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 BstUI 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**

*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’-TACCAAAAATCTAAAAATCCCAATT-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 MgCl2 and 0.2 μM primers with the following cycling conditions: 95°C for 30 s, 50°C for 30 s, 72°C for 1 min, 35 cycles.

*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\textsuperscript{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 IFF).

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 65%), 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\textsuperscript{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 rhabdomyosarcoma mentioned in the paragraph above). The co-twin was not clinically affected with BWS.

Methylation profiling of BWS\textsuperscript{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

\begin{figure}
\centering
\includegraphics[width=\textwidth]{image1.png}
\caption{Loss of methylation at ZAC DMR in a patient with Beckwith–Wiedemann syndrome. Electrophenogram 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).}
\end{figure}
showed no methylation abnormalities at the four loci analysed. As expected, methylation analysis of the group of normal controls with ART (4% of BWS cases versus 1.2% of the general population). Previously, we reported that six of the 149 UK children with BWS had additional LOM was significantly higher in the post-ART group than in the non-ART BWSICD2 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 KvDMRI LOM in 24 of the 25 post-ART BWS patients. Previously, KvDMRI 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 KvDMRI 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; Bliik *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 KvDMRI hypomethylation in post-ART cases might lead to a milder phenotype with a lower incidence of exomphalos, comparison of blood KvDMRI 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
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


D.L., E.R.M.—Writing of first draft of manuscript.


All authors—Critical appraisal and correction of draft manuscript.

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