Analysis of germline CDKN1C (p57^{KIP2}) mutations in familial and sporadic Beckwith-Wiedemann syndrome (BWS) provides a novel genotype-phenotype correlation

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Abstract

Beckwith-Wiedemann syndrome (BWS) is a human imprinting disorder with a variable phenotype. The major features are anterior abdominal wall defects including exomphalos (omphalocele), pre- and postnatal overgrowth, and macroglossia. Additional less frequent complications include specific developmental defects and a predisposition to embryonal tumours. BWS is genetically heterogeneous and epigenetic changes in the IGF2/H19 genes resulting in overexpression of IGF2 have been implicated in many cases. Recently germline mutations in the cyclin dependent kinase inhibitor gene CDKN1C (p57^{KIP2}) have been reported in a variable minority of BWS patients. We have investigated a large series of familial and sporadic BWS patients for evidence of CDKN1C mutations by direct gene sequencing. A total of 70 patients with classical BWS were investigated; 54 were sporadic with no evidence of UPD and 16 were familial from seven kindreds. Novel germline CDKN1C mutations were identified in five probands, 3/7 (43%) familial cases and 2/54 (4%) sporadic cases. There was no association between germline CDKN1C mutations and IGF2 or H19 epigenotype abnormalities. The clinical phenotype of 13 BWS patients with germline CDKN1C mutations was compared to that of BWS patients with other defined types of molecular pathology. This showed a significantly higher frequency of exomphalos in the CDKN1C mutation cases (11/13) than in patients with an imprinting centre defect (associated with biallelic IGF2 expression and H19 silencing) (0/5, $p<0.005$) or patients with uniparental disomy (0/9, $p<0.005$). However, there was no association between germline CDKN1C mutations and risk of embryonal tumours. No CDKN1C mutations were identified in six non-BWS patients with overgrowth and Wilms tumour. These findings (1) show that germline CDKN1C mutations are a frequent cause of familial but not sporadic BWS, (2) suggest that CDKN1C mutations probably cause BWS independently of changes in IGF2/H19 imprinting, (3) provide evidence that aspects of the BWS phenotype may be correlated with the involvement of specific imprinted genes, and (4) link genotype-phenotype relationships in BWS and the results of murine experimental models of BWS.

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BWS is a fetal overgrowth syndrome with variable expression.[1] Clinically, BWS is characterised by pre- and postnatal overgrowth, macroglossia, and abdominal wall defects. Additional but variable complications include organomegaly, hypoglycaemia, hemihypertrophy, genitourinary abnormalities, and a predisposition to embryonic tumours in about 5% of patients. There is convincing evidence that BWS is a human imprinting disorder.[2] Genomic imprinting is a recently discovered epigenetic mechanism whereby gene expression is altered according to the parental origin of each of the two inherited alleles. Imprinted genes appear to have an important role in embryonic growth and development and disordered imprinting occurs in human developmental disorders and in neoplasia.[3–5] The elucidation of the molecular basis of BWS would provide important insights into mechanisms of genomic imprinting and their relevance to normal growth and development.

Imprinted gene(s) involved in the aetiology of BWS have been mapped to chromosome 11p15.5 by family linkage studies and the identification of chromosomal rearrangements in sporadic cases.[6–9] Chromosome 11p15.5 has extensive regions of conserved synteny with the distal region of 7p in the mouse. In both mouse and man several imprinted genes are clustered within this region including the candidate BWS genes IGF2, H19, CDKN1C (p57^{KIP2}), and KCNQ1 (KVLQT1).[10] Approximately 2% of sporadic BWS patients have abnormalities of chromosome 11p15.5, either paternally derived duplications or maternally inherited balanced translocations or inversions.[6 7] The paternally expressed IGF2 lies within the smallest duplicated region and the maternally expressed KCNQ1 is frequently disrupted by BWS associated translocation or inversion breakpoints in 11p15.[11] Paternal uniparental disomy (UPD) for chromosome 11p15.5 (which invariably includes IGF2, H19, CDKN1C, and KCNQ1) is found in ~20% of

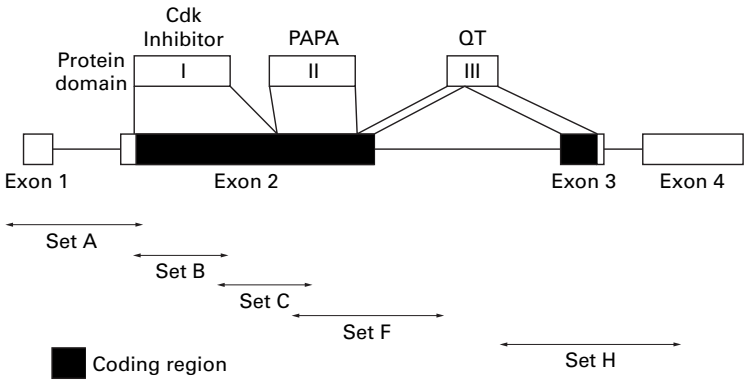

Figure 1 Structure of the CDKN1C (p57^{KIP2}) gene, including coding regions and protein domains. The relative positions of primer pairs used for template preparation in the sequencing reactions are also shown.

BWS cases.[12–14] IGF2 is imprinted and paternally expressed so these findings are consistent with increased dosage of IGF2 protein being implicated in BWS. Overexpression of IGF2 protein during mouse embryogenesis mimics many of the features of BWS.[15] Loss of imprinting (LOI) of IGF2 with biallelic expression occurs in most sporadic patients with BWS and in Wilms tumour.[16–19] The aetiology of LOI of IGF2 is unknown, although there is evidence that IGF2 imprinting is closely linked to that of the reciprocally imprinted H19 gene which maps ~100 kb telomeric. The enhancer competition model has been widely invoked to explain the reciprocal imprinting of H19 and IGF2 and the effects of deletion of the H19 locus in mice.[20] Several groups have reported loss of imprinting of IGF2 in association with decreased H19 expression and altered H19 methylation pattern in Wilms tumour.[18 19] In addition, we have shown that a subgroup of BWS patients (~5-10%) have an abnormal methylation pattern of the IGF2/H19 domain resulting from conversion of the normal maternal allele specific methylation patterns of IGF2 and H19 to a paternal epigenotype.[14 21] By analogy with Angelman and Prader-Willi syndromes, these patients were proposed to have an imprinting centre defect (ICD), which could be a mutation or an epigenetic error.[22] However, some BWS patients exhibit LOI of IGF2 in the presence of normal H19 imprinting.[17] The existence of a H19 independent pathway of IGF2 imprinting is confirmed by the finding of biallelic IGF2 expression with normal H19 methylation and expression in association with a maternally inherited chromosome 11 inversion.[23] In this case the inversion breakpoint maps centromeric to IGF2 and disrupts the KCNQ1 gene.[24] The CDKN1C gene maps centromeric to the chromosome 11 inversion breakpoint studied by Brown *et al*.[22] The CDKN1C gene encodes a cyclin dependent kinase (Cdk) inhibitor and is part of the p21^{CIP2} Cdk inhibitor family. CDKN1C is imprinted in humans and mice with preferential expression of the maternal allele,[25 26] although in humans the imprinting is not complete and a low level of expression from the paternal allele can be detected.[27] Although reduced CDKN1C mRNA expression and loss of heterozygosity have been described in Wilms tumour, mutations have not yet been reported.[28–30] Recently, germline CDKN1C mutations have been reported in BWS patients with variable frequency (0-22%).[31–35] However, it is not known whether CDKN1C mutations cause BWS per se or are associated with changes in IGF2/H19 epigenotype. The purpose of this paper is to (1) investigate the frequency of CDKN1C mutations in a large series of familial and sporadic BWS patients, (2) relate the presence of CDKN1C mutations to the H19/IGF2 epigenotype, and (3) investigate genotype-phenotype relationships in BWS.

Patients and methods

BWS PATIENTS

A diagnosis of BWS was made according to standard diagnostic criteria, either (1) three major features (anterior abdominal wall defects, macroglossia and pre-/postnatal growth >90th centile) or (2) two major plus three or more minor features (characteristic ear signs (ear lobe creases or posterior helical ear pits), facial naevus flammeus, hypoglycaemia, nephromegaly, and hemihypertrophy).[1] Seventy patients with classical BWS were analysed. Results of uniparental disomy analysis and H19 methylation have been reported previously.[13 14 21] In addition, six patients with Wilms tumour and overgrowth (>90th centile) were also investigated.

MOLECULAR GENETIC ANALYSIS

Genomic DNA was isolated from whole blood as described previously.[13 14] The following five sets of primers were used to amplify the entire coding region of the CDKN1C gene and its exon-intron boundaries in overlapping fragments (fig 1). Set A forward primer, 5'-CGTTCCACAGGCCAAGTGCG-3' and reverse primer 5'-GCTGGTGCGCACTAGT ACTG-3'; set B forward primer, 5'-CGTCCC TCCGCAGCACATCC-3' and reverse primer 5'-CCTGCACCGTCTCGCGGTAG-3'; set C forward primer, 5'- TGGACCGAAGTGGA CAGCGA-3' and reverse primer 5'-GGGGCCAGGACCGCGACC-3'; set F forward primer, 5'-CGGAATTCCGGAGC AGCTGCCTAGTGTC-3' and reverse primer 5'- CTTTAATGCCACGGGAGGAGG-3'; set H 5'-CGGCGACGTAAACAAAGCTG-3' and reverse primer 5'-GGTTGCTGCTACATGA CCGG-3'.

The reaction mixtures used for fragments A, B, C, and H were 10 mmol/l Tris-HC1, pH 8.8, 50 mmol/l KC1, 1.0 mmol/l $MgC1_2$, 0.25 mmol/l dATP, dTTP, dCTP, dGTP, 10% dimethyl sulfoxide, and 0.75 units *Taq* polymerase (Amplitaq) in a final volume of 30 µl. For fragment F the reaction mixtures were 1 × LA PCR Buffer II (Takara), 0.2 mmol/l dATP, 7-deaza dGTP, dCTP, dTTP, 5% dimethyl sulfoxide, and 2.5 units *Taq* polymerase in a final volume of 50 µl. A Perkin-Elmer Thermocycler was used for PCR analysis. Temperature conditions comprised: fragment A, a single denaturing step at 95°C for four minutes followed by 35 cycles of 94°C for 30

Table 1 Details of CDKNIC gene mutations and non-coding sequence variants detected in sporadic (S1-S4) and familial (F1-F2) cases. Genomic sequence is numbered as in Genbank Accession no D64137, with the CDKN1C coding sequence starting at nucleotide 1261

Patient ID	*Nucleotide position*	*Nucleotide change*	*Codon No*	*Amino acid change*	*Protein domain*	*Parental origin*
Coding sequence changes						
S1	1435	C→T	70	Proline→leucine	I	New mutation
S2	2739	C→T	316	Arginine→tryptophan	III	Undefined
F1	2638	C→G	282	Nonsense	III	Mother
F2	1726	16 bp Δ	167	Frameshift	I/II	Mother
F3	2611	C→G	—	Splice site mutation	III	Mother
Non-coding sequence changes						
S3	1145	G→A				Mother
S4	1144	G→A				Mother

seconds, 55°C for 30 seconds, and 72°C for one minute, no primer extension step was used; fragment B, a single denaturing step at 95°C for four minutes followed by 35 cycles of 94°C for 30 seconds, 64°C for 30 seconds, and 72°C for one minute, no primer extension step was used; fragment C, a single denaturing step at 95°C for four minutes followed by 35 cycles of 94°C for 30 seconds, 59°C for 30 seconds, and 72°C for one minute, no primer extension step was used; fragment F, a single denaturing step at 95°C for four minutes followed by 40 cycles of 95°C for one minute, 65°C for one minute, and 72°C for 1.5 minutes, and a primer extension step for 72°C for 10 minutes was used; fragment H, a single denaturing step at 95°C for four minutes followed by 35 cycles of 94°C for 45 seconds, 53°C for 30 seconds, and 72°C for one minute, no primer extension step was used.

PCR fragments were purified before sequencing using Amicon columns according to the manufacturer's instructions. Sequencing was performed on an ABI PRISM 377 semiautomated sequencer. Both forward and reverse strands were sequenced in every case using the di-rhodamine dye-terminator chemistry sequencing kit and protocol (Applied Biosystems). Reactions were set up as follows: 4 µl di-rhodamine kit reaction mix, 16 pmol sequencing primer, PCR template (1-5 µl depending upon the product concentration), and sterile distilled water to 10 µl (final volume) and overlayed with mineral oil (20 µl), before thermal cycling on a Omn-E thermal cycler (Hybaid) as follows: 96°C for three minutes followed by 30 cycles of 98°C for 10 seconds, 55°C for 20 seconds, 60°C for four minutes. Samples were then precipitated, washed, resuspended, denatured, and loaded onto the ABI 377 sequencer according to the manufacturer's instructions. CDKN1C genomic sequence numbering is as given in Genbank Accession no D64137. The CDKN1C coding sequence starts at nucleotide 1261.

GENOTYPE-PHENOTYPE ANALYSIS

The clinical features of BWS patients with confirmed germline CDKN1C mutations identified in this study and those by Hatada *et al*[31 32] were compared to the clinical phenotypes of patients we have identified previously with

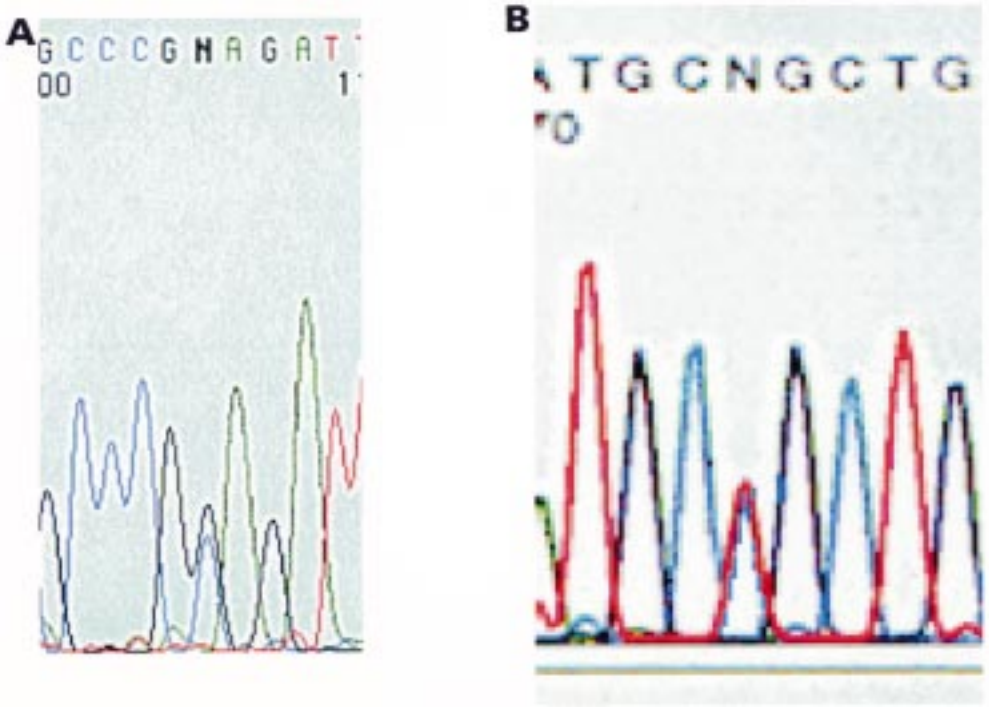

Figure 2 (A) Sequence analysis of patient F3 showing the C→G splice site mutation (see table 1 for details). (B) Sequence analysis for S2 illustrating the C→T transition causing an arginine→tryptophan missense mutation in the QT domain (see table 1 for details).

uniparental disomy or an IGF2/H19 ICD.[13 14 21]

Results

CDKN1C MUTATION ANALYSIS

Seventy patients with classical BWS were examined for germline mutations in the CDKN1C gene by direct sequencing of the coding region and intron-exon boundaries. Fifty four were unrelated patients with sporadic BWS and no evidence of uniparental disomy for chromosome 11p15.5 markers.[13 14] Five of the 54 non-familial patients had H19 hypermethylation suggesting an ICD[14 21] and 49 had a normal biparental H19 methylation pattern. Sixteen of the 70 patients examined were familial cases from seven kindreds. In addition, six patients with Wilms tumour and overgrowth (>90th centile) were analysed.

Germline CDKN1C sequence variants were identified in seven BWS probands. In four cases, the nucleotide change occurred within the coding sequence and was predicted to cause a change in protein sequence (two missense and two protein truncating) (table 1, fig 2). The two protein truncating mutations were from familial cases (F1 and F2), and in both instances the mutation was maternally inherited. The human CDKN1C protein consists of three structurally distinct domains (fig 1): (1) the 5'-amino-terminal, which is significantly similar to regions of p21^{CIP1} and p27^{KIP1} and has been shown to be necessary for Cdk inhibition, (2) a highly polymorphic hexanucleotide repeat encoding a proline-alanine series of repeats, and (3) a highly conserved 3'-QT box containing glutamine and threonine residues that is conserved between CDKN1C (p57^{KIP2}) and p27^{KIP1}. Both familial protein truncating mutations occurred within exon 2. Two non-familial patients (S1 and S2) had previously unreported nucleotide substitutions within the coding region producing putative missense mutations. S1 showed a de novo non-conservative proline to leucine substitution within the conserved Cdk inhibitory domain. The parental transmission of the non-conservative arginine to tryptophan substitution in S2 could not be established with certainty. This mutation arises in the C-terminal amino acid and was not present in the mother of S2, but the father's DNA was unavailable. In a further familial case (F3), a C to G substitution at position -3 of the exon 3 splice acceptor was characterised in the proband, an affected sib, and their mother. This change was not identified in 50 control chromosomes and in a survey of human splice sites,[36] no wild type splice acceptor sites with a G at position -3 were reported. Further supportive evidence that this transversion was likely to be pathogenic was provided when the wild type and mutant sequences were analysed by the Splice Site Prediction Neural Network Program (Reese *et al*), http://www-hgc.lbl.gov/project/splice.html). The program correctly identified all the splice sites within the wild type sequence, but there was a complete failure to recognise the mutant 3' splice acceptor site.

Overall, germline CDKN1C mutations were identified in 43% (three of seven) familial cases analysed, and 4% (two of 55) non-disomic sporadic cases. In addition, two previously unreported nucleotide substitutions in the 5' untranslated region were identified in two sporadic patients (S3 and S4). In both cases the sequence variant was maternally inherited and may represent either a regulatory mutation or a rare polymorphism. None of the putative regulatory mutations in S3 or S4 or the missense mutation in S2 have been detected in 100 control chromosomes (unpublished observations), which, together with the pattern of inheritance in the familial cases, favours the interpretation that these are disease associated mutations. No germline mutations (or sequence variants) were identified in six children with Wilms tumour and overgrowth.

PHENOTYPIC EXPRESSION OF CDKN1C MUTATIONS IN BWS

BWS is genetically heterogeneous and we therefore investigated the relationship between phenotypic expression of BWS and molecular pathology. We compared the phenotype of BWS patients with the CDKN1C coding sequence/splice site mutations identified in this study (n=8) and those reported previously by Hatada *et al*[31 321] (n=5) to that of BWS patients with other types of defined molecular pathology (table 2). A striking difference was a very high incidence of exomphalos (11/13) among patients with CDKN1C mutations. The incidence of exomphalos in CDKN1C mutation cases was significantly higher than in patients with an ICD (0/5) ($p<0.005$) or those cases with UPD (0/9) ($p<0.0005$). However, germline CDKN1C mutations do not account for all BWS patients with exomphalos and 12 BWS cases with exomphalos did not have a detectable CDKN1C mutation. There were no other significant phenotypic differences be-

Table 2 Clinical features in 13 patients with germline CDKN1C coding sequence mutations

Patient	*Source*	*Overgrowth*	*Abdominal wall defect*	*Macroglossia*	*Minor features*
S1	Current paper	+	Umbilical hernia	+	Ear lobe abnormalities, naevus flammeus, hypoglycaemia
S2	Current paper	+	Exomphalos	–	Ear lobe abnormalities, hypoglycaemia, organomegaly
F1	Current paper	+	Exomphalos	+	Ear lobe abnormalities, naevus flammeus
F2a	Current paper	+	–	+	Ear lobe abnormalities, naevus flammeus, organomegaly
F2b	Current paper	+	Exomphalos	+	Ear lobe abnormalities
F3a	Current paper	+	Exomphalos	+	Ear lobe abnormalities, hypoglycaemia, prune belly syndrome
F3b	Current paper	+	Exomphalos	+	Ear lobe abnormalities
F3c	Current paper	–	Exomphalos	+	Ear lobe abnormalities
H1	Hatada *et al*[31]	+	Exomphalos	+	Ear lobe abnormalities, naevus flammeus, hypoglycaemia
H2	Hatada *et al*[31]	+	Exomphalos	+	Ear lobe abnormalities, naevus flammeus, hypoglycaemia
H3a	Hatada *et al*[32]	+	Exomphalos	+	Ear lobe abnormalities, naevus flammeus, organomegaly
H3b	Hatada *et al*[32]	+	Exomphalos	+	Ear lobe abnormalities, mental retardation
H4	Hatada *et al*[32]	+	Exomphalos	+	Ear lobe abnormalities, organomegaly

tween the three molecular subgroups (data not shown). None of the patients with CDKN1C mutations developed Wilms tumour.

Discussion

We have confirmed that germline CDKN1C mutations account for a subgroup of patients with BWS. Our results suggest that the molecular pathology of familial and sporadic BWS differs, such that mutations in CDKN1C account for only a minority of sporadic classical BWS cases but a substantial proportion of familial cases. Unfortunately, it is not possible to elicit the relationship between family history and CDKN1C mutation status in studies by other groups. However, in Japanese BWS patients, Hatada *et al*[31 32] found germline CDKN1C mutations in four (one familial, three sporadic) of 24 cases. Lee *et al*[33] found CDKN1C mutations in two (one familial and one unknown) of 40 BWS patients. Although O'Keefe *et al*[34] identified a germline CDKN1C mutation in the only familial case studied (and in none of four sporadic cases), a full breakdown of family history status is not available for the patients studied by Hatada *et al*[31 32] and Lee *et al*.[33] Finally, Okamoto *et al*,[35] using SSCP analysis, did not detect germline CDKN1C mutations in 40 BWS patients (five of whom were familial). The mutation detection techniques used by us and others would not have identified large CDKN1C deletions or epigenetic silencing of CDKN1C. Thus the frequency of CDKN1C involvement in BWS may have been underestimated. Our observation that germline CDKN1C mutations are significantly more frequent in familial than in sporadic BWS cases is consistent with our previous findings that the majority of sporadic BWS cases have LOI of IGF2.[17] Nevertheless, although CDKN1C mutations are uncommon in sporadic cases, the identification of a germline CDKN1C mutation has major implications for genetic counselling and is therefore of considerable clinical importance.

To date no recurrent CDKN1C mutations have been reported. Familial BWS is characterised by a higher penetrance and more severe phenotypic expression when the disease is maternally transmitted, features that are compatible with the preferential maternal expression of CDKN1C. However, Lee *et al*[33] reported partial features of BWS in a mother of a classical BWS patient who had inherited a CDKN1C mutation from her father.

In our study, all patients with a germline CDKN1C mutation had a normal H19/IGF2 methylation pattern indicating that the CDKN1C gene does not represent the H19/IGF2 regional imprinting control centre inactivated in patients with an ICD. Furthermore, CDKN1C mutations were not identified in the five ICD patients analysed and in 11 sporadic BWS patients with loss of imprinting of IGF2 and normal H19/IGF2 methylation status.[17] Direct analysis of IGF2 imprinting status has not been possible in patients with CDKN1C mutations because of absence of suitable material or uninformativeness of transcribed polymorphisms.

BWS is clinically and molecularly heterogeneous. The variability of the BWS phenotype may be determined by the differential involvement of several imprinted genes according to the precise molecular pathology. This hypothesis is compatible with our findings of genotype-phenotype correlations in BWS. Overall, exomphalos occurs in ~45% of cases[1]; however, we have shown that CDKN1C inactivation is associated with a high risk of exomphalos (87%), while patients with UPD and IGF2/H19 hypermethylation (ICD) have a low frequency of exomphalos. The absence of CDKN1C mutations in non-BWS overgrown patients with Wilms tumour is compatible with these findings. As we might expect UPD to result in reduced CDKN1C expression, these findings are at first sight contradictory. Nevertheless, as UPD cases are mosaic, the presence of a proportion of normal cells may markedly reduce the severity of the phenotype. This may be further offset by the relatively relaxed imprinting of the paternal allele, which expresses detectable levels of CDKN1C mRNA.[37] Consistent with our predictions, BWS patients with a chromosome 11p15.5 duplication do not have exomphalos.[38] It is possible that some patients with exomphalos and no detectable CDKN1C mutation have epigenetic silencing of CDKN1C mRNA expression.

Our genotype-phenotype findings are reinforced by the results of two recent mouse models of BWS. Thus a *cdkn1c* knockout mouse displayed a high frequency of anterior abdominal wall abnormalities including exomphalos, but lacked some other features of BWS (including overgrowth).[39] Furthermore, mice with IGF2 protein overexpression mimic many features of BWS but do not show exomphalos.[15] Exomphalos in BWS has generally been attributed to result from organomegaly and a concomitant increased intra-abdominal pressure. However, the preferential association of exomphalos with CDKN1C mutations suggests that this explanation is insufficient and that additional factors (for example, disturbance of normal cellular migration or adhesion patterns) are likely to be indicated. Genitourinary development abnormalities are common in BWS, but the association with prune belly syndrome (seen in case F3a) is rare. It is not known if this association will be specific to CDKN1C mutation cases or if it is coincidental (although the incidence of isolated prune belly syndrome is ~1 in 3000). Further studies of the molecular pathology of BWS including the imprinting status of IGF2, H19, CDKN1C, and KCNQ1 in patients with different subgroups of molecular lesions, followed by further analysis of genotype/phenotype relationships should provide insight into the pathogenesis of BWS.

The mechanism by which mutations in CDKN1C may cause BWS is unclear. Overexpression of CDKN1C causes cell arrest at G1 and so inactivating mutations might lead directly to abnormal cellular proliferation. In the mouse, IGF2 protein overexpression but not CDKN1C inactivation is associated with

overgrowth.[14 39] As we did not find evidence that CDKN1C mutations directly affect IGF2 imprinting (see above), the most likely explanation for the phenotypic overlap of CDKN1C mutations and IGF2 overexpression is that the two proteins are implicated in a common growth control pathway. If so, this throws a new light on the observation that imprinted genes tend to be clustered in the genome.[2] Within the 11p15.5 imprinted gene cluster, IGF2 and H19 have been functionally linked by the enhancer competition model and further studies of IGF2, CDKN1C, and other 11p15.5 imprinted genes may elucidate functional relationships that provide an explanation for their genomic clustering.

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