ORIGINAL ARTICLE

Silencing of CDKN1C (p57KIP2) is associated with hypomethylation at KvDMR1 in Beckwith–Wiedemann syndrome


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.1,4 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,5,6 and is sometimes associated with hypermethylation and silencing of the H19 gene.7,8 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 (p57KIP2).12–14 Mutations in CDKN1C are found in approximately 40% of familial cases and 5% of sporadic cases.6 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.15–18

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.27 Deletion of the unmethylated paternal allele of the human KvDMR1 locus in somatic cell hybrids resulted in the reexpression of three normally
been shown to have monoallelic expression in mouse extraembryonic tissues. 

The direction of transcription of the maternally expressed 

KCNQ1 

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.

- **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 KvDMR1, which appear to define the extent of the domain, as white boxes with a vertical black stripe. Imprinted expression of TSSC4, T1, 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. 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) is indicated.

- **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 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 cDNA probes were synthesised using the MaxiScript T7/T3 kit (Ambion) and [γ-32P]-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-Cyclophilin 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 53ºC. 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, Hercules, CA, USA). The intensity of protected fragments was quantified using a Phosphorimagery (Piscataway, NJ, USA) and ImageQuant software (Molecular Dynamics, Piscataway, NJ, USA).

  - **Methylation analysis**

    DNA (5 μg) from patients and controls was digested with PpuMI alone or in combination with SacI at 37ºC 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'-GCCAGGATACGGCGGCTCAGCTCACGCTCCAGGG-3' and antisense primer 5'-TGCTTCGCTAGCTCGCTCGCTAGCCC-3', with human genomic DNA as template. These primers amplify a sequence corresponding to positions 116–1007 in accession number D64137. 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 65ºC 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 and internal controls β-actin and GAPDH (Applied Biosystems) was carried out using the standard curve.
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 KvDMR1. In the experiment shown in fig 2, BWS patient cells exhibited an average 93% reduction in KvDMR1. In the experiment shown in fig 2, BWS patient cells exhibited an average 93% reduction in KvDMR1. 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.

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. It has recently been shown, however, that promoter hypermethylation at CDKN1C does occur in a number of malignancies in which this gene is silenced. 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 SacII. A 1.2 kb PpuMI fragment that encompasses the CDKN1C promoter includes two SacII 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 PpuMI fragment was completely digested by SacII 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 SacII 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. 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.

KvDMR1 is normally methylated on the maternally derived chromosome and unmethylated on the paternal allele.
This locus has been shown to function as an ICR in 11p15.5, regulating the imprinted expression of at least six paternally silenced genes. The most frequent alteration (genetic or epigenetic) associated with BWS is an absence or LOM at the maternal allele of KvDMR1. 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. Roughly 50% of familial cases and 5% of sporadic cases of BWS are due to maternally inherited mutations of CDKN1C. Assuming that expression levels in fibroblasts mirror those in fetal tissues during development, the epigenic 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.

KvDMR1 has been shown to function as a chromatin insulator and/or bidirectional silencer in cell culture, and at least the insulator function is abrogated by methylation. 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 of KCNQ1OT1 which appears to be functionally separable from its insulator/silencer activity (our unpublished results). The involvement of non-coding RNAs in gene silencing has been shown for genes on the mammalian inactive X-chromosome and at the Igf2r locus. 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 unmethylated candidate tumour suppressor gene, CDKN1C. This model is also supported by the finding that two other genes normally repressed by unmethylated KvDMR1 in the mouse (TSSC3 and SL22A1L) 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, 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.

ACKNOWLEDGEMENTS

This research utilised core facilities supported in part by Roswell Park Cancer Institute’s Cancer Center Support Grant, CA16056, funded by the National Cancer Institute. This work was supported by a grant from the Association for Research of Childhood Cancer, and by a National Cancer Institute / National Institute of Health grant (CA63333 to MJH).
Silencing of KCNQ1OT1 in Beckwith-Wiedemann syndrome.

Authors’ affiliations
N Diaz-Meyer, C D'ay, K Khato, M J Higgins, Departments of Cancer Genetics, Roswell Park Cancer Institute, Buffalo, NY, USA
E R Maher, W Cooper, Section of Medical and Molecular Genetics, Department of Paediatrics and Child Health, University of Birmingham, UK
W Reik, Laboratory of Developmental Genetics and Imprinting, Babraham Institute, Cambridge, UK
C Junien, Inserm U383, Hospital Necker Enfants Malades, Paris, France
G Graham, Department of Genetics, Children’s Hospital of Eastern Ontario, Ottawa, Canada
E Algar, Department of Paediatrics, University of Melbourne, Victoria, Australia
V M Der Kalousian, McGill University and Department of Medical Genetics, Montreal Children’s Hospital, Montreal, Canada

REFERENCES
Silencing of \textit{CDKN1C} (p57\textsuperscript{KIP2}) is associated with hypomethylation at KvDMR1 in Beckwith–Wiedemann syndrome


\textit{J Med Genet} 2003 40: 797-801

doi: 10.1136/jmg.40.11.797

Updated information and services can be found at:
http://jmg.bmj.com/content/40/11/797

These include:

References
This article cites 42 articles, 26 of which you can access for free at:
http://jmg.bmj.com/content/40/11/797#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Topic Collections
Articles on similar topics can be found in the following collections
Molecular genetics (1254)

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/