CDKN1C expression in Beckwith-Wiedemann syndrome patients with allele imbalance

Elizabeth M Algar, Gillian J Deeble, Peter J Smith

Abstract
In this study, we have examined CDKN1C expression in BWS patients with allele imbalance (AI) affecting the 11p15 region. Two of two informative patients with AI, attributable to mosaic paternal isodisomy, exhibited reduced levels of CDKN1C expression in the liver and kidney, respectively, relative to expression levels in the equivalent tissues in normal controls. Although overall expression was reduced, some expression from the paternally derived CDKN1C allele was evident, consistent with incomplete paternal imprinting of the gene. One patient showed evidence of maternal allele silencing in addition to AI. These findings show for the first time that CDKN1C expression is reduced in BWS patients with AI and suggest that CDKN1C haploinsufficiency contributes to the BWS phenotype in patients with mosaic paternal isodisomies of chromosome 11.

Keywords: CDKN1C; Beckwith-Wiedemann syndrome; allele imbalance

Beckwith-Wiedemann syndrome (BWS) is a complex overgrowth disorder affecting 1 in 14,000 subjects. It has a variable phenotype that includes the three major features of macroglossia, pre- and postnatal overgrowth, and umbilical hernia or omphalocele, and several minor features including ear lobe creases and pits, renal abnormalities, facial naevus flammeus, neonatal hypoglycaemia, and hemihypertrophy. One in 10 patients with BWS develop a tumour within the first seven years of life.

The BWS gene maps to chromosome region 11p15.5. The identification of a subgroup of BWS patients with paternal isodisomy affecting 11p and with partial or complete trisomy 11 in which the paternal chromosome is duplicated has suggested that increased dosage of a paternally expressed, maternally imprinted gene is causative in the syndrome. The maternally imprinted insulin-like growth factor 2 gene (IGF2) maps to 11p15.5 and encodes a mitogenic peptide that is active during embryogenesis and early development. Several pieces of evidence strongly implicate the dysregulated expression or dosage of IGF2 in BWS. A subset of BWS patients exhibit loss of imprinting (LOI) or biallelic expression at the IGF2 locus, and this has been shown both in patients who carry 11p15.5 translocations affecting the region known as BWR1 on the maternal chromosome, mapping proximal to IGF2, and in cytogenetically normal cases. In the latter group, epigenetic inactivation of the maternal allele of the H19 gene, lying 200 kb downstream and telomeric with respect to IGF2, is associated with LOI affecting the IGF2 locus. Recent experiments in which double mutant mice have been generated with an inactivated maternal H19 allele and a deletion of the maternally expressed IGF2R, which normally acts as a sink for degradation of excess IGF2 peptide by receptor mediated endocytosis, have shown that extremely high levels of embryonic IGF2 can elicit several characteristics of BWS including somatic overgrowth, omphalocele, visceromegaly, and placentalomegaly. Furthermore, another study has reported a BWS-like phenotype in ES cell mosaic mice carrying an IGF2 transgene. Although IGF2 expression in these chimaeric animals was shown to be associated with transactivation of the endogenous Igf2 gene, and not the transgene, the animals manifested some of the major stigmata of BWS including prenatal overgrowth, tongue enlargement, and skeletal abnormalities. Although in humans a number of mechanisms have been identified that serve to increase IGF2 dosage in BWS, direct proof that IGF2 is the BWS gene has been lacking. BWS patients do not show raised levels of IGF2 in their serum and biallelic expression of IGF2 has been noted in patients with somatic overgrowth but without additional diagnostic features of BWS, suggesting that an excess of embryonic IGF2 alone is not sufficient to elicit BWS. Furthermore, linkage analysis in familial BWS has excluded the IGF2 locus.

Familial BWS affects 15% of all BWS cases and shows a pattern of maternal inheritance consistent with a pattern of paternal imprinting of a causative gene. This is in contrast to the sporadic cases of BWS in which 20% exhibit paternal isodisomy and 1-2% trisomy with paternal duplication and in which a maternally imprinted, paternally expressed gene is thought to be responsible for the syndrome. Recently, mutations in the paternally imprinted CDKN1C gene, mapping centromeric of IGF2 on 11p15.5, were identified in a subset of BWS patients. Of seven reported mutations, four were found to be maternally inherited, and were predicted to result in the functional inactivation of the maternally expressed allele. BWS cases in which CDKN1C mutations have been identified have a complete phenotype including gigantism, omphalocele, macroglossia, neonatal hypoglycaemia, and earlobe creases and pits. Mice carrying a targeted deletion of the maternally expressing
CDKN1C allele have a phenotype characteristic of some aspects of BWS including omphalocele, renal hyperplasia, enlarged adrenals and skeletal abnormalities. Together these observations suggest that inactivation of CDKN1C plays a role in BWS.

In this report we have examined CDKN1C expression in somatic tissue in BWS patients with mosaic paternal isodisomy. We have shown that CDKN1C expression is reduced in these patients consistent with the predicted effects on CDKN1C expression of mosaic paternal disomy at this paternally imprinted locus. Surprisingly, however, we found that both patients with mosaic disomies expressed CDKN1C from both the paternal and maternal alleles and in one case expression from the paternal allele apparently greatly exceeded that from the maternal allele. This suggests that other, as yet unknown mechanisms affect expression from the CDKN1C locus in BWS in addition to genomic imprinting.

Material and methods

**GENOTYPING**

DNA from peripheral blood or somatic tissue was extracted using standard procedures. Genotyping at TH (11p15.5) and c-SRL-Id6 (11p15.3) was performed using previously described methods. PCR amplification of the TCAT tetranucleotide repeat at the TH locus enabled the identification of five alleles, a to e, ranging in size from 260 to 244 bp. PCR amplification of the GATA repeat at the c-SRL-Id6 marker enabled the identification of five alleles, a to e, ranging in size from 249 to 233 bp. Genotyping at CDKN1C exploited the deletion polymorphisms previously identified in the gene. PCR amplification of CDKN1C generating alleles designated a (331 bp), b (319 bp) and c (307 bp) was routinely done on 200 ng alkali denatured DNA with primers F2 (CGACGTGGCTTAGTGTCGGGTGTC) and R5 (CGAGTGACGCTGTCACGGCAG) using the Expand Long Template PCR system (Boehringer Mannheim). Final primer concentrations were 1 pmol/µl and 0.25 mmol/l dNTPs and 1.5 mmol/l MgCl₂ were present in the reaction; 0.25 mmol/µl deaza dGTP was substituted for dGTP to allow for amplification of this highly GC rich region. Cycling was performed for one cycle at 95°C for five minutes, 69°C for 30 seconds, and 35 cycles at 69°C for one minute, 72°C for one minute, 30 seconds, and 96°C for one minute with a final cycle at 69°C for one minute, 72°C for 10 minutes. In all of the genotyping studies described above, 3HdCTP was incorporated into PCR reactions and allelic products were run out on 6% denaturing polyacrylamide gels containing urea in 1 x TBE buffer. Gels were dried and then subjected to phosphoimage analysis (Molecular Dynamics) or to standard autoradiography. For identification of CDKN1C alleles, and the analysis of allele specific CDKN1C expression, an additional amplification protocol, which was found to generate high levels of PCR product, was developed during the course of this study. Use of this protocol eliminated the need for incorporation of 3HdCTP and allowed allele identification, following staining with ethidium bromide, of PCR products run in non-denaturing 6% polyacrylamide gels containing 10% glycerol. The polymorphic region of CDKN1C was amplified using primers F4 (GCGTTTCTACCCGGAGACG) and R5 in a reaction mix containing the PCR Enhancer system (Life Technologies) in the presence of 2 x enhancer reagent, 0.20 mmol/l dNTPs, 0.2 pmol/µl of each primer, and 1.5 mmol/l MgSO₄. PCR proceeded for one cycle at 95°C for two minutes, 35 cycles at 95°C for 45 seconds, 57°C for 30 seconds, 68°C for one minute, and one cycle at 68°C for 10 minutes. Ten microlitres of the 468 bp PCR products obtained were digested overnight with PvuII, generating alleles a (318 bp), b (306 bp), and c (294) derived from the 12 bp polymorphic CDKN1C repeat region. Fifteen microlitres of the digest mix was loaded onto non-denaturing gels as described above and alleles were separated following electrophoresis at 127 V for 18 hours at room temperature. Allele imbalance was assessed by reproducible deviation of allele ratios from those identified in normal subjects at either of the three 11p15 markers examined. In addition in vitro mixing of known amounts of DNA from homozygotes for respective CDKN1C alleles was also performed to establish the extent of allele skewing owing to selective amplification of shorter alleles.

**SEMIQUANTITATIVE RT-PCR**

RNA extraction from somatic tissues was performed using the Qiagen Rneasy RNA extraction kit. RNA was treated with 0.25 U/µl DNase I (Promega) for 30 minutes at 37°C, followed by heat inactivation of the DNase at 95°C for five minutes. A total of 500 ng of RNA was reverse transcribed in a total reaction volume of 42 µl using random primers and M-MLV-Reverse transcriptase according to standard procedures. For semiquantitative analysis of CDKN1C expression in BWS somatic tissues, the intensity of CDKN1C PCR product generated using primers F4 and R7 was compared relative to the intensity of PCR product amplified from the human phosphoribosyl transferase gene (HPRT) using primers HPRT F and HPRT R, amplified from the PCRs produced using primers F4 and R7 was compared relative to the intensity of PCR product amplified from the human phosphoribosyl transferase gene (HPRT) using primers HPRT F and HPRT R, amplified from the same source of cDNA. PCR reactions for CDKN1C and HPRT were done separately. CDKN1C amplification was performed using the PCR Enhancer system of Life Technologies according to the manufacturer’s instructions and incorporating 3 x enhancer and 0.5 U Expand Taq polymerase (Boehringer Mannheim) into the final reaction mix. Primer sequences were: F4 5'GGGTCTAACCGGAGACG and R7 5'TAAATTGGCTCACCCCGAGCC and amplification was performed for one cycle at 95°C for three minutes and for up to 42 cycles at 95°C for 45 seconds, 56°C for 30 seconds, and 68°C for one minute 30 seconds, followed by a final cycle of 56°C for 30 seconds and 68°C for five minutes. HPRT was amplified using PCR Ready To Go Beads (Pharmacia Biotech) and primers
HPRT F 5’GGCGTCTGATTAGTGTGAT- GATGAACC and HPRT R 5’CTTGGAGC- CTTGACATCTTTAGGA. Cycling was performed for one cycle at 95°C for five minutes, up to 40 cycles at 69°C for one minute, 72°C for one minute, 94°C for one minute, and for one cycle at 69°C for one minute, 72°C for 10 minutes. The exponential phase of the reaction for both CDKN1C and HPRT amplification was determined in preliminary experiments using cDNA prepared as described above from both normal and BWS kidney and from normal and BWS liver. PCR reactions were arrested at selected cycle numbers in the range from 24 up to 42 cycles. The cycle numbers selected at which CDKN1C and HPRT PCR product band intensities were compared from each tissue spanned the exponential reaction phase. Analyses were done in duplicate at the selected cycle numbers.

For identification of the exponential amplification phase and PCR product quantitation, 10 µl of PCR products were run on agarose, Southern blotted in 20 x SSC, and hybridised with probes representing sequence internal to the amplified products. CDKN1C products were probed with an internal gel purified CDKN1C PCR product obtained by amplification using primers F4 and R5 as described above. HPRT products were probed with a gel purified HPRT PCR product. Probes were labelled by random priming using the High Prime reagent (Boehringer Mannheim) according to the manufacturer’s instructions. Filters were probed for one hour in ExpressHyb buffer (Clontech), washed, and subjected to phosphoimaging analysis. Band intensities were quantified using the Peak Finder option of the Imagequant software (Molecular Dynamics).

**MUTATION ANALYSIS**

Sequencing of the entire coding region of the CDKN1C gene was done using direct automated sequencing with the ABI PRISM dye terminator cycle sequencing ready reaction kit, according to the manufacturer’s instructions. Electrophoresis was performed by the DNA Sequencing facility in the Department of Microbiology, Monash University, Clayton, Victoria, Australia. Overlapping CDKN1C PCR fragments for sequencing were generated using the following primer pairs: F7 5’AGCGAGCTAGCAGCAGGCAAT and R8 5’GTGCGCACTAGTACTGGGAA (316 bp), F1 5’CGAGCACATCCACGATGGACGGC and R2 5’GACGGGACACTAG- GCAGCTG (402 bp), F4 5’GCCTTCTAC- CGCGAAGACG and R5 5’CGAGTGCAGCT- GTTCAGCGAG (466 bp), F5 5’CTCAAGAGAGCGCCGAGGAGC and R9 5’TATATGCCACCGGAGGAGG (273 bp), F11 5’AGCTGACCGCCGCAG- GACCT and R12 5’CCCTCCCAAGCTC- CGTGGCC (412 bp). PCR products spanning the polymorphic PAPA repeat region of the gene were amplified with primers F2 5’GACGCTGCTAGTGTGGTGGGC and R5 (331), or F4 and R5, gel purified, A tailed with Promega T4 DNA polymerase, and ligated into pGem T (Promega). Following blue/white selection, clones were miniprepped, re-PCRD with primers F2 and R3, and subjected to automated sequencing.

**IMPRINTING**

Allele specific expression at the CDKN1C locus was examined by amplification of cDNA prepared from normal kidney, Wilms tumour, and liver. DNA free cDNA was prepared as described above. In order to visualise allele specific PCR products from samples expressing low levels of CDKN1C, cDNA was on occasions concentrated by ethanol precipitation according to standard protocols. CDKN1C was amplified using primers F2 and R7, following which PCR products, of maximum size 559 bp, were digested with PvuII, predicted to cut two sites within the fragment, and 15 µl digest aliquots were run out on 6% non-denaturing gels containing 10% glycerol. This procedure generated a constant band of size 237 bp outside the polymorphic repeat region of the cDNA and a variable fragment of maximum size 322 bp. The predicted size of this fragment is 318 bp; however, the 5’ PvuII recognition site begins only one base in from the 5’ end of the F2 primer and PvuII is unable to cut at this site in the generated fragment. CDKN1C was amplified using primers F2 and R7 under either of two reaction conditions. In some instances, CDKN1C was alkali denatured before amplification in a reaction mix containing the Expand Taq polymerase buffer system and incorporating uTP dCTP. Five microlitres of PvuII digested PCR product was run out on 6% non-denaturing polyacrylamide gels containing 10% glycerol and gels were dried and subject to phosphoimaging. In other instances, the PCR enhancer system of Life Technologies was used for PCR, using 3 x enhancer in the mix, and 15 µl of PvuII digests were run out on gels as described above and then stained with ethidium bromide. Images were obtained from these gels using the GelDoc system of Biorad with Multianalyst software. PCR conditions for amplification of CDKN1C using the PCR enhancer system were: one cycle of 95°C for five minutes, 35 cycles of 58°C for 30 seconds, 68°C for one minute 30 seconds, 95°C for 45 seconds, and one cycle of 58°C for 30 seconds, 68°C for five minutes. CDKN1C genotyping of samples used in imprinting studies and of parental DNA was done according to methods described above using CDKN1C primers F4 and R5 and amplification of genomic DNA from either somatic tissues or peripheral blood.

To examine the presence of genomic contamination in the samples used to examine allele specific CDKN1C expression, exon 9 of the WT1 gene zinc finger region was amplified using intronic primers.19

**Results**

**ANALYSIS OF ALLELE IMBALANCE (AI) IN THE BWS POPULATION**

Twenty four patients diagnosed with BWS were examined for allele imbalance affecting the 11p15 region using three previously described polymorphisms. These included the tetranucleotide repeat of the tyrosine hydroxy-
lase gene, the 12 and 6 bp deletion polymorphisms within the CDKN1C gene, and the tetranucleotide repeat of the c-SRL-Id6 marker. DNA for genotyping was sourced from peripheral blood lymphocytes. In two cases, constitutional DNA was also extracted from patient liver and kidney following resection of tumours for clinical indications. Twenty two patients were heterozygous or informative at any one of the three markers. Two patients were homozygous at all three markers. Allele imbalance was diagnosed where there was significant skewing (>30% deviation) of allelic ratios at any of the three polymorphic markers relative to that seen in normal controls. At the CDKN1C locus, no significant allele skewing was observed in normal controls where alleles differed in size by 12 bp (that is, alleles a and b); however, where alleles a and c were present (size difference 24 bp), significant allele skewing was observed in normal controls with the result that the intensity of the shorter allele, c, was 2.5 times that of the a allele. This was shown, by allele mixing experiments, to be the result of selective amplification by PCR of the shorter CDKN1C c allele. Genotyping at TH and c-SRL-Id6 showed no evidence of significant skewing favouring shorter alleles in normal controls. According to these criteria, two patients examined in the population (9%) showed marked allele imbalance in DNA isolated from peripheral blood lymphocytes and in both cases the CDKN1C locus was affected. Fig 1A and B depict the allele imbalance identified in two patients, BWSW and BWS91 respectively, at affected loci. Patient BWSW exhibited AI at all three markers examined; however, patient BWS91 exhibited AI at CDKN1C only, as the TH and c-SRL-Id6 loci were uninformative in this patient. Genotyping at the CDKN1C locus, on DNA isolated from the parental peripheral blood, is also shown for these patients and indicates that the paternally inherited CDKN1C allele is more abundantly represented in the patient peripheral blood DNA than is the maternally inherited allele. These findings suggest that these two patients have mosaic paternal isodisomy of 11p consistent with previous studies on BWS and show directly that the CDKN1C locus is affected. Allele ratios were calculated for each patient by densitometric analysis and show a mean ratio value of paternal to maternal alleles at the CDKN1C locus in BWSW of 2.51 and in BWS91 of 4.9. It is possible that the two patients who were homozygous at all three markers examined may also exhibit allele imbalance with a high percentage of mosaicism, but this is unlikely given the high sensitivity of the genotyping method used and the absence of any evidence for a second allele following prolonged autoradiographic exposure or phosphoimaging.

Both patients with allele imbalance identified in this study had developed a tumour. Patient BWSW was an 11 month old child with a birth weight of 4950 g. He had a marked right sided hemihypertrophy, macroGLOSSIA, and a large umbilical hernia. He did not develop hypoglycaemia in the neonatal period and was noted to have hepatomegaly and renal abnormalities including a distended right ureter and fetal lobulations. He developed a hepatoblastoma at 11 months of age. Patient BWS91 was a 5 year old child with a birth weight of 4960 kg. He had earlobe creases and a left sided hemihypertrophy. He developed a peribiliar Wilms tumour of the right kidney at 3 years of age.
Table 1  Ratios of CDKN1C RT-PCR product band intensities measured at variable cycle numbers spanning the exponential phase, relative to the intensity of the HPRT control gene PCR product measured at 26 cycles, in both a normal liver and kidney and in BWSW liver and BWS91 kidney. The figures in the column on the far right hand side represent the relative ratios of the normalised CDKN1C ratios in BWSW and normal liver and BWS91 and normal kidney.

<table>
<thead>
<tr>
<th>Cycle No</th>
<th>CDKN1C/HPRT Normal liver</th>
<th>CDKN1C/HPRT BWSW liver</th>
<th>CDKN1C/HPRT BWS/normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>0.26</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>38</td>
<td>0.42</td>
<td>0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>42</td>
<td>0.93</td>
<td>0.23</td>
<td>0.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cycle No</th>
<th>CDKN1C/HPRT Normal kidney</th>
<th>CDKN1C/HPRT BWS91 kidney</th>
<th>CDKN1C/HPRT BWS/normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.001</td>
<td>0.00014</td>
<td>0.14</td>
</tr>
<tr>
<td>30</td>
<td>0.032</td>
<td>0.005</td>
<td>0.16</td>
</tr>
<tr>
<td>35</td>
<td>0.140</td>
<td>0.029</td>
<td>0.21</td>
</tr>
</tbody>
</table>

different cycle numbers to establish the exponential phase for both HPRT and CDKN1C and 1 μl of cDNA was amplified in subsequent reactions at selected cycle numbers to enable quantitation of relative CDKN1C expression levels in each sample. CDKN1C expression levels in BWS liver and kidney were compared with levels in kidney and liver from controls, without evidence of BWS or anomalies of 11p. These included a sample of control kidney resected in association with a Wilms tumour, which was found to have a homozygous tumour specific deletion affecting the 3' end of the WT1 gene.2 The presence of this identifiable WT1 lesion suggested that the normal kidney in this patient was unlikely to carry a CDKN1C lesion and that expression levels of CDKN1C in this sample could be considered to approach normal. Both the BWS and control kidney samples were from kidney cortex reducing the possibility that regional variation in kidney CDKN1C expression would affect this comparative analysis. In addition, the samples were age matched as far as practicable, with the control kidney from a child 2 years 9 months of age and the BWS kidney from a 5 year old (BWS91). The control liver was obtained from normal tissue resected in association with a sporadic hepatoblastoma in a 3 year old child. The hepatoblastoma had no evidence of anomalies of chromosome 11 (that is, LOH) and the child had no features of BWS. Although we could not definitively exclude an 11p15 lesion from this liver sample there was no a priori evidence that one existed.

CDKN1C was amplified using a forward primer F4 upstream of the polymorphic PAPA repeat region in exon 2 and a reverse primer located at the 3' end of exon 3 and extending past the stop codon into exon 4 (R7). This RT-PCR was designed to amplify a 696 bp fragment derived from CDKN1C cDNA. Positions of these primers and of primers used in CDKN1C genotyping are depicted in fig 2A. PCR products were Southern blotted and hybridised with gene specific probes as described in Methods. In both the kidney from patient BWS91, and in the liver from BWSW, CDKN1C expression was observed to be significantly less than that in the corresponding control samples when expression levels were normalised to HPRT product intensities obtained following 26 PCR cycles. Table 1 depicts the ratios of CDKN1C band intensities at different cycle numbers spanning the exponential amplification phase in both normal and BWS liver and kidney relative to HPRT levels at 26 cycles. At each CDKN1C cycle number at which a ratio is calculated, the levels of CDKN1C are significantly less in the BWS tissues relative to their control counterpart. Fig 2B and C depicts CDKN1C band intensities observed at different cycle numbers and HPRT intensities at 26 cycles in both BWS and normal liver and kidney tissues respectively. The HPRT levels amplified from the liver cDNA from patient BWSW were approximately 30% of those obtained in control liver in the exponential amplification phase. This situation persisted although several different RNA preparations from BWSW liver were made and identical amounts of RNA were reverse transcribed on each occasion. Similarly, lower levels of a second control gene, B-actin, were also found in this sample. Therefore, we have to conclude that some degradation of the liver tissue had occurred which had affected the quality of the RNA extracted from this sample. However, as the results presented for CDKN1C expression in table 1 are standardised to a control gene level, sample differences in RNA quality will be accounted for. Some variation is also apparent in the relative levels of CDKN1C calculated at the different cycle numbers presented in table 1. The band intensities for CDKN1C were very low at the lower PCR cycle numbers examined and the variation is most likely attributable to the reduced accuracy of band quantitation at low signal levels. These results, however, suggest that there are significant differences in CDKN1C expression levels between the BWS patient and normal control tissues.

To eliminate the possibility that CDKN1C mutations may have contributed to the reduced levels of CDKN1C expression or the phenotype in these patients, the entire coding region of the gene was examined for point mutations or deletions and insertions using direct automated sequencing of overlapping PCR fragments. Primer positions are indicated in fig 2A and nucleotide positions refer to those for the CDKN1C sequence deposited in the NCBI database, accession U48869. Sizes of the generated PCR fragments are described in Methods. Sequence analysis extended from an intronic primer located at position +136 from the first initiation codon and extended to +210 downstream from the stop codon in the fourth exon of the gene. The PCR fragment spanning the polymorphic PAPA repeat region, F2/R5 or F4/R5, was subcloned into the pGemT vector following A tailing of gel purified PCR products, and clones were selected for sequencing. Two alleles, a and b, were identified in each patient as predicted from genotyping analyses. In both patients a single 12 bp deletion was identified corresponding to the 3' APAP deletion previously described by Tokino et al.15 No other sequence anomalies could be identified, although at least two clones corresponding to each allele were sequenced for each sample. Therefore, we excluded the
possibility that a CDKN1C coding mutation was responsible for the reduction in CDKN1C expression observed in these patients.

Allele specific expression of CDKN1C was then examined in the liver of patient BWSW and in the kidney from patient BWS91. Patient RNA was DNase treated and subjected to RT-PCR incorporating [alpha]PdCTP and using primers F2 and R7. This PCR reaction was designed to amplify a 559 bp cDNA product which could be digested at PvuII sites (n=1133, fig 2A) flanking the polymorphic repeat region thereby generating allele specific PCR products which were of a size that could be separated on 6% non-denaturing polyacrylamide gels. Genomic contamination was excluded from the source RNA by checking for contamination using an intron specific PCR for the WT1 gene described previously. In addition no bands of the size predicted from a genomic CDKN1C product were seen on gels. Allele specific expression analysis showed heterogeneous expression of maternal and paternal alleles consistent with this, the result obtained for patient BWS91 showed CDKN1C almost exclusively from the paternal allele with very little expression from the maternal allele (fig 3A). Although the imprinted paternal CDKN1C allele has in some cases been shown to have a “leaky imprint” and the finding of biallelic expression in the liver of BWSW is consistent with this, the result obtained for BWS91 was entirely unexpected given the previously widely reported bias towards paternal imprinting at the CDKN1C locus. This result alone suggests that additional mechanisms play
a role in reducing CDKN1C expression in BWS cases with AI. To show that the results obtained from this analysis were not attributable to a flaw in our experimental design or some unforeseen artefact we showed that both monoallelic and maternal allele specific expression at the CDKN1C locus could be clearly shown in Wilms tumour and normal kidney tissue samples (fig 3B). In the informative example shown, the paternal CDKN1C genotype is a/a, the maternal is a/b, and in the Wilms tumour, which has retained heterozygosity at CDKN1C, the maternally derived CDKN1C allele b, is exclusively expressed.

Discussion
In this study we have shown that BWS patients with mosaic paternal isodisomy affecting 11p15.5 exhibit reduced levels of CDKN1C expression in somatic tissues including the kidney and liver relative to normal controls. In one patient with biallelic CDKN1C expression, BWS91, we were able to show that the relative expression from the paternally inherited CDKN1C allele was reduced relative to that predicted by representation of the allele in the patient’s somatic DNA, consistent with paternal imprinting at the locus. Paternal imprinting at CDKN1C, in association with the reduction in maternal allele representation in patients with paternal isodisomy of 11p, is therefore likely to play a role in reducing overall CDKN1C expression in BWS.

In the second patient examined with mosaic paternal isodisomy, BWS91, CDKN1C expression was lower in the kidney tissue from this patient compared with that in a normal control. However, the CDKN1C transcript exhibited negligible expression from the maternal allele and allelic expression was predominately from the paternal allele. This was contrary to the expected pattern of expression at this paternally imprinted locus. This observation suggests that this patient has an imprinting anomaly, resulting in silencing of the maternal CDKN1C allele, as well as mosaic paternal isodisomy, which affects the expected pattern of allelic expression from the CDKN1C locus. These results, together with previous reports describing heterozygous inactivating mutations affecting the CDKN1C locus in BWS patients, suggest that insufficient levels of CDKN1C play an important role in BWS.

Using semiquantitative RT-PCR, it is difficult to be precise about the extent to which CDKN1C expression is reduced in the two BWS patients. It was not possible to examine the levels of CDKN1C in the patient tissues by other means owing to lack of availability of sufficient material. However, our results show convincing evidence for substantially lower levels of CDKN1C in these patients. Others have also reported biallelic expression of CDKN1C in BWS cases, but it has not previously been shown whether this was associated with mosaic paternal isodisomy in association with a leaky paternal imprint and with reduced levels of CDKN1C expression.23 The result reported here suggests that this may be so.

The predominant CDKN1C expression from the paternal allele in the kidney of patient BWS91 suggests that CDKN1C imprinting in this patient is abnormal. Interestingly, we had previously shown evidence in the kidney tissue from the same patient that the IGF1R locus on chromosome 15q is subject to an unusual application of imprinting where the normally biallelically expressed IGF1R gene is paternally silenced.24 We proposed that this represented an unusual application of imprinting possibly reflecting allelic differences in imprinted genes. If these genes act in trans, then it is possible that imprinting at other loci may also be affected, possibly explaining the silencing of CDKN1C expression from the maternal allele in this patient.

Although we did not establish in this study that the IGF2 locus was affected by AI in the patients investigated, previous studies on BWS have shown that both CDKN1C and IGF2 are affected.25 If this is so in the two cases presented in this study, then the haploinsufficiency affecting the IGF1R in patient BWS91 might be predicted to affect the phenotype of the patient. Although this patient exhibited a significant degree of AI on 11p15, the phenotype of the patient lacked some of the features of complete BWS. For example, neither macroglossia nor exomphalos were reported. Although speculative, this might indicate that the phenotype of this patient is largely the result of CDKN1C insufficiency. This is because haploinsufficiency of the IGF1R in this patient, which we had previously noted was a germline phenomenon, would be predicted to impede mitotic signalling by IGF2, with the net effect of modifying the effects of IGF2 overexpression on early development. The observations that we have described for this patient suggest that genetic background, in addition to 11p15 anomalies, may affect the BWS phenotype.

The suggestion that CDKN1C haploinsufficiency is important in BWS has been difficult to reconcile with the observations that a small proportion of BWS patients (2%) are trisomic for 11p. In these patients, duplication of paternal 11p is not predicted to affect CDKN1C expression. However, if IGF2 and CDKN1C activities are antagonists of cell cycle progression, that is activated by IGF2 mediated signal transduction through the IGF1R, then the net effect of IGF2 overexpression or CDKN1C inactivation will be similar. Convergence between IGF2 and CDKN1C mediated pathways would explain why BWS patients with 11p trisomy, paternal isodisomy, and CDKN1C mutations have overlapping phenotypic features. According to this model, patients with mosaic paternal isodisomies would be predicted to have a more severe phenotype as affected cell lineages would suffer the dual effect of increased IGF2 dosage and CDKN1C insufficiency. However, the presence and severity of phenotypic features would also be affected by the degree of mosaicism in addition to the influence of modifier genes that impact on IGF2 and CDKN1C associated metabolic pathways.
CDKN1C expression in Beckwith-Wiedemann syndrome

We are indebted to the patients and their families who provided material for this study and to the medical and nursing staff of the Royal Children’s Hospital (Melbourne) (RCH) and the Victorian Clinical Genetics Service. This work was conducted with the permission of, and under the guidelines of, the RCH ethics in human research committee (reference number EHRG. 97045A) and was supported by the National Health and Medical Research Council of Australia and the RCH Research Institute.