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ARTICLE

Hypomethylation at multiple maternally methylated imprinted regions including *PLAGL1* and *GNAS* loci in Beckwith–Wiedemann syndrome

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Genomic imprinting is an epigenetic phenomenon restricting gene expression in a manner dependent on parent of origin. Imprinted gene products are critical regulators of growth and development, and imprinting disorders are associated with both genetic and epigenetic mutations, including disruption of DNA methylation within the imprinting control regions (ICRs) of these genes. It was recently reported that some patients with imprinting disorders have a more generalised imprinting defect, with hypomethylation at a range of maternally methylated ICRs. We report a cohort of 149 patients with a clinical diagnosis of Beckwith–Wiedemann syndrome (BWS), including 81 with maternal hypomethylation of the *KCNQ1OT1* ICR. Methylation analysis of 11 ICRs in these patients showed that hypomethylation affecting multiple imprinted loci was restricted to 17 patients with hypomethylation of the *KCNQ1OT1* ICR, and involved only maternally methylated loci. Both partial and complete hypomethylation was demonstrated in these cases, suggesting a possible postzygotic origin of a mosaic imprinting error. Some ICRs, including the *PLAGL1* and *GNAS/NESPAS* ICRs implicated in the aetiology of transient neonatal diabetes and pseudohypoparathyroidism type 1b, respectively, were more frequently affected than others. Although we did not find any evidence for mutation of the candidate gene *DNMT3L*, these results support the hypotheses that trans-acting factors affect the somatic maintenance of imprinting at multiple maternally

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methylated loci and that the clinical presentation of these complex cases may reflect the loci and tissues affected with the epigenetic abnormalities.

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Introduction

The expression of imprinted genes in the mammalian genome is dependent on parental origin. These genes have key roles in the control of foetal growth and many of them have been implicated in human growth disorders and cancer.[1–3] Epigenetic modifications including DNA methylation differentially mark imprinted genes in egg and sperm and lead to the unequal expression of parental alleles in somatic cells.[1] Maintenance of differential DNA methylation between maternal and paternal alleles of specific sequences is crucial to proper imprinting control, and methylation defects at imprinted loci have been described in human imprinting disorders.[4]

Beckwith–Wiedemann syndrome (BWS, OMIM 13650) is a tumour-associated overgrowth disorder that is mainly caused by the dysregulation of a cluster of imprinted genes located at chromosome 11p15.5.[5] Although some familial cases with dominant maternal inheritance have been described,[6,7] BWS cases are mostly sporadic and are characterised by methylation abnormalities at one of the two imprinting control regions (ICRs) (*H19* and *KCNQ1OT1* ICR, also known as IC1 and IC2) or paternal uniparental disomy (UPD) at 11p15.5. Several genes with growth-regulatory properties are part of the 11p15.5 cluster, including *IGF2* and *H19*, which are controlled by the *H19* ICR, and *CDKN1C* and *KCNQ1OT1*, which are controlled by the *KCNQ1OT1* ICR. We recently demonstrated that gain of methylation at the *H19* ICR (IC1 defect, ICD1) is independent of the DNA sequence context in several BWS patients and proposed that these molecular defects generally arise as *de novo* epimutations in early embryogenesis.[8]

A subset of BWS patients with hypomethylation at the *KCNQ1OT1* ICR (IC2 defect, ICD2) have loss of methylation at other imprinted loci.[9] Hypomethylation of multiple maternally methylated ICRs including *KCNQ1OT1* has also been demonstrated in cases of transient neonatal diabetes[10,11] (TND, OMIM 601410). TND is a disease characterised by intrauterine growth retardation and transient hyperglycaemia that results from dysregulation of the imprinted growth inhibitor and antiapoptotic *PLAGL1 (ZAC)* gene located at chromosome 6q24.[12] A minority of TND patients have BWS features, such as macroglossia and abdominal wall defects, suggesting an interaction between the 6q24 and 11p15.5 loci. However, no molecular defect at *PLAGL1* has been reported in BWS patients so far and the relationship between BWS and TND remains undefined.[9,13]

We have further investigated the role of the imprinted genes other than the 11p15.5 cluster in BWS, by analysing DNA methylation at 11 ICRs with multiple techniques and screening a large number of cases through a multicentre study. The results obtained demonstrate that a subset of BWS patients display hypomethylation at multiple maternally methylated ICRs, including those involved in TND and pseudohypoparathyroidism type 1b (PHP1b), and support the hypothesis that multiple maternal hypomethylation may present with different clinical phenotypes.

Patients and methods

Patients

The patients comprised 149 individuals with clinical diagnosis of BWS according to the criteria reported by DeBaun *et al.*[14] These included 105 patients from Italy and 44 from the Netherlands (Table 1). Their clinical features are summarised in Table 2. Genetic analyses of the Italian cohort were performed with the informed consent of the

Table 1 Classification of epigenetic defects found in 149 BWS patients

	Italian cohort		*Dutch cohort*		*Total*		
	No. of patients	*No. of patients with multiple defects*	*No. of patients*	*No. of patients with multiple defects*	*No. of patients*	*No. of patients with multiple defects*	*Fraction*
No defect found	24	0	6	0	30	0	0
UPD11	13	0	5	0	18	0	0
ICD1[a]	16	0	4	0	20	0	0
ICD2	52	9	29	8	81	17	0.21
Total	105	9	44	8	149	17	

BWS, Beckwith–Wiedemann syndrome.
[a]Four of these individuals carried a maternally inherited microdeletion in the *H19* ICR (Sparago *et al*[6]).

Table 2 Clinical features of the BWS patients with multiple maternal hypomethylation

Patients	*Sex*	*Additional genes involved*	*Birthweight centile*[a]	*Postnatal overgrowth*[b]	*Macro-glossia*	*Abdominal wall defect*	*Pits and creases*	*Hypo-glycaemia*	*Poly-hydramnio's*	*Naevus flammeus*	*Hemihyper-trophy*	*Organo-megaly*	*Childhood cancer*	*Born after IVF*	*Others*
BWS1	M	*MEST, PLAGL1, IGF2R, PEG3*	10–25	Y	Y	Y	N	N	N	Y	N	N	N	N	Twin[c], respiratory distress
BWS2	M	*MEST, PLAGL1, IGF2R*	10–50	Y	Y	Y	Y	Y	N	Y	Y	Y	N	N	Cryptorchidism, hypothyroidism
BWS3	F	*MEST*	10–50	Y	N	Y	Y	N	N	N	N	Y	N	N	Speech retardation, seizures
BWS4	M	*MEST, PLAGL1, GNAS, NESPAS*	90	Y	Y	N	N	N	N	Y	N	N	N	N	
BWS5	F	*GNAS, IGF2R*	75	NA	Y	Y	Y	Y	N	N	N	Y	N	N	
BWS6	NA	*PLAGL1, IGF2R, GNAS*	NA	NA	NA	Y	NA	NA	NA	NA	NA	NA	NA		Fetus
BWS7	M	*PLAGL1*	75	Y	Y	Y	N	N	N	N	N	N	N	Y	Cryptorchidism, hypospadias
BWS8	M	*IGF2R, GNAS, NESPAS*	>97	Y	Y	N	N	N	N	N	N	N	N	N	
BWS9	F	*PLAGL1*	90	Y	Y	N	Y	Y	N	N	N	Y	N	N	ASD
BWS10	F	*PLAGL1, NESPAS, GRB10*	10	NA	Y	Y	N	N	N	N	N	N	N	N	
BWS11	M	*MEST*	97	N	Y	Y	Y	Y	Y	Y	Y	Y	N	N	Apnoea, feeding and hearing problems
BWS12	F	*GRB10*	50	N	Y	Y	N	N	NA	NA	N	N	N	N	Premature birth (28 weeks), cryptorchidism
BWS13	F	*MEST, GNAS, NESPAS*	50	Y	Y	N	N	N	N	Y	N	Y	N	N	
BWS14	F	*GNAS, NESPAS*	10	Y	Y	Y	Y	N	Y	N	N	N	N	N	
BWS15	F	*GNAS, NESPAS*	50–90	NA	Y	Y	N	Y	Y	N	N	N	N	N	Macrostomia
BWS16	F	*GNAS, NESPAS*	97	NA	Y	Y	Y	Y	Y	Y	N	Y	N	N	Premature birth (28 weeks)
BWS17	F	*NESPAS*	90	N	Y	N	N	Y	N	Y	N	N	N	N	
Multiple LOM			58 (±35)	9/17 (0.53)	15/16 (0.94)	12/17 (0.71)	7/16 (0.44)	7/16 (0.44)	4/15 (0.27)	7/15 (0.47)	2/16 (0.12)	7/16 (0.44)	0/16 (0.00)	1/17 (0.06)	
KCNQ1OT1 LOM			80 (±21)	18/36 (0.50)	42/47 (0.89)	36/46 (0.78)	31/44 (0.70)	19/42 (0.45)	7/46 (0.15)	33/46 (0.72)	19/46 (0.41)	12/45 (0.27)	1/46 (0.02)	4/46 (0.09)	
P-value[d]			0.06		0.60	0.53	0.06	0.92	0.32	0.08	0.04	0.20	0.55	0.71	

ASD, atrial septal defect; BWS, Beckwith–Wiedemann syndrome; N, no; NA, not available; Y, yes.
[a]Birth weight centiles were corrected for gestational age and twin pregnancy.[15,16]
[b]>90 centile.
[c]Hormone treatment of the mother for infertility.
[d]χ^2 testing was used in all cases, but birthweight centile, for which *t*-testing analysis was used.

parents of the patients. The experimental plan was approved by the ethical committees of the Second University of Naples, Italy. The collection of clinical data in the Netherlands was approved by the ethical committee of the Academic Medical Centre in Amsterdam (MEC 99/030), and the experiments were performed in accordance with the local (ethical) protocols for research on patient material.

DNA methylation analysis of the imprinted loci

The DNA methylation of ICRs at various imprinted loci was analysed in DNA derived from peripheral blood leukocytes using four different techniques. The *H19* ICR and the *KCNQ1OT1* ICR were analysed by Southern blotting hybridisation, as described earlier.[17] The *KCNQ1OT1* ICR and all the other ICRs were analysed by combined bisulphite restriction analysis (COBRA) and methylation-specific PCR (MS-PCR). For the COBRA, 2 μg of genomic DNA was treated with sodium bisulphite, PCR amplified, the PCR product digested with a restriction enzyme containing a CpG dinucleotide in its target sequence and the fragments separated on a polyacrylamide gel. In some individuals, the methylation of the *KCNQ1OT1, MEST (PEG1)* and *PLAGL1* ICRs was also analysed by bisulphite sequencing. In this case, the PCR products were cloned in Topo pCR2.1 vector (Invitrogen) and the clones sequenced. Primer sequences, genomic locations and amplicon sizes of relevant assays are detailed in Supplementary Table 1; further experimental details are available on request. MS-PCR was performed exactly as described.[18]

Methylation analysis of classical satellite DNA

DNA methylation of classical satellites 2 and 3 was analysed by Southern blotting, as described.[19] Two micrograms of DNA were digested overnight with *Hpa*II, *Msp*I and *Mcr*BC, in separate tubes. Blots were hybridised with oligonucleotide probes specific for satellite 2 of chromosomes 1 and 16 and satellite 3 of chromosome 9.

Mutation analysis of the *DNMT3L* gene

The *DNMT3L* gene was analysed by sequencing all exons and their flanking intronic regions. The primers and PCR conditions used are as described;[20] DNA sequencing was obtained from PRIMM (Italy).

Statistical analysis

Fisher's exact testing and χ^2 analysis were used as appropriate. Statistical significance was taken at the 5% level.

Results

DNA methylation defects at multiple imprinted loci in BWS

We investigated 149 individuals affected with BWS for the presence of DNA methylation defects at imprinted loci other than the 11p15.5 cluster. In all cases, leukocyte-derived DNA was analysed. The BWS samples were classified for the presence of hypermethylation at the *H19* ICR (ICD1), hypomethylation at the *KCNQ1OT1 ICR* (ICD2), paternal UPD at 11p15.5 (UPD11) or no epigenetic defect at 11p15.5, as described earlier[5,21,22] (see Table 1 and Supplementary Figure 1). The same samples were then analysed for the presence of methylation defects at other imprinted loci. One ICR normally methylated on the paternal allele, *GTL2-IG* (14q32), and eight maternally methylated ICRs, *PLAGL1* (6q24), *IGF2R* (6q25.3), *GRB10* (7p21), *MEST* (7q32.2), *SNRPN* (15q11), *PEG3* (19q13), *GNAS* (20q13.32) and *NESPAS* (20q13.32), were investigated. At these loci, methylation levels were determined by using both COBRA and MS-PCR (Table 3).

Abnormal methylation at loci other than 11p15.5 was detected in 17 patients with ICD2 (21%, see Table 1). The results were reported as unme/me ratios and named 'loss of methylation values' (Table 3). In patients with multiple methylation anomalies, only maternally methylated ICRs were affected. No methylation defect of imprinted loci other than 11p15 was detected in patients with ICD1, or in those with UPD11, or in those with no detected anomaly of chromosome 11p15.5. No methylation changes were observed in 120 normal controls with MS-PCR and 100 normal controls with COBRA. The number and type of loci involved and the extent of hypomethylation varied from patient to patient (Table 3). Apart from *KCNQ1OT1*, methylation defects were found at *MEST* in six to seven cases, *PLAGL1* in seven cases, *IGF2R* in six cases, *GNAS* in eight cases, *NESPAS* in 10 cases and *GRB10* in four cases. The observed hypomethylation defects were strikingly mosaic in form; individual patients showed epimutations ranging from modest to total hypomethylation at different loci. Generally, MS-PCR appeared to be more sensitive to allelic imbalances. Nevertheless, qualitatively, the results obtained with the two different methods were comparable (Table 3 and Figure 1a and b).

As MS-PCR and COBRA assay the methylation of only a few CpGs at each site, we investigated the extent of the methylation losses, by bisulphite-sequencing analysis of two cases from the Italian cohort showing significant hypomethylation of the *MEST* and *PLAGL1* ICRs (Figure 2). The patient DNA showed essentially complete hypomethylation within the amplicons sequenced. Demonstration of heterozygosity by the analysis of closely linked SNPs and microsatellites excluded the deletion of the methylated allele in the cases with complete absence of methylation at the ICR (data not shown).

The specificity of the methylation defects was investigated by the analysis of repetitive sequences. We analysed the methylation of satellites 2 and 3, by Southern blotting, in four patients with multiple hypomethylation. The results showed that methylation was present at essentially

Table 3 Loss of methylation values of various imprinted loci in BWS patients with multiple maternal hypomethylation

Patient	*KvDMR1*			*MEST*		*PLAGL1*		*GNAS*		*NESPAS*		*IGF2R*		*GRB10*		*PEG3*		*SNRPN*		*H19*		*GTL2*	
	MSP	*SB*	*COBRA*	*MSP*	*COBRA*	*MSP*	*COBRA*	*MSP*	*COBRA*	*MSP*	*COBRA*	*MSP*	*COBRA*	*MSP*	*COBRA*	*MSP*	*COBRA*	*MSP*	*COBRA*	*MSP*	*COBRA*	*MSP*	*COBRA*
BWS1	23	20	6	∞	5	∞	20	1	1	1	ND	38	3	1	1	1	2	1	1	1	1	1	1
BWS2	30	20	3	23	3	∞	9	2	1	6	ND	18	5	1	1	1	1	1	1	1	1	1	1
BWS3	33	20	5	2	2	1	1	1	1	1	ND	1	1	∞	6	1	1	1	1	1	1	1	1
BWS4	3	2	3	3	2	3	2	2	2	3	ND	1	1	1	1	1	1	1	ND	1	1	1	1
BWS5	ND	20	16	ND	1	ND	1	ND	2	ND	ND	ND	3	ND	1	ND	1	ND	1	1	1	1	1
BWS6	ND	∞	13	ND	1	ND	2	ND	2	ND	ND	ND	2	ND	ND	ND	ND	ND	ND	1	ND	1	nd
BWS7	ND	∞	4	ND	1	ND	4	ND	1	ND	ND	ND	1	ND	ND	ND	ND	ND	ND	1	ND	1	nd
BWS8	∞	∞	7	1	1	1	1	4	2	∞	ND	2	2	1	ND	1	ND	ND	ND	1	ND	1	ND
BWS9	ND	∞	2	ND	1	ND	16	ND	1	ND	ND	ND	ND	ND	ND	ND	ND	1	ND	1	ND	1	ND
BWS10	2	4.5	ND	1	ND	∞	ND	3	ND	2	ND	1	ND	3	ND	1	ND	1	ND	1	ND	1	ND
BWS11	∞	11.5	10	3	2	1	1	1	1	1	ND	1	1	1	1	1	ND	1	ND	1	ND	1	ND
BWS12	4.5	3.5	3	1	1	1	1	1	1	3	ND	4	3	3.5	3	1	ND	1	ND	1	ND	1	ND
BWS13	8	5	4	2	2	1	1	2	2	7	ND	1	1	1	1	1	ND	1	ND	1	ND	1	ND
BWS14	∞	∞	10	1	1	1	1	∞	55	∞	ND	1	1	1	1	1	ND	1	ND	1	ND	1	ND
BWS15	8	6	7	1	1	1	1	∞	55	∞	ND	1	ND	1	ND	1	ND	1	ND	1	ND	1	ND
BWS16	9.5	7	8	1	2	1	1	16	3	14	ND	3	2	1	1	1	ND	1	ND	1	ND	1	ND
BWS17	2.5	4	3	1	1	1	1	1	1	2.5	ND	1	1	1	1	1	ND	1	ND	1	ND	1	ND
NC[a]	1 ± 0.17	1.0 ± 0.25	1 ± 0.15	1 ± 0.20	1 ± 0.35	1 ± 0.16	1 ± 0.06	1 ± 0.26	1 ± 0.25	1 ± 0.22		1 ± 0.30	1 ± 0.33	1 ± 0.17	1 ± 0.35	1 ± 0.22	1 ± 0.12	1 ± 0.16	1 ± 0.40	10.18	1 ± 0.15	1 ± 0.30	1 ± 0.15

BWS, Beckwith–Wiedemann syndrome; COBRA, combined bisulphite restriction analysis; MSP, methylation-specific PCR; ND, not done; SB, Southern blotting.
Numbers represent unme/me ratios. The ratios were normalised to the average of 120 control samples with MS-PCR and 100 control samples with COBRA. Numbers greater than 1 indicate loss of methylation.
[a]NC, normal controls.

control levels in all individuals investigated (Supplementary Figure 2).

Overall, these results demonstrate that a subset of BWS patients display a complex but specific hypomethylation defect involving multiple maternally methylated imprinted loci. Interestingly, the hypomethylated loci included the *PLAGL1* locus implicated in TND, and the *GNAS* and *NESPAS* loci implicated in PHP1b.

Clinical characteristics of patients with multiple maternal hypomethylation

The clinical features of the patients with multiple hypomethylation were compared with those of the patients with loss of methylation restricted to *KCNQ1OT1* (Table 2). The birth weight of the patients with multiple hypomethylation was on average lower than that of the subgroup with isolated *KCNQ1OT1* hypomethylation (58th ± 35 *vs* 79th ± 21 centile). This difference is just over the limits of statistical significance ($P = 0.06$). However, the low frequency of neonatal macrosomia was particularly evident among the cases with severe hypomethylation (38th ± 34, see Table 3, in bold). Intriguingly, some of these individuals showed postnatal overgrowth. The frequencies of nevus flammeus and hemihypertrophy among the patients with multiple defects were lower than those observed among the patients with only *KCNQ1OT1* hypomethylation ($P = 0.03$ and $P = 0.04$, respectively). In addition, some patients with multiple hypomethylation displayed characteristics not usually associated with BWS, such as speech retardation (BWS3), peri/postnatal apnoea, feeding difficulties, hearing problems (BWS11) and premature birth (28 weeks of gestation, BWS12 and BWS16). Overall, these data suggest that maternal hypomethylation in addition to that at 11p15.5 may modify the clinical presentation of BWS.

Mutation analysis of *DNMT3L*

In the mouse, *Dnmt3L* is required for establishing maternal imprints.[23] We, therefore, looked for mutations at the *DNMT3L* locus in the mothers of two patients (BWS1 and BWS2) with severe hypomethylation at multiple imprinting loci. We sequenced all exons and flanking intronic regions. Only four single-nucleotide variations with respect to the reference sequence (NT_011515) were detected, but all of them corresponded to polymorphisms also found in the normal population (db8129767, db2014457, db762424 and db2014264).

Discussion

Rossignol *et al*[9] reported that a subgroup of BWS patients displayed maternal methylation defects at imprinted loci in addition to the *KCNQ1OT1* ICR, raising the hypothesis that genes other than those located at chromosome 11p15.5 were involved in the pathogenesis of BWS. In a larger cohort of patients, we have demonstrated that the

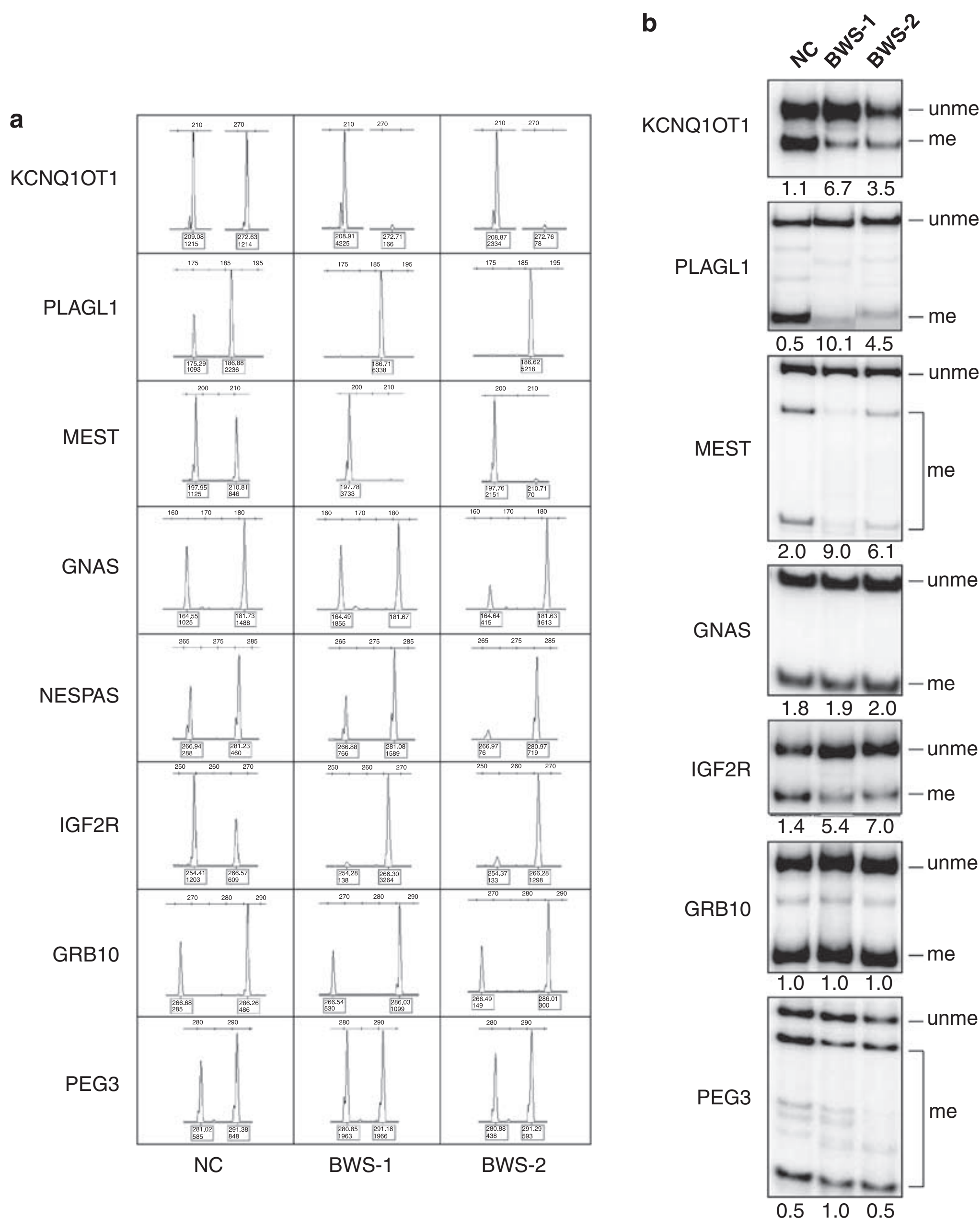

Figure 1 Examples of the assays used for the methylation analysis. The methylation of nine maternally methylated ICRs was analysed by MS-PCR (**a**) and COBRA (**b**) in the DNAs of a normal individual and two BWS patients with multiple methylation defects (BWS1 and BWS2). The peaks of the MS-PCR corresponding to unmethylated and methylated DNA are indicated by blue and red filled circles, respectively, and the bands of the COBRA corresponding to the unmethylated and methylated DNA are indicated at the right side of the panels. The area of the MS-PCR peaks and the non-normalised unme/me ratios of intensities of the COBRA bands are also indicated below each panel. Details are given in Patients and methods and Supplementary Tables 1 and 2. Note that the results obtained with the two techniques are highly concordant.

epigenetic defects at loci other than 11p15.5 were limited to loss of methylation at maternally methylated ICRs, and that these abnormalities were restricted to the individuals with loss of *KCNQ1OT1* methylation. We also observed that a specific set of ICRs were found more frequently affected than others and that the patients with multiple methylation defects often displayed atypical BWS phenotypes.

The percentage of ICD2 patients with multiple hypomethylation was reported by Rossignol *et al*[9] to be 25% in a study of the *KCNQ1OT1*, *IGF2R*, *MEST* and *SNRPN* loci in 40 BWS cases. We have found a 20% frequency of multiple

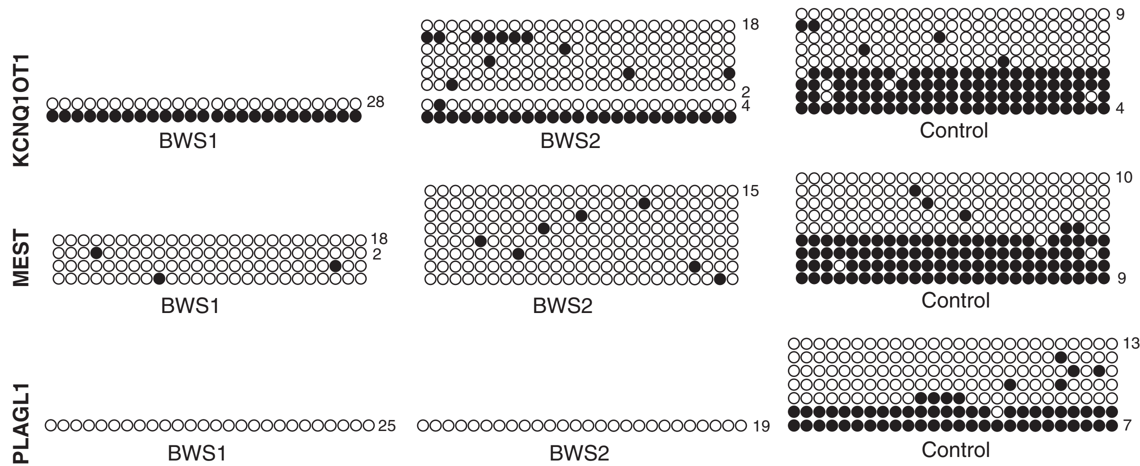

Figure 2 Multiple hypomethylated ICRs, as analysed by bisulphite sequencing. The methylation of the three maternally methylated ICRs was analysed by bisulphite sequencing in the DNAs of a normal individual and two BWS patients with multiple methylation defects (BWS1 and BWS2). Twenty-five CpGs of the *KCNQ1OT1* ICR, 26 CpGs of the *PLAGL1* ICR and 59 CpGs (only the first 25 CpGs are shown) of the *MEST* ICR were analysed. Each line corresponds to a single template DNA molecule cloned; each circle corresponds to a CpG dinucleotide. Filled circles designate methylated cytosines; open circles, unmethylated cytosines. Numbers at the right border of the CpG circles indicate multiple sequenced clones with identical methylation pattern. Note that the hypomethylation extends over the entire amplicons sequenced.

methylation among nine maternally methylated and two paternally methylated loci analysed, including a hypomethylation frequency of only 11% among the loci investigated by Rossignol *et al.*[9] This lower frequency may reflect variations in the clinical inclusion criteria for the two cohorts investigated; this is particularly significant bearing in mind that multiple hypomethylation cases may have a BWS presentation modified by the effects of loci other than 11p15.5. Differently from Rossignol *et al,*[9] we used multiple techniques to analyse DNA methylation. Notably, there was a broad concordance between the results of methylation analysis by Southern blotting, COBRA and MSP. In some cases, there was a variation in the degree of hypomethylation detected by the different techniques (for example, the hypomethylation of *KCNQ1OT1* in BWS1–4). In two cases (MEST, BWS16 and GNAS, BWS2), the discrepancy caused an apparently divergent determination of hypomethylation, with MSP detecting marginal hypomethylation not detected by COBRA. In general, it appears that MSP is more sensitive than COBRA to allelic imbalance. As MSP is based on simultaneous amplification of methylated and non-methylated DNAs with different primers, it is possible that the different species take different times to reach exponential phase, and this emphasises the differences between methylated and non-methylated DNAs. Aside from this, small discrepancies in calculated methylation ratios may reflect the different positions of the target CpG dinucleotides assayed by the different tests.

The loci most frequently affected in our patient cohort were the *NESPAS* and *GNAS* ICRs on chromosome 20, followed by the *MEST*, *PLAGL1* and *IGF2R* ICRs, whereas *GRB10*, *PEG3* and *SNRPN* were frequently spared. This may represent clinical ascertainment bias among the patient cohort. Alternatively, the range of loci implicated may indicate underlying mechanistic aetiologies primarily affecting specific groups of imprinted loci. It has been recently demonstrated that TND patients with gene mutations in *ZFP57* had imprinting defects chiefly affecting the *PLAGL1*, *PEG3* and *GRB10* loci, whereas other loci including *KCNQ1OT1*, *MEST*, *NESPAS* and *GNAS* were generally spared.[18] We suggest, therefore, that there may be an aetiological divergence between that patient group and the cohort described here.

Rossignol *et al*[9] did not identify any significant difference between the clinical features of patients with multiple hypomethylation and those with hypomethylation restricted to 11p15.5. In a more extensive characterisation, we have found that patients with multiple defects were on average smaller at birth than those with ICD2 alone. In addition, some of the individuals with multiple hypomethylation had unusual BWS phenotypes. Similarly to a case of the French study, one patient of our cohort (BWS3) had developmental delay and primary speech retardation associated with *MEST* hypomethylation. Another patient with multiple methylation defect (BWS11) showed apnoea and feeding and hearing problems. In addition, two patients of our cohort (BWS12 and BWS16) and one patient of the French cohort were born preterm. Therefore, it is possible that dysregulation of additional genes may modify the typical phenotype of BWS. The tumour incidence (low in the BWS cases with ICD2), however, was not affected by the presence of multiple methylation defects.

It is striking that some individuals manifested imprinting defects that would be expected in association with clinical features of another imprinting disorder. For example, four patients (BWS8, BWS14, BWS15 and BWS16) had total or near-total hypomethylation of *GNAS* and/or *NESPAS*. The *GNAS* locus on chromosome 20q13 is a complex imprinted cluster with multiple ICRs and gene products.[24] Hypomethylation at these ICRs has also been described in several patients affected with PHP1b.[25] Although further investigation is probably needed to exclude all features of PHP1b, no alteration of calcium metabolism has been reported in our BWS patients with multiple hypomethylation so far. However, the two patients with more marked *GNAS* hypomethylation were not macrosomic at birth (10th and 50–90th centiles, respectively), suggesting that abnormal expression at the *GNAS* locus may modify the BWS phenotype. Four patients (BWS1, BWS2, BWS9 and BWS10) in this cohort displayed complete or near-complete hypomethylation of *PLAGL1* ICR, which is normally associated with TND. Although neonatal diabetes was not described among these cases, they were not macrosomic; this suggests that *PLAGL1* hypomethylation may alter the clinical presentation of BWS. Interestingly, TND patients with marked *KCNQ1OT1* hypomethylation displayed macroglossia and abdominal wall defects, features typical of BWS.[10] These observations suggest that the clinical presentation of these complex cases may be modified to an extent depending on their mosaic epigenotype. Possibly, the lack of presentation reflects the somatic mosaicism of the patients, with critical target tissues being spared. Alternatively, the interaction of genetic pathways affected by these different imprinted genes gives rise to a primary clinical presentation, with other clinical disorders being ameliorated. Also, it is not possible to exclude that the observed loss of methylation at ICRs other than *KCNQ1OT1* is limited to blood leukocytes. Clearly larger cohort studies, with extensive clinical and epigenetic characterisations, are warranted.

Consistent with the French study, hypomethylation was incomplete in most cases, indicating epigenetic mosaicism, probably arising postfertilisation.[9] It cannot be excluded, however, that an imprinting defect originating in the gametes results in unstable methylation of the ICRs afterwards. The loss of methylation may arise stochastically or as a consequence of defective trans-acting factor or environmental cause. The use of assisted reproduction technology (ART) can be excluded as a cause, as the frequency of ART-associated cases is not increased among the patients with multiple hypomethylation in both the French[9] and our studies (Table 2). Our results also exclude defects in the *DNMT3L* gene as a common cause of multiple hypomethylation in the BWS. The multi-zinc finger *ZFP-57* gene has been implicated in the aetiology of multiple hypomethylation in TND.[18] This protein, which is enriched in undifferentiated ES cells, may be required for the maintenance of DNA methylation at specific ICRs in early embryogenesis. It is possible that a defect in a similar regulatory protein is involved in multiple hypomethylation in BWS.

Functional interactions between imprinted genes controlling embryo growth have been proposed on the basis of similar expression patterns.[13,26] In addition, the product of the *PLAGL1* gene has been shown to bind *in vitro* the *KCNQ1OT1* ICR and activate the expression of the non-coding *KCNQ1OT1* RNA.[13] Also, extensive networks of intra- and interchromosomal interactions were demonstrated among imprinted domains.[27] As a consequence of these interactions, it is possible that epigenetic alterations at one locus may result in abnormalities at other loci.

In conclusion, hypomethylation at multiple maternally methylated imprinted loci, including *KCNQ1OT1*, *MEST*, *GNAS*/*NESPAS*, *PLAGL1* and *IGF2R,* is associated with atypical BWS presentations. These cases may be an example of a group of more generalised imprinting disorders resulting from defects in the imprinting maintenance factors. Further investigation will be necessary to identify the involved gene(s) and better define the phenotypes associated with these complex disorders.

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Conflict of interest

The authors had no conflict of interest in connection with this paper.

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