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Discordant *KCNQ1OT1* imprinting in sets of monozygotic twins discordant for Beckwith–Wiedemann syndrome

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Beckwith–Wiedemann syndrome (BWS) presents with visceromegaly, macroglossia, tumor predisposition and other congenital abnormalities, and is usually associated with abnormalities of chromosome 11p15. A number of identical twin pairs, mostly female, have been reported to be discordant for BWS. We show here that the incidence of female monozygotic twins among patients with BWS is dramatically increased over that of the general population. A cluster of imprinted genes within 11p15 is thought to be coordinately regulated via the imprinted expression of *KCNQ1OT1*, which encodes an untranslated RNA. In skin fibroblasts from five monozygotic twin pairs discordant for BWS, each affected twin had an imprinting defect at *KCNQ1OT1* on 11p15, whereas the unaffected twin did not. Five additional monozygotic twin pairs, for whom only blood was available, also displayed an imprinting defect at *KCNQ1OT1.* It is possible that discordance for BWS in MZ twins is due to unequal splitting of the inner cell mass during twinning, thereby causing differential maintenance of imprinting at *KCNQ1OT1.* Alternatively, we propose that *KCNQ1OT1* is especially vulnerable to a loss of imprinting event, caused by a lack of maintenance DNA methylation at a critical stage of preimplantation development, and that this loss of imprinting predisposes to twinning as well as to discordance for BWS. These data underscore the importance of continued surveillance of children born following assisted reproductive technologies that impact the preimplantation embryo.

INTRODUCTION

Beckwith–Wiedemann syndrome (BWS) (1–3) is a phenotypically variable overgrowth syndrome that is characterized by pre- and postnatal overgrowth, visceromegaly, macroglossia, abdominal wall defects, ear abnormalities, hemihyperplasia and neonatal hypoglycemia. Children with BWS are at increased risk (7–21%) of developing childhood tumors (4–7).

The molecular etiologies described for BWS are heterogeneous and all involve a cluster of genes on 11p15 (8–10). Although most cases of BWS are sporadic, dominant inheritance with preferential maternal transmission is observed in some 10–15% (1,11,12). For some of these families, linkage to chromosome 11p15 has been demonstrated (13,14). Although chromosome abnormalities rarely occur in BWS, such cases include maternally derived translocations and inversions of chromosome 11p15 and paternally derived partial

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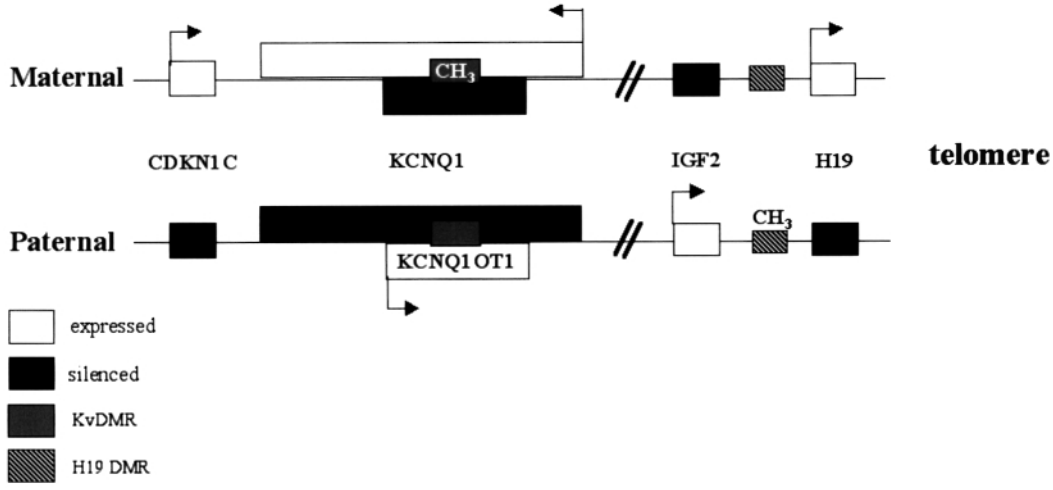

Figure 1. Genes and their imprinting status on human chromosome 11p15 (not all genes are shown). Expressed genes are indicated by unfilled boxes and silenced genes by black boxes. The differentially methylated regions H19 DMR and KvDMR1 are indicated. CH_3 indicates the methylated allele. Alternate names for the following approved gene names are: CDKN1C = $p57^{KIP2}$, KCNQ1 = KvLQT1, and KCNQ1OT1 = LIT1 and KvLQT1-AS.

trisomy of chromosome 11p15 (15–17). These parent-of-origin effects, together with the finding of paternal uniparental disomy (UPD) of chromosome 11p15 in 20% of BWS cases (18–22), all suggest that abnormal genomic imprinting plays an important role in the etiology of BWS (Fig. 1 and Table 1).

BWS patients have been shown to have mutations or epimutational abnormalities in several genes on 11p15 (Fig. 1 and Table 1). Coding mutations in the CDKN1C gene, a maternally expressed cyclin-dependent kinase inhibitor, have been found in 5% of sporadic BWS cases and approximately 30–50% of dominantly transmitted cases of BWS (23–29). Epimutations affecting both transcription and methylation of imprinted genes have been shown to occur in BWS. Such alterations have been documented for three genes in 11p15: KCNQ1OT1, H19 and insulin-like growth factor 2 (IGF2). KCNQ1OT1 encodes a paternally expressed untranslated RNA transcribed in antisense orientation and overlapping with KCNQ1 (30,31). Biallelic expression of KCNQ1OT1 is seen in 50% of BWS patients (30). Such BWS patients show partial or complete loss of maternal allelic-specific methylation at KvDMR1, a CpG island upstream of KCNQ1OT1 (30–32).

H19, which has a differentially methylated promoter region, is a maternally expressed gene coding for an untranslated RNA (33,34). The IGF2 gene, which is co-regulated with H19, encodes a paternally expressed growth factor. In 1–2% of BWS cases, the H19 promoter region is biallelically methylated with loss of H19 expression and biallelic IGF2 expression (35). An additional 30% of BWS cases show biallelic IGF2 expression that is accompanied by normal methylation and transcription of H19 (36,37).

Genotype and phenotype discordance in monozygotic twins can have a variety of causes, including non-random X inactivation (38). The excess of female discordant monozygotic twins reported for BWS has prompted investigators to speculate that monozygous twinning, genomic imprinting and X inactivation may be mechanistically as well as temporally related (39).

We report that monozygotic twins with BWS are greatly increased in frequency over that in the general population. Also, we show that discordance for BWS in such monozygotic twins is associated with differences in the imprinting status at KCNQ1OT1 and the methylation status at KvDMR1. Furthermore, discordance for BWS could not be associated with non-random X-inactivation patterns.

Table 1. Chromosome 11p15 genetic and epigenetic alterations associated with Beckwith–Wiedemann syndrome[a]

Genetic/epigenetic alteration	Frequency in Beckwith–Wiedemann syndrome cases	Frequency in Beckwith–Wiedemann syndrome twin pairs (n = 10)
11p15 translocations/duplications	1–2%	0/10
11p15 uniparental disomy	20%	0/10
Hypermethylation of H19	1–2%	0/10
CDKN1C mutation	5%[b]	0/10
Loss of KvDMR1 methylation/ biallelic expression of KCNQ1OT1[a]	50%	10/10
Unknown	20%	0/10

[a]Note that approximately 85% of BWS cases are sporadic with normal karyotypes.
[b]5% of sporadic cases; 30–50% of autosomal dominant cases.

RESULTS

In our sample of 250 BWS patients, there were 20 sets of monozygotic twins discordant for BWS and 2 sets of dizygotic twins. Monozygosity was established (probability > 99.9%) by analysis of polymorphic microsatellite markers (data not shown). These data demonstrate a highly significant excess of monozygotic twins (8%) compared with the general population [0.3–0.4%; $P < 0.0001$; 95% confidence interval (CI) = (4.6%, 11.3%)] (40,41) and a significant excess of female (16) over

Table 2. Incidence and sex ratios of control twins and twins with Beckwith–Wiedemann syndrome

	Dizygotic (DZ)	Monozygotic (MZ)	Sex ratio of MZ (female : male)
Normal population	0.7–1.1%	0.3–0.4%	1 : 1
Study population	0.8%	8%[a]	4 : 1 (16 : 4)
Data from literature	0	17%	2.4 : 1 (12 : 5)

[a]Only one of these twin pairs has been reported in the literature.

male (4) monozygotic twins [$P < 0.007$; 95% CI = (62.4%, 97.5%)] (Table 2) (42). However, the incidence of dizygotic twins (0.8%) was not significantly different from that in the general population (43) [0.7–1.1%; $P = 0.65$; 95% CI = (0%, 1.9%)]. These findings are consistent with the numerous reports of monozygotic twins discordant for BWS (Table 2) (39,44–53).

To investigate the molecular basis for the phenotypic discordance for BWS within monozygotic twin pairs, we examined patient samples from discordant twin pairs for five different genetic and epigenetic lesions known to be associated with BWS (Fig. 1 and Table 1). We first analyzed the methylation status of KvDMR1 using the differentially methylated NotI site in the CpG island upstream of KCNQ1OT1. Hypomethylation at KvDMR1 was defined as a methylation index of 0.42 or less based on previous normative data (54). Previous studies by Smilinich et al. (32), Mitsuya et al. (31), and Lee et al. (30) have demonstrated in somatic cell hybrids that the maternal allele is methylated and the paternal allele is non-methylated at KvDMR1. Then, allele-specific transcription of the imprinted transcript KCNQ1OT1 was assayed using RT–PCR-based assays of three single-nucleotide polymorphisms (SNPs) (30).

We found that for each of the five twin pairs for whom fibroblast cultures could be obtained, the affected twin showed loss of methylation at KvDMR1. Four of these showed concomitant biallelic expression at KCNQ1OT1; one was uninformative for all available SNPs. The unaffected twins of the discordant pairs maintained normal methylation at KvDMR1 and normal monoallelic expression of KCNQ1OT1 (Fig. 2B,C,F,G and Table 3). In contrast, four sets of

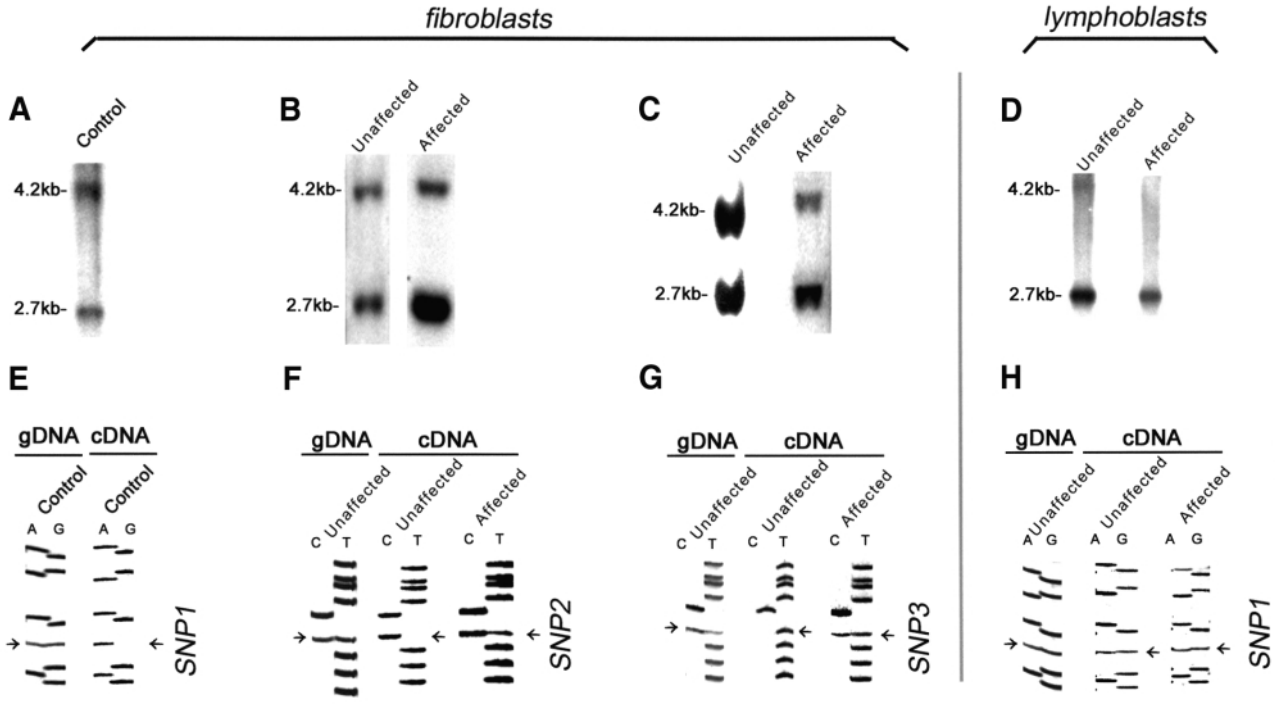

Figure 2. Analysis of methylation at KvDMR1 and allelic expression of KCNQ1OT1 in monozygotic twins discordant for BWS. (A) Southern blot of KvDMR1 for a control monozygous twin (Control 3) with a normal methylation index of 0.50. The upper band (4.2 kb) is methylated and of maternal origin, the lower band (2.7 kb) is non-methylated and of paternal origin. (B) Southern blots of KvDMR1 for the twin pair MZ10. The unaffected twin has a normal methylation index of 0.55 and the affected twin shows loss of maternal methylation with a methylation index of 0.20. (C) Southern blots of KvDMR1 for fibroblasts from the monozygotic twin pair MZ1. The unaffected twin has a normal methylation index of 0.50 and the affected twin shows loss of maternal methylation with a methylation index of 0.29. (D) Southern blots of KvDMR1 for lymphoblasts of twin pair MZ7. Both the unaffected and affected twins show loss of maternal methylation with methylation indices of 0.01 and 0 respectively. (E) Sequencing results for a control monozygous twin heterozygous in genomic DNA for SNP1 (gDNA, left-hand arrow) and showing normal monoallelic KCNQ1OT1 expression (cDNA, right-hand arrow). Only the informative lanes for the nucleotide sequencing reactions (C and T or A and G) are shown in (E–H). (F) The sequence of genomic DNA for the unaffected twin of twin pair MZ10. The affected twin is also heterozygous for SNP2 (data not shown). Allele-specific expression for KCNQ1OT1 is shown for both twins. The sequencing results for cDNA of the unaffected twin show normal monoallelic expression of KCNQ1OT1, whereas the affected twin shows biallelic expression. (G) (gDNA) Twin pair MZ1 is heterozygous for SNP3 (only the unaffected twin is shown). Allele-specific expression for KCNQ1OT1 in the unaffected twin (lane 2) shows normal monoallelic expression, whereas expression for the affected twin (lane 3) is biallelic. (H) Allele-specific expression of KCNQ1OT1. The gDNA lanes show that the twins are heterozygous for SNP1 (only the unaffected twin is shown). Both the affected and unaffected twins show biallelic expression of KCNQ1OT1 (lanes 2 and 3 respectively).

Table 3. Methylation status at KvDMR1 and transcription status of KCNQ1OT1 in fibroblasts of control twins and monozygotic twins discordant for Beckwith–Wiedemann syndrome

Twin pairs	Sex	Methylation at KvDMR1 (MI[a])		Allelic transcription of KCNQ1OT1	
Controls		Twin 1	Twin 2	Twin 1	Twin 2
Control 1	F	0.50	0.52	Monoallelic	Monoallelic
Control 2	F	0.50	0.47	Uninformative	Uninformative
Control 3	F	0.48	0.50	Monoallelic	Monoallelic
Control 4	F	0.50	0.53	Monoallelic	Monoallelic
Beckwith–Wiedemann syndrome twins		Unaffected twin	Affected twin	Unaffected twin	Affected twin
MZ1	F	0.50	0.29	Monoallelic	Biallelic
MZ2	F	0.52	0.08	Monoallelic	Biallelic
MZ3	F	0.48	0.05	Monoallelic	Biallelic
MZ4	F	0.50	0.15	Uninformative	Uninformative
MZ10	M	0.55	0.20	Monoallelic	Biallelic

[a]Methylation index is defined in Materials and Methods.

monozygotic control non-BWS twins showed normal methylation of KvDMR1 and allele-specific transcription of KCNQ1OT1 (Fig. 2A,E and Table 3).

We also analyzed lymphoblastoid cell lines from eight monozygotic twin pairs who were discordant for BWS (Table 4 and Fig. 2D,H). We found decreased methylation of KvDMR1 and/or biallelic expression of KCNQ1OT1 for all eight twin pairs, i.e. 16 twins. Studies in fibroblasts for three of these twin pairs (MZ1, MZ3 and MZ10) had shown an epigenetic change only in the affected twin (Table 3). However, in the hematopoietic cells of these twins, the imprinting defects were variably reflected in both the affected and unaffected individuals of the twin pair (Fig. 2D,H). This is most likely due to fetal sharing of circulations, as commonly occurs during monozygotic twin development (55). Furthermore, the high rate of loss of imprinting observed in the blood of these twin pairs may indicate a selective growth advantage for hematopoietic cells having loss of imprinting at KvDMR1/KCNQ1OT1, thereby leading to overrepresentation of such lymphoid progenitors.

It is unlikely that the above observations are due to spurious parent-of-origin effects that have been observed in methylation of chromosome 7 in cultured lymphoblast cell lines (56), especially since we found no changes in methylation at KvDMR1 in 23 lymphoblast lines from normal controls, including three sets of normal monozygotic twins.

The discordant BWS twin pairs showed no evidence of the four other chromosome 11 abnormalities commonly associated with BWS, namely cytogenetic rearrangements, uniparental disomy for 11p15, hypermethylation at H19 or mutations in the coding regions of CDKN1C lesions (data summarized in Table 1). The imprinting status of one chromosome 11 imprinted gene and one non-chromosome 11 imprinted gene were assessed. We examined the methylation status of the H19 differentially methylated region in the lymphoblast DNA from the twin pairs discordant for BWS, and found normal methylation indices in both the affected and unaffected twins (data not shown). We also evaluated the methylation pattern of SNRPN, an imprinted gene on chromosome 15 (57), and found it to be normal in all 10 discordant twin pairs (data not shown).

Our data show that whereas 50% of sporadic BWS cases demonstrate loss of imprinting at KCNQ1OT1, all 10 of 10 monozygotic twin pairs tested exhibit this imprinting defect either in the non-hematopoietic tissue of the affected twin or in the hematopoietic tissue of one or both twins. The fact that all 10 cases of BWS in monozygotic twins have a common epigenetic alteration is significantly different [$P < 0.01$; 95%

Table 4. Methylation status at KvDMR1 and transcription status of KCNQ1OT1 in lymphoblasts of monozygotic twins discordant for Beckwith–Wiedemann syndrome

Beckwith–Wiedemann syndrome twin pairs	Sex	KvDMR1 (MI[a])		KCNQ1OT1	
		Unaffected twin	Affected twin	Unaffected twin	Affected twin
MZ1	F	0.48	0.32	Monoallelic	Biallelic
MZ3	F	0.23	0.12	Biallelic	Biallelic
MZ5	F	0.30	0.18	Biallelic	Biallelic
MZ6	F	0.22	0.05	Biallelic	Biallelic
MZ7	F	0.01	0.00	Biallelic	Biallelic
MZ8	F	0.10	0.27	Uninformative	Uninformative
MZ9	F	0.24	0.04	Biallelic	Biallelic
MZ10	M	0.45	0.32	Monoallelic	Biallelic

[a]Methylation index is defined in Materials and Methods.

CI = (0.7%, 1.0%)] from the genetic heterogeneity commonly observed in BWS (Table 1).

The large number of female monozygotic twins discordant for BWS has previously prompted investigators to suggest that the X-inactivation process may be intimately involved with an anomalous imprinting process and monozygotic twinning (39). Therefore, we analyzed X-inactivation patterns in the fibroblast samples from female monozygotic twins discordant for BWS using a methylation-dependent analysis of the androgen receptor gene (58). Normally, X inactivation is a stochastic process resulting in allele ratios for the two X's randomly distributed around 50 : 50; skewed X-inactivation patterns of 80 : 20 or greater are uncommon in normal individuals but variably reported in female monozygotic twins (59). Reciprocal skewing of X inactivation has been reported in female monozygotic twins discordant for X-linked recessive disorders (38); the affected twin preferentially expresses the mutated allele, whereas the normal twin preferentially expresses the normal allele.

Skewing of X inactivation has been observed in control female monozygotic twins as previously reported (59), as well as in female monozygotic twins discordant for BWS (Fig. 3 and Table 5). In normal singlet females, we find a range of X inactivation ratios up to 100%. However, the majority of females have X inactivation ratios with less than 80% inactivation of one allele. In a sample of 60 such normal female individuals tested, 51 (85%) had X inactivation ratios that were 80% or less skewing for one allele (data not shown). As shown in Table 5, four twin pairs discordant for BWS were tested for X inactivation status. In three of the BWS twin pairs, X inactivation skewing of 80% inactivation or more was seen in one twin while the other twin had a more balanced X inactivation pattern (MZ1, MZ2 and MZ4). In two of these families, the twin with BWS had more skewing, while in one

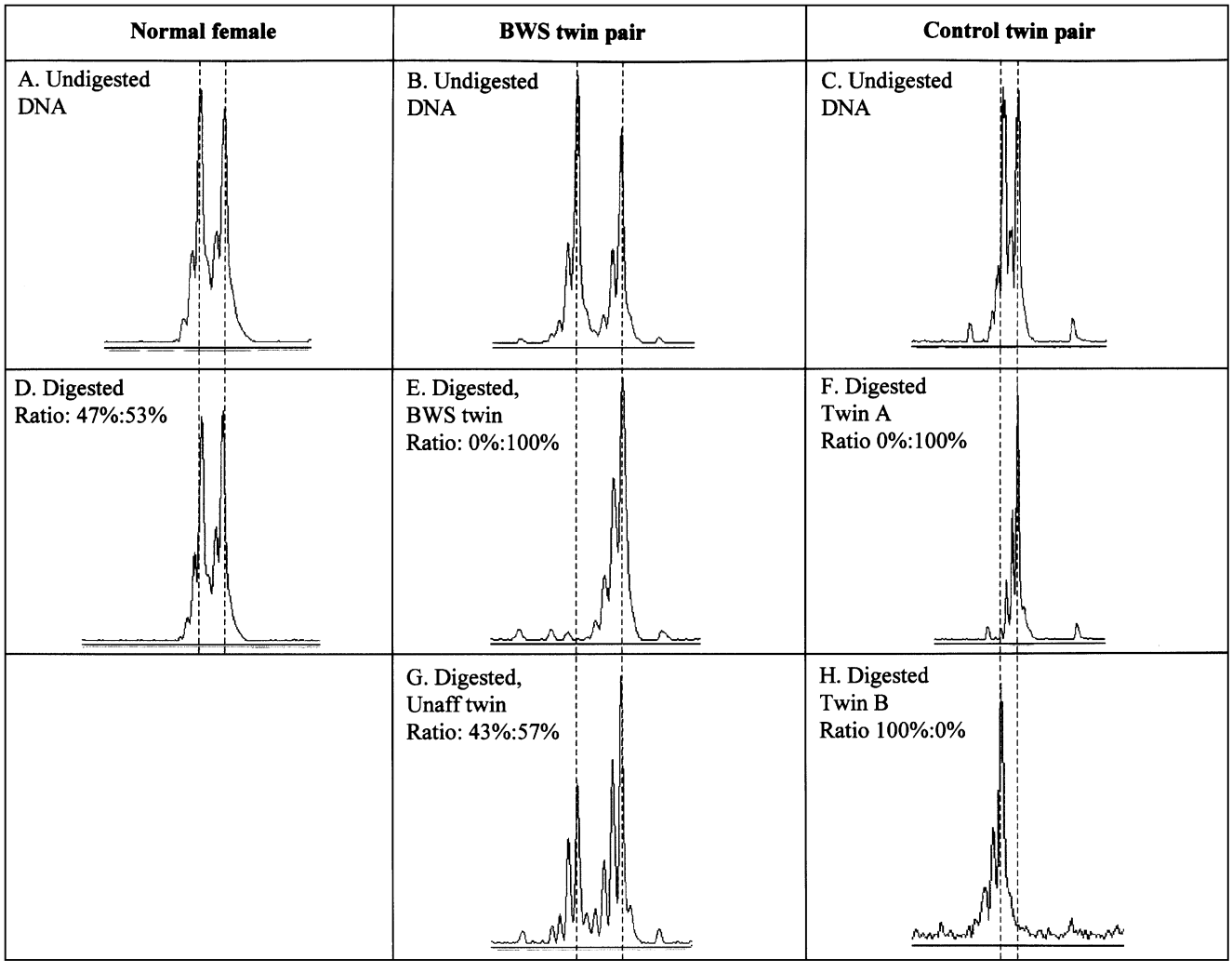

Figure 3. Analysis of X chromosome inactivation using methylation analysis of the androgen receptor gene (AR). (A–C) Results from amplification of undigested genomic DNA to indicate AR alleles. (D–H) Results from amplification of HhaI/HpaII/BamHI-digested DNA as described in Materials and Methods. These panels show a normal female with approximately 50% : 50% X inactivation (A, D), a BWS twin pair with 0% : 100% inactivation in one twin and 43% : 57% inactivation in the other (family 4 of Table 5: B, E, G), and a control twin pair with 0% : 100% inactivation in one twin and 100% : 0% inactivation in the other (family 7 of Table 5: C, F, H).

Table 5. X chromosome inactivation in fibroblasts of control monozygotic twins and monozygotic twins with Beckwith–Wiedemann syndrome

Twin pairs	Percentage inactive X chromosome (allele 1 : allele 2)	
Controls	Twin 1	Twin 2
Control 1	84% : 16%	0% : 100%
Control 2	0% : 100%	76% : 24%
Control 3	59% : 41%	84% : 16%
Control 4	86% : 14%	15% : 85%
Control 5	0% : 100%	100% : 0%
Beckwith–Wiedemann syndrome twins	Unaffected twin	Affected twin
MZ1	88% : 12%	50% : 50%
MZ2	43% : 57%	0% : 100%
MZ3	71% : 29%	24% : 76%
MZ4	40% : 60%	15% : 85%

family, the unaffected twin had more skewing. In one twin pair discordant for BWS (MZ3), neither twin showed X inactivation skewing.

The five control MZ twin pairs tested showed the same patterns of X inactivation seen in the MZ twins discordant for BWS (Table 5). In two of the control MZ twin pairs (C2 and C3), one twin showed skewing of 80% inactivation or greater, while the other had inactivation of less than 80% for one allele. Three control MZ twin pairs showed skewing in both twins (C1, C4 and C5).

DISCUSSION

Discordant BWS in monozygotic twins provides an exceptional opportunity to investigate the molecular and biological bases of BWS and monozygotic twinning. In fact, our work has demonstrated that the incidence of monozygotic twinning in BWS is dramatically increased. The majority of this increase is for female monozygotic twins. In skin fibroblasts from five such twin pairs, only the affected twin showed altered maternal methylation of KvDMR1, the CpG island upstream of KCNQ1OT1, as well as biallelic expression of KCNQ1OT1. We found that similar abnormalities were present in the hematopoietic cells from both the affected and unaffected monozygotic twins. Since sharing of blood circulation is a common feature of monozygotic twinning (55), it is not surprising that the imprinting alterations at KvDMR1/KCNQ1OT1 are observed in both affected and unaffected twins of such monozygotic twin pairs discordant for BWS.

Given the genetic and molecular heterogeneity usually associated with BWS, our finding that 10 monozygotic twin pairs all exhibit the same imprinting defect at KCNQ1OT1 suggests that the monozygotic twinning in these cases is in some way mechanistically linked to the imprinting error found in such twin pairs. That is, such epigenetic alterations may increase the chance of monozygotic twinning or conversely the monozygotic twinning process may predispose to epigenetic alterations at KvDMR1.

Previous studies have addressed genotypic and phenotypic discordance in monozygotic twin pairs. As Machin (38) notes, most monozygotic twins are not identical, since there may be major discordance for birth weight, congenital anomalies and genetic disease. He speculates that there may be unequal allocation of early progenitor cells to the monozygotic twins that may have important implications for the cascade of developmental events during embryogenesis.

Postzygotic events thus may lead to the formation of two or more cell clones in the inner cell mass and early embryo that actually stimulate the monozygotic twinning event. In monozygotic twin pairs, if two different cell clones exist in one early zygote, mutual 'recognition and repulsion' could result in monozygotic twinning. It is possible, therefore, that a group of cells carrying an imprinting alteration at KCNQ1OT1 could preferentially increase the growth rate of this group of cells compared with the normal cells, thereby generating asymmetry of the entire cell mass and increasing the chance of separation of the epigenotypically distinct cell clones.

Since the timing of monozygotic twinning and X inactivation overlap in the pre-implantation stage of blastocyst development (60,61), the female monozygotic twinning process may affect X inactivation or the distribution of inactivated X chromosomes. There have been reports regarding the occurrence of skewed X inactivation both in normal female monozygotic twins and in female monozygotic twins discordant for X-linked disorders (38,60). In our study, more skewed X inactivation was observed for monozygotic twins than in control singletons. However, the presence of non-random X inactivation did not correlate with the presence or absence of BWS in these twins, indicating that skewing of X inactivation is associated with monozygotic twinning but not with the acquisition of BWS per se as proposed previously (39).

However, the significant female preponderance in monozygotic twins discordant for BWS and an imprinting defect on chromosome 11 could be related to a variety of other sex-related factors. One possibility is that a developmental error occurs with equal frequency in male and female embryos, with a lethal effect on the male monozygotic twins. Alternatively, it may be that the lag in early development of female embryos as compared with males (62), possibly secondary to the X inactivation process, may make female monozygotic twin embryos more susceptible to certain developmental errors.

In this regard, an attractive explanation for BWS discordance in monozygotic twins is a failure of maintenance methylation during a single cell cycle at (or just prior to) the twinning event. The resulting hemimethylated daughter duplex would be converted in the next S phase to a fully methylated and an unmethylated sister chromatid, which would segregate to different blastomeres, and could then separate in the twinning event. Depending on the timing of twinning, it is possible that failure of maintenance methylation could result in mosaicism or complete discordance for imprinting defects in one or both twins.

A plausible mechanism by which maintenance methylation could be perturbed for one cell cycle in the early embryo has emerged from genetic studies of the Dnmt1 gene in mice (63). $Dnmt1_o$, a specialized oocyte-specific form of the major maintenance DNA methyltransferase, is required specifically for maintenance methylation of imprinted single copy sequences during the fourth S phase. In the absence of $Dnmt1_o$, one-half of the normally imprinted alleles are demethylated,

and a loss of imprinting phenotype consistent with mosaic reactivation of normally silenced imprinted alleles is observed. We suggest that KvDMR1 is less efficiently methylated by the human orthologue of $Dnmt1_o$ than are other DMRs, and that BWS arises when maintenance methylation of KvDMR1 fails to occur. $Dnmt1_o$ or Dnmt1 is also required for X inactivation, and association of DNMT1 with one of the X chromosomes in females may reduce the amount available for maintenance methylation of KvDMR1, thereby accounting for the sex bias.

The preimplantation phase of embryonic development appears from our study to be crucial for imprint maintenance at KCNQ1OT1. Epigenetic imprinting defects in monozygotic twins are reminiscent of similar epigenetic errors identified in cloning experiments in animals. An example of this is the epigenetic alteration in the growth-regulatory gene IGF2R associated with large offspring syndrome in sheep embryos (64). Studies of assisted reproductive technologies in humans show an increase in the rate of monozygotic twinning (65), and a recent study reports a case of BWS following in vitro fertilization (66). These data suggest that close scrutiny of new reproductive technologies is warranted, given that manipulation of preimplantation conditions can affect both the rate of monozygotic twinning and epigenetic programming, with consequent risks for developmental anomalies and tumor predisposition.

MATERIALS AND METHODS

Patient material

250 cases of BWS have been accrued in our database from medical centers across Canada and the USA. We confirmed the diagnosis of BWS in one of each of the 22 twin pairs. Our diagnosis was based on the presence of at least three of the following features: macrosomia, macroglossia, hemihyperplasia, ear creases/pits, abdominal wall defect, omphalocele, umbilical hernia or embryonal tumor. A diagnosis of BWS was also made if at least two of the preceding features plus one of neonatal hypoglycemia, abdominal organomegaly or renal malformation were present.

Of the 22 twin pairs who were discordant for BWS, microsatellite analysis showed that 20 twin pairs were monozygotic and 2 pairs were dizygotic. We obtained adequate samples to undertake comprehensive molecular testing in only 10 of these 22 discordant twin pairs. In this series of 10 twin pairs available for molecular testing, the unaffected twins had no features of BWS. In one case (MZ10), the unaffected twin had three hemangiomas, but no other findings consistent with a diagnosis of BWS. None of these BWS patients had positive family histories. For two of the monozygotic twin pairs discordant for BWS (MZ2 and MZ4), only fibroblasts were available, and for five twin pairs (MZ5, MZ6, MZ7, MZ8 and MZ9), only lymphoblasts/lymphocytes were available. For three twin pairs (MZ1, MZ3 and MZ10), both cell types were obtained. Parental samples were available for all 10 families. Control samples were obtained from normal monozygotic twins as well as from monozygotic twins with osteogenesis imperfecta. This study was approved by the Research Ethics Board of the Hospital for Sick Children, Toronto, Canada.

Zygosity studies of twins

We performed zygosity testing using PCR amplification of eight microsatellite markers (D5S818, vWF, D13S317, THO1, D7S820, TPOX, D16 and CSF1PO) and the AMELX/Y sex-differentiation marker. An estimate of the probability of monozygosity is given based on the population frequency of the alleles present in both twins, with a value approaching greater than 99.9%.

Cell lines

Lymphoblast lines were maintained in RPMI 1640 media supplemented with 15% fetal calf serum. Fibroblast strains were maintained for fewer than 10 passages in α-MEM supplemented with 10% fetal calf serum.

Analysis of methylation and transcription on chromosome 11

DNA or RNA was obtained from patient samples for analyses of chromosome 11 uniparental disomy, methylation status at H19 and KvDMR1, and allelic expression of KCNQ1OT1 (29,54). Briefly, Southern blots of DNA samples that were digested with NotI and EcoRI were hybridized with a [^{32}P]dCTP-labeled DMR probe (Fig. 2A–D) (54). Allele-specific expression of KCNQ1OT1 was determined by DNA sequencing of gel-purified RT–PCR products from lymphoblasts or fibroblasts of patients informative for SNP1, SNP2 and SNP3 polymorphisms as described previously (30). The genomic sequences for these SNPs are as follows: for SNP1, AGCTCTGACC(G/A)TCAGACCCCC; for SNP2, GAAATGTGTA(C/T)GGCATGTTGT; and for SNP3, GTAGACAGTG(C/T)GGCCCTCTCC.

Analysis of methylation at SNRPN locus on chromosome 15

To assess the methylation status of a non-chromosome 11 imprinted gene, we analyzed methylation at the SNRPN locus by Southern blot analysis using the restriction enzymes XbaI and NotI and a probe containing exon 1 of the SNRPN gene (67).

Analysis of androgen receptor trinucleotide repeat for X inactivation

The X inactivation assay used is a modification of the human androgen receptor (AR) gene assay described by Allen et al. (58). The androgen receptor $(CAG)_n$ repeat region at Xq11–q12 is used as a marker of X inactivation. Genomic DNA is first amplified using primers that flank the AR repeat to determine the different $(CAG)_n$ repeat alleles carried by each individual to use as markers for each of the X chromosomes. Then, two methylation-sensitive enzymes HhaI and HpaII are used to digest genomic DNA, while a third restriction enzyme, BamHI is used to pre-cut genomic DNA to improve PCR efficiency. The HhaI and HpaII enzymes digest unmethylated DNA in the AR $(CAG)_n$ repeat region on active, unmethylated X chromosomes and do not cut methylated sites on inactive, methylated X chromosomes. The PCR primers flanking the AR $(CAG)_n$ repeat region are then used again to amplify the DNA

after digestion. Only the undigested, inactive X chromosome's AR allele will be amplified, allowing an estimation of the degree of X inactivation of each X chromosome. PCR products were run on an automated sequencer to generate a ratio comparing the digested versus undigested PCR-amplified DNA from the same patient for comparison. X inactivation patterns are described as skewed if the ratio of the active to inactive X chromosome is less than 20% or greater than 80%.

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