ORIGINAL ARTICLE

Silencing of *CDKN1C* (*p57^{KIP2}*) is associated with hypomethylation at KvDMR1 in Beckwith–Wiedemann syndrome

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Context: Beckwith–Wiedemann syndrome (BWS) arises by several genetic and epigenetic mechanisms affecting the balance of imprinted gene expression in chromosome 11p15.5. The most frequent alteration associated with BWS is the absence of methylation at the maternal allele of KvDMR1, an intronic CpG island within the *KCNQ1* gene. Targeted deletion of KvDMR1 suggests that this locus is an imprinting control region (ICR) that regulates multiple genes in 11p15.5. Cell culture based enhancer blocking assays indicate that KvDMR1 may function as a methylation modulated chromatin insulator and/or silencer.
Objective: To determine the potential consequence of loss of methylation (LOM) at KvDMR1 in the development of BWS.
Methods: The steady state levels of *CDKN1C* gene expression in fibroblast cells from normal individuals, and from persons with BWS who have LOM at KvDMR1, was determined by both real time quantitative polymerase chain reaction (qPCR) and ribonuclease protection assay (RPA). Methylation of the *CDKN1C* promoter region was assessed by Southern hybridisation using a methylation sensitive restriction endonuclease.
Results: Both qPCR and RPA clearly demonstrated a marked decrease (86–93%) in the expression level of the *CDKN1C* gene in cells derived from patients with BWS, who had LOM at KvDMR1. Southern analysis indicated that downregulation of *CDKN1C* in these patients was not associated with hypermethylation at the presumptive *CDKN1C* promoter.
Conclusions: An epimutation at KvDMR1, the absence of maternal methylation, causes the aberrant silencing of *CDKN1C*, some 180 kb away on the maternal chromosome. Similar to mutations at this locus, this silencing may give rise to BWS.

Imprinted genes are expressed from one allele only, depending on whether it was inherited from the mother or the father. Among the genes in the 1000 kb 11p15.5 imprinted domain, five (*TSSC3, SLC22A1L, CDKN1C, KCNQ1, H19*) are expressed exclusively or preferentially from the maternally derived chromosome, whereas the insulin-like growth factor 2 gene (*IGF2*) is predominantly expressed from the paternally derived chromosome (fig 1).[1] Alterations in one or more of these imprinted genes result in Beckwith–Wiedemann syndrome (BWS), a generalised overgrowth condition affecting about 1 in 10 000 live births. Importantly, children with BWS are 1000 times more likely to develop cancer, with 7–10% of patients developing embryonal tumours, including Wilms' tumour, adrenocortical carcinoma, hepatoblastoma, and rhabdomyosarcoma.[2]

The aetiology of BWS is complex, with patients being divided into a number of molecular subgroups defined by different genetic and epigenetic alterations in the imprinted domain.[3 4] Approximately 20% of cases result from paternal uniparental disomy (UPD) for 11p15.5, whereas paternally derived duplications and maternally derived translocations of 11p15.5 account for 1–2% of cases. Loss of imprinting (LOI) resulting in biallelic expression of *IGF2* has been shown to occur in 20–50% of individuals with BWS,[5–7] and is sometimes associated with hypermethylation and silencing of the *H19* gene.[6 8] All of these mechanisms result in an imbalance in the expression of one or more imprinted genes in this region. In this regard it is notable that mouse models that overexpress *Igf2* recapitulate several aspects of BWS, including overgrowth, macroglossia, and organomegaly, in a dose dependent fashion.[9–11] The only known examples of where a genetic alteration in a single gene causes BWS are mutations in the maternally derived allele of the cyclin dependent kinase inhibitor *CDKN1C* (*p57^{KIP2}*).[12–14] Mutations in *CDKN1C* are found in approximately 40% of familial cases and 5% of sporadic cases.[8] Significantly, although maternally inherited deletions of *Cdkn1c* in the mouse result in perinatal death, these mice also have some features of BWS, including abdominal wall defects.[16–18]

UPD, duplications and translocations of 11p15.5, and mutations in *CDKN1C* together account for only about half the sporadic cases of BWS. We and others have shown that the majority of remaining cases are associated with an absence or loss of methylation of KvDMR1, a maternally methylated CpG island in 11p15.5, shown in fig 1 (B).[7 19–25] KvDMR1 contains the promoter for a paternally expressed antisense RNA, termed *KCNQ1OT1* and formerly called *KvLQT1-AS* or *Lit1*.[26–28] Interestingly, this transcript is incorrectly activated on the maternal allele in persons with BWS who have LOM at this locus.[7 29] Deletion of the unmethylated paternal allele of the human KvDMR1 locus in somatic cell hybrids resulted in the reexpression of three normally

Abbreviations: BWS, Beckwith–Wiedemann syndrome; LOI, loss of imprinting; UPD, uniparental disomy; RPA, ribonuclease protection assay; ICR, imprinting control region; LOM, loss of methylation; RT-PCR, reverse transcription coupled polymerase chain reaction; qPCR, quantitative PCR

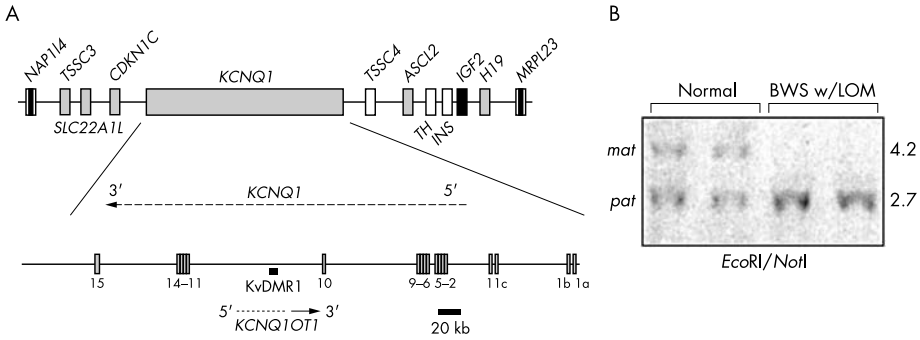

Figure 1 The chromosome 11 imprinted domain and loss of methylation at KvDMR1 in BWS. (A) Genomic map showing the location of imprinted genes in human chromosome 11p15.5. Maternally expressed genes are indicated as grey boxes, paternally expressed genes as black boxes, non-imprinted (biallelic) genes within the imprinted domain as white boxes, and *NAP1L4* and *MRPL23*, which appear to define the extent of the domain, as white boxes with a vertical black stripe. Imprinted expression of *TSSC4*, *TH*, and *INS* has not yet been demonstrated in human tissue, but *Tssc4* and *Ins2* have been shown to have monoallelic expression in mouse extraembryonic tissues.[1] Below the map is an enlargement of the *KCNQ1* locus showing its exon-intron structure and the position of KvDMR1. The direction of transcription of the maternally expressed *KCNQ1* gene and the paternally expressed antisense transcript (*KCNQ1OT1*) are indicated. (B) Southern analysis of fibroblast DNA from normal individuals and from patients with Beckwith–Wiedemann syndrome (BWS) and loss of methylation (LOM) at KvDMR1, showing the absence of the 4.2 kb methylated fragment in the patients.

paternally repressed genes, including *CDKN1C*.[26] More recently, we have shown that paternal inheritance of a KvDMR1 deletion in the mouse results in the derepression in *cis* of *CDKN1C* and five other normally silent (imprinted) genes on the paternal chromosome, as well as a growth deficiency phenotype.[27] KvDMR1 has been shown to function as a position dependent chromatin insulator[30] or position independent silencer,[28 31 32] depending on the enhancer blocking assay and cell culture system. Importantly, the position dependent chromatin insulator activity is abrogated by methylation.[30] Thus KvDMR1 appears to function as an imprinting control region (ICR) which, in its unmethylated form, silences genes on the paternal chromosome, thereby giving rise to maternal specific imprinted expression.

Individuals with BWS who have mutations in CDKN1C and those with BWS who have LOM at KvDMR1 have an increased incidence of exomphalos.[15 23 25] This observation and the results of KvDMR1 knockouts suggest that demethylation of KvDMR1 on the maternal chromosome during development leads to BWS through the pathological silencing of *CDKN1C*.[3] Using RT-PCR and a ribonuclease protection assay (RPA), we show that the steady state level of *CDKN1C* is dramatically downregulated in cases of BWS where methylation at KvDMR1 is lost.

MATERIALS AND METHODS

Quantitative RT-PCR

Fibroblast cell lines cultured in DMEM and 10% fetal calf serum were harvested while subconfluent, and total RNA was isolated using a guanidine thiocyanate method (Totally RNA kit, Ambion, Austin, TX, USA). First strand cDNA was synthesised from 4 µg total RNA using RetroScript reverse transcriptase (Ambion) and oligo (dT) primer. Quantitative PCR was carried out on an ABI Prism 7700 Sequence Detector using a TaqMan assay for *CDKN1C*[33] and internal controls *β-actin* and *GAPDH* (Applied Biosystems, Foster City, CA, USA). For each TaqMan probe assay, a single 96 well plate contained triplicate cDNA samples from BWS cases and normal controls, as well as serial dilutions of human fetal kidney cDNA to generate a standard curve.

Ribonuclease protection assay

Radioactive RNA probes were synthesised using the MaxiScript T7/T3 kit (Ambion) and ^{32}P-UTP (800 Ci/mmol) (Perkin-Elmer Life Sciences, Boston, MA, USA). The probe used for the *CDKN1C* was from Pharmingen, San Diego, CA, USA and the Tri-Cyclophillin human probe was from Ambion. RPA was carried out with 30 µg total RNA using the RPAIII kit as described by the manufacturer (Ambion) with hybridisation at 55°C. RNase digestion was carried out with a 1/100 dilution of the RNaseA/T1 mix. Conditions were such that all probes were in excess. Protected fragments were electrophoresed in 5% TBE-Urea precast Ready Gels (BioRad, Heracles, CA, USA). The intensity of protected fragments was quantified using a Phosphorimager (Piscataway, NJ, USA) and ImageQuant software (Molecular Dynamics, Piscataway, NJ, USA).

Methylation analysis

DNA (5 µg) from patients and controls was digested with *Ppu*MI alone or in combination with *Sac*II at 37°C for 18 hours. Digests were electrophoresed in 0.9% agarose and transferred to Hybond N$^+$ (Amersham, Little Chalfont, Bucks, UK) as suggested by the manufacturer. A hybridisation probe for the *CDKN1C* promoter region was prepared by PCR using sense primer 5′-CGCAGATAGCGGCTTCAGACTCCAGCTCC AGGG-3′ and antisense primer 5′- TGCTGGCTAGCTCGCT CGCTCAGGCCTGGC-3′, with human genomic DNA as template. These primers amplify a sequence corresponding to positions 116–1007 in accession number D64137.[34] The 892 bp PCR product was cloned into pCRII TOPO and labelled by random priming. Washes were twice for 10 minutes at room temperature in 2X SSC, and twice for 30 minutes at 65°C with 0.2X SSC, 0.5% SDS.

RESULTS

To test the hypothesis that LOM at KvDMR1 is associated with downregulation of *CDKN1C*, we analysed the steady state expression level of this gene in fibroblast cell lines from five persons with BWS who had complete LOM at KvDMR1 (fig 1(B) and data not shown). Since, in principle, *CDKN1C* could be downregulated by more than one mechanism in BWS, patient expression levels were compared with fibroblast cells from normal individuals instead of those from other BWS subgroups (patients with normal methylation at KvDMR1). Total RNA was extracted from subconfluent cultures of patient and control fibroblasts, and first strand cDNA was synthesised. RT-PCR using a TaqMan assay for *CDKN1C*[33] and internal controls *β-actin* and *GAPDH* (Applied Biosystems) was carried out using the standard curve

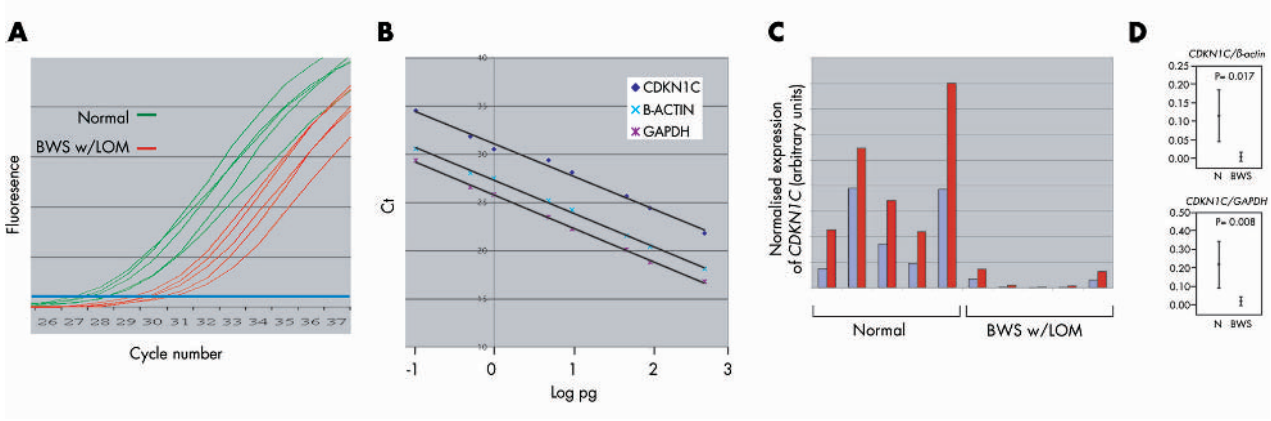

Figure 2 RT-PCR analysis of BWS patients with LOM at KvDMR1. (A) Corrected amplification plots generated by plotting intensity *v* cycle number. Each line represents the average of triplicate assays from each normal (green) or patient (red) sample. For illustrative purposes, each plot was normalised to a common internal control signal. (B) Representative standard curves used for quantification generated by plotting the threshold cycle (Ct) for different amounts of input cDNA. (C) Chart showing *CDKN1C* expression levels determined by RT-PCR normalised with respect to β-actin (blue bars) and *GAPDH* (red bars). (D) Results of Student's *t*-tests comparing normalised expression levels of *CDKN1C* of normal controls (N) with those of individuals with BWS and LOM at KvDMR1.

method. Each experiment for a given TaqMan assay consisted of triplicate samples from subjects with BWS and normal controls, as well as serial dilutions of human fetal kidney cDNA to generate a standard curve, on the same 96 well plate.

Representative amplification plots for *CDKN1C* from one experiment using fibroblast cDNA from normal individuals (green lines) and from persons with BWS who had LOM at KvDMR1 (red lines) are shown in fig 2(A). For illustrative purposes, each amplification plot was normalised with respect to the same internal control Ct value. Note that in all cases, amplification plots corresponding to the persons with BWS crossed the fluorescence threshold at a higher cycle number (lower expression level) than those corresponding to normal individuals. Fig 2(B) shows typical standard curves used for quantification and demonstrates that each PCR assay was linear over four orders of magnitude. The histogram in fig 2(C) shows results from a representative experiment where the expression level of *CDKN1C* was normalised to *β-actin* and *GAPDH*. Despite a wide range of expression levels in fibroblasts from normal individuals, we were able to show that *CDKN1C* was significantly down-regulated in fibroblasts from BWS cases with LOM at KvDMR1. In the experiment shown in fig 2, BWS patient cells exhibited an average 93% reduction in *CDKN1C* expression when normalised to either *β-actin* or *GAPDH*. Five independent experiments were carried out with similar results.

These findings were confirmed in a RPA using radioactive single stranded RNA probes for *CDKN1C* and cyclophilin. Despite roughly equivalent intensities of the cyclophilin protected fragment across all samples, and a large degree of variation in *CDKN1C* expression among normal samples, an obvious reduction in the intensity of the *CDKN1C* protected fragment was observed in cases of BWS with LOM at KvDMR1, as evident in fig 3(A). Following quantification by PhosphorImager analysis, and normalisation to the cyclophilin signal, the average *CDKN1C* expression level in fibroblasts from persons with BWS who had LOM at KvDMR1 was reduced by 86% compared with the level in fibroblasts from normal individuals (fig 3(B)), consistent with the results from the RT-PCR assay. A residual signal indicating low level expression of *CDKN1C* was observed in RNA from most BWS patient cell lines. Since cell lines from patients' parents were not available, we could not determine the allelic origin of these transcripts. Although incomplete repression of the maternal allele of *CDKN1C* in patient fibroblasts cannot be ruled out, the residual signal seen in these samples may represent low level expression of *CDKN1C* from the paternal allele.[35 36]

Unlike the mouse *Cdkn1c* gene, which is differentially methylated on the silent paternal allele, the human locus is devoid of methylation both in the promoter region and in the gene body.[35] It has recently been shown, however, that promoter hypermethylation at *CDKN1C* does occur in a number of malignancies in which this gene is silenced.[37 38] To determine whether promoter methylation at *CDKN1C* accompanies its silencing in association with LOM at KvDMR1, Southern analysis was carried out using the methylation sensitive restriction endonuclease *Sac*II. A 1.2 kb *Ppu*MI fragment that encompasses the *CDKN1C* promoter includes two *Sac*II sites which are located in a region containing multiple potential transcription factor binding sites (fig 4). In fibroblast DNA from normal individuals, the 1.2 *Ppu*MI fragment was completely digested by *Sac*II into 500 bp and 700 bp fragments, indicating a lack of methylation at these restriction sites. The same cleavage pattern was observed in DNA from subjects with BWS and LOM at KvDMR1, with no trace of higher molecular weight (methylated) bands detectable. These results demonstrate that, at least for the four CpG's assessed by the two *Sac*II sites, no promoter methylation changes accompany silencing of *CDKN1C* in persons with BWS and LOM at KvDMR1.

DISCUSSION

Multiple genetic and epigenetic mechanisms affecting the integrity or expression of imprinted genes in human band 11p15.5 can give rise to BWS.[3 4] Most of these mechanisms result in either two active copies of the *IGF2* gene which encodes a potent fetal growth factor—for example, paternal UPD, paternal duplication, LOI—or the absence (paternal UPD) or mutation of the active maternal allele of *CDKN1C*, a cyclin dependent kinase inhibitor. Animal models involving increased expression of *Igf2* or null mutation in *Cdkn1c* suggest that abnormal expression of these two genes is the major contributor to the BWS phenotype. Indeed, in one mouse model, *Igf2* and *Cdkn1c* appeared to function in an antagonistic manner during the development of certain organ systems.[39]

KvDMR1 is normally methylated on the maternally derived chromosome and unmethylated on the paternal allele.[7 19–21]

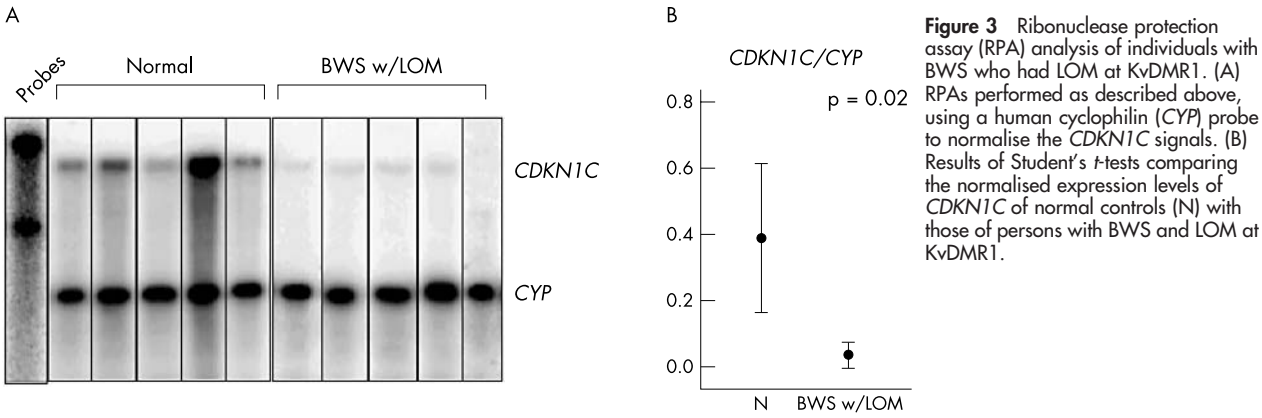

Figure 3 Ribonuclease protection assay (RPA) analysis of individuals with BWS who had LOM at KvDMR1. (A) RPAs performed as described above, using a human cyclophilin (*CYP*) probe to normalise the *CDKN1C* signals. (B) Results of Student's *t*-tests comparing the normalised expression levels of *CDKN1C* of normal controls (N) with those of persons with BWS and LOM at KvDMR1.

This locus has been shown to function as an ICR in 11p15.5 (and in the mouse counterpart distal chromosome 7), regulating the imprinted expression of at least six paternally silenced genes.[26 27] The most frequent alteration (genetic or epigenetic) associated with BWS is an absence or LOM at the maternal allele of KvDMR1.[22–25] Here we show, both by RT-PCR and RPA, that LOM at KvDMR1 is associated with a dramatic reduction in the steady state expression level of *CDKN1C*. Our results are consistent with the recent demonstration that *Cdkn1c* was silenced in ES cells deficient for *Dnmt3L*, a DNA methyltransferase family member shown to be essential for the establishment of maternal imprints.[40] Roughly 50% of familial cases and 5% of sporadic cases of BWS are due to maternally inherited mutations of *CDKN1C*.[20] Assuming that expression levels in fibroblasts mirror those in fetal tissues during development, the epigenetic repression of *CDKN1C* observed in fibroblasts from individuals with BWS who have LOM at KvDMR1 could be functionally equivalent to an inactivating mutation and, if so, is the likely cause of the syndrome. These results also explain the high frequency of exomphalos observed in cases of BWS with LOM at KvDMR1 or with germline mutations in *CDKN1C*.[15 23 25]

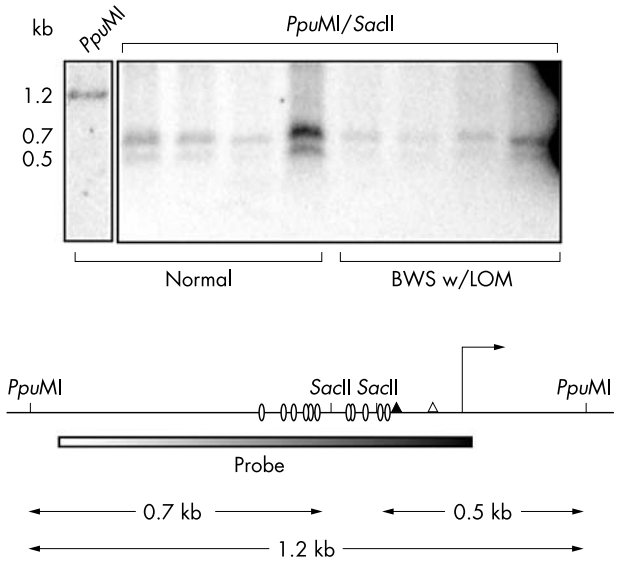

Figure 4 Methylation analysis of *CDKN1C* promoter. Fibroblast DNAs from normal individuals, and from persons with BWS and LOM at KvDMR1, were digested with *Ppu*MI alone (first lane only) or with *Ppu*MI plus *Sac*II; electrophoresed in agarose; and transferred to a nylon membrane. The location of the hybridisation probe is shown on the genomic restriction map. Also indicated on the map are the putative transcription start site (arrow), TATA and CAT boxes (open and filled triangles, respectively), and 11 Sp1 binding sites as determined by Tokino *et al*.[34]

KvDMR1 has been shown to function as a chromatin insulator and/or bidirectional silencer in cell culture,[28 30 31] and at least the insulator function is abrogated by methylation.[30] However, at present, it is not known whether this locus operates in a similar fashion to silence paternal copies of maternal specific genes. KvDMR1 also contains the promoter for *KCNQ1OT1/Kcnq1ot* which appears to be functionally separable from its insulator/silencer activity (our unpublished results).[26–28] The involvement of non-coding RNAs in gene silencing has been shown for genes on the mammalian inactive X-chromosome[41] and at the *Igf2r* locus.[42] Although the functional significance of *KCNQ1OT1* in gene silencing has yet to be demonstrated, it is possible that KvDMR1 is a bipartite regulatory locus which utilises more than one mechanism to—for example—silence different genes. Regardless of the manner in which the unmethylated copy of KvDMR1 results in gene silencing, our present findings support the model that loss of methylation at KvDMR1 inappropriately activates this repressive function on the maternal chromosome which, in turn, leads to the pathological silencing of the imprinted candidate tumour suppressor gene, *CDKN1C*.[3] This model is also supported by the finding that two other genes normally repressed by unmethylated KvDMR1 in the mouse (*TSSC3* and *SLC22A1L*)[27] are at least partially silenced in cases of BWS with LOM at KvDMR1 (ND-M's and MJH's unpublished results). However, we cannot exclude the possibility that LOM at KvDMR1 and decreased expression of *CDKN1C* and other genes are manifestations of a widespread epimutation in the 11p15.5 imprinted domain that are not directly related mechanistically. In this regard, however, it is significant that a preliminary analysis demonstrated no aberrant methylation at the *CDKN1C* promoter in cells from individuals with BWS and LOM at KvDMR1 (fig 4). Thus, unlike the situation in a variety of cancers,[37 38] repression of *CDKN1C* in cases of BWS with LOM at KvDMR1 does not appear to be associated with promoter methylation. Nevertheless, formal proof of a causative relationship between LOM at KvDMR1 and silencing of *CDKN1C* and other genes awaits the generation of a mouse model with biallelic unmethylated copies of KvDMR1 and features of BWS.

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