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ORIGINAL INVESTIGATION

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Epimutation of the TNDM locus and the Beckwith–Wiedemann syndrome centromeric locus in individuals with transient neonatal diabetes mellitus

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Abstract Transient neonatal diabetes mellitus (TNDM) is characterised by intra-uterine growth retardation, while Beckwith–Wiedemann syndrome (BWS) is a clinically heterogeneous overgrowth syndrome. Both TNDM and BWS may be caused by aberrant loss of methylation (LOM) at imprinted loci on chromosomes 6q24 and 11p15.5 respectively. Here we describe two patients with a clinical diagnosis of TNDM caused by LOM at the maternally methylated imprinted domain on 6q24; in addition, these patients had LOM at the centromeric differentially methylated region of 11p15.5. This shows that imprinting anomalies can affect more than one imprinted locus and may alter the clinical presentation of imprinted disease.

Keywords Transient neonatal diabetes mellitus · Beckwith–Wiedemann syndrome · Imprinting · Loss of methylation · Methylation-specific PCR · Pyrosequencing

Abbreviations BWS: Beckwith–Wiedemann syndrome · DMR: Differentially methylated region · IUGR: Intra-uterine growth retardation · LOM: Loss of methylation · MS-PCR: Methylation-specific PCR · MLPA: Multiplex ligation-dependent probe amplification · TNDM: Transient neonatal diabetes mellitus · UPD: Uniparental disomy

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Introduction

Transient neonatal diabetes mellitus (TNDM) is a rare imprinting disorder characterised by intra-uterine growth retardation (IUGR), transient diabetes and, in some cases, macroglossia and abdominal wall defects. The TNDM locus on chromosome 6q24 contains one maternally-methylated differentially methylated region (DMR), and ~85% of TNDM cases show loss of methylation at that DMR, which was shown to lead to paternal overexpression of the genes at that locus (Mackay et al. 2002). Three genetic causes have been identified: paternal uniparental disomy of chromosome 6 (UPD6), 6q24 duplication of paternal origin, and loss of methylation (LOM) at the TNDM DMR (Temple and Shield 2002).

It is well known that several imprinted genes are regulators of growth, and that mutations or epimutations of these genes cause growth disorders (Robertson 2005). While IUGR is a major feature of TNDM, the imprinting disorder Beckwith–Wiedemann syndrome (BWS) is chiefly characterised by foetal and/or postnatal overgrowth, with variable features including hemihypertrophy, macroglossia, abdominal wall defects and transient hypoglycaemia (reviewed in Weksberg et al. 2005; Cooper et al. 2005). The BWS locus is an imprinted region of chromosome 11p15 which contains two differentially methylated regions (the *H19* and *KCNQTOT1* DMRs); a minority of cases involve gene mutation or chromosomal rearrangement, but the major cause (~50%) is epigenetic: LOM at the centromeric *KCNQTOT1* DMR which lies within the gene *KCNQ1*. The *KCNQTOT1* DMR regulates several genes including the maternally-expressed cell cycle control gene *CDKN1C*, and LOM at this DMR is particularly associated with clinical features of BWS including macroglossia and anterior abdominal wall defects.

It has recently become apparent that mutations and epimutations in the human BWS region can cause growth retardation phenotypes as well as overgrowth. Maternally-derived duplications of chromosome 11p15 were reported in patients with growth retardation (Fisher et al. 2002; Eggermann et al. 2005a), while some patients with a clinical diagnosis of Silver-Russell syndrome (a syndrome including IUGR) exhibited LOM of the *H19* DMR (Gicquel et al. 2005; Eggermann et al. 2005b). In addition, Arima et al. (2005) reported LOM at the *KCNQTOT1* DMR in two TNDM patients, one with paternal UPD6 and one with LOM at 6q24, and proposed that the imprinted genes in the BWS and TNDM regions might contribute to a growth-regulatory network.

We analysed the methylation of the *H19* and *KCNQTOT1* DMRs in a cohort of TNDM patients, and identified two patients with loss of maternal methylation at both the TNDM DMR and *KCNQTOT1* DMR.

Methods

Patients

The TNDM cohort (a subset of that described in Mackay et al. 2005) comprised 43 individuals with a clinical diagnosis of TNDM including low birth weight and hyperglycaemia developing within 6 weeks of birth and resolving within 18 months. The cohort comprised seventeen individuals with paternal uniparental disomy of chromosome 6, 9 with duplication of 6q24, 13 with loss of methylation at the TNDM DMR, and 4 with no identified cause of disease (Mackay et al. 2005). One hundred and twenty anonymised DNA samples from normal individuals were used as controls, and DNA samples from 16 anonymised individuals with a molecular diagnosis of BWS were tested to indicate the range of methylation variation at 11p15.5 in these individuals (results summarised in Table 1).

Bisulphite treatment

Genomic DNA derived from peripheral blood lymphocytes (2 μg) was bisulphite treated using the EZ DNA Methylation kit (Zymo Research) and resuspended in 50 μl.

Methylation specific PCR of *KCNQTOT1* DMR

Methylation-specific PCR (MS-PCR) utilised the divergent sequence changes deriving from bisulfite treatment of differentially methylated DNA, yielding differently-sized products in a ratio reflecting that of the starting material. The reaction contained a forward primer and divergent reverse primers, encompassing 6CpG dinucleotides, from a region between –52nt and +33nt with respect to the putative transcriptional start site of *KCNQ1OT1* (Du et al. 2004). The amplification reaction contained 1 μl DNA, 0.2 mM dNTP, 5 pmol of each primer, HotStar Taq (Qiagen) and buffer containing 1.5 mM Mg^{++}, in a final volume of 10 μl. Primer sequences were: K-common (6-Fam) ccacctcacacccaac caatacctcata; K-meth ttcgtcgttgt cgacgtggcgatcgttttt; K-unmeth tttgtgttgaggtgatgtggtgattgt tttgtt. Reaction conditions were 95°C 15 min > [95°C 20″/60°C 20″/72°C 20″]$_{28}$ > 72°C 5 min. Methylated (maternal) and unmethylated (paternal) product sizes were 210 and 270 bp respectively. PCR products were visualised on an ABI 3100 or ABI 3130xl genetic analyser. Peaks were inspected and peak heights < 100 or > 8,000 pixels were discarded, and the degree of methylation calculated as paternal/maternal peak heights and normalised against normal controls (> 6 per experiment). At least 3 separate amplifications were carried out on all patients in the cohort, and atypical results were confirmed in repeat bisulphite reactions. MS-PCR of BWS DMR1 was performed on all samples in a similar manner (data not shown; primers and conditions available on request).

Quantitation of *KCNQTOT1* DMR methylation by pyrosequencing

Pyrosequencing primers were designed to interrogate CpG dinucleotides at −10 and −16nt relative to the putative transcriptional start site of *KCNQ1OT1* (Du et al. 2004). Pyrosequencing was carried out according to standard methods (Dupont et al. 2004).

Microsatellite analysis of chromosome 11

Microsatellite analysis was carried out using primer sets: TH (2.15 Mb from pter), D11S1318 (2.29 Mb),

Table 1 Methylation ratiometry of BWS *KCNQOT1* DMR controls, BWS patients and TNDM patients by MS-PCR and Pyrosequencing

	KCNQOT1 DMR Methylation ratio: MS-PCR[a]	*KCNQOT1* DMR %T: pyrosequencing[a]
BWS LOM *KCNQOT1* DMR ($n=7$)[b]	12.1 ± 9.3 (range 2.49–21.2)[b]	79.2 ± 14.9 (range 56.9–100)
BWS duplication 11p15.5 ($n=1$)[c]	2.54	68.2
BWS UPD 11 ($n=3$)[b,c]	1.88 ± 0.25 (range 1.59–2.23)[2]	72 ± 5.7 (range 60–79)
TNDM1	2.10	70.4
TNDM2	3.46	76.6
TNDM other ($n=41$)	1.00 ± 0.20	47.9 ± 4.5
Normal controls[d]	1.00 ± 0.17	55.9 ± 6.8

[a]Methylation ratios were derived from at least three MS-PCR reactions performed in duplicate. The pyrosequencing results were derived from triplicate samples in a single experiment
[b]The range of methylation ratios seen in BWS LOM and BWS UPD patients presumably reflects the variation in the extent of mosaicism present in their blood
[c]In the case of the BWS UPD and duplication cases, diagnosis had previously been made by microsatellite analysis, cytogenetics and MLPA
[d]For control methylation ratios, MS-PCR was performed on 120 samples in duplicate from single bisulphite-treated DNA samples. For pyrosequencing, 75 samples were tested once

D11S4088 (2.71 Mb) and D11S4146 (3.70 Mb). One primer of each pair was fluorescently-labelled, and after 28 cycles of PCR amplification, products were resolved on an ABI 3100 genetic analyser and inspected for evidence of altered allele ratio indicative of UPD11 or deletion/duplication of chromosome 11p15.

Multiplex ligation-dependent probe amplification

Multiplex ligation-dependent probe amplification (MLPA) analysis was performed to seek evidence of duplication or deletion of chr11p15.5. Six probes between 1.97 and 3.02 Mb from pter were used, including sequences within *H19*, *IGF2*, *KCNQOT1* and *CDKN1C*. Probe design and MLPA were performed according to standard methods (Schouten et al. 2002) and validated using known 11p15.5 duplication and deletion controls. Probe sequences are available on request.

Results

Among the TNDM patients tested, we found maternal LOM at the *KCNQOT1* DMR in two patients (Patients 1 and 2), both of whom also exhibited maternal LOM at the TNDM DMR (Mackay et al. 2005). Figure 1 shows typical examples of MS-PCR results from a normal control (Fig. 1a), from BWS patients (Fig. 1b–e), and from the two TNDM patients with LOM at *KCNQOT1* DMR (Fig. 1f, g). Results of MS-PCR were verified by quantitative pyrosequencing of bisulphite-induced C/T polymorphisms (Fig. 2). MS-PCR and pyrosequencing results are summarised in Table 1.

In neither patient was there any evidence of a change in paternal:maternal allele ratios of microsatellites at 11p15.5, excluding mosaic paternal UPD11 or allele copy number change in that region. In addition, MLPA analysis of six probes within 11p15.5 showed no evidence of copy number change in the two TNDM patients, whereas both deletion and duplication were readily detectable in control patients (data not shown).

No LOM at the *KCNQOT1* DMR was observed in other members of the TNDM cohort, including 17 individuals with paternal UPD6, nor in 120 normal controls. No member of the TNDM or control cohorts showed methylation variation at BWS DMR1, while some BWS patients, as expected (Cooper et al. 2005; Weksberg et al. 2005), did exhibit gain of methylation at BWS DMR1 (data not shown). To determine whether the LOM at the *KCNQOT1* DMR extended to other imprinted genes, these patients were epigenotyped by MS-PCR for the Prader–Willi/Angelman syndrome imprinted region on chromosome 15 (Zeschnigk et al. 1997). Their methylation profile in this region was within normal limits.

Table 2 compares the clinical features of patients 1 and 2 with those of 25 out of the remaining 41 tested TNDM patients, for whom these data were available.

Discussion

We describe two individuals with loss of methylation epigenotypes at both 6q24 and 11p15.5, who presented with intra-uterine growth retardation and TNDM. One intriguing feature of these cases is that they did not present with overgrowth, despite molecular diagnosis of a BWS epimutation in lymphocyte-derived DNA. Both, however, had features occasionally found in both BWS and TNDM, including moderate macroglossia and abdominal wall defects; patient 1 had an umbilical hernia, while patient 2 underwent surgery for umbilical hernia and persistent omphalo-enteric duct. The patients did not share distinctive birth weights: patient 1 was among the smallest term infants (1,940 g), and patient 2 among the largest (2,430 g), in the LOM group of

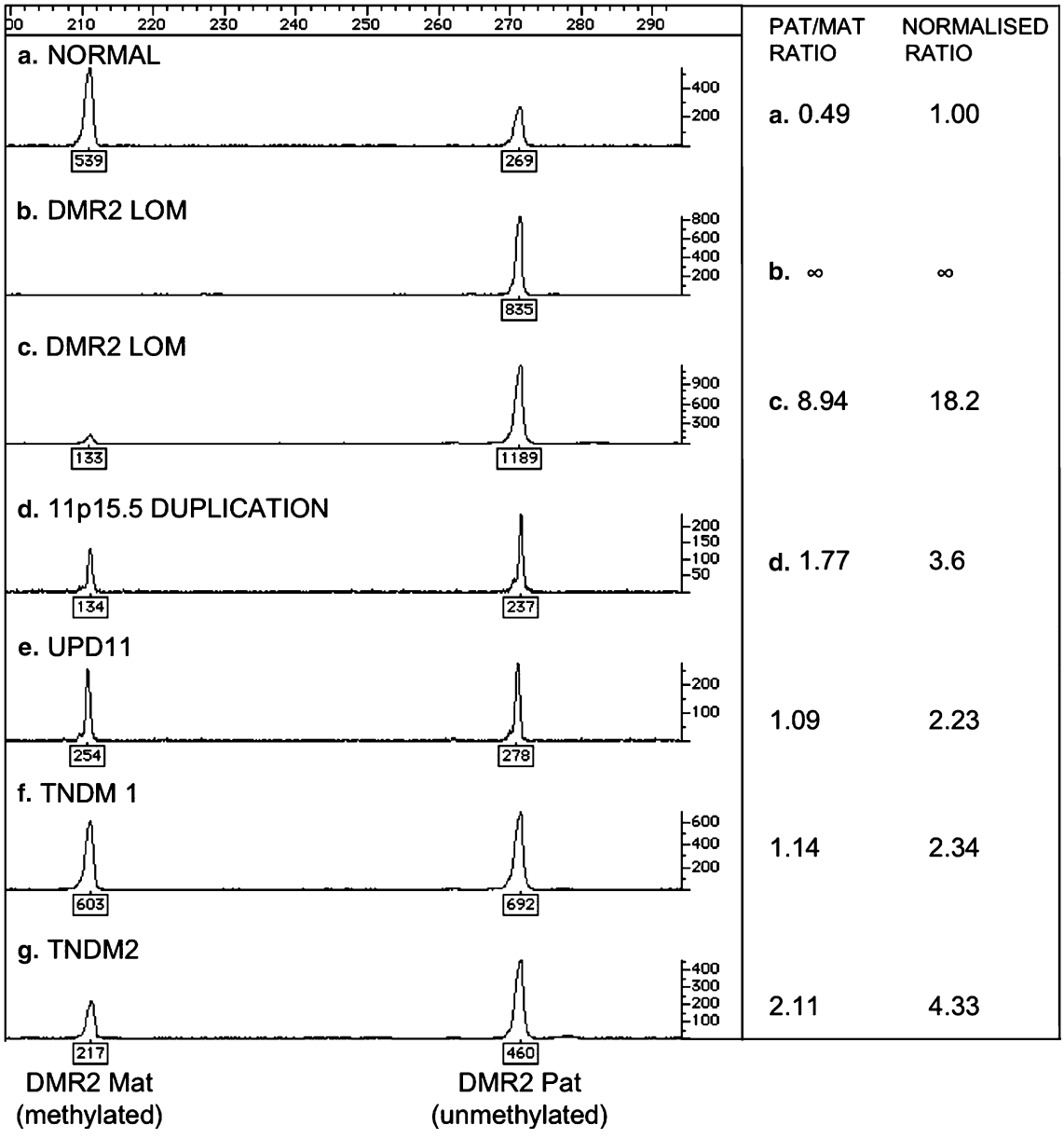

Fig. 1 Examples of methylation-specific PCR analysis of BWS DMR2. X-axis scale represents calculated product size (bp), *Y*-axis indicates peak height, as do figures under each peak. The ratio (T/C) was calculated as the peak height ratio of unmethylated versus methylated amplification products and normalised to the average of at least 6 control samples. **a** Normal control, **b**, **c** BWS patients with LOM at *KCNQOT1* DMR; **d** BWS patient with duplication of 11p15.5; **e** BWS patient with mosaic UPD11; **f**, **g** TNDM patients 1 and 2

TNDM patients (Table 2). Patient 2 seems therefore to exhibit features of both TNDM and BWS although features of the latter condition are less marked, while patient 1 has a possible but less obvious overlap between the two conditions.

From our results we cannot explain the presence of macroglossia and umbilical hernia in TNDM cases on the basis of additional 11p involvement, as other cases in our cohort from all aetiological groups have been reported with these features and have no demonstrable aberrations at 11p15.5. Both features are likely to have several aetiologies. Umbilical hernia, for example, is common in the general population. Gross macroglossia of the sort reported in BWS has never been reported in TNDM and to our knowledge no patient with TNDM has had to undergo tongue reduction surgery. The finding of these two features in both TNDM and BWS is intriguing and it is possible that the methods we used are insufficiently sensitive, or the DNA samples from inappropriate tissue, to detect mosaic 11p15.5 aberrations in other patients.

Arima et al. (2005) found LOM at the *KCNQOT1* DMR in 2/18 TNDM patients, one with LOM at 6q24 and one with UPD6, and hypothesised that the two imprinted clusters were functionally related. However, in our series, which included 17 UPD6 cases, we detected

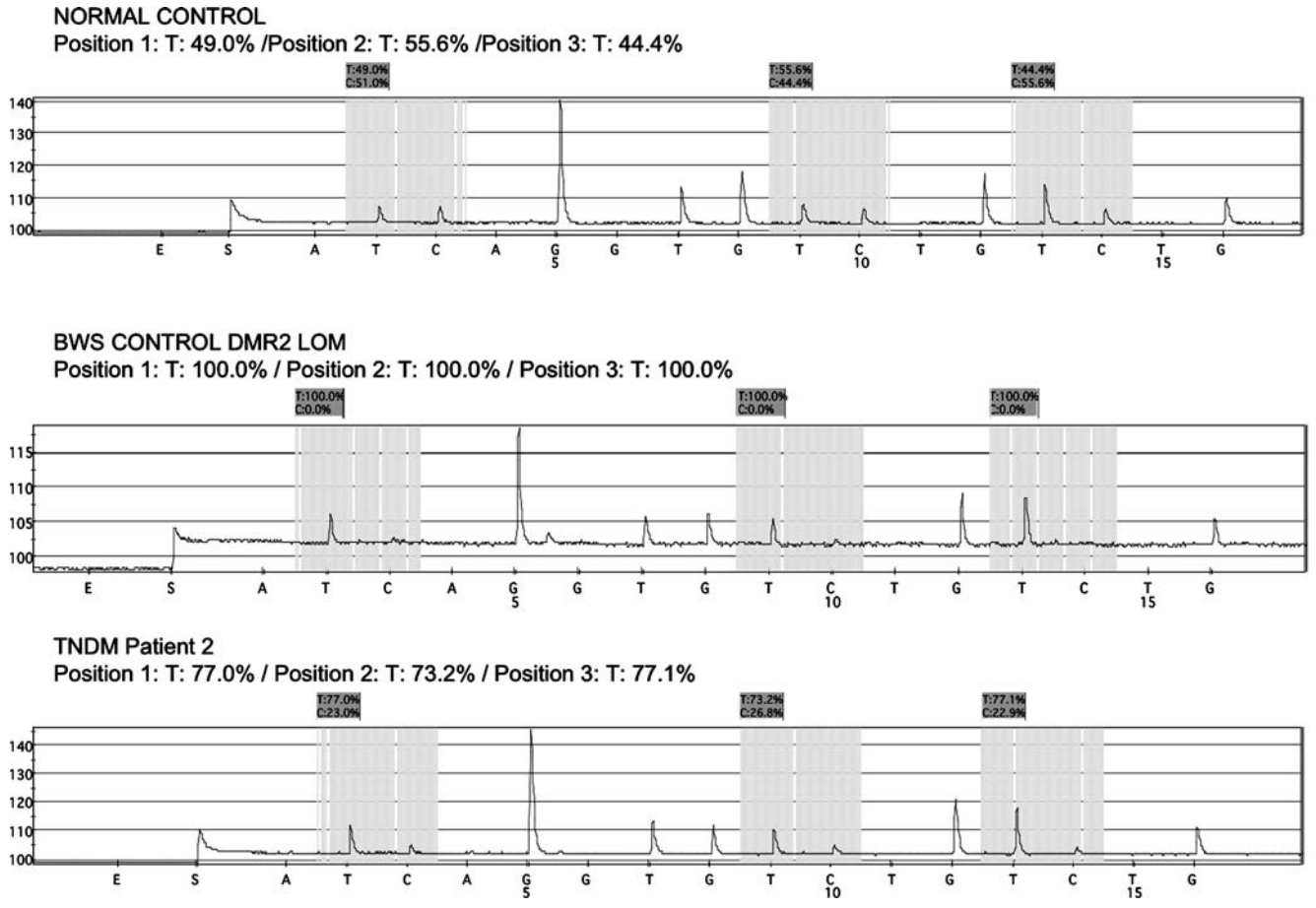

Fig. 2 Examples of quantitative pyrosequencing of bisulphite-induced C/T polymorphisms in *KCNQOT1* DMR. *Shaded areas* represent C/T polymorphisms; *shaded boxes* above represent calculated % C/%T at each polymorphism. *Top trace* normal control; *second trace* BWS patient with LOM at *KCNQOT1* DMR (same patient as in Fig. 1b; Table 1 row 2); third trace, TNDM patient 2 (same patient as Fig. 1b, Table 1 row 6, Table 2 row 2)

Table 2 Clinical characteristics noted at birth of TNDM patients

Chr6 anomaly	*N*	Macroglossia	Abdominal wall defect	Birth weight (g)	Birth weight mean ± standard deviation	Birth weight median	Birth weight range (g)
Patient 1		Yes	UH	1,940			–
Patient 2		Yes	UH + exomphalos	2,430			–
LOM	8	5	0		2,260 ± 357	2,253	1,690–2,955
Paternal UPD	10	5	1 (UH)		2,020 ± 334	1,970	1,800–2,500
6q24 duplication	7	1	1 (UH)		1,880 ± 232	1,840	1,600–2,275

The Table summarises clinical findings for term infants within the Wessex cohort for whom these data are available. Mean birth weights, standard deviation and range of the mean are given for the three aetiological groups in rows 4–6
UH umbilical hernia

LOM at *KCNQOT1* DMR only in patients with LOM at chr6q24, and an alternative hypothesis is that the two sites are related not necessarily because of the function of the imprinted genes but because a common mechanism underlies their loss of methylation. We did not identify LOM at 15q11–13 but it is possible that LOM is present at other imprinted regions, and that it is the phenotype of this particular cohort of patients that has preselected which locations are involved. In keeping with this hypothesis it is interesting to find that in patients 1 and 2, 6q24 TNDM LOM was complete as far as could be ascertained, while *KCNQOT1* DMR LOM was not complete, and might have gone undetected by techniques less sensitive than MS-PCR and pyrosequencing (Figs. 1, 2), suggesting that LOM was mosaic. The degree of LOM was comparable to that seen in BWS cases due to UPD11, which is always found in mosaic form, or to mosaic LOM (Table 1). Our observations are therefore

compatible with mosaicism, arising in the early zygote and being variably present in somatic tissues, the relative methylation levels in different tissues explaining why the individuals presented with TNDM and not BWS. If this is so, then (i) or (a) the clinical presentation, including birth weight and the extent of macroglossia/abdominal wall defect, may reflect the extent of the BWS and TNDM imprinting anomalies in different tissues, and (ii) or (b) this defect may be heritable.

It is possible that one imprinting error created a predisposition to the other, though that seems unlikely since both would be expected to arise within a short time (a few hours or days) within the early zygote (Bestor 2003). However, TNDM and BWS have both been described in discordant monozygotic multiple births (Weksberg et al. 2002; Kant et al. 2005), indicating that methylation errors occur in the same timeframe as twinning, and suggesting that imprinting errors that give rise to discrepant growth rates between zygotic cells may also cause embryo fragmentation (Bestor 2003). In monozygotic discordant twins with either TNDM or BWS, each twin shows evidence of LOM in blood-derived DNA, because of foetal circulation sharing, whereas only the affected twin manifests the anomaly somatically. It is possible that the dual LOM observed in lymphoblastoid DNA of our patients is a relic of a complex, mosaic epigenetic error. If this is so then (i) or (a) the severity of the *KCNQOT1* DMR anomaly in fibroblast DNA may differ from that seen in blood-derived DNA and (ii) or (b) some individuals with BWS and *KCNQOT1* DMR LOM may exhibit LOM at TNDM DMR. We are currently investigating these possibilities.

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