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Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies

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BACKGROUND: There is an increased incidence of rare imprinting disorders associated with assisted reproduction technologies (ARTs). The identification of epigenetic changes at imprinted loci in ART infants has led to the suggestion that the techniques themselves may predispose embryos to acquire imprinting errors and diseases. However, it is still unknown at what point(s) these imprinting errors arise, or the risk factors.

METHODS: In 2009 we conducted a Japanese nationwide epidemiological study of four well-known imprinting diseases to determine any association with ART. Using bisulfite sequencing, we examine the DNA methylation status of 22 gametic differentially methylated regions (gDMRs) located within the known imprinted loci in patients with Beckwith-Wiedemann syndrome (BWS, $n = 1$) and also Silver-Russell syndrome (SRS, $n = 5$) born after ART, and compared these with patients conceived naturally.

RESULTS: We found a 10-fold increased frequency of BWS and SRS associated with ART. The majority of ART cases showed aberrant DNA methylation patterns at multiple imprinted loci both maternal and paternal gDMRs (5/6), with both hyper- and hypomethylation events (5/6) and also mosaic methylation errors (5/6). Although our study may have been limited by a small sample number, the fact that many of the changes were mosaic suggested that they occurred after fertilization. In contrast, few of the patients who were conceived naturally exhibited a similar pattern of mosaic alterations. The differences in methylation patterns between the patients who were conceived naturally or after ART did not manifest due to the differences in the disease phenotypes in these imprinting disorders.

CONCLUSION: A possible association between ART and BWS/SRS was found, and we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable.

Key words: assisted reproduction technologies / genomic imprinting / DNA methylation / gametic differentially methylated regions / genomic imprinting disorders

Introduction

Human assisted reproduction technologies (ARTs) are used in the treatment of infertility and involve the manipulation of eggs and/or sperm in the laboratory. Several recent studies have identified an increased incidence of some normally very rare imprinting disorders after ART, including Beckwith-Wiedemann syndrome (BWS: ONIM 130650), Angelman syndrome (AS: ONIM 105830) and Silver-Russell syndrome (SRS: OMIM 180860) but not Prader-Willi syndrome (PWS: OMIM 176270; DeBaun *et al*., 2003; Gosden *et al*., 2003; Svensson *et al*., 2005). Additionally, there are several reports suggesting that epigenetic alterations (epimutations) at imprinted loci occur during the *in vitro* manipulation of the gametes, with both IVF and ICSI approaches implicated (Cox *et al*., 2002; DeBaun *et al*., 2003; Gicquel *et al*., 2003; Maher *et al*., 2003; Moll *et al*., 2003; Orstavik *et al*., 2003; Ludwig *et al*., 2005; Rossignol *et al*., 2006; Bowdin *et al*., 2007; Kagami *et al*., 2007). However, some studies do not support a link between ART and imprinting disorders (Lidegaard *et al*., 2005; Doornbos *et al*., 2007).

Epigenetic marks laid down in the male or female germ lines, and which are inherited by the embryos, establish the imprinted expression of a set of developmentally important genes (Surani, 1998). Because imprinted genes are regulated by these gametic epigenetic marks, and by further epigenetic modifications in the somatic cell, they are particularly vulnerable to environmentally induced mutation. One of the best studied epigenetic marks is DNA methylation. DNA methylation is established in either the maternal or paternal germline at discrete genomic loci. This methylation is preserved in the fertilized embryo to generate differentially methylated regions (DMRs) which then signal to nearby genes to establish domains of imprinted chromatin by mechanisms that are not fully understood (John and Lefebvre, 2011). These germline or gametic DMRs (gDMRs) can orchestrate the monoallelic expression of genes over megabases of DNA (Tomizawa *et al*., 2011) and are reset with every reproductive cycle (Lucifero *et al*., 2002; Obata and Kono, 2002).

The increased frequency of epimutation(s) at imprinted loci in ART infants has led to the suggestion that ART procedures may induce imprinting error(s). However, these studies are confounded because ART populations are, by their very nature, different from populations who were conceived without the use of ART, with a low fertility rate, an increased frequency of reproductive loss and usually of advanced age, all of which are associated with increased occurrence of fetal and neonatal abnormalities. Furthermore, it is difficult to determine the causality of imprinting errors in any specific abnormality reported after ART. Both IVF and ICSI appear to be associated with an increased relative risk of imprinting disorders (Savage *et al*., 2011). These procedures are often undertaken for unexpected infertility and require ovarian stimulation, oocyte collection and *in vitro* culture before the embryos are implanted. It has been suggested that infertility and any resulting ovarian stimulation may predispose to epigenetic errors (Sato *et al*., 2007). Animal studies suggest that *in vitro* embryo culture may be associated with epigenetic alterations. In particular, the large offspring syndrome in cattle undergoing ART is associated with the loss of maternal allele methylation at insulin-like growth factor 2 receptor (*IGF2R*) gDMR (Young *et al*., 2001) and has phenotypic similarity to BWS. It is still unknown when these imprinting errors arise and what factors predispose to epigenetic changes. Previously, Chang *et al*. (2005) reported no phenotypic differences between BWS patients who were conceived after ART and naturally. However, Lim *et al*. (2009) reported that patients who were conceived after ART had a significantly lower frequency of exomphalos and higher risk of non-Wilms tumor neoplasia. Phenotypic differences between patients who were conceived after ART and naturally are largely unreported, while any changes to phenotype may be altered by the frequency and the degree of epimutations. Studies revealed that some patients with BWS born after ART presented with epimutations that were not restricted to the 11p15 region (Rossignol *et al*., 2006; Bliek *et al*., 2009; Lim *et al*., 2009). Further analysis of abnormal methylation patterns in imprinting disorders may provide clues as to the cause of disease and identify the ART-related risk factor(s).

To address these questions in this study, we engaged in a nationwide epidemiological study of the Japanese population to determine the frequency of four imprinting disorders after natural conception and after ART. We then analyzed the DNA methylation status of 22 gDMRs in BWS and SRS patients conceived by the two routes. Finally, we compared the abnormal methylation patterns and the phenotypes reported for both sets of patients. As a result we found that both BWS and SRS were more frequent after ART and that ART patients exhibited a higher frequency of aberrant DNA methylation patterns at multiple loci with, in some cases, mosaic methylation errors.

Materials and Methods

Nationwide investigation of imprinting disorders

The protocol was established by the Research Committee on the Epidemiology of Intractable Diseases. The protocol consisted of a two-stage postal survey. The first-stage survey was used to estimate the number of individuals with any of the four imprinting diseases: BWS, SRS, PWS and AS. The second-stage survey was used to identify the clinico-epidemiological features of these syndromes.

In the first-stage survey, the pediatric departments of all hospitals were identified based on a listing of hospitals, as at 2008, supplied by the R&D Co. Ltd (Nagoya, Japan). Hospitals were classified into seven categories according to the type of institution and the number of hospital beds. The survey was mailed to a total of 3158 departments in October 2009 with letters of request for participation in recording these diseases. A simple questionnaire was used to ask about the number of patients with any of the four imprinting disorders. Diagnosis was determined by karyotype analyses, genetic analyses and clinical phenotypes by their clinical doctors. In December 2009, a second request was sent to departments that had not responded to the earlier deadline (at the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

The second questionnaires were forwarded to the departments that had reported patients with the imprinting disorders on the first questionnaires. Detailed clinical information for the patients with these imprinting disorders was collected, including the age, gender, growth and development pattern, the methods of the diagnosis, the presence of infertility treatment and the methods of ART where applicable. Duplicate results were excluded using the information regarding the patient's age and gender where available. The study was approved by the Ethics Committee of Tohoku University School of Medicine.

Estimation of prevalence of imprinting disorders

The number of patients, who were diagnosed by genetic and cytogenetic testing and by clinical phenotypes, was obtained from data from the departments who responded to the first survey. The 95% confidence interval (CI) was calculated as previously described (Wakai *et al*., 1997). The prevalence was determined, based on the population of Japan in 2009 (127 510 000) with data from the Statistics Bureau of the Ministry of Internal Affairs and Communications.

DNA preparation

Genomic DNA was obtained from blood or buccal mucosal cell samples from patients with one of the imprinting disorders using standard extraction methods (Kobayashi *et al*., 2007). For control DNAs, DNA was prepared from the sperm and cord blood samples from unaffected individuals. The study was performed after obtaining patients or their parents' consent.

Bisulfite-treatment PCR including the SNPs

We first searched for single nucleotide polymorphisms (SNPs) within 22 previously reported human gDMRs (Kikyo *et al*., 1997; Smith *et al*., 2003; Kobayashi *et al*., 2006, 2009; Wood *et al*., 2007) using 20 control Japanese blood DNA samples. PCR primer sets were designed to span these SNPs (Supplementary data, Table SI) and human sperm DNA and blood DNA was used to confirm that these PCR assays detected the methylation status of the 22 DMRs. Paternal DMRs were shown to be fully methylated in sperm DNA, maternal DMRs were fully unmethylated and in blood DNA, both paternal and maternal DMRs showed ~50% methylation (Supplementary data, Fig. S1). The human gDMRs and the non-imprinted repetitive long interspersed nucleotide element (*LINE1*) and *Alu* repetitive sequences were examined by bisulfite sequencing using established protocols (Kobayashi *et al*., 2007). Briefly, PCR products were purified and cloned into the pGEM-T vector (Promega, Madison, WI, USA). Individual clones were sequenced using M13 reverse primer and an automated ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). On average, 20 clones were sequenced for each sample.

Statistics

The frequency of the manifestation in patients who were conceived after ART was compared with that observed in patients conceived naturally using Fisher's exact test.

Results

Frequency of four imprinting disorders and their association with ART

We first investigated the nationwide frequency of four imprinting disorders (BWS, AS, PWS and SRS) in Japan in the year 2009. Of a total of 3158 departments contacted, 1602 responded to the first-stage survey questionnaire (50.7%). The total number of cases was calculated using a second-stage survey ensuring the exclusion of duplicates (Table I). Using this information, and taking into account the number of patients with suspect clinical signs but without a formal diagnosis, we identified 444 BWS patients (95% CI: 351–538), 949 AS patients (95% CI: 682–1217), 2070 PWS patients (95% CI: 1504–2636) and 326 SRS patients (95% CI: 235–416). From these figures (and using the 2009 population of Japan: 127 510 000) we estimated the prevalence of these syndromes to be 1 in 287 000, 1 in 134 000, 1 in 62 000 and 1 in 392 000, respectively, for BWS, AS, PWS and SRS. Further details are given in Supplementary data, Table SII and Supplementary data, Fig. S2.

Table I The 2009 frequency of four imprinting diseases in Japan in relation to use of assisted reproduction techniques (ART).

Imprinting disorders	Total estimated patient number (95% CI)	The total prevalence of the syndrome	The number of patients after ART/ total (%)
BWS	444 (351–538)	1 in 287 000	6/70 (8.6)
AS	949 (682–1217)	1 in 134 000	2/123 (1.6)
PWS	2070 (1504–2636)	1 in 62 000	4/261 (1.5)
SRS	326 (235–416)	1 in 392 000	4/42 (9.5)

Results of a nationwide epidemiological investigation of four imprinting disorders in Japan, under the governance of the Ministry of Health, Labor and Welfare of the Japanese government. Precise diagnosis was performed using fluorescence *in situ* hybridization and DNA methylation analyses. The type of ART, obtained from the questionnaires, was compared with the frequencies of these diseases and the epimutation rates. BWS, Beckwith-Wiedemann syndrome, AS, Angelman syndrome, PWS, Prader-Willi syndrome; SRS, Silver-Russell syndrome.

Between 1997 and 2008, the period during which the ART babies in this study were born, 0.64–0.98% of the total number of babies born in Japan were born as a result of IVF and ICSI. We ascertained the frequency of ART procedures in the cases of BWS, AS, PWS and SRS via the questionnaire sent to doctors (Table I, Supplementary data, Table SIII). The numbers of patients with PWS and AS we identified was low; however, the frequency of ART in these cases was not dissimilar to that expected, based on the population rate of ART use, with 2/123 (1.6%) cases of AS and 4/261 (1.5%) cases of PWS born after ART. In contrast, for BWS and SRS the frequency of ART was nearly 10-fold higher than anticipated with 6/70 (8.6%) BWS and 4/42 (9.5%) SRS patients born after ART.

After analyzing the second questionnaire, the blood or buccal mucosal cell samples were obtained from 15 individuals with BWS, 23 with SRS, 73 with AS and 29 with PWS. Using polymorphic bisulfite-PCR sequencing, we examined the methylation status of gDMRs within these samples at the imprinted regions implicated in these syndromes. For BWS we assayed *H19* and *KCNQ1OT1* (*LIT1*) gDMRs, for SRS we assayed the *H19* gDMR and for PWS and AS we assayed the *SNRPN* gDMR. For all patients (conceived naturally and with ART), the frequencies of DNA methylation errors (epimutations) corrected were 7/15 (46.7%) for BWS, 9/23 (39.1%) for SRS, 6/73 (8.2%) for AS and 2/29 (6.9%) for PWS. When looking at the ART cases exclusively, epimutation rates were 3/5 (BWS), 3/7 (SRS), 0/2 (AS) and 0/2 (PWS).

Abnormal methylation patterns in the ART and naturally conceived SRS patients with epimutations.

While hypomethylation of *H19* at chromosome 11 is known to be a frequent occurrence in SRS (Bliek *et al*., 2006), various additional loci at chromosomes 7, 8, 15, 17 and 18 have been implicated as having a

role in this syndrome (OMIM 180860). We first identified SNPs in the previously reported 22 human DMRs using genomic DNA isolated from human sperm and blood from unaffected individuals, which could then be used in bisulfite-PCR methylation assays to assign methylation to the parental allele. We next collected a total of 15 SRS samples, including previously collected samples (ART: 2, naturally conceived: 4), which had DNA methylation errors at the paternal gDMR at *H19*. Five of these were born from ART and 10 were from natural conceptions. We analyzed and compared the DNA methylation status of the 3 other paternal gDMRs and the 19 maternal gDMRs (Supplementary data, Fig. S3, Table, Supplementary data, Table SIV). In four out of the five ART cases, DNA methylation errors were not restricted to the *H19* gDMR, and were present at both maternally and paternally methylated gDMRs. These four cases showed a mixture of hyper- and hypomethylation with mosaic (partial) patterns. In contrast, only 3 of the 10 naturally conceived patients showed DNA methylation errors at loci other than *H19* gDMR.

To determine whether DNA methylation errors occurred in patients at a broader level in the genomes, we assessed the methylation profiles of the non-imprinted *LINE1* and *Alu* elements. We examined a total of 28 CpG sites in a 413-bp fragment of *LINE1* and 12 CpG sites in a 152-bp fragment of *Alu* (Supplementary data, Table SIV**)**, and no significant differences were found in the methylation ratios between patients conceived by ART and naturally.

The abnormal methylation pattern in BWS patients with epimutations

In BWS, hypermethylation of *H19* or hypomethylation of *KCNQ1OT1(LIT1)* at human chromosome 11 are both frequently reported (Choufani *et al*., 2010). We collected seven BWS samples with DNA methylation errors of the *LIT1* gDMR, one of which was derived from ART patient and six from naturally conceived patients (Supplementary data, Fig. S3, Table II, Supplementary data, Table SIV). In the one ART (ICSI) case, we identified four additionally gDMR methylation errors, again present at both maternally and paternally methylated gDMRs and with mixed hyper- and hypomethylation patterns. Furthermore, the methylation error at the *NESPAS* DMR was mosaic in this patient. One of the six naturally BWS cases had similar changes. Although we had only one BWS case conceived by ART, widespread methylation errors were similar to those for the DNA methylation error pattern in SRS.

Phenotypic differences between ART patients and those conceived naturally

The increased frequency of DNA methylation errors at other loci in the ART cases suggested that the BWS and SRS cases born after ART might exhibit additional phenotypic characteristics. However, when we compared in detail the clinical features from both categories of conception (Supplementary data, Table SV), we found no major differences between ART and naturally conceived patients with BWS and SRS.

Discussion

Our key finding from this study was a possible association between ART and the imprinting disorders, BWS and SRS. We did not find a similar association with PWS and AS but our numbers were quite low in this study and a larger due to the questionnaire return rate and relative rarity of the diseases, international study will be required to reach definitive conclusions. Furthermore, factors such as PCR and/or cloning bias in the bisulfite method and correction for changing rate of ART over time must be considered when analyzing any results.

In addition to the possible association between ART and BWS/SRS, we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART shown by us and others is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable. The process of ART exposes the developing epigenome to many external influences, which have been shown to influence the proper establishment and maintenance of genomic imprints, including hormone stimulation (Sato *et al*., 2007), *in vitro* culturing (DeBaun *et al*., 2003; Gicquel *et al*., 2003; Maher *et al*., 2003), cryopreservation (Emiliani *et al*., 2000; Honda *et al*., 2001) and the timing of embryo transfer (Shimizu *et al*., 2004; Miura and Niikawa, 2005). Furthermore, we and others have also shown that some infertile males, particularly those with oligozoospermia, carry pre-existing imprinting errors in their sperm (Marques *et al*., 2004; Kobayashi *et al*., 2007; Marques *et al*., 2008) which might account for the association between ART and imprinting disorders.

Imprinting syndromes and their association with ART

We report the first Japanese nationwide epidemiological study to examine four well-known imprinting diseases and their possible association with ART. We found that the frequency of ART use in both BWS and SRS was higher than anticipated based on the nationwide frequency of ART use at the time when these patients were born. Several other reports have raised concerns that children conceived by ART have an increased risk of disorders (Cox *et al*., 2002; DeBaun *et al*., 2003; Maher *et al*., 2003; Orstavik *et al*., 2003; Ludwig *et al*., 2005; Lim and Maher, 2009). However, the association is not clear in every study (Lidegaard *et al*., 2005; Doornbos *et al*., 2007). The studies reporting an association were mainly from case reports or case series whereas the studies where no association was reported were cohort studies. Therefore, the differences in the epidemiological analytical methods might accounts for the disparity in findings.

Owing to the rare nature of the imprinting syndromes, statistical analysis is challenging. In addition, the diagnosis of imprinting diseases is not always clear cut. Many of the syndromes have a broad clinical spectrum, different molecular pathogenesis, and the infant has to have reached a certain age before these diseases become clinically detectable. It is therefore likely that some children with these diseases are not recorded with the specific diagnosis code for these syndromes. Nonetheless, in this study we were examining the relationship between ART and the imprinting syndromes and these confounding factors are likely to apply equally to both groups.

Both BWS and SRS occurred after ART but our numbers for PWS and AS were low, precluding any definitive conclusion for these two disorders. However, while most cases of BWS and SRS are caused by an epimutation, epimutations are very rare in PWS and AS (only 1–4%) and ART would not be expected to increase chromosome 15

Table II Abnormal methylation in patients with SRS and BWS.

Case	ART	Abnormal methylation			
SRS					
SRS-1	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated	PEG10 hypermethylated (mosaic)	GRB10 hypermethylated; ZNF597 hypomethylated
SRS-2	IVF-ET	H19 hypomethylated (mosaic)			
SRS-3	IVF-ET	H19 hypomethylated (mosaic)	PEG1 hypermethylated (mosaic)		
SRS-4	IVF-ET	H19 hypomethylated	GRB10 hypermethylated		
SRS-5	IVF-ET	H19 hypomethylated (mosaic)	INPP5F hypermethylated		
SRS-6		H19 hypomethylated			
SRS-7		H19 hypomethylated (mosaic)	ZNF597 hypermethylated (mosaic)	ZNF331 hypomethylated (mosaic)	
SRS-8		H19 hypomethylated			
SRS-9		H19 hypomethylated (mosaic)			
SRS-10		H19 hypomethylated			
SRS-11		H19 hypomethylated (mosaic)	PEG1 hypermethylated		
SRS-12		H19 hypomethylated			
SRS-13		H19 hypomethylated (mosaic)	FAM50B hypomethylated		
SRS-14		H19 hypomethylated			
SRS-15		H19 hypomethylated			
BWS					
BWS-1	ICSI	LIT1 hypomethylated	ZDBF2 hypermethylated	PEG1 hypermethylated	NESPAS hypomethylated (mosaic)
BWS-2		LIT1 hypomethylated			
BWS-3		LIT1 hypomethylated			
BWS-4		LIT1 hypomethylated			
BWS-5		LIT1 hypomethylated			
BWS-6		LIT1 hypomethylated	ZDBF2 hypomethylated	ZNF331 hypomethylated (mosaic)	
BWS-7		LIT1 hypomethylated			

ET, embryo transfer. Summary of the abnormal methylation patterns in the ART conceived and naturally conceived patients with Silver-Russell syndrome (SRS) and Beckwith-Wiedemann syndrome (BWS) with epimutations. Numbers in parentheses show the results of the methylation rates obtained using bisulfite-PCR sequencing. The % of DNA methylation of 22 gDMRs in all patients with SRS and BWS examined are presented in Supplementary data, Table SIV. Depictions in red represent DMRs normally exclusively methylated on the maternal allele, while blue represent paternally methylated sites.

deletions or uniparental disomy, consistent with our findings. Prior to this investigation, there was some evidence for an increased prevalence of BWS after ART but less evidence for an increased prevalence of SRS, with five SRS patients reported linked to ART (Svensson *et al*., 2005; Bliek *et al*., 2006; Kagami *et al*., 2007; Galli-Tsinopoulou *et al*., 2008). Our population-wide study provides evidence to suggest that both BWS and SRS occur more frequently after ART in the Japanese population.

Mechanisms of epimutation in the patients conceived by ART

By performing a comprehensive survey of all the known gDMRs in a number of patients with BWS and SRS, we found that multiple loci were more likely to be affected in ART cases than those conceived naturally. Lim *et al*. (2009) have reported a similarly increased frequency of multiple errors after ART, with 37.5% of patients conceived with ART and 6.4% of naturally conceived patients displaying abnormal methylation at additional imprinted loci. However, while Bliek *et al*. (2009) reported alterations in multiple imprinted loci in 17 patients out of 81 BWS cases with hypomethylation of *KCNQ1OT1*(*LIT1*) ICR, only 1 of the cases with multiple alterations was born after ART. Similarly, Rossignol *et al*. (2006) reported that 3 of 11 (27%) ART-conceived patients and 7 of 29 (24%) naturally conceived patients displayed abnormal methylation at additional loci. In these four earlier studies, not all gDMRs were assayed and it may be that by doing so, these incongruities will be resolved.

The pattern of cellular mosaicism we observed in some patients suggested that the imprinting defects occurred after fertilization rather than in the gamete as DNA methylation alterations arising in the gamete would be anticipated to be present in every somatic cell. This suggested the possibility that the DNA methylation errors occurred as a consequence of impaired maintenance of the germline imprints rather than a failure to establish these imprints in the germline or a loss of these imprints in the sperm or oocytes *in vitro*. Furthermore, some patients conceived by ART with SRS and BWS showed

alterations at both maternally and paternally methylated gDMRs suggesting that the defects were not limited to one parental germline. The mechanisms controlling the protection of imprinted loci against demethylation early in the development remain unclear. Our data suggested that this protection may fail in ART resulting in the tissue-specific loss of imprints, though it remains unclear if this ever occurs naturally. Potential factors involved could include the culture conditions for the newly fertilized oocyte and the length of exposure to specific media or growth factors, as part of the ART procedure. Some of the naturally conceived patients also had abnormal methylation at both maternally and paternally methylated gDMRs, which were in some cases mosaic. This could indicate that fertility issues arise as a consequence of pre-existing mutations in factors required to protect and maintain imprints early in life and it may therefore be possible to identify genetic mutations in these factors in this group of patients.

Clinical features

In our large-scale epidemiological study, we found differences in the frequency of some classic features of SRS and BWS between patients conceived by ART and those conceived naturally. We found that 7/7 (100%) ART conceived SRS patients showed body asymmetry, whereas only 30/54 (55.5%) who were conceived naturally possessed this feature. Similarly in BWS, earlobe creases were present in 4/7 (57.1%) ART conceived cases and 44/89 (49.4%) naturally conceived, bulging eyes in 3/7 (42.8%) versus 21/89 (23.6%), exomphalos in 6/7 (85.7%) versus 61/89 (68.5%) and nephromegaly in 2/7 (28.6%) versus 18/89 (20.2%), respectively. It is therefore possible that the dysregulation of the additional genes does modify the typical SRS and BWS phenotypes (Azzi *et al.*, 2010). BWS patients with multiple hypermethylation sites have been reported with complex clinical phenotypes (Bliek *et al.*, 2009) and a recently recognized BWS-like syndrome involving overgrowth with severe developmental delay was reported after IVF/ICSI (Shah *et al.*, 2006).

In our study patients with diagnosed imprinting disorders that presented with defects at additional loci (i.e. other than the domain responsible for that disorder) did not display additional phenotypes not normally reported in BWS or SRS. Since we were effectively selecting for classic cases of BWS and SRS in the first instance, it is possible that there are individuals born through ART showing entirely novel or confounding phenotypes that were not identified in our survey. Alternatively, as many of the alterations we observed showed a mosaic pattern, it is possible that mosaic individuals have more subtle phenotypes. In light of this new information on mosaicism, we may be able to use our knowledge of the individual's epigenotype to uncover these subtle changes.

This study, and the work of our colleagues, highlights the pressing need to conduct long-term international studies on ART treatment and the prevalence of imprinting disorders, particularly as the use of ART is increasing worldwide. It remains to be seen if other very rare epigenetic disorders will also have a possible association with the use of ART. Furthermore, it is not yet known what other pathologies might be influenced by ART. For example, in addition to general growth abnormalities, many imprint methylation errors also lead to the occurrence of various cancers (Okamoto *et al.*, 1997; Cui *et al.*, 1998). Further molecular studies will be required to understand the pathogenesis of these associations, and also to identify preventative methods to reduce the risk of occurrence of these syndromes following ART.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

H.H., H.O., N.M., F.S. and A.S. performed the DNA methylation analyses. M.K., K.N. and H.S. collected the samples of the patients. K.N. did the statistical analyses. H.H., M.V.D.P., R.M.J. and T.A. wrote this manuscript. All authors have read and approved the final manuscript.

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Conflict of interest

None declared.

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