ORIGINAL ARTICLE

Alternative mechanisms associated with silencing of *CDKN1C* in Beckwith–Wiedemann syndrome

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Background: Mutations in the imprinted gene *CDKN1C* account for approximately 10% of Beckwith–Wiedemann syndrome (BWS) cases. Fibroblasts from BWS patients with loss of methylation (LOM) at the imprinting control region (ICR) KvDMR1 have reduced *CDKN1C* expression. Another group of BWS patients with downregulated *CDKN1C* expression but with normal methylation at KvDMR1 has been identified.
Objective: To investigate the mechanism of *CDKN1C* silencing in BWS in these two classes of patients.
Methods: The *CDKN1C* promoter region was analysed for changes in DNA methylation using bisulphite sequencing, and for alterations in chromatin structure using the chromatin immunoprecipitation (ChIP) assay.
Results: There was only spurious CpG methylation of the *CDKN1C* promoter in fibroblast DNA from both normal individuals and patients with BWS, irrespective of the methylation status of KvDMR1. There was no detectable change in chromatin structure at the *CDKN1C* promoter in patients with LOM at KvDMR1. BWS patients with downregulated *CDKN1C* and normal methylation at KvDMR1 had depletion of dimethylated H3-K4 and enrichment of dimethylated H3-K9 and HP1γ at the *CDKN1C* promoter, suggesting that in these cases gene silencing is associated with repressive chromatin changes.
Conclusions: *CDKN1C* may be downregulated by multiple mechanisms including some that do not involve promoter methylation. In BWS patients with normal methylation at KvDMR1 and reduced expression of *CDKN1C*, repressive chromatin may play a role, but the absence of methylation and repressive chromatin structure at the *CDKN1C* promoter in BWS patients with LOM at KvDMR1 argues for a direct role of this epimutation in silencing *CDKN1C*.

An imbalance in the expression of imprinted genes in human chromosome band 11p15.5 (fig 1A), either by mutation or deregulation of genomic imprinting, results in Beckwith–Wiedemann syndrome (BWS), an overgrowth condition where patients have a 1000-fold increased risk of developing embryonal tumours.[1] *CDKN1C* (also known as $p57^{KIP2}$) is one of the genes in human chromosome 11p15.5 that is implicated in BWS. It encodes a cyclin dependent kinase inhibitor involved in the negative regulation of the cell cycle[2 3] and is critical during mouse embryogenesis.[4 5] *CDKN1C* is imprinted and primarily expressed from the maternal allele; however, in humans some expression (5–30%) is observed from the paternal chromosome.[6 7] In BWS, mutations of *CDKN1C* have been found in 40% of familial cases and in roughly 5% of sporadic cases.[8–10]

Chromosome 11p15.5 contains at least two imprinting control regions (ICRs), which are differentially methylated in the germline and which regulate the imprinted expression of two or more genes. The *H19* ICR controls the imprinted expression of the *H19* and *IGF2* genes (and the *Ins* gene in mouse) (see the paper by Arney[11] and references therein), whereas KvDMR1 functions by silencing at least eight maternally expressed genes on the paternal chromosome.[12–14] The exact mode of action of KvDMR1 is still unknown. Enhancer blocking assays suggest that KvDMR1 may function as a methylation sensitive insulator or silencer.[15–18] KvDMR1 is normally methylated on the maternally inherited allele and unmethylated on the paternal allele.[19 20] However, a large proportion (~50%) of non-UPD BWS patients suffer an epimutation at KvDMR1, namely loss of methylation (LOM) at the maternally inherited allele.[19–21] These patients have a high occurrence of omphalocele similar to BWS patients with mutations in *CDKN1C*.[10 21] This observation suggested that LOM at the maternally inherited KvDMR1 allele in BWS may result in the downregulation of maternally expressed genes including *CDKN1C*. Indeed, we have recently demonstrated a strong correlation between LOM at KvDMR1 and reduced expression of *CDKN1C* in BWS patients.[22] Although we postulate that the silencing of *CDKN1C* in these patients is a direct consequence of this epimutation at KvDMR1, we cannot exclude the possibility that these two epigenetic phenomena are mechanistically unrelated and simply represent two manifestations of domain-wide epigenetic disruption. In this regard, we have identified some BWS patients with reduced expression of *CDKN1C* but with apparently normal methylation at KvDMR1.

To gain a better understanding of the mechanisms by which *CDKN1C* is silenced in BWS, we analysed DNA methylation and chromatin structure in the promoter region of *CDKN1C* in fibroblasts from normal individuals and patients. We found that the promoter region of *CDKN1C* was unmethylated in all the normal and BWS patient samples analysed. However, the two subgroups of BWS patients with downregulated *CDKN1C* were distinguishable on the basis of their chromatin structure at the *CDKN1C* promoter, indicating different silencing mechanisms.

Abbreviations: BWS, Beckwith–Wiedemann syndrome; ChIP, chromatin immunoprecipitation; ICR, imprinting control region; LOI, loss of imprinting; LOM, loss of methylation; MI, methylation index; RPA, ribonuclease protection assay

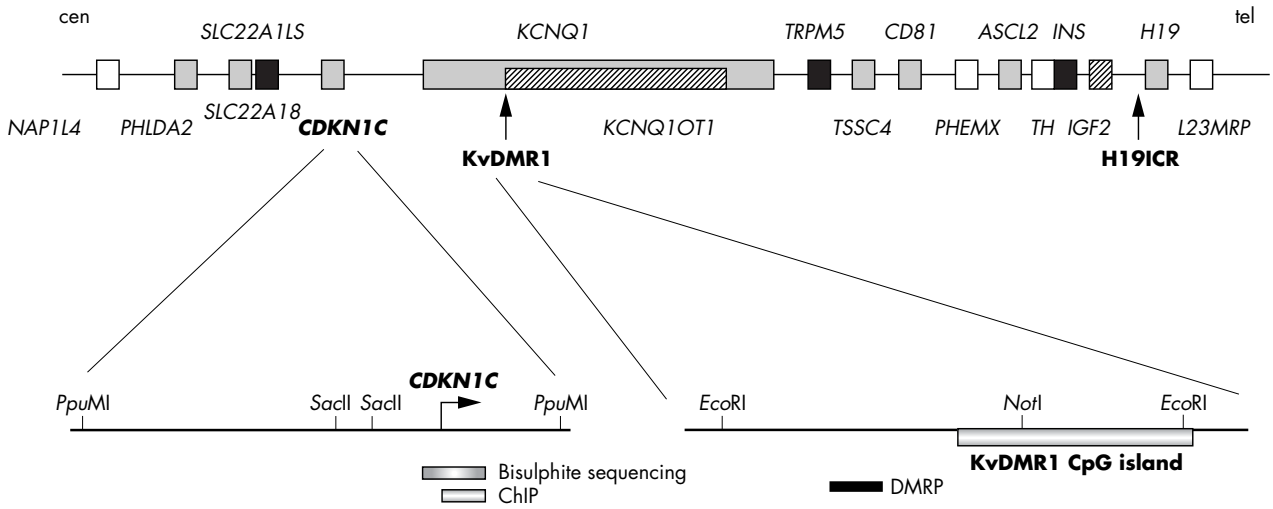


Figure 1 Map of the chromosome 11p15.5 imprinted domain. Physical map of the 1 Mb imprinted cluster on human chromosome region 11p15.5. The imprinting status is shown: paternally expressed genes (diagonally stripped box), maternally expressed genes (grey), biallelically expressed genes (white), and unknown (black). Also shown are imprinting control regions (ICR): *H19* ICR and KvDMR1. Below the map are enlargements of the *CDKN1C* (on the left) and KvDMR1 regions (on the right) showing the relative positions of sequences analysed by bisulphite sequencing and chromatin immunoprecipitation (ChIP), and the location of the probe used for Southern blot analysis of KvDMR1. The putative transcription start site for *CDKN1C*[23] is shown with an arrow.

METHODS

Methylation analysis of KvDMR1 and the *H19* ICR region by Southern hybridisation

Genomic DNA of fibroblast cell lines from normal individuals and BWS patients was digested with *Eco*RI and the methylation sensitive enzyme *Not*I. Blots were hybridised with the DMRP probe (fig 1) as described previously.[20] Hybridisation signals were detected using a Typhoon PhosphorImager and quantitated using ImageJ software (http://rsb.info.nih.gov/ij/). The methylation status (that is, normal or LOM) of a patient was determined by comparing their methylation index (MI; intensity of methylated band/intensity of unmethylated band) to that of normal controls. For the Southern blot in fig 2A, the mean (SD) MI of three normal samples was 0.81 (0.09). Thus methylation at KvDMR1 in BWS patients was considered normal if the MI was 0.81 $\pm$ 2SD or from 0.63 to 0.99, which is comparable to previous studies.[21 24 25] For analysis of CTCF binding site No 6 of the *H19* ICR and the proximal promoter of *H19*, DNA was digested with *Sau96*I plus *Hha*I or with *Pst*I plus *Sma*I, respectively. The probe for CTCF site No 6 was a 318 base pair (bp) *Bgl*II-*Nco*I fragment corresponding to nucleotides 7795–8113 of AF125183. The probe for the proximal promoter region of *H19* was a cloned polymerase chain reaction (PCR) product encompassing nucleotides 9231–10386 of AF125183.

Bisulphite sequencing

Genomic DNA was digested with *Bam*HI (Fermentas), which flanks the region analysed by bisulphite sequencing.[26] Following digestion, DNA was purified by phenol-chloroform extraction and resuspended in 50 µl H_20. Two micrograms of digested genomic DNA were denatured with 5.5 µl of fresh 2M NaOH and incubated at 37℃ for 10 minutes. Bisulphite treatment was carried out at 50℃ under mineral oil for 16 hours with 30 µl of 10 mM hydroquinone (Sigma, St Louis, Missouri, USA) and 520 µl of sodium (meta)bisulphite (Sigma) at pH 5.0. Reactions were desalted using the Qiaquick Gel Extraction kit (Qiagen, Valencia, California, USA). Bisulphite treated DNA was eluted in 50 µl of water, desulphonated with 5.5 µl of 3M NaOH for five minutes at room temperature, followed by ethanol precipitation and resuspension in 40 µl of water.

Reagents for a 20 µl PCR included the following: 1 µl of bisulphite treated DNA, 1 µM PAGE purified primers (IDT DNA) specific for bisulphite treated DNA, 0.2 mM dNTP, 1× GeneAmp PCR buffer II, 1.5 mM $MgCl_2$, 2.5% DMSO, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, California, USA). Primers for amplification of the *CDKN1C* promoter region of DNA treated with bisulphite were p57_C.F GGTTGGGYGTTTTATAGGTTA and p57_C.R ACCTAACTATCCGATAATAAACTCTTC, as previously described.[27] The following ''touchdown'' PCR program was used: one cycle of 94℃ for 10 minutes; 10 ''touchdown'' cycles of 94℃ for one minute, 65℃ (−1.2℃ per cycle) for one minute, 72℃ for one minute; 35 cycles of 94℃ for one minute, 53℃ for one minute, and 72℃ for one minute; followed by a final extension cycle of 72℃ for 10 minutes. Before cloning, amplification was confirmed by running a portion of the PCR products on a 2% agarose gel. To decrease the chance of stochastically amplified PCR products of bisulphite treated DNA, products from two independent PCRs for each sample were mixed together and cloned into the pCR4-TOPO vector following the manufacturer's recommendations (Invitrogen, San Diego, California, USA). Plasmid DNA was isolated by miniprep using the Qiaprep 96 Turbo BioRobot kit (Qiagen) or the High Pure Plasmid isolation kit (Roche, Indianopolis, Indiana, USA). For every sample analysed by bisulphite sequencing, 10 clones were sequenced with T3 primers using an ABI PRISM 3700 DNA analyser (Applied Biosystems). Sequencing was done by Macrogen (Seoul, Korea). In the alignment process, vector sequences were removed and clone sequences were aligned. A compilation of individual sequenced clones was saved in FASTA format. Methylation status was determined by using the MethTools software (http://genome.imb-jena.de/methtools).

Ribonuclease protection assay (RPA)

RPA was carried out as described previously.[22] Briefly, radioactive RNA probes were synthesised using the MaxiScript T7/T3 kit (Ambion, Austin, Texas, USA) and ^{32}P-UTP (800Ci/mmol) (Perkin-Elmer Life Sciences, Norwalk, Connecticut, USA). The probe used for *CDKN1C* was from Pharmingen (San Diego, California, USA) and the human

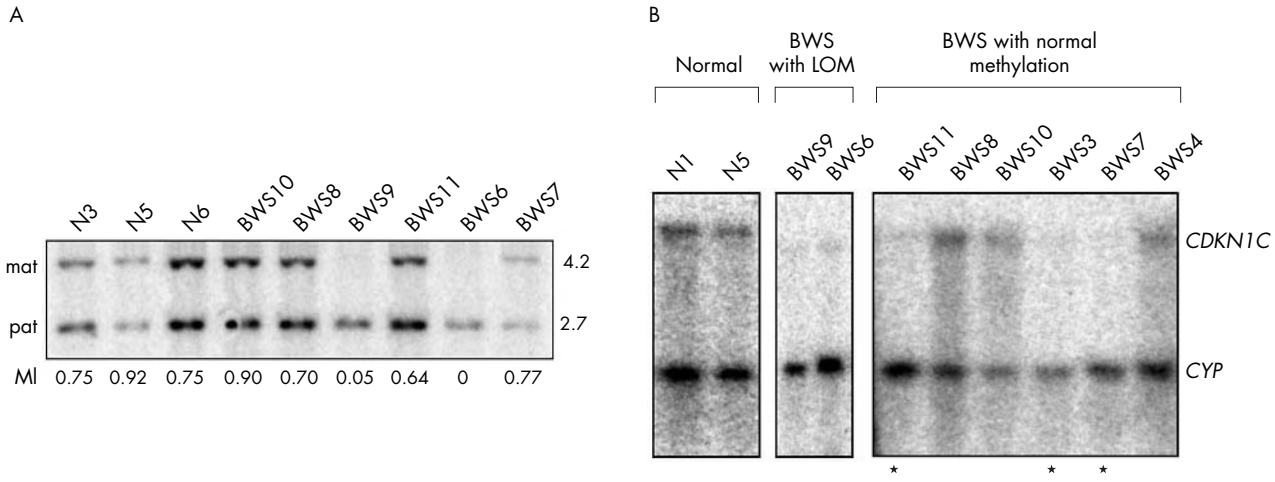


Figure 2 (A) Analysis of methylation status of KvDMR1 by Southern hybridisation. DNA from Beckwith–Wiedemann patient fibroblast cell lines (BWS) and from normal individuals (N) was digested with *Eco*RI and *Not*I. Blots were hybridised with the DMRP probe (Smilinich *et al*[20], fig 1B). The Southern blot analysis differentiates between the methylated (4.2 kb) and unmethylated (2.7 kb) alleles. Densitometry analysis of the bands allowed the samples to be classified as normal methylation (methylation index (MI) 0.81 ± 2SD or from 0.63 to 0.99) or loss of methylation at KvDMR1 (MI <0.63). (B) Downregulation of *CDKN1C* in BWS patients. Ribonuclease protection assay was undertaken as described by Diaz-Meyer *et al*[22] with *CDKN1C* and *CYCLOPHILIN* (CYP) RNA probes. Asterisks indicate BWS patient samples with normal methylation at KvDMR1 that show downregulation of *CDKN1C*. For comparison, the results of two BWS patients with LOM at KvDMR1 are included (BWS6 and BWS9).[22]

tri-cyclophillin 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 done with a 1/100 dilution of the RNaseA/T1 mix. Conditions were such that both probes were in excess. Protected fragments were electrophoresed in 5% TBE-urea precast Ready Gels (BioRad, Hercules, California, USA).

Chromatin immunoprecipitation (ChIP)

We used the ChIP assay kit from Upstate Biotechnology (Santa Cruz, California, USA). Between one million and five million cells were fixed with 1% formaldehyde for 10 minutes at 37°C in cell culture media. The formaldehyde cross linked cells in 0.6 ml sodium dodecylsulphate lysis buffer were sonicated for 180 seconds (10 seconds on, and five seconds off) on ice using a Branson sonicator with a 2 mm microtip, and settings of 40% for output control and 90% for duty cycle. Sonicated chromatin (200–1000 bp) was centrifuged at 20 000 × *g* for 10 minutes at 4°C, aliquoted, snap frozen in liquid nitrogen, and stored at −70°C. Antibodies obtained from Upstate Biotechnology (Waltham, Massachusetts, USA) included anti-dimethyl histone H3 (lys4) (catalogue No 07–030), and anti-dimethyl histone H3 (lys9) (catalogue No 07–212). Antibody against HP1-γ (catalogue No MAB3450) was from Chemicon International Inc (Temecula, California, USA). Sonicated chromatin was diluted 10-fold in ChIP dilution buffer (200 µl), pre-cleared with 80 µl salmon sperm DNA/protein A agarose for one hour at 4°C with rotation. A portion of the protein A purified chromatin (20 µl) was used to prepare DNA for the ''input'' sample. Antibodies (2–5 µl) were added to the clarified chromatin (180 µl) and incubated overnight with rotation. Sixty microlitres of protein A agarose were added to the antibody–chromatin mix and incubated at 4°C for one hour with rotation. The complex was collected by gentle centrifugation and washed three times with ChIP buffers (Upstate Biotechnology), and the bound chromatin was eluted in 500 µl of elution buffer. After adding 20 µl of 5 M NaCl, protein–DNA cross linking was reversed by heating at 65°C for four hours. Samples were treated with proteinase K, purified by MiniElute PCR purification kit (Qiagen), and then eluted in 100 µl of low TE buffer (1 mM Tris, 0.1 mM EDTA). Chromatin modifications were determined using *LDHA* and *β globin* as internal controls. All the primes were located in the promoter regions or the first exon. The primers used are the following: CDKN1C.F AGGCCTGA GCGA GCGAGCTAGCCA and CDKN1C.R CTGTCCGGTGGT GGACTCTTCTGCGT; LDHA.F GGGTATGGTTGAGACTCGAGA TGAG and LDHA.R TTGCAGGGTGACTCAGTTCAAATGTA; β globin.F GTACTGATGGTATGGGGCCAAGAGA and β globin.R GGTCTAAGTGATGACAGCCGTACCTG.

RESULTS

CDKN1C expression is reduced in some BWS patients with apparently normal methylation at KvDMR1

We have previously observed complete concordance between LOM at KvDMR1 and the reduction of *CDKN1C* expression in fibroblasts from BWS patients.[22] From an expression analysis of six BWS patients with normal methylation at KvDMR1 (fig 2A and data not shown), three samples (BWS3, BWS7, BWS11) were identified with decreased expression of *CDKN1C* using either a ribonuclease protection assay (RPA) (fig 2B) or real time reverse transcriptase PCR (RT-PCR, data not shown). The degree of *CDKN1C* repression was similar to that observed in BWS patients with LOM at KvDMR1 (fig 2B, BWS6 and BWS9).[22] These patients all had a normal karyotype and the finding of normal methylation at KvDMR1 makes paternal UDP unlikely. Though unexpected, these results suggest an additional mechanism for down-regulation of *CDKN1C* in these BWS patients, independent of KvDMR1 methylation.

Silencing of *CDKN1C* in BWS is not associated with hypermethylation at the promoter region

A large CpG island containing the promoter region and most of the gene body of *CDKN1C* is differentially methylated in mouse but not in humans.[6] However, it has been shown that *CDKN1C* becomes biallelically methylated in several human cancers and that this correlates with downregulation of *CDKN1C* expression.[27–30] In our earlier study, we showed by Southern blot that methylation is absent at two *Sac*II sites in the promoter region of *CDKN1C* (fig 1) in BWS patients with loss of methylation at KvDMR1 and downregulation of

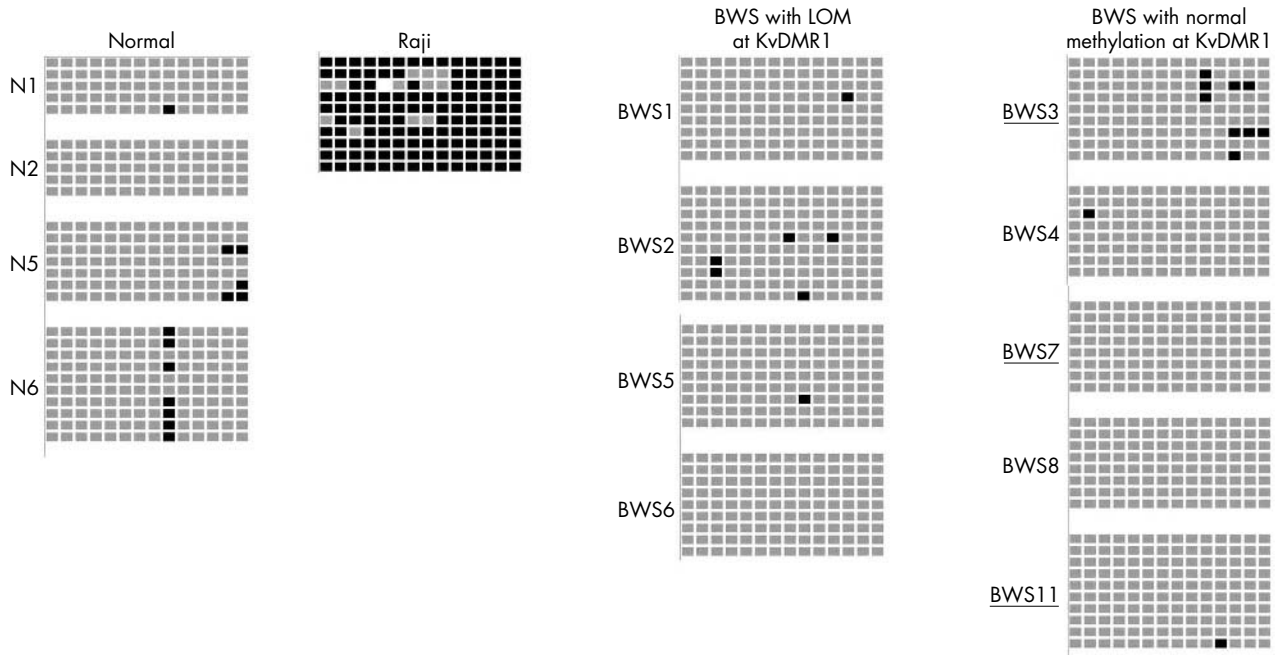


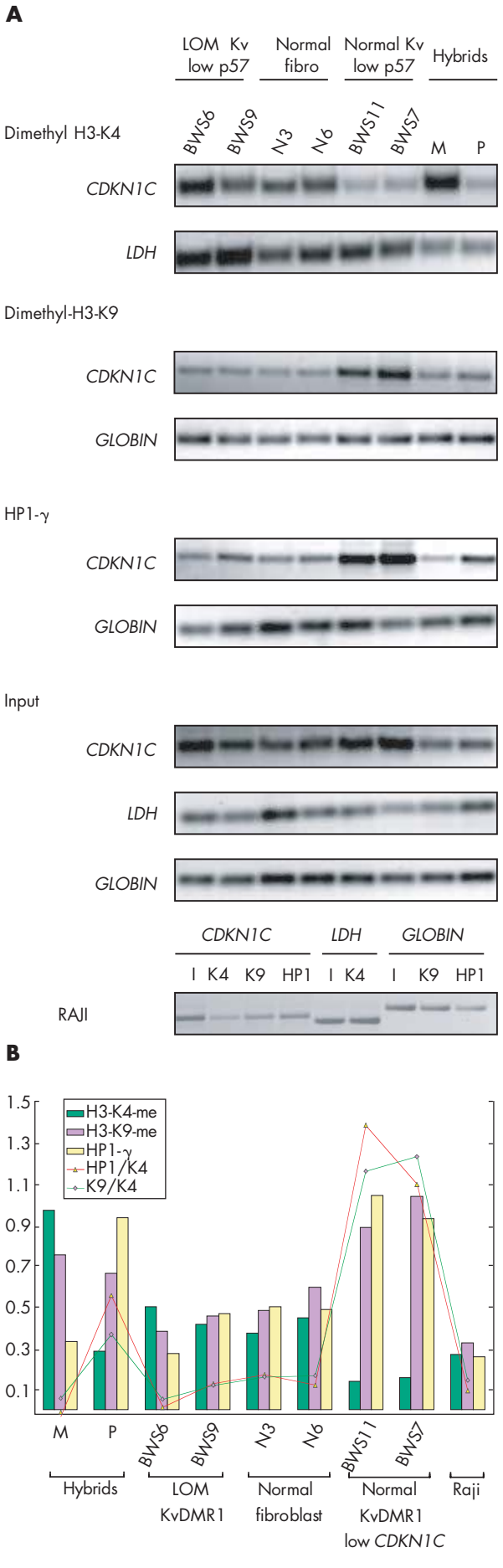
Figure 3 Methylation analysis of the promoter region of *CDKN1C* by bisulphite sequencing. Bisulphite sequencing was carried out on a region corresponding to the promoter and part of the first exon of the *CDKN1C* gene and spans a total of 155 bp containing 14 CpGs (fig 1). For a given sample, each line represents the results for a single cloned DNA molecule. Black rectangles represent methylated CpGs, while grey rectangles represent unmethylated CpGs. Except for the tumour cell line Raji, samples were fibroblast DNA from normal individuals and non-UPD Beckwith–Wiedemann syndrome (BWS) patients with and without loss of methylation (LOM) at KvDMR1. BWS cases with reduced expression of *CDKN1C* but normal methylation at KvDMR1 are underlined.

CDKN1C.[22] To rule out the possibility that downregulation of *CDKN1C* observed in BWS patients simply reflects methylation elsewhere at the *CDKN1C* promoter, we carried out a comprehensive methylation analysis of the promoter using bisulphite sequencing.

PCR primers were chosen that amplified a 155 bp fragment containing 14 CpG dinucleotides spanning the proximal promoter and transcription start site of *CDKN1C*, and which is consistently methylated in tumour cell lines where the *CDKN1C* gene is silenced (fragment C in Kikuchi *et al*[27]). PCR products were cloned and 10 clones were sequenced for each sample. As a positive control, the same region of *CDKN1C* was analysed in Raji, a Burkitt's lymphoma cell line previously shown to be densely methylated at the *CDKN1C* promoter.[27] In agreement with the earlier study, all 10 molecules analysed from Raji DNA showed primarily methylated CpG dinucleotides (fig 3). In contrast all clones from four normal fibroblast lines that gave reliable sequencing information indicated that the *CDKN1C* proximal promoter region is predominantly unmethylated (fig 3). As no polymorphisms were identified in the *CDKN1C* promoter region to assess PCR bias, a mixing experiment was undertaken using different amounts of Raji and normal DNA to confirm that we were able to detect methylation at the *CDKN1C* promoter region in the presence of the unmethylated allele (that is, to rule out PCR bias towards the unmethylated allele). Using COBRA analysis,[31] methylated alleles were easily detected in PCR products from a 1:9 (Raji:normal) DNA mixture (data not shown), indicating that, if a bias did exist, it favoured amplification of methylated alleles. Despite some sporadic methylation, we found no significant differences in methylation patterns in BWS patients compared with normal controls (fig 3), regardless of KvDMR1 methylation status. Importantly, this confirms that there does not appear to be aberrant DNA methylation at the *CDKN1C* promoter region in BWS patients who show downregulation of *CDKN1C* associated with LOM at KvDMR1.[22] Considering that we observed primarily unmethylated alleles in the normal and BWS samples, silencing mechanisms other than the hypermethylation observed in some cancers[27–30] appear to be operating in BWS.

Repressive chromatin in BWS patients with downregulation of *CDKN1C* but normal methylation at KvDMR1

As BWS patients with loss of methylation at KvDMR1, and some with normal methylation at KvDMR1, both show downregulation of *CDKN1C* in a promoter methylation independent fashion, we postulated that chromatin modifications associated with gene silencing might be involved. We first sought to determine whether allele specific differences in chromatin structure were detectable at the *CDKN1C* promoter by carrying out chromatin immunoprecipitation (ChIP) experiments on somatic cell hybrids containing a single human chromosome 11 of known parental origin. We have previously shown that these hybrids maintain both allele specific gene expression and methylation.[32] The advantage of these cell lines is that one can examine the chromatin structure of each parental allele independently, even in the absence of a polymorphism. As internal controls, we used the promoter regions of the housekeeping gene *LDHA* and the erythroid specific *β globin* gene, two chromosome 11 genes assumed to be active and silent, respectively, in the somatic cell hybrids. Figure 4 shows that, compared with the hybrid carrying the paternal chromosome, the *CDKN1C* promoter in the maternal hybrid was highly enriched in dimethyl H3-K4, a histone modification associated with active genes,[33] and depleted in HP1γ, a protein associated with the chromatin of inactive promoters.[34] In contrast, the hybrid containing the paternally derived chromosome 11 had a threefold lower level of H3-K4 dimethylation, but was enriched for HP1γ at the *CDKN1C* promoter. These results are consistent with the imprinted expression of *CDKN1C*, which is expressed predominantly from the maternal allele.[8] Somewhat



surprisingly, we found little difference between the maternal and paternal *CDKN1C* promoters with respect to dimethyl H3-K9 levels, a histone modification associated with silent chromatin.[35](fig 4A and 4B).

ChIP analysis using the same antibodies detected no differences at the *CDKN1C* promoter between fibroblasts from normal individuals and BWS patients with loss of methylation at KvDMR1 (fig 4A and 4B). The same analysis was carried out on two of the three BWS samples with reduced *CDKN1C* expression but normal methylation at KvDMR1 (the third sample was not available for analysis). Strikingly, cells from these patients showed both decreased levels of H3-K4 dimethylation and enrichment of dimethyl H3-K9 and HP1γ (fig 4A and 4B). These data suggest that, compared with normal controls and BWS patients with LOM at KvDMR1, a repressive chromatin structure exists at the *CDKN1C* promoter in this BWS subgroup which is probably associated with gene silencing. Raji, the Burkitt's lymphoma cell line that is hypermethylated at the *CDKN1C* promoter (fig 4),[27] shows a ChIP profile similar to the fibroblast cell lines from normal individuals for all three antibodies analysed. This indicates that even though there is a reduction in *CDKN1C* expression levels, no change in histone modification was identified. More importantly, this suggests that DNA methylation alone (or in combination with DNA methylation binding proteins) is capable of downregulating *CDKN1C* without accompanying chromatin changes typical of repressed chromatin. Furthermore, Raji shows normal methylation at the *Not*I site in KvDMR1 as determined by Southern blot (not shown), suggesting that KvDMR1 is not associated with the silencing of *CDKN1C* by hypermethylation.

Downregulation of *CDKN1C* is not associated with hypermethylation at *H19*

Several mouse models have been constructed that partially recapitulate the complex phenotype of BWS, the most complete being a mouse line having both a null mutation of *Cdkn1c* and loss of imprinting for *Igf2* (see Caspary *et al*[36] and references therein). Analysis of this double mutant suggested that *Cdkn1c* and *Igf2* function in the same developmental pathway and are antagonistic. A corollary of this, which is consistent with the high degree of similarity between the mutant mouse phenotype and BWS, is that *CDKN1C* and *IGF2* may synergise to produce some characteristics of this condition. In BWS, loss of imprinting (LOI) at

Figure 4 Analysis of chromatin structure at the promoter region of *CDKN1C* by chromatin immunoprecipitation (ChIP). (A) ChIP polymerase chain reaction (PCR) results obtained using antidimethyl H3-K4, antidimethyl H3-K9, and anti HP1-γ antibodies. Samples analysed correspond to: (1) fibroblast cell lines from Beckwith–Wiedemann syndrome (BWS) patients (BWS6 and BWS9) with loss of methylation at KvDMR1 and with low expression of *CDKN1C* (LOM Kv/low p57); (2) fibroblast cell lines from normal individuals (N3 and N6); (3) fibroblast cell lines from BWS patients (BWS7 and BWS11) with normal methylation at KvDMR1 and low expression of *CDKN1C* (normal Kv/lowp57); (4) human–mouse somatic cell hybrids containing either a maternally derived human chromosome 11 (M) or a paternally derived human chromosome 11 (P). The Burkitt's lymphoma cell line, Raji, was also analysed. Input (I) corresponds to PCR of DNA isolated from chromatin before immunoprecipitation. (B) Histograms show the relative enrichment (left vertical axis values) of dimethyl H3-K4 (green), dimethyl H3-K9 (purple), and HP1-γ (yellow) in the different samples analysed. The relative enrichment for each chromatin modification was determined by comparing the normalised intensity (relative to input) of the bands for *CDKN1C* dimethyl H3-K4 with that of *LDHA*, and dimethyl H3-K9, or HP1-γ, with that of *β globin*. Lines in the graph show the enrichment (right vertical axis values) of silencing associated factors, HP1-γ (red) and dimethyl H3-K9 (green) relative to dimethyl H3-K4. Note the dramatic enrichment of HP1-γ and dimethyl H3-K9 with respect to dimethyl H3-K4 in the paternal chromosome hybrid and in BWS patients with normal methylation at KvDMR1 and downregulation of *CDKN1C*.

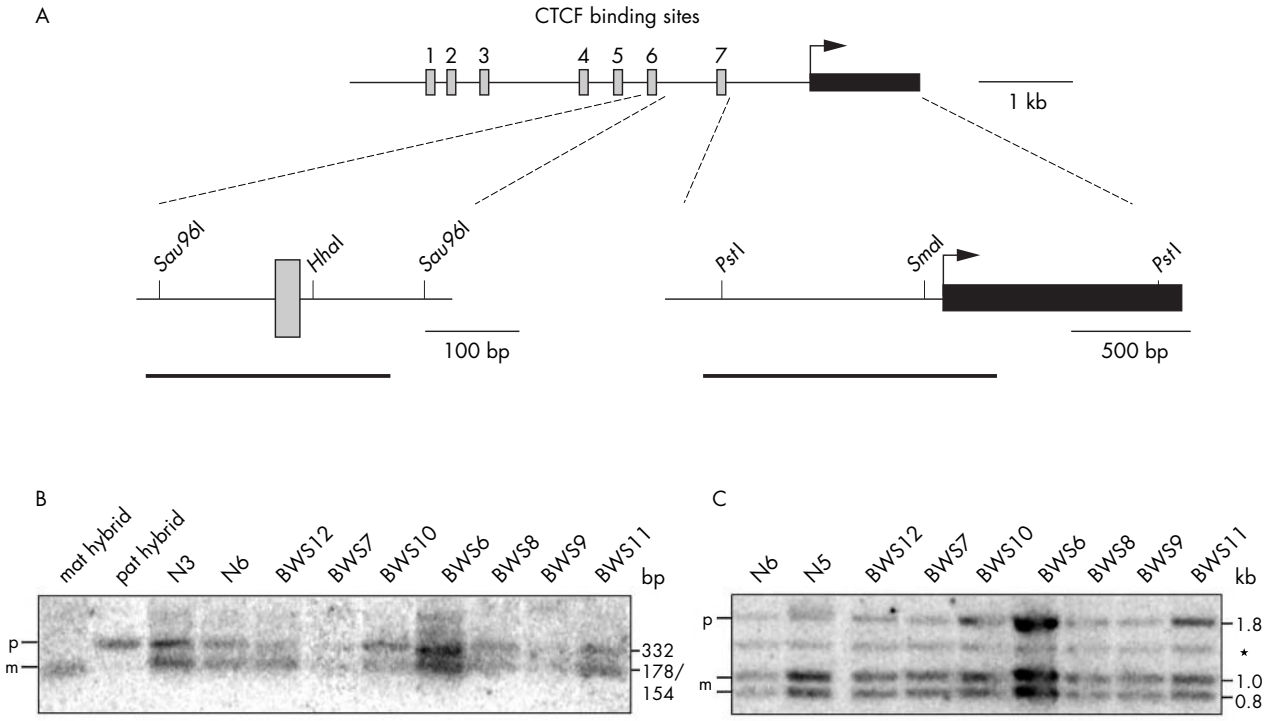


Figure 5 Methylation analysis of the region upstream of the *H19* gene. (A) Map of the upstream region of the human *H19* gene showing the locations of the seven CTCF binding sites and the positions of the two regions analysed for methylation; enlargements of these regions are shown below the map indicating the position of the methylation sensitive restriction enzymes (*Hha*I and *Sma*I) used in the analysis. The heavy black lines below the enlargements represent the location of the hybridisation probes. (B) DNA from the indicated cell lines was digested with *Sau96*I and *Hha*I, electrophoresed and hybridised with a probe encompassing CTCF binding site No 6. The *Hha*I site was cleaved (unmethylated) in DNA from the somatic cell hybrid containing a maternally derived human chromosome 11 giving rise to a unresolvable doublet of 178/154 base pairs. The same site was resistant to cleavage (methylated) in DNA from a hybrid carrying a paternally derived chromosome 11 resulting in a 332 bp *Sau96*I fragment. All normal and patient samples showed both alleles suggestive of normal differential methylation at this site. (C) DNA from the indicated cell lines was digested with *Pst*I and *Sma*I, electrophoresed, and hybridised with a probe encompassing the promoter region and part of exon 1 of the *H19* gene. In each sample, the uncut 1.8 kb paternal allele as well as the cleavage products (1.0 and 0.8 kb) of the maternal allele were visible, with no indication of hypermethylation. The band marked with an asterisk represents hybridisation of the probe to an adjacent *Pst*I fragment.

IGF2 is usually associated with hypermethylation upstream of the *H19* gene.[37] Therefore we determined whether BWS patients with downregulated expression of *CDKN1C* also suffered an epimutation at the *H19/IGF2* locus. Of the seven potential binding sites for the insulator protein CTCF in the human *H19/IGF2* ICR (fig 5A), only site No 6 appears to be differentially methylated in normal tissue, and methylation at this site largely correlates with expression of *IGF2* in somatic cell hybrids treated with 5-aza-2′-deoxycytidine.[38] Consistent with these results, we found that a single site for the methylation sensitive restriction enzyme *Hha*I next to CTCF binding site No 6 was unmethylated (cleaved) in a somatic cell hybrid carrying a maternally derived human chromosome 11, but methylated (uncut) in a hybrid containing a paternally derived chromosome 11 (fig 5B). Both digested and undigested alleles were observed in fibroblast DNA from normal individuals and BWS patients, including both subgroups with downregulation of *CDKN1C*, with no evidence of hypermethylation in any case (fig 5B). Also consistent with the lack of an epimutation in the *H19* ICR in these patients was the finding of normal differential methylation at a *Sma*I site just upstream of the *H19* start site (fig 5C), hypermethylation of which is associated with loss of imprinting at *IGF2*.[37]

DISCUSSION

Our results suggest the existence of at least three silencing mechanisms for *CDKN1C*. These alternative methods of inactivation of *CDKN1C* could all be functionally equivalent to loss of function mutations of *CDKN1C*. Regardless of the mechanism, downregulation of *CDKN1C* appears to be a common occurrence that may lead to BWS[22] or, in some cases, contribute to cancer.[7 27–30 39–41] It should be noted, however, that reduced expression of *CDKN1C* in BWS patients does not generally appear to be associated with predisposition to cancer. Although one report suggests that LOM at KvDMR1, and presumably downregulation of *CDKN1C*, may be related to the incidence of non-Wilms' embryonyl tumours in BWS patients,[42] several other studies suggest that biallelic expression of *IGF2* associated with hypermethylation at the *H19* ICR tracks with tumour development.[21 24 25]

CDKN1C is silenced in association with promoter methylation in several tumour types.[27–30] This represents one mechanism for epigenetic silencing of *CDKN1C*. Although it is a possible mechanism for silencing *CDKN1C* in BWS, we did not observe hypermethylation of *CDKN1C* in any of the BWS patient samples available; however, a larger BWS patient sample size should be analysed to extend these results. Together, our results suggest the presence of distinct mechanisms in cancer and BWS for silencing of *CDKN1C*. It is evident that neither DNA methylation nor chromatin structure (at least as studied here) at the *CDKN1C* promoter region is altered in patients with LOM at KvDMR1 and reduced expression of *CDKN1C*. Evidence complementing our results was recently provided by Soejima *et al*,[41] who showed that downregulation of *CDKN1C* expression in some oesophageal cancers correlates with loss of CpG methylation and loss of histone H3 lysine 9 methylation at KvDMR1, but does

not correlate with methylation or with histone modification at *CDKN1C*. Together, these results strongly support the view that LOM at KvDMR1 is mechanistically related to the silencing of *CDKN1C* in these patients, and that these two epigenetic alterations are not simply independent manifestations of a domain-wide epimutation in 11p15.5.

The molecular mechanisms whereby KvDMR1 regulates imprinting, in particular *CDKN1C* imprinted expression, are not yet understood. It has been shown previously that loss of methylation at KvDMR1 leads to biallelic expression of *KCNQ1OT1*, an antisense (with respect to *KCNQ1*) non-coding RNA transcript (fig 1).[19] It is possible that *KCNQ1OT1* may function in a manner analogous to the *Air* transcript in the *Igf2r* locus, perhaps by facilitating the establishment and spreading of a domain-wide repressive chromatin structure.[43] Indeed, Thakur *et al* have shown that, at least in an episomal context, the *Kcnq1ot1* RNA may play a role in gene silencing.[44] Enhancer blocking assays suggest that KvDMR1 may function as a methylation sensitive insulator or as a bidirectional silencer, or both.[15–18] Our finding that the chromatin structure of the *CDKN1C* promoter does not appear to be altered in BWS patients with LOM at KvDMR1 and downregulation of *CDKN1C* argues against the establishment of domain-wide repressive chromatin on the maternal chromosome caused by the epimutation at KvDMR1. One could therefore speculate that, at least in some instances, KvDMR1 may function like the *H19* ICR (that is, as a chromatin insulator).

On the other hand, *CDKN1C* can be silenced by a KvDMR1 independent mechanism. We identified three patients with normal methylation at KvDMR1 but with low levels of *CDKN1C* expression. In the two patients analysed, we observed repressive chromatin modifications at the *CDKN1C* promoter region. Our analysis included antibodies for only three chromatin modifications, namely dimethyl H3-K4, dimethyl H3-K9, and HP1γ; nevertheless, enrichment of dimethyl H3-K9 and HP1γ, and depletion of dimethyl H3-K4 were clearly seen in this class of BWS patients. We therefore propose that the altered chromatin structure at the *CDKN1C* promoter region is associated with the downregulation of *CDKN1C* levels in these two patients. Whether reduced expression is caused by the acquisition of repressive chromatin at the promoter, or if the altered chromatin is a result of silencing by another mechanism, remains to be determined. Although studies both in cell culture and in the mouse indicate that reduction in the expression level of *CDKN1C* can be associated with excess *IGF2* levels,[36 45] real time RT-PCR analyses do not show increased levels of *IGF2* expression in these patients, nor in BWS patients with LOM at KvDMR1 (data not shown). Furthermore, Southern analysis at two loci known to be associated with LOI at *IGF2* demonstrated normal methylation in both groups of BWS patients with reduced *CDKN1C* levels. These results argue that the repression of *CDKN1C* expression in BWS patients with normal methylation at KvDMR1 is not related to overexpression of *IGF2*. Moreover, these finding suggest that it is not necessary to disrupt the expression of both *CDKN1C* and *IGF2* in order to manifest BWS.

The aetiology of BWS is complex, with several different genetic and epigenetic mechanisms.[1] However, the molecular defect in 10–20% of patients has yet to be established. Although the sample size is small, our finding of reduced levels of *CDKN1C* expression in three of six non-UPD BWS patients with normal methylation at KvDMR1 suggests that a substantial proportion of these cases may show an aberrant chromatin structure at the *CDKN1C* promoter. Further studies are necessary to fully understand the mechanisms responsible for the change in histone modification in the *CDKN1C* promoter region and the impact of these modifications on chromatin structure and transcriptional regulation.

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