Loss of imprinting of *IGF2* is linked to reduced expression and abnormal methylation of *H19* in Wilms' tumour

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The insulin-like growth factor-II (*IGF2*) and *H19* genes are imprinted in mouse and human, with expression of the paternal *IGF2* and maternal *H19* alleles. *IGF2* undergoes loss of imprinting (LOI) in most Wilms' tumours (WT). We now show that: (i) LOI of *IGF2* is associated with a 80-fold down regulation of *H19* expression; (ii) these changes are associated with alterations in parental-origin-specific, tissue-independent sites of DNA methylation in the *H19* promoter; and (iii) loss of heterozygosity is also associated with loss of *H19* expression. Thus, imprinting of a large domain of the maternal chromosome results in a reversal to a paternal epigenotype. These data also suggest an epigenetic mechanism for inactivation of *H19* as a tumour suppressor gene.

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One of the most provocative recent discoveries in mammalian genetics is genomic imprinting, or differential expression of parental alleles in normal development. The first molecular evidence was derived from studies of transgenes in mice[1], some of which are imprinted and associated with methylation of the nonexpressed allele[2]. Several genes are imprinted in mice[3] including the insulin-like growth factor-II gene[4] (*Igf2*), an autocrine growth factor in many tumours[5–7] and *H19* (ref. 8), a gene that may act as an RNA and whose normal function is unknown. *IGF2* and *H19* map to chromosome 11p15.5 (ref. 9), a region involved in paternal uniparental disomy (UPD) in Beckwith-Wiedemann syndrome[10] (BWS), which also predisposes to a wide variety of childhood cancers including Wilms' tumour (WT).

Recently, both *IGF2* and *H19* have been shown to be imprinted in humans, with reciprocal expression of the paternal *IGF2* (refs 11–13) and maternal *H19* alleles[11,14,15]. In addition, *IGF2* (refs 11,12) and *H19* (ref. 11) undergo loss of imprinting (LOI) in most WT's. Because *IGF2* is an autocrine growth factor, it has been proposed that LOI of *IGF2* leads to overexpression of *IGF2* and Wilms' tumourigenesis. Alternatively, *H19* has been proposed to act as a tumour suppressor gene, since it suppresses tumourigenicity when overexpressed using an expression construct in WT and rhabdomyosarcoma cells[16]. However, the relationship of LOI to tumour suppressor activity is unclear, particularly considering the low frequency of imprinting changes of *H19* (29%) compared to *IGF2* (77%)[11].

To investigate the relationship of LOI to deregulated gene expression, we examined Wilms' tumours with and without LOI for expression of *IGF2* and *H19*. Surprisingly, while *IGF2* expression was variably increased in tumours showing LOI of *IGF2*, LOI of *IGF2* was invariably associated with abrogation of *H19* expression. LOH, which also affects the maternal allele, similarly led to loss of *H19* expression. Finally, LOI of *IGF2* was associated with increased methylation of five parental-origin-specific *Hpa*II sites upstream of *H19*. These data suggest that LOI involves reversal of an imprinting domain on the maternal chromosome, and that LOI and LOH share a common final pathway of reduced expression of *H19* in WT.

IGF2 and *H19* expression in LOI

Twelve WT's heterozygous for transcribed polymorphisms in *IGF2* or *H19* (and thus informative for imprinting status) were examined for expression of these genes by northern blot analysis. Of these tumours, eight showed LOI of *IGF2*, and one also showed LOI of *H19*. In addition, three fetal kidneys and normal tissue from six kidneys resected from children with WT were also examined for expression of these genes. Of these nine kidneys, eight showed normal imprinting. One kidney derived from a patient with BWS had undergone LOI in normal tissue, consistent with LOI seen in nontumour cells of some BWS patients[17,18].

WT's with LOI expressed on average twice as much *IGF2* as WT's without LOI (1.82 ± 0.35 versus 0.96 ± 0.46, normalized to *GAPDH*), as would be predicted by loss of imprinting of the normally nonexpressed allele (Fig. 1, Table 1). However, these differences were not statistically significant, because of the wide range of expression of *IGF2* among the tumours of each group. While normal kidney tissue removed from WT patients is not developmentally similar to WT precursor cells[19], these

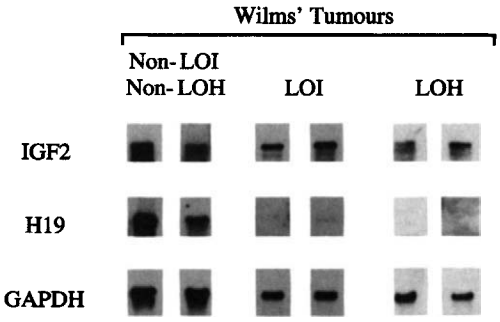

Fig. 1 Northern blot analysis of six WTs for expression of *IGF2*, *H19* and *GAPDH*. RNA from two WTs without loss of heterozygosity (LOH) or loss of imprinting (LOI), two WTs with LOI, and two WTs with LOH was electrophoresed, transferred to nylon membranes and hybridized with *IGF2*, *H19* and *GAPDH* probes (see Methodology). The filters were stripped after each hybridization.

tissues also showed a wide range of *IGF2* expression (Table 1).

In contrast, expression of *H19* in all tumours showing LOI of *IGF2* was barely detectable (0.022 ± 0.007, normalized to *GAPDH*), approximately 80-fold lower than that seen in tumours with normal imprinting of *IGF2* (1.79 ± 0.68, normalized to *GAPDH*; Fig. 1, Table 1). One tumour showed LOI of *H19* and *IGF2*, and *H19* expression was barely detectable in this tumour, as well. These differences in expression were highly statistically significant ($p<0.0001$). The ratio of *IGF2* to *H19* expression was also highly significant ($p < 0.0001$), 168 ± 66 for WT's with LOI, compared to 0.640 ± 0.195 for WT's without LOI or LOH.

Thus, tumours with LOI of *IGF2*, while activating the normally silent maternal allele, showed a two-fold but statistically insignificant increase in *IGF2* expression, compared to tumours without LOI. However, LOI of *IGF2* was significantly associated with silencing of the normally expressed *H19* allele on that chromosome. These data suggest that decreased expression of *H19* might be the pathologically significant result of *IGF2* LOI in WT. We thus hypothesized tumours with loss of heterozygosity (LOH) of 11p would also show down regulation of *H19* expression, because LOH preferentially involves the maternal allele. Indeed, preferential maternal LOH originally led to the imprinting hypothesis in WT[20–22]. In all four tumours with LOH examined, *H19* showed little or no expression (0.008 ± 0.007, normalized to *GAPDH*; Fig. 1, Table 1), and this result was also statistically highly significant ($p <0.0001$). The *IGF2* to *H19* ratio in these tumours was 1,902 ± 701 ($p \pm 0.0001$). Thus, down regulation of *H19* expression appears to be a common final pathway of both LOI and LOH.

Parental-origin-specific methylation of *H19*

Recently, Ferguson-Smith *et al.*[23] found parental-origin-specific methylation of the murine *H19* gene by examining experimentally derived embryos maternally disomic for *H19* (MatDi7). They observed that the *H19* promoter is hemimethylated in normal embryos and unmethylated in MatDi7 embryos, indicating that the maternal allele is normally unmethylated and the paternal allele is normally methylated (after fertilization, as it is unmethylated in sperm[23]). Paternal-specific methylation has also been shown by examination of interspecific hybrid mice[24].

Because tumours with LOI of *IGF2* showed down regulation of *H19* expression in our study, we sought to determine whether the human *H19* promoter also undergoes parental-origin-specific methylation, and if

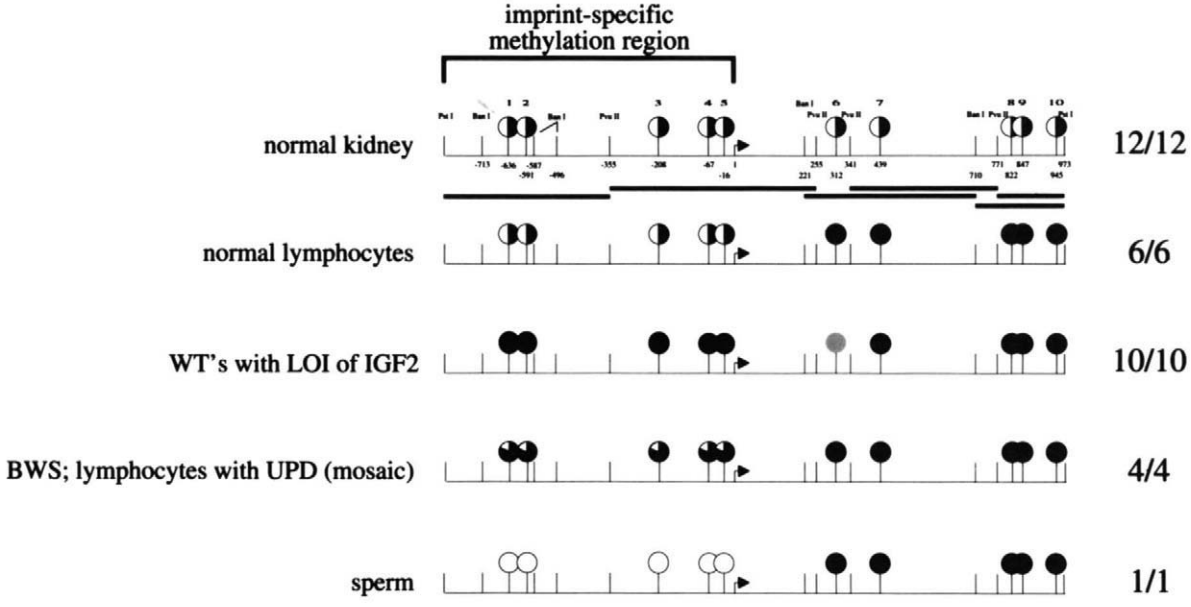

Fig. 2 Methylation status of 10 *Hpa*II sites in the promoter and 5' portion of the *H19* gene. *Hpa*II sites are numbered 1 through 10 and are indicated by circles which are filled in black according to the degree of methylation. Methylation status of *Hpa*II sites that are filled in grey was not determined. Numbers on the right of each methylation pattern indicate number of samples showing a given methylation pattern/number of samples tested. A restriction map is shown for *Pst*I, *Ban*I and *Pvu*II. The probes used are shown as horizontal lines under the first methylation map. The transcription start site is indicated by an arrow.

so, whether the methylation pattern is altered in tumours with LOI. Zhang *et al.* recently observed hypomethylation of *H19* in ovarian teratomas, which are parthenogenetic, suggesting that the two alleles are differentially methylated[14].

We therefore hybridized 15 tumours and 22 nonmalignant tissues with six distinct probes derived from the promoter and 5' portion of *H19*. These probes recognize specifically all 10 *Hpa*II sites in this region, corresponding to the region known to exhibit parental-origin-specific variation in the mouse (Fig. 2). Sites 6–10 showed hemimethylation in kidney but were completely methylated in lymphocytes, which did not express *H19*. Therefore these sites showed tissue-specific variation as is seen with most genes showing tissue-specific expression. However, all five *Hpa*II sites in the promoter showed hemimethylation in normal kidney, normal lymphocytes and in six other fetal tissues (adrenal, brain, heart, lung, spleen and tongue), independent of *H19* expression in those tissues (Fig. 3*a*). These results suggest that *H19*

Table 1 Expression of *IGF2* and *H19* and methylation of the *H19* promoter in 41 normal tissues and Wilms' tumours

Tissue	Patient	*IGF2*-ApaI DNA	*IGF2*-ApaI RNA	*IGF2*-DR DNA	*IGF2*-DR RNA	*H19*-RsaI DNA	*H19*-RsaI RNA	Expression *IGF2/GAPDH*	Expression *H19/GAPDH*	Methylation *H19* promoter 1	2	3	4	5
Fetal kidney														
FK	1	b/b		a/b		a/b		0.54	1.1	0	0	0	0	0
FK	2	b/b		b/b		a/b	a/-		0.96					
FK	3	b/b		b/b		b/b				0	0	0	0	0
FK	4	a/b	-/b	b/c	-/c	b/b		0.4	0.82					
WT's with LOI														
NK	5	b/b		a/b		a/b	a/-			0	0	0	0	0
WT	5	b/b		a/b	a/b	a/b	a/-	1.09	0.01	+	+	+	+	+
NK	6	a/b	-/b	b/c		a/b	a/-	0 0	0 0	0				
WT	6	a/b	a/b	b/c		a/b		0.71	0.03	+	+	+	+	+
NK	7	a/b	a/-	a/c		a/b	-/b			0	0	0	0	0
WT	7	a/b	a/b	a/c		a/b	-/b			+	+	+	+	+
NL	8	a/b		a/c		a/b				0	0	0	0	0
WT	8	a/b	a/b	a/c	a/c	a/b	a/b	3.25	0.01	+	+	+	#	#
NK	9	a/b	a/-	a/c	-/c			1.69	0.76	0	0	0	0	0
WT	9	a/b	a/b	a/c	a/c	b/b		2.46	0	+	+	+	+	+
NK	10	b/b		b/c				0.43	0.16	0	0	0	0	0
WT	10	b/b		b/c	b/c	b/b		1.91	0.02	+	+	+	+	+
NK	11	a/b		b/c		a/b		2.39	3.68	0	0	0	0	0
WT	11	a/b	a/b	b/c		a/b		1.03	0.06	+	+	+	+	+
WT	12	b/b		a/b	a/b	a/b		2.31	0.02	+	+	+	+	+
WT	13	a/b	a/b	b/c		b/b				+	+	+	+	+
BWS with LOI														
NK	14	a/b	a/b	b/c		a/b				+	+	0	0	0
WT	14	a/b	a/b	b/c		a/b		0.45	0	+	+	+	+	+
WT's with LOH														
NK	15	a/b		b/c	b/-	b/b								
WT	15		b					3.37	0					
NK	16	a/b		a/b		a/b								
WT	16					a		4.18	0.03					
NK	17	a/a		c/c		a/b								
WT	17	a/?		c/?		b		2.6	0					
NK	18	b/b		a/b		a/a				0	0	0	0	0
WT	18	b/?		a		a/?		1.5	0	+	+	+	+	+
WT's without LOI or LOH														
NK	19	a/b		b/c		a/b				0	0	0	0	0
WT	19	a/b		b/c	b/-	a/b	a/-	0.14	0.23	0	0	0	0	0
NK	20	a/b		e/e		b/b				0	0	0	0	0
WT	20	a/b	-/b	e/e		b/b		0.37	3.04	0	0	0	0	0
NK	21	a/b		a/b		b/b			0.22	0	0	0	0	0
WT	21	a/b	-/b	a/b		b/b		1.13	1.08	0	0	0	0	0
NK	22	b/b		b/b		a/b	-/b			0	0	0	0	0
WT	22	b/b		b/b		a/b	-/b			0	0	0	0	0
WT	23	a/b	-/b	b/b		a/a		2.2	2.81					

Four fetal kidneys (FK) and 19 WT's with corresponding normal kidney (NK) or lymphocytes (NL) were examined for expression of *IGF2* and *H19*, as well as methylation of *Hpa*II sites 1-5 in the *H19* promoter (Fig. 2). Tissues are grouped according to their genetic abnormality: LOI, LOH or neither. The genotypes that are boxed are informative for either imprinting status or LOH. Methylation status is scored as follows: 0 = normal hemimethylation; + = 90-100% methylation; # = site 4 or site 5 but not both are fully methylated in WT8 (cannot be distinguished because of the fragment size).

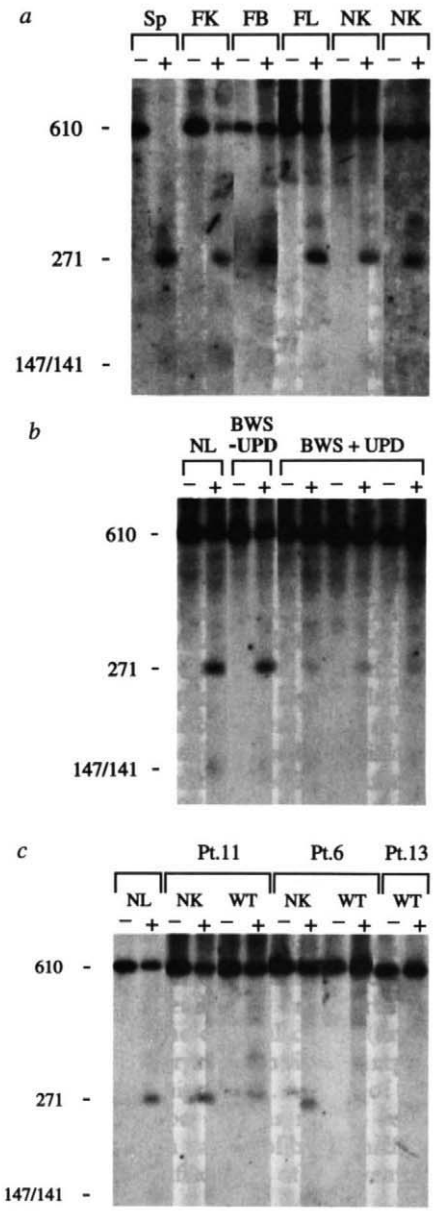

Fig. 3 Methylation analysis of *Hpa*II sites 3, 4 and 5 of the *H19* promoter. *a*, normal tissues; *b*, BWS patients with paternal uniparental disomy of 11p15.5; *c*, Wilms' tumours, showing loss of *IGF2* imprinting. DNA was digested sequentially with either *Pvu*II and *Pst*I alone (–), or *Pvu*II, *Pst*I and *Hpa*II (+). The digests were electrophoresed, transferred to nylon membranes and hybridized with a 610 bp fragment that spans *Hpa*II sites 3–5 (Fig. 2) (see Methodology). Fragment sizes are indicated on the left in base pairs. *a*, Sp, sperm; FK, fetal kidney; FB, fetal brain; FL, fetal lung; NK, normal kidney. *b*, NL, normal lymphocytes (non-BWS); BWS-UPD, BWS patient without UPD; BWS+UPD, BWS patients with UPD (numbers 1, 2 and 3 in Table 2). *c*, DNA samples are numbered as in Table 1.

shows parental-origin-specific tissue-independent methylation of the promoter region.

We sought to confirm this observation more rigorously, in a manner similar to that employed by Ferguson-Smith *et al.*[23], (that is, using a genome derived from a single parent), but without using teratomas (which are parthenogenetic) or hydatidiform moles (which are androgenetic), since it is well established that tumours undergo substantial alterations in DNA methylation[25–27]. We therefore obtained blood samples from 25 patients with BWS in order to identify those with uniparental disomy (UPD, two paternal copies of 11p15). Four such patients were found (Table 2), consistent with previous estimates of UPD frequency[28]. If methylation of the *H19* promoter were parental-origin-specific, then the BWS patients with UPD should show increased methylation of this region. Indeed, sites 1–5, which were hemimethylated in tissues of normal patients, showed increased methylation in all four BWS patients with paternal UPD (Figs 2,3*b*). The degree of methylation (80–90%) was consistent with the somatic mosaicism of disomic and non-disomic cells in these and other patients[28]. In contrast, the methylation pattern of sites 6–10 (which showed tissue variation in normal patients) was unchanged in BWS patients with UPD (Fig. 2). Thus, just as in the mouse, where parthenogenetic animals could be derived experimentally[23], patients with BWS and UPD provide strong evidence that the *H19* promoter undergoes parental-origin-specific methylation in humans.

In order to exclude the possibility that these methylation differences were allele-specific and were not caused simply by the fact that the patients had BWS, we examined five patients with BWS not showing UPD. In four of five cases, the methylation pattern was indistinguishable from that seen in normal individuals, as was the case for sites 6–10 (Fig. 3*b*). Thus, in these individuals, the pattern of methylation in the promoter of *H19* was parental-origin-specific, in that the paternally derived copy was methylated in a tissue-invariant manner.

One BWS patient not showing UPD nevertheless showed increased methylation of three of the five parental-origin-specific methylation sites in the *H19* promoter in normal tissue, thus partially resembling the pattern seen in tumours with LOI (see below). However, analysis of RNA from this patient revealed that his normal tissue had undergone loss of imprinting, which we have observed in one-third of BWS patients (data not shown). Finally, as in mice[23,24], parental-origin-specific methylation of the promoter must have occurred postzygotically, because this region was unmethylated in sperm DNA (Figs 2,3*a*).

Abnormal methylation of the H19 promoter

Because tumours with LOI of *IGF2* showed reduced expression of *H19*, and because normal imprinting of *H19* is associated with methylation of the paternal allele, we asked whether the methylation pattern of *H19* was disturbed in these tumours. In nine cases showing LOI of *IGF2*, the *H19* promoter showed 90–100% methylation at the sites normally unmethylated on the maternally inherited allele (Fig. 3*c*, Table 1). Thus, the maternal allele had acquired a paternal pattern of methylation. This is consistent with the fact that the *IGF2* gene on the same (maternally derived) chromosome was expressed in these tumours (Table 1), as occurs normally only on the paternally derived chromosome. In contrast, four tumours without LOI of *IGF2* showed no change in the methylation of *H19* (Table 1), indicating that these changes were

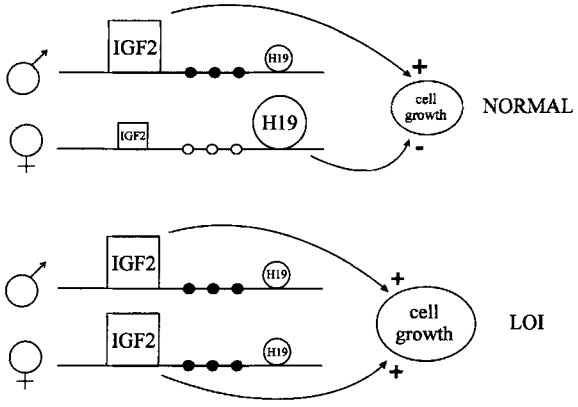

Fig. 4 Model of loss of imprinting of *IGF2*, *H19*, and methylation of the *H19* promoter in Wilms' tumour. In normal cells, the paternal *IGF2* and maternal *H19* genes are expressed (shown large). Several sites upstream of *H19* are methylated on the paternal allele (filled circles) and unmethylated on the maternal allele (open circles). In tumours with LOI, the maternal chromosome reverses to a paternal epigenotype, with a paternal pattern of methylation of the *H19* promoter, *IGF2* turned on, and *H19* turned off, causing increased cell growth. LOI of *H19* on the maternal chromosome, when it occurs, could occur independently or could be influenced by events on the paternal chromosome.

related to abnormal imprinting and not malignancy *per se*. Some tumours show LOI of both *IGF2* and *H19* (ref. 11). In the one tumour here with LOI of both genes, the maternally inherited *H19* allele was partially methylated, with four of the five sites affected (Table 1), consistent with abnormal methylation on both chromosomes in this tumour.

Discussion

Imprinted genes in humans and loss of imprinting in cancer are novel observations whose implications in normal development and disease are only beginning to be understood. As a first step, we have tested the hypothesis that LOI is related to abnormal expression of the affected genes in Wilms' tumour. We have found an approximately twofold increase in *IGF2* levels in tumours with LOI, consistent with what one might expect from activation of a normally silent allele. Far more striking, however, was the virtual abolition of *H19* expression in tumours with LOI of *IGF2*. This change in expression appears to be related to LOI specifically, since it was not seen in tumours with normal imprinting. LOH was also associated with loss of *H19* expression. Thus, loss of *H19* expression may be a common final pathway of LOH and LOI and may explain why LOH preferentially involves the maternal allele. Consistent with this hypothesis, Hao *et al.* recently observed suppression of tumorigenicity when *H19* was introduced into and expressed at high levels in a WT and rhabdomyosarcoma cell line[16]. Thus, our data are consistent with their hypothesis that *H19* is a tumour suppressor gene. Nevertheless, we have also mapped a rhabdomyosarcoma suppressor gene, using subchromomal transferable fragments (STFs) from 11p15 (ref. 29), to a region between *D11S12* and *D11S724*, thus excluding *IGF2* and *H19*. LOH mapping of WT also places the common region of overlap to the same region defined by STFs (C. Junien, personal communication), centromeric to *IGF2* and *H19*. The most likely explanation is that there are multiple genes on 11p15 important in WT, as there are for other tumours on other chromosomes[30].

Our data also show that the *H19* promoter region undergoes parental-origin-specific DNA methylation as in the mouse[23,24]. We examined 10 specific *Hpa*II sites in this region and found five that show parental-origin-specific, tissue-independent methylation, and five that show more typical tissue-specific variation. These data are generally consistent with those of Zhang *et al.*[14], who found hypomethylation of *H19* in ovarian teratomas and allele-specific methylation of *H19* in normal tissue.

We sought to examine the *H19* promoter region directly in normal tissue by identifying BWS patients with paternal UPD, which allowed us to distinguish paternal from maternal methylation patterns. This approach permitted

Table 2 Identification of BWS patients with uniparental disomy

	D11S16 *MspI*	*CALCA* *TaqI*	*HBG1* *HindIII*	*HBG2* *HindIII*	*D11S551* *MspI*	*INS* *PvuII*	*IGF2* *SstI*	*IGF2* *ApaI*	*IGF2* DR	*H19* *RsaI*	*H19* *TaqI*	*HRAS1* *TaqI*	*HRAS1* *MspI*
1-M	1/3	2/2	1/2	2/2	2/2	1/1	1/2	1/2	1/3	1/1	2/2	4/4	4/4
1-F	2/2	2/2	1/1	1/2	1/1	3/3	2/2	2/2	n.d.	2/2	1/2	3/4	3/4
1-P	2/2	2/2	1/1	2/2	1/1	3/3	2/2	2/2	2/2	2/2	1/1	3/3	3/3
2-M	1/2	n.d.	n.d.	n.d.	n.d.	4/6	1/2	1/2	2/3	1/2	2/2	4/5	1/4
2-F	2/2	n.d.	n.d.	n.d.	n.d.	3/5	2/2	2/2	1/2	2/2	1/1	3/4	1/4
2-P	1/2	n.d.	n.d.	n.d.	n.d.	3/3	2/2	2/2	2/2	2/2	1/1	3/3	1/1
3-M	1/3	1/2	1/1	1/	n.d.	/3	2/2	2/2	2/2	1/2	1/2	1/4	4/4
3-F	1/3	2/2	1/1	1/1	n.d.	1/3	1/2	1/2	2/3	1/2	1/1	3/4	3/4
3-P	1/3	2/2	1/1	1/1	n.d.	3/3	1/1	1/1	3/3	2/2	1/1	4/4	4/4
4-M	1/3	2/2	1/1	1/2	n.d.	3/3	2/2	2/2	2/2	1/2	2/2	4/4	4/4
4-P	1/3	2/2	1/1	2/2	n.d.	2/2	2/2	2/2	2/2	1/1	1/1	4/4	4/4

Genotypes in thick-lined boxes are those for which the patients conclusively showed paternal uniparental disomy. Genotypes for which the patients were not informative are indicated by thin-lined boxes. Alleles are numbered according to decreasing size. M, mother; F, father; P, patient; n.d., not determined.

precise mapping of parental-origin-specific methylation to the region immediately upstream from, but not beyond, the transcriptional start site. We also found that this region was hypomethylated with the exception of sperm DNA, as in mouse[23], implying that the methylation pattern is established after fertilization.

What of the 29% of tumours that show LOI of both *IGF2* and *H19*? In the tumour studied here, *H19* expression was still low, although one of the five parental origin- specific *Hpa*II sites was unmethylated. LOI of *H19* has been seen only when *IGF2* is also affected. An interesting possibility is that a factor normally involved in expression of the maternal *H19* allele, when that allele is inactivated in cancer, could leak onto the paternal chromosome if the chromosomes are paired as in *Drosophila*[31]. Alternatively, H19 overexpression could be deleterious to the cell, and thus LOI of H19 on the paternal chromosome is found only when H19 expression is down regulated.

Studies of isodisomic mice[32] and evolutionary models of divergent selective pressures on parental gametes[33,34] both suggest that paternally imprinted chromosomes stimulate growth, and maternally imprinted chromosomes inhibit growth; the paternal expression of *IGF2* and maternal expression of *H19* are consistent with this idea. Concordant LOI of *IGF2*, inactivation of *H19*, and switch in the methylation pattern suggest a model in which a large domain of the maternal chromosome has reversed to a paternal epigenotype, leading to increased growth in which both *IGF2* and *H19* may be important (Fig. 4). The initial event in this switch could be a change in expression of either gene. In normal development, expression of one gene may be influenced by expression of the other, by competition for a common factor[24]. The initial event could also involve DNA methylation. More than ten years ago, Feinberg and Vogelstein reported abnormal DNA methylation in cancer[25], and knockout mice lacking a normal DNA methyltransferase gene show altered DNA methylation and abnormal imprinting[35]. Thus, abnormal methylation may play a causal role in this switch, which could have implications for cancer treatment.

Methodology

Nucleic acid preparation. Genomic DNA was prepared by treating minced tissues or peripheral blood lymphocytes with 0.5 mg m^{-1} proteinase K in 1% SDS, followed by phenol/chloroform extraction and ethanol precipitation. Poly A^+ RNA was isolated using the FastTrack mRNA isolation kit (Invitrogen).

Analysis of uniparental disomy. To detect paternal UPD in patients with BWS, DNA was obtained from 25 BWS patients and their parents, and analysed using 13 RFLPs on 11p15. 5 μg of DNA was digested overnight with 20 U of the appropriate restriction enzyme, electrophoresed on a 1% agarose gel, transferred[35] to Hybond-N^+ (Amersham), and hybridized[36] with one of the following probes: *D11S16* (ref. 37), to detect an *Msp*I polymorphism; pTT42 (ref. 38), which detects a *Taq*I polymorphism[39] in the calcitonin gene; JW151 (ref. 40), which detects two *Hin*dIII polymorphisms in the β-globin gene cluster[41]; *D11S551*, which detects an *Msp*I polymorphism[42]; pHINS310, which detects a variable-length polymorphism[43], in the insulin gene; p*IGF2*/8-1 (ref. 44), which detects an *Sst*I polymorphism[45] in the insulin-like growth factor-II gene; *D11S813E* (ref. 46), which detects a *Taq*I polymorphism[47] in the *H19* gene; and pEJ6.6 (ref. 48), which detects a variable-length polymorphism[49] in the c-Ha-ras-1 (*HRAS1*) gene. Three additional RFLP analyses were performed using PCR[50]: An *Apa*I polymorphism in *IGF2* (ref. 51), a dinucleotide repeat polymorphism in *IGF2* (ref. 52), and *Rsa*I polymorphism in *H19* (ref. 53).

Methylation analysis of the *H19* promoter. A 1.8 kb *Pst*I fragment of the *H19* gene[46], including 825 bp upstream and 973 bp downstream of the transcriptional start site, and containing 10 *Hpa*II sites, was analysed for DNA methylation by digestion with the following restriction endonucleases (Fig. 2): for all but site 6, *Pvu*II + *Pst*I ± *Hpa*II; for sites 6, 8, 9 and 10, *Ban*I + *Pst*I ± *Hpa*II. In each case, 5 μg of DNA was digested overnight with 20 U of enzyme, followed by an additional 4 h incubation with another 20 U. DNA was precipitated after each digest and redissolved in the appropriate restriction buffer. The digested DNA was electrophoresed on a 3% NuSieve agarose (FMC)/1% agarose gel and transferred to Hybond-N^+ (Amersham). The *Pvu*II/*Pst*I/*Hpa*II-digested DNA was hybridized to the following *Pvu*II/*Pst*I fragments of the 1.8 kb *H19* probe: the 470 bp *Pvu*II/*Pst*I fragment (for analysis of *Hpa*II sites 1 and 2), the 610 bp fragment (for analysis of sites 3, 4 and 5), the 401 bp fragment (for analysis of site 7), and the 200 bp fragment (for analysis of sites 8, 9 and 10) (Fig. 2). The *Ban*I/*Pst*I/*Hpa*II-digested DNA was hybridized to the following *Ban*I/*Pst*I fragments of the 1.8 kb *H19* probe: the 489 bp fragment (for analysis of sites 6 and 7) and the 263 bp fragment (for analysis of sites 8, 9 and 10) (Fig. 2). Signals were quantitated in a PhosphorImager (Molecular Dynamics).

Analysis of tissues for LOI of *IGF2* and H19. To determine whether tissues showed LOI of *IGF2* and *H19*, they were analysed for mono- or bi-allelic expression of these genes. DNA and reverse-transcribed mRNA from each tissue was analysed as described in ref. 16 by the following RFLP assays using the polymerase chain reaction: an *Apa*I polymorphism[51] and a dinucleotide repeat polymorphism[16,52] in the *IGF2* gene, and a *Rsa*I polymorphism[53] in *H19*.

Northern blot analysis. 0.1–0.5 μg of mRNA was electrophoresed on a 1% agarose/5% formaldehyde gel, transferred to GeneScreen (DuPont), and hybridized[54] with the following probes: *GAPDH* (gift of Paul Killian); the 1.8 kb *Pst*I fragment of *H19* (Fig. 2); an *Eco*RI cDNA fragment of *IGF2* (ref. 44). Gene expression was quantified on a PhosphorImager (Molecular Dynamics), and normalized for total mRNA by rehybridizing with *GAPDH*. All of the blots were hybridized simultaneously with a given probe to preclude differences in probe quality.

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