ORIGINAL ARTICLE

The epigenetic imprinting defect of patients with Beckwith–Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region

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Background: Genomic imprinting refers to an epigenetic marking resulting in monoallelic gene expression and has a critical role in fetal development. Various imprinting diseases have recently been reported in humans and animals born after the use of assisted reproductive technology (ART). All the epimutations implicated involve a loss of methylation of the maternal allele (demethylation of KvDMR1/*KCNQ1OT1* in Beckwith–Wiedemann syndrome (BWS), demethylation of *SNRPN* in Angelman syndrome and demethylation of DMR2/*IGF2R* in large offspring syndrome), suggesting that ART impairs the acquisition or maintenance of methylation marks on maternal imprinted genes. However, it is unknown whether this epigenetic imprinting error is random or restricted to a specific imprinted domain.

Aim: To analyse the methylation status of various imprinted genes (*IGF2R* gene at 6q26, *PEG1/MEST* at 7q32, *KCNQ1OT1* and *H19* at 11p15.5, and *SNRPN* at 15q11–13) in 40 patients with BWS showing a loss of methylation at *KCNQ1OT1* (11 patients with BWS born after the use of ART and 29 patients with BWS conceived naturally).

Results: 3 of the 11 (27%) patients conceived using ART and 7 of the 29 (24%) patients conceived normally displayed an abnormal methylation at a locus other than *KCNQ1OT1*.

Conclusions: Some patients with BWS show abnormal methylation at loci other than the 11p15 region, and the involvement of other loci is not restricted to patients with BWS born after ART was used. Moreover, the mosaic distribution of epimutations suggests that imprinting is lost after fertilisation owing to a failure to maintain methylation marks during pre-implantation development.

Genomic imprinting refers to an epigenetic marking of certain genes, resulting in monoallelic expression in a parent-of-origin-dependent manner. Target gene expression on imprinted regions involves changes in chromatin structure and specific patterns of DNA methylation and post-translational histone modifications such as acetylation and methylation.¹ Imprinting control elements are characterised by differentially methylated regions (DMR), in which the imprinted allele is methylated and the other parental allele is unmethylated. Imprinting is established during the development of germ cells and must be maintained at a critical stage of pre-implantation development when the rest of the genome is subjected to a wave of demethylation.¹

Imprinted genes have a crucial role in mammalian development particularly in fetal growth. Aberrant imprinting results in numerous human genetic disorders, including behavioural disorders and cancer. Syndromes involving epigenetic changes have recently been reported in animals and humans conceived by assisted reproductive technology (ART). These syndromes include large offspring syndrome in ruminants,² and the Beckwith–Wiedemann syndrome (BWS; MIM 130650)^{3–6} and Angelman syndrome (MIM 105830)^{7–8} in humans. Many genetic and epigenetic mechanisms are involved in Angelman syndrome and BWS, but, remarkably, in all imprinting disorders observed after the use of ART, the epimutation involves a loss of methylation at maternally imprinted methylated imprinting control elements (*IGF2R* DMR2 (MIM 147280) in large offspring syndrome, KvDMR1

of the *KCNQ1OT1* gene (MIM 604115) in patients with BWS and *SNURF-SNRPN* exon 1/promoter (MIM 182279) in those with Angelman syndrome). The demethylation of these imprinting control elements is responsible for a maternal to paternal switch, with activation of non-coding RNA on the maternal allele.

The precise mechanism underlying the association of imprinted disorders and ART is unknown. The gamete and embryo manipulations used in ART may interfere with genomic imprinting by altering the acquisition or maintenance of imprints during germ-cell maturation or early embryogenesis. In mice, maternal methylation marks are acquired sequentially by the various maternal imprinted loci during oocyte growth.⁹ Alternatively, ART may alter the maintenance of methylation imprints in pre-implantation embryos. No specific procedure has yet been implicated in the epigenetic risk of babies conceived by ART. Indeed, the ART used to conceive patients with Angelman syndrome and BWS involved various procedures, including classical in vitro fertilisation, intracytoplasmic sperm injection (ICSI), embryo cryopreservation and early or late embryo transfer.^{3–8} However, two recent studies^{10–11} have suggested that ovarian stimulation may itself increase the risk of Angelman syndrome and BWS. Moreover, the epigenetic change in patients with BWS conceived by ART is the same as that

Abbreviations: ART, assisted reproductive technology; BWS, Beckwith–Wiedemann syndrome; DMR, differentially methylated regions; ICSI, intracytoplasmic sperm injection

found in monozygotic twins with BWS. The prevalence of monozygotic twinning is particularly high in patients with BWS.¹² Twins are discordant and the affected twin always shows demethylation of *KCNQ1OT1*,^{12,13} suggesting that the *KCNQ1OT1* locus is vulnerable to demethylation at a critical stage of pre-implantation development.

No attempt has yet been made to determine whether the epigenetic imprinting error after the use of ART is restricted to a specific imprinted domain or is randomly distributed. In this study, we analysed the methylation status of other imprinted loci in patients with BWS conceived by ART. We showed that some patients with BWS born after using ART and some patients with BWS conceived naturally displayed abnormal methylation at loci other than the 11p15 centromeric domain.

PATIENTS AND METHODS

Patients with BWS

A total of 40 patients with BWS showing demethylation at the KvDMR1/*KCNQ1OT1* locus were included in this study. Eleven (patients A–K; six female and five male patients) were born after using ART (ICSI (n = 3), classical in vitro fertilisation (n = 8); embryo transfer on day 2 (n = 7), day 3 (n = 1), day 5 (n = 2) and day 6 (n = 1); cryopreservation (n = 1)). Two of these cases occurred in a pair of dizygotic twins and, in both cases, the other twin displayed no BWS phenotype. Twenty nine patients (patients 1 to 29; 14 female and 15 male patients) were conceived naturally. Four of these patients (three female and one male patients; patients 26–29) were monozygotic twins.

We obtained informed consent from all patients (or their parents) in accordance with national ethics rules and the ethical board of the Trousseau Hospital, University of Paris 6, France.

Methods

Southern-blot analysis

We used methyl-sensitive Southern blotting to analyse the methylation status of five genes mapping to four imprinted loci (6q26, 7q32, 11p15.5 and 15q11–13). The methylation status of the *KCNQ1OT1* and *H19* genes at 11p15.5, *IGF2R* DMR2 at 6q26 and the *SNRPN* CpG island at 15q11–13 was analysed as described previously.^{13–15}

For the methylation analysis of *PEG1/MEST* (MIM 601029) at 7q32, genomic DNA was digested with *FokI* and the methylation-sensitive enzyme, *SmaI*, subjected to electrophoresis in 1.3% agarose gels and blotted on Gene Screen Plus membranes (NEN Life Science Products, Boston, Massachusetts, USA). The probe used for hybridisation was obtained using polymerase chain reaction with primers (sense primer PEG1AS: 5'-AGGGGTCTGCTGTTTTGCC-3' and 3' anti-sense primer PEG1AAS: 5'-AGGGTGAGACCAGGGTCATT-3') amplifying an 889-bp fragment in the CpG island (GeneBank accession number AC045582, nt 3124–4012). The upper band (2 kb) is methylated and corresponds to the maternal allele. The lower band (0.9 kb) is unmethylated and corresponds to the paternal allele.

Methylation indexes were determined by densitometry of autoradiographs using a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

Bisulphite sequencing of *IGF2R* DMR2 and *PEG1/MEST* DMR

Genomic DNA (1 µg) was treated with sodium bisulphite, as described previously.¹⁶ The primers used for the analysis of *IGF2R* DMR2 were sense primer IGF2RS: 5'-GGTATGTTGGGGATAGGTTTTGGGAGTTG-3' and 3' anti-sense primer IGF2RAS:

5'-CAAACACACTAACAACCACTACATCCCTC-3', which amplified a 217 bp fragment (containing 17 CpGs) in DMR2 (GeneBank accession number X83701, nt 2023–2239). The primers used for the analysis of *PEG1/MEST* DMR were sense primer PEG1BS: 5'-GTTTTGTTTATTGAGGAGGGGG-3' and 3' anti-sense primer PEG1BAS: 5'-AAAATCCTAAATCATACTACAACAA-3', which amplified a 270 bp fragment (containing 9 CpGs) in the CpG island (GeneBank accession number AC045582, nt 3835–4104).

Methylation analysis of the classical satellites

The DNA methylation status of the classical satellites of chromosomes 1 and 16 and of the α -satellites of chromosomes 6 and 11 was analysed using Southern blotting as described previously¹⁷ for seven of the 11 patients (patients A, C, D, E, G, I and K) born after the use of ART.

Statistical analysis

We used χ^2 tests to compare qualitative data. The significance threshold was set at 5%.

RESULTS

Allele-specific methylation of different imprinted regions

Control participants

Southern-blot analysis of leucocyte DNA from 50 (*IGF2R* DMR2, *PEG1/MEST* and *SNRPN*) and 100 (*KCNQ1OT1*) control participants showed two bands of equal intensity indicating that the two parental alleles were differentially methylated. The mean (standard deviation (SD)) methylation indices were 50.3% (2.7%), range 44–57.2 for *IGF2R* DMR2; 53.6% (2.1%), range 47.9–57 for *PEG1/MEST*; 51.6% (2.5%), range 45–56 for *KCNQ1OT1*; and 49.1% (3.1%), range 44–54 for *SNRPN*.

Patients with BWS

Three of the eleven patients with BWS conceived by ART displayed abnormal methylation patterns of at least one locus other than 11p15 (table 1). Two patients (patients C and F) showed demethylation of the maternal DMR2 of the *IGF2R* gene (methylation indices of 25% and 14%, respectively; fig 1A). One patient (patient E) showed partial demethylation of the *SNRPN* CpG island (methylation index 39%; fig 1B). The methylation status of *PEG1/MEST* was normal in all patients born after the use of ART (fig 1C). A comparison of ART procedures between the three patients with abnormal methylation involving various loci and the eight patients with a methylation defect restricted to *KCNQ1OT1* identified no specific procedure, including late embryo transfer, to be responsible.

Of the 29 patients with BWS who were conceived naturally, 7 patients also displayed abnormal methylation patterns at a locus other than 11p15 (table 1): *IGF2R* DMR2 in four cases (patients 5, 13, 28 and 29 with methylation indices of 9%, 28%, 28% and 32%, respectively; fig 1A) and *PEG1/MEST* for the other three (patients 6, 7 and 25 with methylation indices of 3%, 3% and 29%, respectively; fig 1C).

After polymerase chain reaction amplification and direct sequencing of the bisulphite-converted DNA, we observed a demethylated pattern of the CpG sites for patient C (*IGF2R* DMR2 locus) and patients 6 and 7 (*PEG1/MEST* locus; fig 1D).

Methylation analysis of classical and α -satellites

The classical satellite DNA of the juxtacentromeric regions of chromosomes 1 and 16 and the α -satellites of the centromeric regions of chromosomes 6 and 11 were normally methylated in seven analysed patients conceived by ART (fig 2).

Figure 2 Southern-blot analysis of the methylation patterns of the classical and α -satellites of patients with Beckwith–Wiedemann syndrome after the use of assisted reproductive technology. DNA was digested with *Hind*III (H), *Hind*III+*Hpa*II (H+H) and *Hind*III+*Msp*I (H+M). The Sat 2 probe is specific for chromosomes 1 and 16.

Clinical presentation of patients with demethylation at various imprinted loci

The phenotypes of patients with BWS with methylation defects involving various imprinted genes were compared with those of patients with BWS with methylation defects restricted to *KCNQ1OT1*. No phenotypic differences were observed between children with a methylation defect involving various loci and children with a defect restricted to *KCNQ1OT1* (table 2). Nevertheless, the frequency of macrosomia at birth was higher in patients with involvement of various imprinted loci than in those with defects restricted to *KCNQ1OT1*, although this difference was not significant (71% *v* 43%).

One patient conceived by ART displayed demethylation at *SNRPN* (patient E; table 1). This patient was a girl born

prematurely (32 weeks) with severe hypotonia. Brain magnetic resonance imaging was suggestive of prenatal stroke and the patient died shortly after birth.

Interestingly, two patients with BWS who were conceived naturally and displaying loss of methylation at different loci displayed atypical BWS phenotypes. One of these patients (patient 6, table 1, with a loss of methylation at *KCNQ1OT1* and *PEG1/MEST*) was a boy born without macrosomia (birth height -1.5 SD, birth weight -1.4 SD, head circumference -0.3 SD), displaying mild macroglossia, an umbilical hernia, micropenis and clinodactyly of the fifth fingers. His growth rate gradually increased from the age of 6 months, and he reached 2.5 SD in height and 5 SD in weight by the age of 6 years. During this period, he was diagnosed with developmental delay, expressed primarily in the form of non-specific speech retardation.

The second patient (patient 13, table 1, with a loss of methylation at *KCNQ1OT1* and *DMR2/IGF2R*) was a boy born prematurely (29 weeks) with severe intrauterine growth retardation (birth height -4.2 SD, birth weight -2.5 SD, head circumference -0.4 SD) and facial dysmorphic features suggestive of fetal alcohol syndrome. Body asymmetry and mild macroglossia at birth led to molecular testing for BWS. The patient died at the age of 2.5 months from respiratory failure and infection. On postmortem examination, hepatomegaly was observed, with a multinodular tumour identified as a hepatoblastoma on pathological analysis.

DISCUSSION

Epigenetic defects (demethylation of *KCNQ1OT1* or hypermethylation of *H19*) account for about 70% of molecularly proved cases of BWS. Some of these cases display a microdeletion of the centromeric¹⁸ or telomeric^{19,20} imprinting centres of the 11p15 region. Recent reports^{3–6,11} have highlighted an increase in the risk of BWS in children born after the use of ART. The molecular defect in patients with BWS conceived by ART consists of demethylation of the *KvDMR1/KCNQ1OT1* locus, suggesting that ART impairs the acquisition (during oocyte maturation) or maintenance (after fertilisation) of the maternal methylation marks of the centromeric imprinting centre of the 11p15 region. The timing of methylation mark acquisition by maternal imprinted genes during oocyte maturation has been studied only in mice, in which it was found to be asynchronous.⁹ However, the timing of *KvDMR1/KCNQ1OT1* methylation in mice is unknown. Little is known about the acquisition of methylation marks in humans, but the studies carried out to date have suggested that various key aspects of the timing of DNA methylation

Table 2 Clinical characteristics of patients with Beckwith–Wiedemann syndrome (BWS) with a methylation defect involving various loci and patients with BWS with a methylation defect restricted to *KCNQ1OT1*

	Patients with BWS with involvement of various imprinted genes (n = 10)	Patients with BWS with involvement of <i>KCNQ1OT1</i> (n = 30)	p* Value
Sex	6F/4M	15F/15M	
Delivery (weeks): median (range)	37 (29–38)	36 (21–41)	
Macrosomia†	71%	43.5%	NS
Macroglossia	100%	100%	NS
Organomegaly	22%	46%	NS
Abdominal wall	70%	73%	NS
Exomphalos (n)	4/7	14/22	NS
Hemihyperplasia	20%	20%	NS
Ear abnormalities	60%	56%	NS
Hypoglycaemia	60%	46%	NS

NS, non-significant.

* χ^2 test

†Birth weight or birth height ≥ 2 SD according to Usher and McLean charts. Twin patients (n = 6) were not included.

acquisition and maintenance are conserved in the germ line and early embryo.^{21–23} Thus, maternal methylation marks are probably acquired sequentially in human oocytes, as in mouse oocytes.

The methylation status of imprinted loci other than the 11p15 region has not been investigated in patients with BWS. In particular, no previous study has attempted to determine whether the epigenetic imprinting error after the use of ART is restricted to a specific imprinted domain or randomly distributed. In this study, we compared methylation status at various imprinted loci in patients with BWS born after the use of ART and in patients with the same epigenetic defect (ie, demethylation of *KCNQ1OT1*) conceived naturally.

We found that some patients with BWS conceived by ART displayed abnormal methylation at loci other than 11p15. Analysis of the ART procedures used in the three patients with abnormal methylation of several loci implicated no specific ART procedure. The methylation defect involved imprinted loci and the methylation pattern of constitutive heterochromatin was normal in patients with BWS conceived by ART. Some naturally conceived patients with BWS also displayed abnormal methylation at loci other than 11p15. It indicates that ART procedures are not specifically involved in loss of methylation at various imprinted loci.

The loss of methylation at the various imprinted loci was only partial, suggesting mosaicism of the epimutation. This mosaic pattern suggests that the epigenetic error occurs after fertilisation, during early development, rather than during oocyte maturation. Similar results were observed in mice.²⁴ There are therefore two possibilities:

- the mechanism involved in the epigenetic error is different in patients with a methylation defect involving various loci and in patients with a defect restricted to *KCNQ1OT1*; or
- the mechanism is the same in these two sets of patients, but the degree of expression is different, ranging from demethylation of the most vulnerable locus (*KCNQ1OT1*) to the demethylation of several imprinted loci.

Evidence in favour of this second possibility is provided by the observation of variable levels of demethylation in people displaying a loss of methylation at various loci (table 1). The high incidence of monozygotic twinning with the affected twin displaying a loss of methylation at *KCNQ1OT1*^{12–13} suggests that the *KCNQ1OT1* locus is particularly vulnerable to epigenetic errors during early development.²⁵ The mechanisms safeguarding imprinting marks during the wave of demethylation that occurs during early pre-implantation development remain poorly characterised. DNA methyltransferases and methyl-binding domain proteins are probably key regulators in this process, and a model based on errors in trafficking of the oocyte isoform of DNMT1 has been proposed to explain the genetics of BWS in monozygotic twins.²⁵ Recently, Arnaud *et al*²⁶ showed that a maternal imprint can be acquired in the absence of Dnmt3L in female germ cells. This incomplete penetrance of Dnmt3L deficiency was neither locus nor embryo specific, but stochastic suggesting that in the absence of Dnmt3L other factors can mark individual DMRs.

Surprisingly, patients with defects at loci other than 11p15 do not display other phenotypes. However, the slight difference in the frequency of macrosomia, although non-significant, could be accounted for by the involvement of other loci. *PEG1/MEST* is believed to be involved in fetal growth, although its precise function is unknown. Disruption of the *PEG1/MEST* gene causes embryonic growth retardation when paternally transmitted.²⁷ A loss of maternal methylation marks at this locus may therefore result in the biallelic expression of *PEG1/MEST* and fetal overgrowth. Regarding

the *IGF2R* gene, differential methylation of maternal and paternal DMR2 region is maintained in humans²⁸ and some humans do appear to display transcriptional imprinting of *IGF2R*.²⁹ *IGF2R* is responsible for the clearance and inactivation of IGF2. A loss of maternal *IGF2R*/DMR2 methylation would probably decrease *IGF2R* expression, thereby increasing IGF2 availability and fetal growth.

Recently, some patients with a clinical diagnosis of transient neonatal diabetes mellitus (MIM 601410) caused by loss of methylation at the transient neonatal diabetes mellitus maternally methylated imprinted domain on 6q24 were described with a partial loss of methylation at *KCNQ1OT1*.^{30–31} As in our series, the phenotype of patients with involvement of both loci (6q24 and 11p15) was not very different from the phenotype of patients with the involvement of only 6q24.³¹

In this study, we searched for imprinting defects involving loci other than the *KCNQ1OT1* locus in patients born after the use of ART. Our original hypothesis that other loci are involved only in patients conceived by ART was not confirmed. Instead, we showed the involvement of other imprinted loci in some naturally conceived patients with BWS. Moreover, the pattern of cellular mosaicism observed suggests that the imprinting defect occurs after fertilisation via a mechanism impairing the maintenance of maternal imprints. The mechanisms controlling the protection of imprinted loci against demethylation remain unclear, but our data suggest that this protection fails in these patients, resulting in a loss of maternal imprints.

WEB RESOURCES

The accession number and URLs for data presented herein are as follows: GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/> (for *PEG1/MEST* genomic sequence (accession number AC045582)).

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for BWS, transient neonatal diabetes mellitus, Angelman syndrome, *KCNQ1OT1*, *IGF2* receptor, *PEG1/MEST* and *SNRPN*).

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