were compared with the non-ART group. Clinical information was collected by a standard questionnaire, inspection of hospital notes or direct examination.

Molecular analysis

DNA was extracted from peripheral blood lymphocytes by standard procedures. After the exclusion of paternal isodisomy of chromosome 11p15, KvDMR1 methylation status was performed as previously described with PCR amplification of bisulphite modified DNA and digestion with restriction enzyme *Bst*U1 yielding different sized fragments which is separated using ABI377 or 3730 (Cooper *et al*., 2005). The methylation index is then calculated as the ratio of methylated to unmethylated DNA. In addition, a cohort of 55 BWS IC2 defect patients (including eight post-ART cases), in whom sufficient DNA was available, was analysed for methylation status at up to three additional DMRs at the Transient Neonatal Diabetes Mellitus (TNDM) locus at 6q24 (*ZAC*), 7q32 (*PEG1*) and the Angelman/Prader-Willi locus at 15q13 (*SNRPN*). The methylation status of the *DLK1*-IG DMR at 14q32 was analysed in patients found to have multi-DMR LOM. Methylation at these DMRs was assessed by methylation-specific PCR (*ZAC* and *DLK1*), direct sequencing of bisulphite modified DNA (*PEG1*) or pyrosequencing of bisulphite modified DNA (*SNRPN*). The methylation status of these four DMRs were also analysed in a group of 20 normal controls.

Bisulphite modification

Genomic DNA (2 μg) derived from peripheral blood lymphocytes was bisulphite modified using the EZ DNA Methylation Gold kit (Zymo Research).

Methylation analysis

6q24 (ZAC) and 14q32 (DLK1) methylation-specific PCR

Methylation status of the TNDM CpG island at 6q24 (*ZAC*) and *DLK1* IG-DMR at 14q32 were analysed using methylation-specific PCR (MS-PCR) as previously described (Mackay *et al*., 2005; Temple *et al*., 2007). The methylated:unmethylated area ratio was calculated for 20 normal controls to establish a normal range to compare the test data. Hypomethylation is defined as a ratio of more than two standard deviations below the mean value.

7q32 (PEG1) bisulphite sequencing

Primers were designed for the analysis of *PEG1* DMR at 7q32 using the Methyl Primer Express software by Applied Biosystems (sense primer 5′-AGTTGGGGTTGTTTTTGG-3′ and 3′ anti-sense primer 5′-TACCAAAATCTAAAAATCCCAATT-3′). This amplified a 264 bp fragment which contains 15 CpGs. PCR was performed with Hot Star *Taq* and buffer (Qiagen) with final concentrations of 0.2 mM dNTP, 2 mM $MgCl_2$ and 0.2 μM primers with the following cycling conditions: $95°15' \rightarrow [95°20''/56°10''/72°10'']_{35} \rightarrow 72°5'$.

15q13 (SNRPN) pyrosequencing

The methylation status at the Prader-Willi/Angelman locus at 15q13 (*SNRPN*) was performed by pyrosequencing using the commercially available PyroMark kit by Biotage according to the manufacturer's protocol.

Statistical analysis

Fisher exact testing, Wilcoxon–Rank sum, *t*-testing and Kaplan–Meier analysis were used as appropriate. Statistical significance was taken at the 5% level.

Results

Patient demographics

In the post-ART group, there were 25 affected children from 23 pregnancies with 10 twins (two affected twin pairs and six twins with no clinical evidence of BWS in their co-twins). In the non-ART group, there were 87 affected children including two twin and one triplet pregnancies (all co-twins and co-triplets were clinically unaffected). There were a total of 14 male and 11 female patients in the post-ART group ($n = 25$, mean age 3.4 years) and 42 males and 45 females in the non-ART group ($n = 87$, mean age 6 years). In the post-ART group, the mean maternal age was 36.7 years and the mean paternal age was 41.8 years. Except for one patient, all had a molecular genetic diagnosis of BWS and two or more clinical features. The one patient without a molecular abnormality in the post-ART group had features of macroglossia, macrosomia, umbilical hernia, earlobe creases and mild speech and language delay.

Clinical features of post-ART and non-ART children with BWS^{ICD2}

The 25 post-ART cases were conceived by IVF ($n = 12$) or ICSI ($n = 13$). Molecular genetic analysis revealed that 24 of the 25 post-ART children had LOM at KvDMR1 (no molecular cause was found in one post-ART child conceived by IVF).

In view of the known genotype–phenotype correlations of BWS (see Cooper *et al*., 2005 and references within), we compared the phenotypes of the 24 post-ART BWS children with KvDMR1 LOM to those of the 87 non-ART BWS children with KvDMR1 LOM. The mean methylation index for KvDMR1 in the post-ART and non-ART group were 4.6% (range 0–18) and 7.6% (range 0–13), respectively, with no statistically significant difference ($P = 0.6$). A methylation index of <20% is used as a cut-off point for diagnosis of KvDMR1 LOM. This value is the operational diagnostic threshold used in the diagnostic laboratory at the West Midlands Regional Genetics Service following robust validation comparing normal controls and known positive controls. The frequencies of neonatal hypoglycaemia (ART 44% versus non-ART 50%), macroglossia (90 and 87%), macrosomia (in singleton births) (70 and 79%), ear creases (56 and 65%) and hemihypertrophy (13 and 16%, respectively) were similar. However, facial naevus flammeus was more common in the ART patients (90 versus 46%, $P = 0.0004$) and exomphalos was less common (43 versus 69%, $P = 0.029$).

None of the non-ART BWS^{ICD2} children (mean age 6.0 years) developed a neoplastic lesion, but two of the post-ART children (mean age 3.4 years) had developed an embryonal tumour. One post-IVF child developed a hepatoblastoma at 8 months and one post-ICSI child a rhabdomyosarcoma at age 9 months. Kaplan–Meier analysis of tumour risk in the ART and non-ART demonstrated a significantly increased risk of tumours in ART cases (log rank $\chi^2 = 10.18$, $P = 0.0014$).

In the cohort of 13 twins/triplets, the overall incidence of exomphalos was 80% with 50% (five of 10) in the post-ART group and 100% (three of three) in the non-ART group but this did not reach statistical significance. Only one twin in the post-ART group developed a tumour (a rhabdomysarcoma mentioned in the paragraph above). The co-twin was not clinically affected with BWS.

Methylation profiling of BWS^{ICD2} children at *ZAC, PEG1, SNRPN and DLK1*

Six cases tested had loss of methylation at *ZAC* (two of 55 tested), *PEG1* (four of 55 tested) or *SNRPN* (one of 55 tested) DMRs (Figs 1–3). In the ART group, three children had additional LOM. One ICSI child demonstrated LOM at both *PEG1* and *SNRPN*. Another ICSI child and one IVF child had single locus LOM at *ZAC* and *PEG1*, respectively. In the non-ART group, three children had single locus LOM with two children at *PEG1* and one child at *ZAC*. In two of the three non-ART cases with LOM at other imprinted loci, there were no reports of fertility problems in the parents or the use of ovarian stimulation but we do not have information regarding the use of ovarian stimulation in the third couple apart from the child was conceived naturally. We went on to test the methylation status of the *DLK1* IG-DMR in these six cases with hypomethylation and found normal methylation levels. No major phenotypic differences

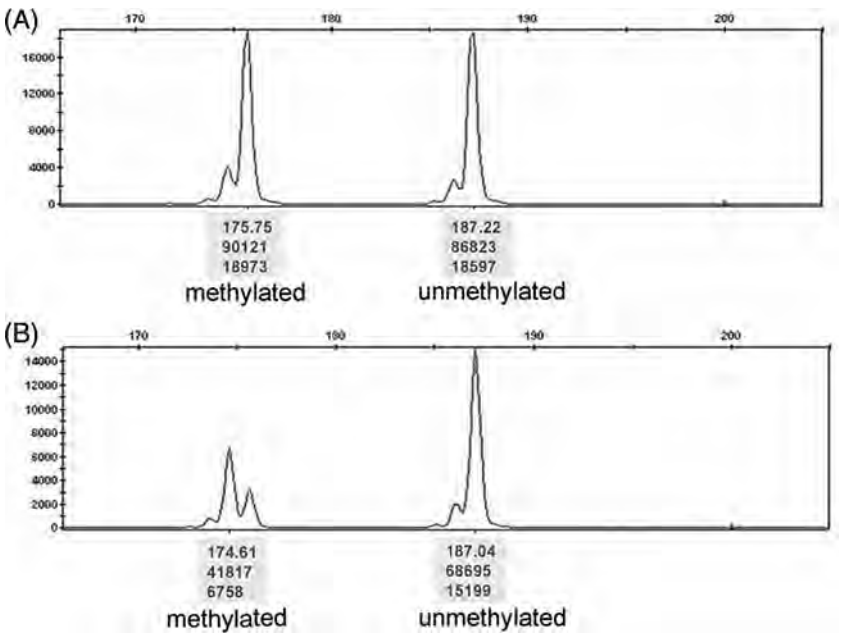

Figure 1 Loss of methylation at *ZAC* DMR in a patient with Beckwith–Wiedemann syndrome.
Electropherogram of amplification products of MS-PCR. The X-axis represents the calculated product size (in bp and also represented as the top number in the box). The Y-axis represents the peak height (bottom number in the box). The methylated to unmethylated ratio was calculated as the area under the curve (middle number in the box) of methylated versus unmethylated amplified products. (**A**) Normal Control (ratio 1.04), (**B**) patient with LOM at *ZAC* (ratio 0.61).

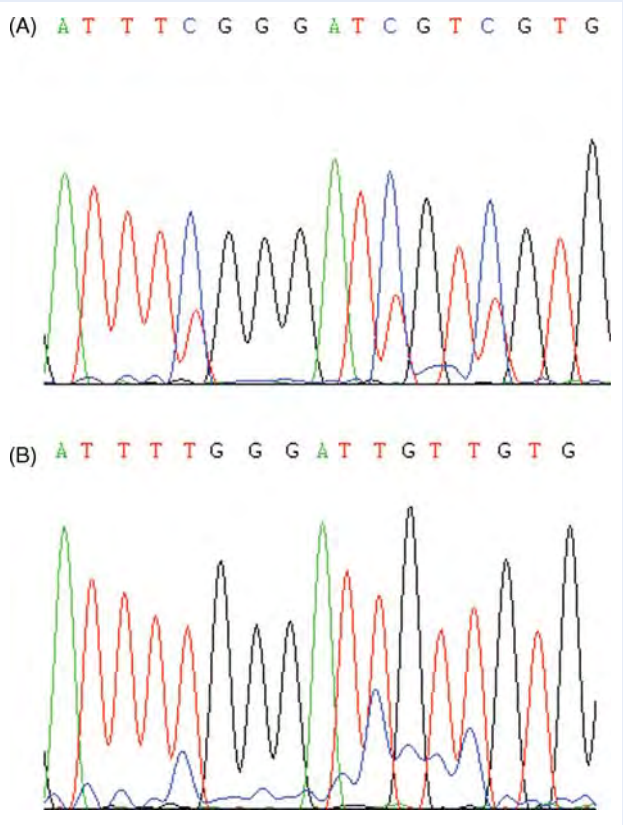

Figure 2 Loss of methylation at *PEG1* DMR in a patient with Beckwith–Wiedemann syndrome.
Bisulphite genomic sequencing trace at the *PEG1* DMR. (**A**) Normal control. DNA sequencing shows both a cytosine and a thymine peak at CpG sites 11, 12 and 13, representing methylated and unmethylated alleles, respectively, with taller cytosine peaks. (**B**) Patient with LOM at *PEG1*. DNA sequencing shows reversal of the expected peaks with taller thymine peaks at CpG sites 11, 12 and 13, indicating a loss of methylation.

were apparent between children with LOM at additional loci and those without this epigenotype (Table I). DNA from the two children with tumours was not available for analysis. The frequency of additional LOM was significantly higher in the post-ART group than in the non-ART BWS^{ICD2} patients (37.5 versus 6.4%, $P = 0.034$). As expected, methylation analysis of the group of normal controls showed no methylation abnormalities at the four loci analysed.

Discussion

Previously, we reported that six of the 149 UK children with BWS had a history of ART (4% of BWS cases versus 1.2% of the general population, $P = 0.009$) (Maher *et al*., 2003). Similar findings were also reported from USA and France (DeBaun *et al*., 2003; Gicquel *et al*., 2003). A case–control study in an Australian population estimated the risk of BWS after ART is approximately nine times greater than that for natural conceptions (Halliday *et al*., 2004). In addition, two studies suggested a link between Angelman syndrome and ICSI (Cox *et al*., 2002; Orstavik *et al*., 2003). We identified KvDMR1 LOM in 24 of the 25 post-ART BWS patients. Previously, KvDMR1 LOM was described in 11 of the 12 post-ART BWS described by DeBaun *et al*. (2003) and Gicquel *et al*. (2003). Thus, a much higher than expected proportion of post-ART BWS patients have KvDMR1 LOM (in unselected up to 50% would be expected) (Cooper *et al*., 2005). This observation provides further evidence of a causal link between ART and IC2 epimutations. Nevertheless, the precise cause of this association is unclear. Both IVF and ICSI are often undertaken for unexplained infertility and require ovarian stimulation, oocyte collection and *in vitro* culture before the embryos are implanted in the womb. Although ICSI also requires an additional step (direct injection of sperm into the ovum), both IVF and ICSI appear to be associated with an increased relative risk (although the absolute risk is small) of imprinting disorders. Animal studies suggest that *in vitro* embryo culture may be associated with epigenetic alterations and, in particular, the large offspring syndrome in sheep and cattle undergoing ART has phenotypic similarities to BWS and, in some cases, is associated with loss of maternal allele methylation at an *IGF2R* DMR (Reik *et al*., 1993; Dean *et al*., 1998; Khosla *et al*., 2001; Young *et al*., 2001). However, it has also been suggested that infertility and ovarian stimulation may predispose to epigenetic errors (Ludwig *et al*., 2005).

Previously, we and others have reported significant differences between Wilms tumour risk and the frequency of exomphalos in children with different molecular subtypes of BWS (Lam *et al*., 1999; Engel *et al*., 2000; Bliek *et al*., 2001; Gaston *et al*., 2001; Weksberg *et al*., 2001; DeBaun *et al*., 2002; Cooper *et al*., 2005; Sparago *et al*., 2007). Thus, Wilms tumour has not been reported in those with IC2 defects or *CDKN1C* mutations but UPD and IC1 defects are associated with a significant risk of Wilms tumour. In contrast, exomphalos is rare in BWS patients with UPD or IC1 defects but is common in those with IC2 defects and *CDKN1C* mutations. Previously, Chang *et al*. (2005) reported no phenotypic differences between post-ART and naturally conceived BWS patients. However, as almost all post-ART BWS children have IC2 defects, we compared these children to non-ART BWS cases with IC2 defects (and not an unselected group of non-ART BWS). We found that post-ART cases had a significantly lower risk of exomphalos and a higher risk of non-Wilms tumour neoplasia. The increased risk of neoplasia, although statistically significant, is based on only two cases and must be considered a preliminary finding. Hence we hope that this finding will prompt other groups to examine their data to better define the relationship between ART and multilocus hypomethylation in BWS children with non-Wilms tumour neoplasia. We note that a childhood tumour was present in two of the 19 post-ART BWS children reported by Chang *et al*. (2005). Two of the 11 children with loss of methylation at multiple loci reported by Rossignol *et al*. (2006) had developed a tumour (a rhabdomyosarcoma and a hepatoblastoma) but neither of these was conceived by ART. The reasons for the phenotypic differences between post-ART and non-ART BWS IC2 defects cases are uncertain. Although it might be suggested that less severe KvDMR1 hypomethylation in post-ART cases might lead to a milder phenotype with a lower incidence of exomphalos, comparison of blood KvDMR1 methylation indices in the two groups did not show any significant difference. However, methylation patterns may differ in different tissues. Nevertheless, such an explanation would not seem to account for the apparent higher risk of neoplasia in post-ART cases. Recently, Rossignol *et al*. (2006) reported that

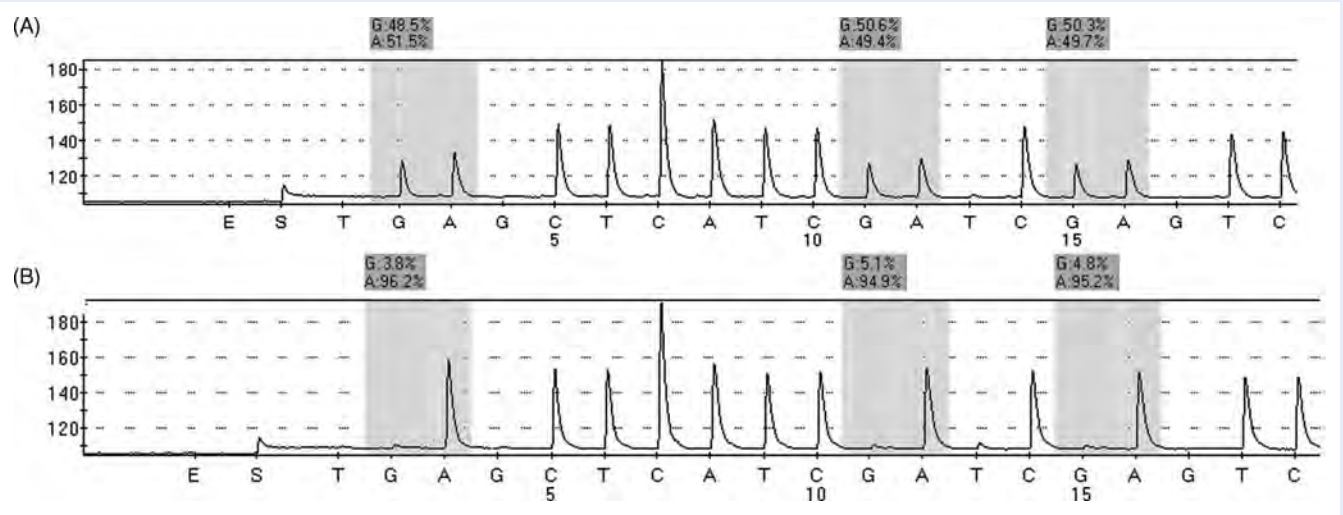

Figure 3 Loss of methylation at *SNRPN* DMR in a patient with Beckwith–Widemann syndrome.
Reverse strand Pyrosequencing trace. Percentage of methylated cytosines is represented as the percentage of guanine (G) and the percentages of unmethylated cytosines which normally will be represented by thymine is represented by alanine (A) on the reverse strand. (**A**) Normal control, (**B**) patient with LOM at *SNRPN*.

Table 1 Clinical and molecular characteristics of Imprinting Centre 2 defect Beckwith–Wiedemann syndrome patients with additional loss of methylation at other imprinted loci

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Sex	M	F	M	F	M	M
ART	IVF	ICSI	ICSI	No	No	No
Pregnancy	Singleton	Singleton	Twin	Singleton	Singleton	Singleton
Macrosomia	No	NR	No	No	Yes	Yes
Exomphalos	Yes	No	No	Yes	Yes	No
Umbilical Hernia	No	Yes	Yes	No	No	Yes
Macroglossia	No	Yes	Yes	Yes	Yes	Yes
Hemihypertrophy	Yes	No	No	No	No	Yes
Embryonal Tumour	No	No	No	No	No	No
Ear creases	Yes	No	Yes	NR	Yes	Yes
Neonatal Hypoglycaemia	No	Yes	Yes	Yes	Yes	Yes
Facial Naevus Flammeus	Yes	Yes	Yes	No	Yes	Yes
6q24 (ZAC) methylation	Normal	Normal	LOM	Normal	Normal	LOM
MI *N* = (0.65 – 1.78)	1.25	1.23	0.55	1.22	0.93	0.61
7q32 (PEG1) methylation	LOM	LOM	Normal	LOM	LOM	Normal
15q13 (SNRPN) methylation	Normal	LOM	Normal	Normal	Normal	Normal
MI *N* = (0.55 – 1.1)	0.84	0.05	0.98	0.82	0.77	0.89
14q32 (DLK1) methylation	Normal	Normal	Normal	Normal	Normal	Normal
MI *N* = (0.5 – 1.4)	0.91	0.82	0.83	0.85	0.76	0.88
11p15.5 KvDMR1 methylation	LOM	LOM	LOM	LOM	LOM	LOM
MI	0.04	0.0	0.0	0.12	0.0	0.02

M, male; F, female; IVF, *in vitro* fertilization; ICSI, intra-cytoplasmic sperm injection; LOM, loss of methylation, MI, methylation index, NR, not recorded, *N*, normal range.

BWS children with IC2 defects might also display loss of methylation at other non-11p15.5 imprinting region DMRs. We found significantly higher frequencies of loss of methylation at DMRs unlinked to 11p15.5 in ART cases than in non-ART cases. This contrasts with the results of Rossignol *et al*. (2006) who found similar rates in both groups, but could be consistent with the hypothesis that differences in phenotype between ART and non-ART IC2 defect BWS patients might be caused by epigenetic differences at non-11p15.5 loci.

Analysis of more extensive cohorts of patients at a larger number of DMRs should provide further information on the relative frequency of hypomethylation at different loci in ART and non-ART BWS patients. Due to the limited amount of DNA available, we only tested the methylation status at the *DLK1*-IG DMR in the six patients with additional loci hypomethylation who are more likely to have a methylation abnormality. In addition, a previous study looking at the methylation status in this paternally methylated DMR in TNDM cases did not find any methylation abnormality at this locus (Mackay *et al*., 2006).

We did not detect any marked differences between the phenotype of non-ART patients with and without additional DMR hypomethylation (LOM+ cases) but the numbers were small. Clearly, it will be interesting to compare the phenotype of BWS LOM+ children to other groups including the subgroup of patients with TNDM who display maternal allele hypomethylation at multiple loci and who are reported to have some phenotypic differences (including a higher birth weight) from TNDM patients without additional DMR hypomethylation (Mackay *et al*., 2006). Recently, mutations in the *ZFP57* gene were identified in some TNDM patients with hypomethylation at multiple imprinted loci (Mackay *et al*., 2008). Finally, our finding that multiple DMR hypomethylation is more frequent in ART cases raises the possibility that some cases of developmental defects or abnormal growth in ART children might be caused by variable combinations of epigenetic alterations at imprinted DMRs.

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Author's roles

E.R.M, D.L, S.C.B.—Study design.
D.L, E.R.M.—Writing of first draft of manuscript.
D.L., E.R.M.—Data interpretation.
D.L., L.T., F.M.—Molecular genetic analysis.
D.L., G.A.K., S.C.B., E.B., A.F., W.L., C.O., T.C., L.A.B., E.R.M.—Patient recruitment, clinical information and sample collection.
All authors—Critical appraisal and correction of draft manuscript.

References

Bliek J, Maas SM, Ruijter JM, Hennekam RC, Alders M, Westerveld A, Mannens MM. 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* 2001;**10**:467–476.

Chang AS, Moley KH, Wangler M, Feinberg AP, Debaun MR. Association between Beckwith-Wiedemann syndrome and assisted reproductive technology: a case series of 19 patients. *Fertil Steril* 2005;**83**:349–354.

Cooper WN, Luharia A, Evans GA, Raza H, Haire AC, Grundy R, Bowdin SC, Riccio A, Sebastio G, Bliek J *et al.* Molecular subtypes and phenotypic expression of Beckwith–Wiedemann syndrome. *Eur J Hum Genet* 2005;**13**:1025–1032.

Cox GF, Burger J, Lip V, Mau UA, Sperling K, Wu BL, Horsthemke B. Intracytoplasmic sperm injection may increase the risk of imprinting defects. *Am J Hum Genet* 2002;**71**:162–164.

Dean W, Bowden L, Aitchison A, Klose J, Moore T, Meneses JJ, Reik W, Feil R. Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. *Development* 1998;**125**:2273–2282.

DeBaun MR, Niemitz EL, McNeil DE, Brandenburg SA, Lee MP, Feinberg AP. Epigenetic alterations of H19 and LIT1 distinguish patients with Beckwith–Wiedemann syndrome with cancer and birth defects. *Am J Hum Genet* 2002;**70**:604–611.

DeBaun MR, Niemitz EL, Feinberg AP. Association of in vitro fertilization with Beckwith–Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am J Hum Genet* 2003;**72**:156–160.

Diaz-Meyer N, Day CD, Khatod K, Maher ER, Cooper W, Reik W, Junien C, Graham G, Algar E, Der Kaloustian VM *et al.* Silencing of CDKN1C (p57KIP2) is associated with hypomethylation at KvDMR1 in Beckwith–Wiedemann syndrome. *J Med Genet* 2003;**40**:797–801.

Engel JR, Smallwood A, Harper A, Higgins MJ, Oshimura M, Reik W, Schofield PN, Maher ER. Epigenotype-phenotype correlations in Beckwith–Wiedemann syndrome. *J Med Genet* 2000;**37**:921–926.

Gaston V, Le Bouc Y, Soupre V, Burglen L, Donadieu J, Oro H, Audry G, Vazquez MP, Gicquel C. Analysis of the methylation status of the KCNQ1OT and H19 genes in leukocyte DNA for the diagnosis and prognosis of Beckwith–Wiedemann syndrome. *Eur J Hum Genet* 2001;**9**:409–418.

Gicquel C, Gaston V, Mandelbaum J, Siffroi JP, Flahault A, Le Bouc Y. In vitro fertilization may increase the risk of Beckwith–Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene. *Am J Hum Genet* 2003;**72**:1338–1341.

Halliday J, Oke K, Breheny S, Algar E, Amor DJ. Beckwith–Wiedemann syndrome and IVF: a case–control study. *Am J Hum Genet* 2004;**75**:526–528.

Khosla S, Dean W, Brown D, Reik W, Feil R. Culture of preimplantation mouse embryos affects fetal development and the expression of imprinted genes. *Biol Reprod* 2001;**64**:918–926.

Lam WW, Hatada I, Ohishi S, Mukai T, Joyce JA, Cole TR, Donnai D, Reik W, Schofield PN, Maher ER. Analysis of germline CDKN1C (p57KIP2) mutations in familial and sporadic Beckwith–Wiedemann syndrome (BWS) provides a novel genotype-phenotype correlation. *J Med Genet* 1999;**36**:518–523.

Lee MP, DeBaun MR, Mitsuya K, Galonek HL, Brandenburg S, Oshimura M, Feinberg AP. Loss of imprinting of a paternally expressed transcript, with antisense orientation to KVLQT1, occurs frequently in Beckwith–Wiedemann syndrome and is independent of insulin-like growth factor II imprinting. *Proc Natl Acad Sci USA* 1999;**96**:5203–5208.

Ludwig M, Katalinic A, Gross S, Sutcliffe A, Varon R, Horsthemke B. Increased prevalence of imprinting defects in patients with Angelman syndrome born to subfertile couples. *J Med Genet* 2005;**42**:289–291.

Mackay DJ, Temple IK, Shield JP, Robinson DO. Bisulphite sequencing of the transient neonatal diabetes mellitus DMR facilitates a novel diagnostic test but reveals no methylation anomalies in patients of unknown aetiology. *Hum Genet* 2005;**116**:255–261.

Mackay DJ, Boonen SE, Clayton-Smith J, Goodship J, Hahnemann JM, Kant SG, Njolstad PR, Robin NH, Robinson DO, Siebert R *et al.* A

maternal hypomethylation syndrome presenting as transient neonatal diabetes mellitus. *Hum Genet* 2006;**120**:262–269.

Mackay DJ, Callaway JL, Marks SM, White HE, Acerini CL, Boonen SE, Dayanikli P, Firth HV, Goodship JA, Haemers AP *et al.* Hypomethylation of multiple imprinted loci in individuals with transient neonatal diabetes is associated with mutations in ZFP57. *Nat Genet* 2008;**40**:949–951.

Maher ER, Brueton LA, Bowdin SC, Luharia A, Cooper W, Cole TR, Macdonald F, Sampson JR, Barratt CL, Reik W *et al.* Beckwith–Wiedemann syndrome and assisted reproduction technology (ART). *J Med Genet* 2003;**40**:62–64.

Niemitz EL, DeBaun MR, Fallon J, Murakami K, Kugoh H, Oshimura M, Feinberg AP. Microdeletion of LIT1 in familial Beckwith–Wiedemann syndrome. *Am J Hum Genet* 2004;**75**:844–849.

Orstavik KH, Eiklid K, van der Hagen CB, Spetalen S, Kierulf K, Skjeldal O, Buiting K. Another case of imprinting defect in a girl with Angelman syndrome who was conceived by intracytoplasmic semen injection. *Am J Hum Genet* 2003;**72**:218–219.

Reik W, Romer I, Barton SC, Surani MA, Howlett SK, Klose J. Adult phenotype in the mouse can be affected by epigenetic events in the early embryo. *Development* 1993;**119**:933–942.

Rossignol S, Steunou V, Chalas C, Kerjean A, Rigolet M, Viegas-Pequignot E, Jouannet P, Le Bouc Y, Gicquel C. The epigenetic imprinting defect of patients with Beckwith–Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region. *J Med Genet* 2006;**43**:902–907.

Smilinich NJ, Day CD, Fitzpatrick GV, Caldwell GM, Lossie AC, Cooper PR, Smallwood AC, Joyce JA, Schofield PN, Reik W *et al.* A maternally methylated CpG island in KvLQT1 is associated with an antisense paternal transcript and loss of imprinting in Beckwith–Wiedemann syndrome. *Proc Natl Acad Sci USA* 1999;**96**:8064–8069.

Sparago A, Russo S, Cerrato F, Ferraiuolo S, Castorina P, Selicorni A, Schwienbacher C, Negrini M, Ferrero GB, Silengo MC *et al.* Mechanisms causing imprinting defects in familial Beckwith–Wiedemann syndrome with Wilms' tumour. *Hum Mol Genet* 2007;**16**:254–264.

Temple IK, Shrubb V, Lever M, Bullman H, Mackay DJ. Isolated imprinting mutation of the DLK1/GTL2 locus associated with a clinical presentation of maternal uniparental disomy of chromosome 14. *J Med Genet* 2007;**44**:637–640.

Weksberg R, Nishikawa J, Caluseriu O, Fei YL, Shuman C, Wei C, Steele L, Cameron J, Smith A, Ambus I *et al.* Tumor development in the Beckwith–Wiedemann syndrome is associated with a variety of constitutional molecular 11p15 alterations including imprinting defects of KCNQ1OT1. *Hum Mol Genet* 2001;**10**:2989–3000.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I, Sinclair KD. Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nat Genet* 2001;**27**:153–154.

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