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 paternal allele is responsible for the syndrome. 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 placental enlargement. 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 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 expression in Beckwith-Wiedemann syndrome

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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 disomy 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.16 17 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.18 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 using previously described primers.16 PCR amplification of the CDKN1C repeat region. Fifteen microlitres of 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 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 PCR from homozygotes for respective CDKN1C alleles was also performed to establish the extent of allele skewing owing to selective amplification of shorter alleles.

Semi-quantitative RT-PCR

RNA extraction from somatic tissues was performed using the Qiagen Rneasy RNA extraction kit. RNA was treated with 0.25 U µl−1 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 phosphobovyl 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’GGTTCCTACCCCCAGAGCAGG and R7 5’AAATGGGCTACCCGCAGGCG 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.
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HPRT F 5'GGCGTCGTAGTATTACGTAG- 
GATGAACC and HPRT R 5'CCTGGGAC- 
CTTGACATCTTTGGA. Cycling was per- 
formed 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 reac- 
tions for both CDKN1C and HPRT amplifica- 
tion was determined in preliminary exper- 
iments 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 reac- 
tion phase. Analyses were done in duplicate 
at the selected cycle numbers.

For identification of the exponential amplifi- 
cation 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 amplifi- 
cation using primers F2 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 
GATGAACC and HPRT R 5'CTTGCGAC- 
GCTGCC (412 bp). PCR products spanned 
the polymorphic PAPA repeat region of 
the 11p15 region using three previously 
described polymorphisms. These included the 
tetranucleotide repeat of the tyrosine hydroxy- 
MUTATION ANALYSIS

Sequencing of the entire coding region of the 
CDKN1C gene was done using direct auto-
mated 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'AGCGAGCTAGCCAGGCGAT and R8 
5'GTTGCGCAGTACTCTGAGAA (316 bp), F1 
5'CAGCACATCCGATGGAGCG and R7 
5'GGTGCGCACTAGTACTGGGA (316 bp), F11 
5'AGCTGACCCGCCGCG (412 bp), F4 and R5 under either of two reaction conditions. In 
some instances, cDNA was alkali denatured 
before amplification in a reaction mix contain- 
ing the Expand Taq polymerase buffer system 
and incorporating α³²P dCTP. Microdrops of 
PvuII digested PCR product was run out on 
6% non-denaturing polyacrylamide gels con- 
taining 10% glycerol and gels were dried and 
subjected to phosphoimage analysis. In 
other instances, the PCR enhancer system of Life Technologies was used for PCR, using 3 × 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 for 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- 

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 express- 
ing low levels of CDKN1C, cDNA was on 
occasions concentrated by ethanol precipita-
tion according to standard protocols. 
CDKN1C was amplified using primers F2 and 
R7, following which PCR products, of maxi-
mum 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, cDNA was alkali denatured 
before amplification in a reaction mix contain- 
ing the Expand Taq polymerase buffer system 
and incorporating α³²P dCTP. Microdrops of 
PvuII digested PCR product was run out on 
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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 
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from either somatic tissues or peripheral blood.

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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 isodismy 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 2.31 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 perilobar Wilms tumour of the right kidney at 3 years of age.

Figure 1 Allele imbalance in lymphocyte DNA at informative loci tested in BWS patients BWSW and BWS91. (A) Genotyping at three informative 11p15 loci in patient BWSW. Allele imbalance is indicated at c-SRL-Id6, CDKN1C, and TH. Allele sizes are allele c (319 bp) and allele d (331 bp), CDKN1C, allele a (260 bp) and allele b (248 bp), and TH, alleles a (260 bp) and d (248 bp). Genotyping at the CDKN1C locus is also shown for parental DNA: maternal (M) and paternal (P). Allele imbalance is indicated at all three loci examined. (B) Genotyping at a single informative locus, CDKN1C, in patient BWS91 (allele a, 331 bp, allele b, 319 bp). Other loci examined were homozygous (data not shown). Parental genotype is also shown, maternal (M) and paternal (P). Allele imbalance is indicated by greater abundance of paternal allele a in the patient lymphocyte DNA.

CDKN1C EXPRESSION IN BWS PATIENTS WITH AI

As both patients in whom AI at the CDKN1C locus had been identified had developed tumours we examined both the gross level of CDKN1C expression using a semiquantitative RT-PCR analysis and the allele specific CDKN1C expression in normal kidney and liver tissues resected in these patients in association with the Wilms tumour and hepatoblastoma. Gross levels of CDKN1C expression were examined in the patient liver and kidney and expression levels normalised relative to the expression level of an internal control gene, HPRT, according to the principles outlined in Golay et al. These were compared with the normalised CDKN1C expression levels in both liver and kidney samples from non-BWS subjects. Quantitation of the relative CDKN1C expression levels between normal and BWS tissues was examined at several different PCR cycle numbers spanning the exponential phase of amplification. RNA isolated from liver and kidney tissues was DNase treated and 500 ng was reverse transcribed in a total volume of 42 µl; 1 µl of cDNA derived from the RT reaction was PCR amplified at
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. 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 5’ APAP deletion previously described by Tokino et al. 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

<table>
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<th>Cycle No</th>
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<th>Cycle No</th>
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<th>CDKN1C/HPRT BWS91 kidney</th>
<th>CDKN1C/HPRT BWS/normal</th>
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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 rates of the normalised CDKN1C ratios in BWSW and normal liver and BWS91 and normal kidney.
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 $^{32}$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=815, n=1133, fig 2A) flanking the polymorphic repeat region thereby generating allelic 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. In order to visualise allele specific expression in the patient samples, liver cDNA from patient BWSW was concentrated before RT-PCR. CDKN1C alleles were predicted to run at 318 bp (allele a) and 306 bp (allele b) respectively. However, the 5' end PvuII restriction site (n=815) lies at the end of the F2 primer and this site was found to be refractory to digestion as allelic products of slightly greater size (allele a 322 and allele b 310) were consistently obtained. Using this approach we found that patient BWSW liver expressed CDKN1C bi-allelically and showed evidence of incomplete imprinting of the paternal allele (fig 3A) and that patient BWS91 expressed 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.

![Figure 2](http://jmg.bmj.com/)

**Figure 2** (A) Graphic showing positions of primers used in the study. Broken lines denote intronic sequence and boxes represent exons. Bases are numbered according to the CDKN1C sequence in the NCBI database (Accession U48869). PvuII restriction sites are indicated by filled triangles. The first translation initiation codon and the stop codons are indicated by thin and thick vertical lines, respectively. The polymorphic PAPA repeat region is indicated by a grey box. (B) CDKN1C expression in normal liver and in BWS liver examined by semi-quantitative RTPCR. Lanes 1 and 2 show bands obtained following amplification of CDKN1C for 36 cycles, lanes 3 and 4 for 38 cycles, and lanes 5 and 6 for 42 cycles from normal liver and BWS liver (normal liver, odd numbered lanes; BWSW liver, even numbered lanes). Lanes 7 and 8 depict bands from amplification of the HPRT gene for 26 cycles in normal and BWS liver respectively. The band intensities in lanes 7 and 8 have been scaled back for visual clarity and do not reflect the band intensity values used in deriving the data presented in table 1. (C) CDKN1C expression in normal kidney and BWS91 kidney examined by semi-quantitative RTPCR. Lanes 1 and 2 show bands obtained following amplification of CDKN1C for 25 cycles, lanes 3 and 4 for 30 cycles, and lanes 5 and 6 for 33 cycles from both normal kidney and BWS91 kidney (normal kidney, odd numbered lanes; BWS91 kidney, even numbered lanes). Lanes 7 and 8 depict bands from amplification of the HPRT control gene for 26 cycles from normal kidney and BWS91 kidney respectively. The band intensities in lanes 7 and 8 have been scaled back for visual clarity and do not reflect the band intensity values used in deriving the data presented in table 1.

![Figure 3](http://jmg.bmj.com/)

**Figure 3** (A) Allele specific expression at the CDKN1C locus in BWS patients. Lane 1, CDKN1C, showing AI, amplified from BWSW liver genomic DNA using primers F2 and R5. Lane 2, CDKN1C amplified from cDNA from BWSW liver showing biallelic expression. Lane 3, CDKN1C, showing AI, amplified from BWS91 kidney genomic DNA using primers F4 and R5 and digested with PvuII. Lanes 4 and 5, CDKN1C amplified from cDNA from BWS91 kidney and a normal kidney control respectively showing predominant expression from the paternal allele, a, in BWS91 kidney. The sizes of the CDKN1C bands generated by amplification from genomic DNA vary according to the primer pairs used (primers F2/R5: allele a, 331 bp, allele b, 319 bp; primers F4/R5: allele a 318 bp, allele b, 306 bp, compare lanes 1 and 3). CDKN1C amplified from cDNA using primers F2 and R7, followed by digestion with PvuII, generates a constant band of size 237 bp and allelic products of 322 bp (allele a) and 310 bp (allele b). (B) Imprinting at the CDKN1C locus in normal kidney and Wilms tumour samples. Lane 1, pGEM markers of size 350 and 222 bp. Lanes 2 and 3, amplification of parental DNA at the CDKN1C locus pertaining to normal kidney, WK75 (lane 2, paternal; lane 3, maternal). Lane 4, amplification of CDKN1C from genomic DNA in kidney WK75. Lane 5, amplification of CDKN1C from cDNA isolated from kidney WK75 showing predominantly monoallelic expression. As both parental DNA samples from WK75 were heterozygous it was not possible to determine the parental origin of the expressed CDKN1C allele in this sample. Lanes 6 and 7, amplification of parental DNA at the CDKN1C locus pertaining to Wilms tumour, NP50 (lane 6, paternal; lane 7, maternal). Lane 8, amplification of CDKN1C from genomic DNA in NP50 showing maintenance of monoallelic expression. Lane 9, amplification of CDKN1C from cDNA isolated from NP50 showing monoallelic expression of the 310 bp maternally inherited allele. Genomic DNA was amplified with primers F4 and R5 and products then digested with PvuII, generating bands of 318 bp (allele a) and 306 bp (allele b). cDNA was amplified with primers F2 and R7 and products digested with PvuII generating allelic bands of 322 bp (allele a) and 310 bp (allele b) and a constant band of 237 bp.
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, BWSW, 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. 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. We proposed that this represented an unusual application of imprinting possibly reflecting allelic differences in imprintor 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. 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 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.
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