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Coding Mutations in p57^{KIP2} Are Present in Some Cases of Beckwith-Wiedemann Syndrome but Are Rare or Absent in Wilms Tumors

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Summary

The Beckwith-Wiedemann syndrome (BWS) is marked by fetal organ overgrowth and conveys a predisposition to certain childhood tumors, including Wilms tumor (WT). The genetics of BWS have implicated a gene that maps to chromosome 11p15 and is paternally imprinted, and the gene encoding the cyclin-cdk inhibitor p57^{KIP2} has been a strong candidate. By complete sequencing of the coding exons and intron/exon junctions, we found a maternally transmitted coding mutation in the cdk-inhibitor domain of the *KIP2* gene in one of five cases of BWS. The BWS mutation was an in-frame three-amino-acid deletion that significantly reduced but did not fully abrogate growth-suppressive activity in a transfection assay. In contrast, no somatic coding mutations in *KIP2* were found in a set of 12 primary WTs enriched for cases that expressed KIP2 mRNA, including cases with and without 11p15.5 loss of heterozygosity. Two other 11p15.5 loci, the linked and oppositely imprinted *H19* and *IGF2* genes, have been previously implicated in WT pathogenesis, and several of the tumors with persistent KIP2 mRNA expression and absence of *KIP2* coding mutations showed full inactivation of *H19*. These data suggest that *KIP2* is a BWS gene but that it is not uniquely equivalent to the 11p15.5 "WT2" tumor-suppressor locus.

Introduction

The Beckwith-Wiedemann syndrome (BWS) is characterized by somatic overgrowth of various tissues, including kidney, liver, adrenal and skeletal muscle, and by a predisposition to embryonal tumors of these organs (Wilms tumor [WT], hepatoblastoma, adrenal cortical carcinoma, and embryonal rhabdomyosarcoma). The tumor predisposition is quite significant relative to the baseline population but is moderate in absolute terms: <10% of affected individuals will develop a childhood neoplasm (reviewed in Junien 1992; Mannens et al. 1994; Weksberg and Squire 1996). BWS is often diagnosed on the basis of the presence of variably prominent findings of hemihypertrophy, macroglossia, omphalocele, and ear creases and pits, and this condition must be clinically distinguished from certain other overgrowth syndromes (Weksberg and Squire 1996). A number of observations have suggested that the BWS gene(s) are on chromosome 11 and that at least one of these genes is in band 11p15.5 and is subject to parental imprinting. Familial BWS shows linkage to chromosome 11p15 (Koufos et al. 1989; Ping et al. 1989), and there appears to be an excess of maternal transmissions; some cases of BWS are associated with uniparental paternal disomy of this chromosomal region, and a third class of cases are associated with chromosomal translocations with breakpoints on chromosome 11p, sometimes although not always localizing to 11p15.5 (Mannens et al. 1994).

Some cases of BWS show pathological biallelic expression of the maternally imprinted 11p15.5 gene *IGF2* in some tissues (Weksberg et al. 1993), and at least some such cases have shown abnormal biallelic CpG hypermethylation of the closely linked and oppositely imprinted *H19* gene (Reik et al. 1995). Several cases have also been described of a severe generalized overgrowth syndrome similar to BWS, with nephromegaly and a predisposition to WT but without prominent macroglossia or omphalocele, in which the hypertrophied tissues have shown biallelic hypermethylation of *H19* and biallelic expression of *IGF2* (Morison et al. 1996).

Imprinted genes on chromosome 11p15.5 are also implicated in sporadic WTs: 45% of WTs show 11p15.5 loss of heterozygosity (LOH) because of mitotic recombination, and invariably there is loss of the maternal alleles and reduplication of the paternal alleles—that is, a bipaternal endpoint (reviewed in Moulton et al. 1996). Among the remaining WTs, which show 11p15.5 retention of heterozygosity (ROH), approximately half undergo epigenetic changes in specific imprinted 11p15.5

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genes, which lead to a "bipaternal epigenotype." These tumors show complete inactivation of the paternally imprinted *H19* gene, with CpG hypermethylation of the previously active maternal allele (Moulton et al. 1994; Steenman et al. 1994; Taniguchi et al. 1995), and, since the *H19* and *IGF2* promoters compete for enhancer elements in *cis* (Leighton et al. 1995), the inactivation of *H19* invariably correlates with a reciprocal activation of the maternal allele of *IGF2* in these tumors. The inactivation of *H19* may contribute to WT formation either via this *cis* effect on *IGF2* expression, or via elimination of a *trans* growth–inhibiting effect of H19 RNA (Hao et al. 1993), or via both mechanisms.

With regard to both BWS and WT, much recent attention has focused on the *KIP2* gene. This gene encodes the cyclin-cdk inhibitor $p57^{KIP2}$ (Matsuoka et al. 1995), maps to chromosome 11p15.5 in the vicinity of some although not all of the BWS translocation breakpoints (Hoovers et al. 1995), and is paternally imprinted in mice and humans, albeit with variable "leaky" expression of the paternal allele in humans (Hatada and Mukai 1995; Chung et al. 1996; Hatada et al. 1996*a*; Kondo et al. 1996; Matsuoka et al. 1996). A large subset of WTs show transcriptional inactivation of *KIP2* (Chung et al. 1996; Hatada et al. 1996*a*), but some cases, both those with and those without 11p15.5 LOH, show persistent expression of this gene (Chung et al. 1996; Hatada et al. 1996*a*; Overall et al. 1996). Here we describe our sequence analysis of the complete coding region of the *KIP2* gene in several cases of BWS and in a large series of WTs with characterized 11p15.5 allelic status and *KIP2* and *H19* expression.

Patients, Material, and Methods

Patients and Tumors

All five index BWS cases were evaluated at the clinical genetics service of Babies Hospital, Columbia University. All were females. Cases BWS1, BWS2, and BWS4 were diagnosed on the basis of the presence of omphalocele, macroglossia, and ear creases and pits. The omphaloceles in BWS1 and BWS2 required surgical correction, and the macroglossia in BWS2 also required surgical correction by partial glossectomy. BWS2 also showed hemihypertrophy. BWS3 was a large infant, with a birthweight and placental weight both >90th percentile and with macroglossia, ear creases, and an umbilical hernia, but there was no omphalocele or hemihypertrophy. BWS5 was diagnosed on the basis of omphalocele, capillary hemangiomas over her eyelids bilateraly, and macroglossia. In this case there was also severe hypoglycemia after birth, which resolved during the postnatal period. Only BWS1 had a positive family history: A sister of the proband had macroglossia and severe omphalocele and died, during the perinatal period, of complications unrelated to BWS. Two other siblings were normal. In all cases, other known overgrowth syndromes were judged to be unlikely, by clinical criteria; for example, the X-linked Simpson-Golabi-Behmel syndrome, which can also manifest some signs in females (Weksberg and Squire 1996), was clinically ruled unlikely, by the absence of skeletal abnormalities and coarse facies. Standard karyotypes were obtained for BWS1, BWS2, and BWS3, and all were 46,XX; a karyotype was not available for BWS4 and BWS5. BWS1, BWS2, BWS3, and BWS5 survived the perinatal period, but BWS4 died perinatally and did not survive long enough for surgical correction of a severe omphalocele, which contained portions of bowel, liver, and kidney. None of the BWS cases have shown clinical or pathological evidence of neoplasms (BWS1 is 14 mo old, BWS2 is 3 years old, BWS3 is 16 mo old, and BWS5 is 5 mo old). Tumors and paired nonneoplastic kidney samples were obtained from WT patients at surgery. All were primary unilateral tumors with typical nonanaplastic histologies. None of the WT patients showed clinical evidence of BWS; only WT537 and WT540 were familial (Moulton et al. 1996).

KIP2 *and* H19 *Expression and* H19 *CpG Methylation*

KIP2 mRNA was quantitated by RNAse protection assay (RPA), and H19 RNA was quantitated by northern blotting as described elsewhere (Chung et al. 1996). As has been described elsewhere, KIP2 mRNA quantitations by northern analysis and RPA are highly concordant (Chung et al. 1996). Allelic CpG methylation of *H19* genomic DNA was assessed by digestion with methylation-sensitive restriction enzymes and Southern blotting, as described elsewhere (Moulton et al. 1994; Chung et al. 1996).

Reverse-Transcription–PCR (RT-PCR) for KIP2 mRNA

RT-PCR was done as described elsewhere (Chung et al. 1996), except that the upstream primer was primer 13, in the 5′ end of exon 2, and the downstream primer was primer 12, in the 3′ end of exon 4 (primers are listed below). The PCR therefore spanned two introns; nested primers was not required, since placenta expresses higher levels of KIP2 mRNA than is seen in most other organs. The PCR products were digested with *Apa*I prior to sizing analysis, to better visualize the BWS1 deletion.

SSCP Analysis

SSCP was performed with 32P-labeled PCR products spanning the coding exons of *KIP2*, by means of a described protocol elsewhere (Tycko et al. 1996) but with electrophoresis at room temperature and with 400–500 V. This was found to improve the quality of band patterns with the C/G-rich *KIP2* sequences. Where neces-

sary, the labeled PCR products were digested with restriction enzymes prior to SSCP, to reduce the fragment sizes to <350 bp.

PCR Primers and Genomic Sequencing

The PCR sequencing strategy for analysis of the *KIP2* gene is shown in figure 1. The primers used for PCR were pairs 1+3, 2+6, 4+8, 7+9, 10+11, and 10+12. Each primer except primer 1 was also used for direct sequencing, and primer 5 was used for additional sequencing of the 4+8 PCR product. Since the 4+8 product includes the insertion/deletion polymorphism, plasmid cloning and sequencing of six clones per sample was also performed for this PCR product. Primer sequences were as follows: 1, CTACATTATGCTAATCGCGG; 2, TCTTCTCGCTGTCCTCTCCT; 3, AGGAGAGGACAGCGAGAAGA; 4, CCGAAGTGGACAGCGACTCG; 5, CGGTCGCGGTGGCTGTCA; 6, CGCCCCACCTGCACCGTCT; 7, TCAAGAGAGCGCCGAGCAG; 8, CTGGTCAGCGAGAGGCTCCT; 9, GCGGGCCCTTTAATGCCAC; 10, TCTCCCGGCCCCCTCTCGG; 11, TCAGCAAAGCCGGCGGGGA; 12, CAAAACCGAACGCTGCTCTG; and 13, CCTTCCCAGTACTAGTGCGC.

Colony-Suppression Assay

Oligonucleotide primers were designed to amplify the major mRNA species of KIP2 (Tokino et al. 1996) incorporating artificial *Eco*RI and *Sal*I restriction sites (underlined) (5′ primer CAGGAATTCCACGATGGAGCGTC and 3′ primer GGGCTGTCGACTGGCTCACCG, respectively). Normal KIP2 cDNA and the BWS1 KIP2 cDNA deletion constructs were generated by RT-PCR of normal placental RNA and BWS1 placental RNA, respectively. The resulting products were cloned in the *Eco*RI and *Sal*I sites of pBluescript KS and subsequently were subcloned into the *Kpn*I/*Sac*I sites of pBKCMV (Stratagene). SAOS-2 cells were maintained in Iscove's modified Dulbecco's medium containing 10% FCS at 37°C in 5% CO_2. Transfections were performed when the cells were at 40% confluency in 10-cm-diameter plates, by use of Lipofectin™ (Gibco-BRL Life Technologies) according to the manufacturer's instructions. In brief, the indicated plasmids (4 or 8 μg) were incubated with 40 μl of Lipofectin prior to transfection. Cells were incubated with the Lipofectin-DNA complexes in serum-free media overnight, after which time the medium was replaced with the cells' usual medium. After 48 h, the cells were selected in maintenance medium containing 600 μg/ml Geneticin (Sigma). After 15 d in selection, the plates were washed and fixed in 5% formalin in PBS and were stained with Giemsa. Colonies were counted within a 10-cm^2 standard grid applied to each plate.

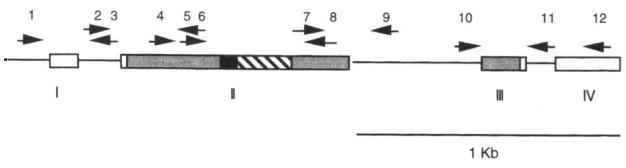

Figure 1 Sequencing strategy for the *KIP2* gene. The PCR sequencing primers are indicated by arrows; the coding region is denoted by gray shading; the PAPV-repeat deletion/insertion polymorphism is denoted by the blackened solid box; and the GC-rich repeat is denoted by hatching. Direct sequencing was used for all of the regions, and this was supplemented by plasmid sequencing for the polymorphic region between primers 4 and 8.

Results

The entire *KIP2* coding region and splice-donor and -acceptor signals were examined in 5 individuals with clinical BWS and in a set of 12 WTs enriched for cases that expressed KIP2 mRNA. The clinical characteristics of the BWS cases are listed above (see Patients, Material, and Methods section). Of the five cases, one, BWS1, showed a *KIP2* coding-region mutation. This was a deletion of nine nucleotides, leading to a predicted deletion of three amino acids, as well as a change of one amino acid, in the cdk-inhibitor domain of the protein (table 1 and fig. 2*A*). This deletion, which interrupts a sequence that is colinear and well conserved in the murine *Kip2* gene, was not observed in any of 105 unaffected individuals (210 chromosomes). That the deletion was present on the active (nonimprinted) allele of the affected child was confirmed by RT-PCR analysis of cDNAs from the placenta (fig. 2*B*). As for the other members of the family, the germ-line deletion was present in the mother and the affected child from whom DNA was available and was absent in the father and an unaffected child (fig. 2*B*). Three siblings of the mother, as well as the maternal grandfather, were also examined, but none carry the mutated *KIP2* allele (data not shown). None of the other four BWS probands showed *KIP2* coding or splice-site mutations, and a bipaternal genotype via uniparental disomy for chromosome 11p15.5 was also excluded for each of these cases, by genotyping with a series of highly polymorphic chromosome 11p15.5 markers (data not shown).

To determine whether the *KIP2* allele with the three-amino-acid deletion present in BWS1 was functionally altered, we cloned it and the normal cDNA encoding the major transcript of *KIP2* into the pBKCMV expression vector and introduced the constructs into SAOS-2 cells. Elsewhere, these cells have been shown to respond to overexpression of $p57^{KIP2}$ in transient-transfection assays, by accumulating in the G1 phase of the cell cycle (Matsuoka et al. 1995). We used a stable transfection assay to determine the ability of the *BWS1* deletion allele

Table 1

***p57/KIP2* Sequence Analysis of WTs and BWS, and Comparison of KIP2 and H19 RNA Expression in WTs**

Case[a]	Status of 11p15 Alleles	*H19* CpG Methylation[b]	H19 RNA[c]	KIP2 RNA[c]	*KIP2* Polymorphism(s)[d]	KIP2 Mutation
WT251	LOH	+/+	<.05	.18	3, 5, 7	None
WT540	LOH	+/+	<.05	.40	1, 2, 3, 4	None
WT802	LOH	+/+	<.05	.22	3, 4	None
WT667	LOH	+/+	<.05	.11	4, 5, 6, 8	None
WT753	LOH	+/+	<.05	.20	3, 4	None
WT616	LOH	+/+	<.05	.06		
WT521	LOH	+/+	ND	<.05		
WT903	LOH	+/+	<.05	<.05		
WT428	ROH	+/+	<.05	<.05		
WT511	ROH	+/+	<.05	<.05		
WT515	ROH	+/+	<.05	.07		
WT516	ROH	+/+	<.05	.25	3, 4	None
WT517	ROH	+/+	<.05	<.05		
WT899	ROH	+/+	<.05	<.05		
WT931	ROH	+/+	<.05	.19	1, 3, 4	None
WT537	ROH	+/−	>1	.45		None
WT564	ROH	+/−	>1	.61	3, 4	None
WT650	ROH	+/−	>1	.05	4, 5	None
WT914	ROH	+/−	.80	.42	3	None
WT933	ROH	+/−	>1	<.05	3	None
BWS 1	NA	NA	NA	NA		Deletion of 9 nt (445–453 cDNA)
BWS 2	NA	NA	NA	NA		None
BWS 3	NA	NA	NA	NA		None
BWS 4	NA	NA	NA	NA	3, 9	None
BWS 5	NA	NA	NA	NA		None

NONE.—NA = not applicable; and ND = not done.

[a] The entire series of WTs is shown, to illustrate the lack of a strict correlation between *H19* and *KIP2* expression and the selection to enrich for *KIP2*-expressing cases for sequencing. Cases selected for sequencing are underlined.

[b] +/+ = Biallelic DNA hypermethylation; and +/− = monoallelic hypermethylation.

[c] RNA expression is relative to that of whole fetal kidney.

[d] Referenced to nucleotide positions in the genomic and DNA sequences (GenBank D64137 and U22398). 1 = T→C (839 cDNA); 2 = T→C (667 cDNA); 3 = C→T (815 cDNA); 4 = four-amino-acid PAPV-repeat deletion/insertion (del760–771 cDNA); 5 = insertion of G (2774 genomic, third intron); 6 = CT→TC (772–773 cDNA); 7 = T→C (725 cDNA); 8 = G→A (866 cDNA); and 9 = C→T (1053 genomic, 5′ UTR). All of the single-nucleotide polymorphisms except 6 are neutral with respect to the amino acid sequence; polymorphism 6 is a conservative ala→val change within the PAPV repeat. In addition, all samples showed both a C instead of a G at position 2079 and an insertion of a G after C2600 of the genomic sequence; both of these changes are in the second intron and may reflect either errors in the GenBank sequence or rare sequence polymorphisms in the population.

to confer colony suppression. The results indicated a reproducible partial loss of biological activity of this allele. Specifically, the ability of $p57^{KIP2}$ to suppress colonies was almost although not entirely abrogated by the 9-bp deletion (table 2), and there were also differences between the morphology of the colonies generated by the two constructs and that of the vector-alone control (fig. 3). The *KIP2* deletion allele generated colonies that were somewhat smaller than those generated by vector alone, but the wild-type *KIP2* control had far fewer colonies, and these were very small (<20 cells) and consisted of flat cells that did not pile up (table 2 and fig. 3). That the deletion in the *KIP2* allele impairs biological activity is not suprising, since the deleted DYDF motif is conserved (with the same or similar amino acids) between other Kip/Cip family members, $p27^{KIP1}$ and $p21^{Cip1}$. By analogy with the crystal structure determined for $p27^{KIP1}$, the DYDF sequence is predicted to form part of a beta-hairpin structure in a region responsible for binding to and inducing structural changes in Cdk2 (Russo et al. 1996).

Although none of the cases of BWS in our series has developed a neoplasm, at least a subset of individuals with the clinical syndrome of BWS are predisposed to the development of WT. Elsewhere we have characterized a series of tumors including 19 histologically typical and nonpretreated WTs for *H19* and *KIP2* expression (Chung et al. 1996). From this series, supplemented by two additional cases, we chose 10 WTs with persistent KIP2 mRNA, defined as >10% of whole fetal kidney

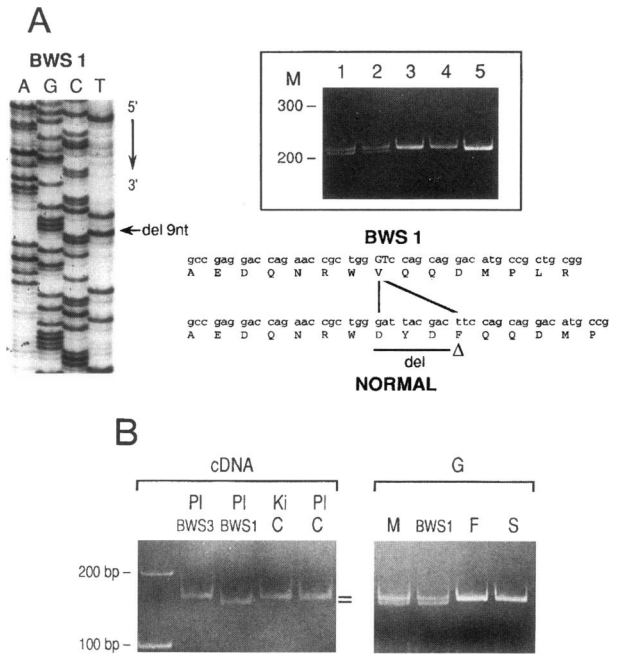

Figure 2 Germ-line *KIP2* coding mutation in a family with BWS. *A*, Plasmid sequencing of the mutated allele in proband BWS1. There is in exon 2 a 9-nucleotide deletion that deletes three amino acids and changes the identity of one amino acid in the CDK-inhibitor domain. The inset shows *RsaI* fragments of genomic PCR with primers 2+6, revealing the deletion in the affected child (1) and the mother (2) and its absence in three unrelated individuals (a total of 40 controls lacked the deletion). M = size markers (in bp). *B*, Occurrence of the deletion on the active (nonimprinted) maternal allele. The left-hand panel shows RT-PCR products (cDNA) from the region of *KIP2* exon 2 containing the deletion. The lower (i.e., deletion) band is present in the placental (Pl) cDNA from BWS1 but not in the placental cDNA from BWS3 or in control (C) placenta and kidney (Ki). BWS1 also shows a faint upper band representing leaky expression from the imprinted allele; this was seen more clearly on radiolabeling (not shown). The RT-PCR products were digested with *ApaI* prior to electrophoresis; only the smaller (i.e., 5′) fragments, containing the deleted region, are shown. The right-hand panel shows genomic PCR products (G) from the mother (M), from BWS1, from the father (F), and from an unaffected sibling (S). The size markers that apply to the left-hand panel do not apply to this panel; the genomic PCR fragments are slightly larger, as indicated in *A*.

levels, as well as 2 WTs that showed very low or undetectable *KIP2* expression by RPA, for SSCP analysis and complete genomic sequencing of the *KIP2* coding region and intron-exon boundaries. Consistent with the "leakiness" of the *KIP2* paternal imprint (Chung et al. 1996; Kondo et al. 1996; Matsuoka et al. 1996), five of the expressing tumors showed 11p15.5 LOH; this finding of persistent KIP2 mRNA in WTs with 11p15.5 LOH has also been described in another study (Overall et al. 1996). Our rationale for enriching for expressing cases was that most coding-region mutations would be expected to have functional significance in such cases; our rationale for also including two nonexpressing tumors was that a minor class of coding-region mutation or splice-site mutation can, in principal, destabilize the mRNA and lead to an apparent lack of expression.

Sequencing revealed nine neutral or conservative sequence polymorphisms in *KIP2* exons and introns, including the previously identified PAPV-repeat length polymorphism in exon 2 (Orlow et al. 1996; Tokino et al. 1996) but also including several novel polymorphisms, as well as, within the second intron, two uniform discrepancies with the GenBank sequence. None of the WTs contained *KIP2* coding or splice-signal mutations (table 1). The SSCP analyses were consistent with the sequence data, since they showed a pattern of bands that was polymorphic from case to case but that did not show evidence of variability within normal/tumor pairs—that is, somatic mutations (fig. 4 and data not shown). On the basis of the combined expression and sequence data, we conclude that, although *KIP2* is definitely transcriptionally repressed in a large subset of WTs, somatic coding mutations in this gene are rare in tumors with persistent KIP2 mRNA expression, as indicated by their complete absence in our series. We wish to emphasize that in this study we have not excluded the possibility of regulatory mutations in promoter/enhancer elements of the *KIP2* gene in cases of WT that lack *KIP2* expression.

The rarity of *KIP2* coding mutations in sporadic WTs, together with the previous findings of dysregulated expression of both this gene and other 11p15.5 imprinted genes in these tumors, favors the hypothesis that the 11p15.5 "WT2" tumor-suppressor locus may in fact consist of multiple genes. Also consistent with this is the

Table 2

Functional Analysis of the *p57/KIP2* Deletion Allele, by a Colony-Suppression Assay in Saos-2 Cells

	No. of Colonies Counted in 10^2-cm Grid			
	Experiment 1: 5 μg Plasmid/ Plate		Experiment 2: 8 μg Plasmid/ Plate	
	Plate 1	Plate 2	Plate 1	Plate 2
Vector alone	273	374	512	547
BWS1 deletion *KIP2* cDNA	288	202	494	464
Normal *KIP2* cDNA	130	151	289	250

NOTE.—A third plate was used for preparation of genomic DNA, and duplicate slot blots of genomic DNA from parallel plates of cells after selection were hybridized with a vector-derived (CMV-promoter) probe and a total-human-DNA probe. The results indicated that the amount of plasmid stably transfected was equal for each of the three constructs in a given experiment (data not shown).

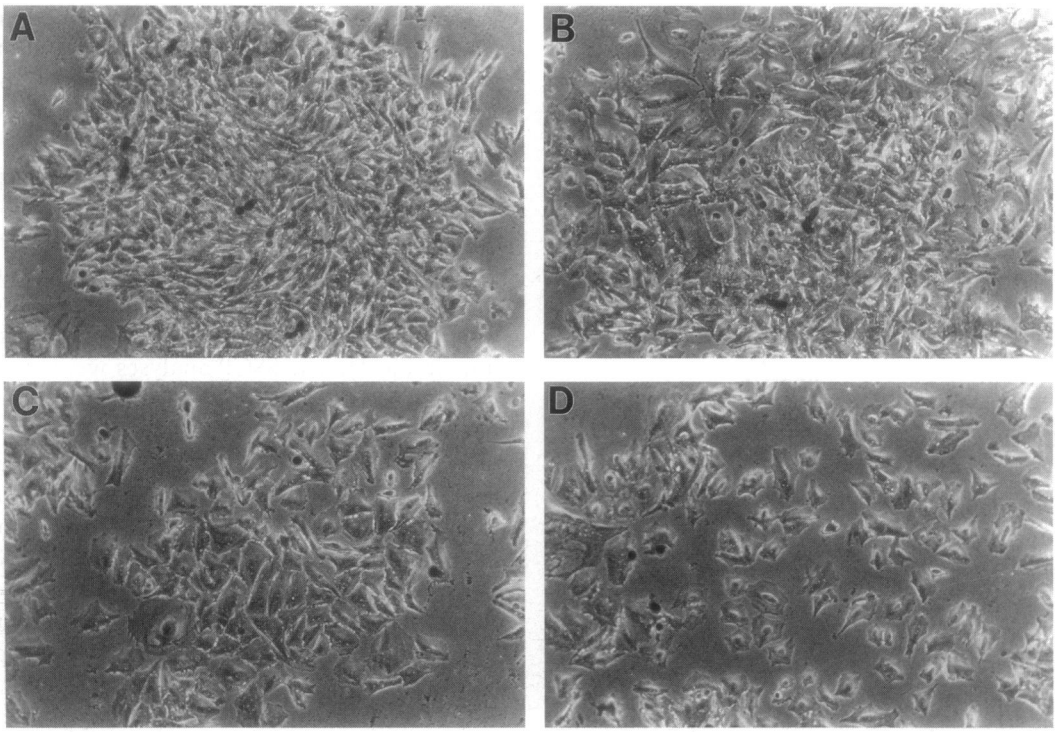

Figure 3 Morphology of $p57^{KIP2}$ Saos-2 transfectants. *A*, Typical colony obtained with the control CMV/neo vector. *B*, Typical colony obtained with the BWS1 p57KIP2 deletion construct. *C*, Typical colony obtained with the normal wild-type $p57^{KIP2}$ construct. In addition to the reduction in colony numbers, the colonies that formed contained fewer and flatter cells, compared with those obtained with the vector control and BWS1 deletion constructs. *D*, Isolated nonproliferating cells seen with the normal p57KIP2 construct after 2 wk of selection. These were not seen in the vector control or BWS1 deletion construct plates.

lack of an absolute correlation between transcriptional inactivation of *H19* and *KIP2* in our series of WTs. In our expanded series, among seven WTs with ROH and biallelic hypermethylation and complete ($<.05$ vs. fetal kidney) inactivation of *H19*, five showed very low *KIP2* expression, but two showed easily detectable KIP2 mRNA at ~20% of the fetal kidney level; conversely, among five WTs with persistent high *H19* expression, three also showed high KIP2 mRNA levels, whereas two showed complete inactivation of *KIP2* (table 1). This lack of an absolute correlation between the inactivation of *KIP2* and *H19* is consistent with these two loci being physically linked but, at least in some cases, functionally independent targets of inactivation. Also consistent with this, DNA methylation may play a more important role in the functional imprinting of *H19*, compared with that of human *KIP2* (Chung et al. 1996). Thus, the mechanisms of inactivation of these two genes in WTs may not be identical.

Discussion

These findings have implications for the genetic basis of BWS and for the role of *KIP2* and other imprinted chromosome 11p15.5 genes in WTs. With regard to BWS, the presence of a germ-line mutation in the *KIP2* gene in one of five affected children suggests that *KIP2* is a BWS gene. The paternal imprinting of this gene (Hatada and Mukai 1995; Chung et al. 1996; Hatada et al. 1996*a*; Kondo et al. 1996; Matsuoka et al. 1996), the tissue distribution of its expression, and the predicted growth-inhibiting effects of its product (Matsuoka et al. 1995) are all consistent with this. However, since most of our BWS cases lacked coding mutations, our data suggest that *KIP2* is not the only gene involved in this syndrome. Our data are concordant with a previous report from Hatada et al. (1996*b*), which described *KIP2* germ-line mutations in two of nine BWS cases. Interestingly, of the two mutations in that study, one was a frameshift resulting in disruption of the QT domain, which would leave the cyclin/cdk binding and inhibitory region of the protein intact (Matsuoka et al. 1995). These reports combined suggest that ~20%, or perhaps even a lesser percentage, of BWS cases are attributable to coding mutations in *KIP2* and that these mutations may tend to preserve partial biological activity of $p57^{KIP2}$ protein. We speculate that complete abrogation of $p57^{KIP2}$-protein function may not be compatible with full-term fetal survival and that either (*a*) there may be selection for

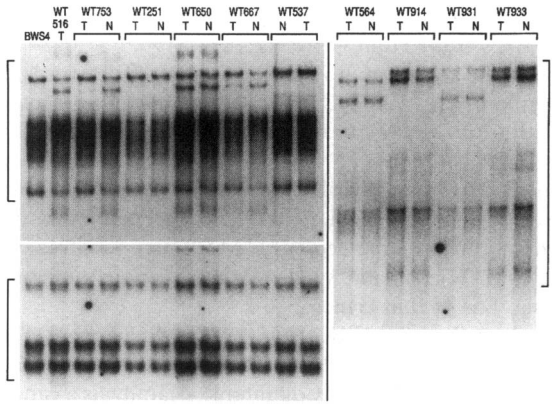

Figure 4 Representative *KIP2* SSCP analyses in BWS and WTs. The region of *KIP2* exon 2 containing the PAPV length polymorphism and several neutral single base polymorphisms was amplified with primers 4+8, internally radiolabeled, and digested with *Pvu*II to yield fragments of 320 and 164 bp. The left-hand panel shows the resulting SSCP band patterns for both fragments (*upper and lower vertical brackets*), and the right-hand panel shows the results for the larger fragment only, after electrophoresis at a higher voltage. Although bands vary between individuals, comparisons of normal (N) and tumor (T) lanes show no evidence of somatic mutations. LOH for the length polymorphism is seen in some of the tumor/normal pairs; in every case this was consistent with LOH at other 11p15.5 markers.

nontruncating mutations with residual activity or (*b*) in cases with more severe mutations, survival may depend on leaky expression from the nonmutated imprinted allele (Chung et al. 1996).

The "nonmutation" cases of BWS might still be caused by down-modulation of *KIP2* expression, via uniparental paternal disomy (Henry et al. 1991, 1993), or perhaps by other imprinting aberrations, but it remains possible that genetic or epigenetic lesions in other imprinted 11p15.5 genes with growth-regulating activity might underlie these cases. Uniparental disomy was not present in any of our BWS cases, and, in one of our nonmutation cases (BWS3) in which we were able to examine RNA of the hypertrophied placenta, northern blots showed abundant KIP2 mRNA with a normal transcript size (data not shown). The extent to which BWS is a genetically heterogeneous disorder should become evident with further study.

With regard to 11p15.5 genes and WT, the question arises whether the identification of *KIP2* as a BWS gene also identifies it as the sole or primary 11p15 tumor-suppressor gene in WTs—that is, as the WT2 locus. *KIP2* expression is clearly significantly down-modulated in a majority of WTs, but the lack of somatic mutations in this gene in expressing tumors, both in our series and in other studies of WTs (studies in which the gene was subjected to partial sequenicing [Orlow et al. 1996; Overall et al. 1996]), is a negative finding that argues against a unique assignment of *KIP2* as WT2. All of the expressing WTs in our series showed amounts of KIP2 mRNA that were moderately reduced compared with those in whole 16–23-wk fetal kidney levels. This most likely reflects a real but moderate transcriptional down-regulation, but, alternatively, since published in situ hybridization data suggest that *Kip2* is most highly expressed in differentiating condensed or tubular structures, as opposed to proliferating blastema, in the mouse fetal kidney (Matsuoka et al. 1995), in some cases it might reflect appropriately low expression due to the early stage of cell differentiation in the blastemal component of these tumors.

In addition to *KIP2,* other imprinted chromosome 11p15.5 genes are transcriptionally dysregulated in WTs. Inactivation of *H19,* which occurs in 70% of WTs, could promote tumorigenesis by loss of H19 RNA (Hao et al. 1993) and/or by *cis*-activation of the linked and reciprocally imprinted *IGF2* gene (Rainier et al. 1993; Moulton et al. 1994; Steenman et al. 1994; Taniguchi et al. 1995). In our series, seven of the *KIP2*-expressing WTs that lacked coding mutations showed complete loss of *H19* expression. Moreover, the inactivation of *H19* in WTs is clearly not related to the stage of cellular differentiation in these tumors (Moulton et al. 1994) and is manifested as a pathological pattern of biallelic DNA hypermethylation in this gene, a situation that has not been encountered in numerous control non-neoplastic kidney tissues from fetuses and adults outside the setting of WT or somatic-overgrowth syndromes (Moulton et al. 1994; Steenman et al. 1994; Reik et al. 1995; Taniguchi et al. 1995; Morrison et al. 1996; Moulton et al. 1996). Since, in the large subset of WTs with 11p15.5 ROH, there is no absolute correlation between *KIP2* down-modulation and *H19* inactivation, we suggest that *H19* and *KIP2* are independently involved, via epigenetic inactivation, as WT-suppressor genes. In cases with 11p15.5 LOH both of these imprinted genes are down-modulated by a single "hit"—mitotic recombination, whereas in the non-LOH cases the tumor-precursor cells may be under selective pressure to epigenetically target both loci for inactivation.

Although BWS conveys a marked predisposition to WT, relative to the risk in the general population, the absolute percentage of BWS affecteds who develop tumors is small, and no neoplasms have developed in the cases in our series. Data from a limited number of cases have suggested that, among persons with BWS, the predisposition to WT might be highest in those who have uniparental paternal disomy for the entire chromosome 11p15 region (Henry et al. 1993). If this is confirmed in larger series, then the fact that these individuals are predicted to have coordinate dysregulation of multiple imprinted 11p15 genes may be consistent with the notion of WT2 as a multigene locus.

Finally, since there is increasing evidence both for a domainlike clustering of imprinted genes and for the involvement of imprinted genes in cellular-growth control, chromosome 11p15 should continue to be explored for additional imprinted genes involved in the pathogenesis of WT and BWS.

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