Epigenetic lesions at the *H19* locus in Wilms' tumour patients

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To test the potential role of *H19* as a tumour suppressor gene we have examined its expression and DNA methylation in Wilms' tumours (WTs). In most WTs (18/25), *H19* RNA was reduced at least 20-fold from fetal kidney levels. Of the expression-negative tumours ten retained 11p15.5 heterozygosity: in nine of these, *H19* DNA was biallelically hypermethylated and in two cases hypermethylation locally restricted to *H19* sequences was also present in the non-neoplastic kidney parenchyma. *IGF2* mRNA was expressed in most but not all WTs and expression patterns were consistent with *IGF2/H19* enhancer competition without obligate inverse coupling. These observations implicate genetic and epigenetic inactivation of *H19* in Wilms' tumorigenesis.

The *H19* gene produces a spliced and polyadenylated RNA which shows high expression in many developing organs, including fetal kidney, and lower but persistent expression in certain human post-natal organs, including juvenile and adult kidney[1–8]. Several features of this gene have singled it out for investigation: first, while H19 transcription is highly regulated and *H19* RNA is a major transcript in expressing tissues, the lack of long or evolutionarily conserved translational reading frames and the failure to identify peptide products have hindered attempts to predict a biological function. Tilghman and coworkers have suggested that the biologically active product might be the RNA itself[9], placing *H19* in a small group of RNA polymerase-II transcription units, including *XIST*[10], which may share this characteristic. Second, *H19* is subject to parental imprinting in mice and humans, with the inactive paternal allele fully methylated at numerous CpG dinucleotides in the body of the gene and in the promoter region and with the active maternal allele unmethylated at all or most of these sites[11–17]. Third, *H19* maps to chromosome 11p15.5, a region subject to loss of heterozygosity (LOH) in WTs, with a nearly complete bias toward the loss of maternal alleles[18]. Fourth, inappropriate expression of an *H19* transgene in mice caused fetal mortality[19] and expression of human *H19* in certain human embryonal tumour lines had growth-inhibitory or anti-clonogenic and anti-tumourigenic effects[6].

Based on these considerations, we have proposed *H19* as a candidate WT suppressor gene[18]. According to this hypothesis, the monoallelic expression of this gene would render it susceptible to functional deletion by "one-hit" kinetics. If H19 is in fact a WT suppressor gene, then it would be predicted to be inactivated not only in the roughly 50 percent of WTs which show 11p15.5 LOH, but also in at least some WTs which retain 11p15.5 heterozygosity. We have now tested this by assessing *H19* expression, CpG-methylation, and 11p15.5 allelic status in primary WTs. Our results confirm the predicted lack of expression in a high proportion of WTs and also reveal a highly specific and localized pattern of hypermethylation in the non-neoplastic kidney parenchyma of a subset of these patients.

As the *IGF2* gene, located within 200 kilobases (kb) of *H19* and subject to opposite imprinting, has been proposed as a candidate dominant oncogene in WT[14,20] and since inverse regulation of *H19* and *IGF2* by an enhancer competition mechanism has been suggested[16], we have also examined expression of this gene. As predicted from previous studies[21,22], *IGF2* mRNA levels were high in most WTs and, consistent with enhancer competition, *IGF2* imprinting was relaxed in tumours with biallelic *H19* hypermethylation, but proliferating epithelial cells from three tumours lacked both *H19* and *IGF2* expression.

Levels of *H19* RNA in WTs

A northern blot survey of 17-week human fetal organs showed that *H19* RNA is expressed most abundantly in adrenal, followed by liver and fetal kidney (data not shown). This agrees with *in situ* hybridization data, which show high *H19* expression in differentiating renal blastema, the presumptive precursor tissue to WT[8]. In our series of 25 WTs, northern analysis showed very low (at least 20-fold reduced from the levels in fetal kidneys by densitometry) or undetectable *H19* RNA in 18 cases (Figs 1, 4*b* and Table 1). Of the remaining cases, three showed

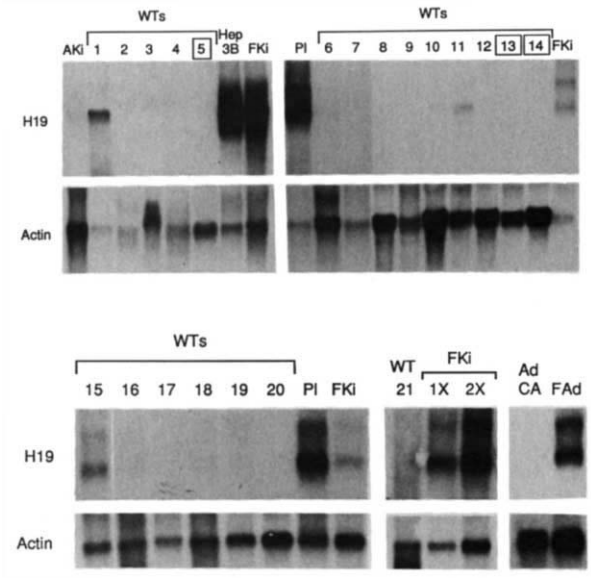


Fig. 1 Northern analysis of *H19* RNAs in WTs and in a congenital AdCa. AKi, adult kidney. FKi, fetal kidney; the sample run on the left half of the upper blot is from a 20-week fetus; the other samples are from a 17-week fetus. FAd, 17-week fetal adrenal. Pl, term placenta. Hep3B, hepatocellular carcinoma cell line. See Fig. 4*b* for analysis of WTs 23–25. Cases analysed as short-term tumour explants are indicated by the boxes. Note the underloading of the 17-week fetal kidney sample in the top panel, as indicated by the β-actin signal.

detectable *H19* expression at a level about 10-fold reduced from fetal kidney and four showed expression equivalent to fetal kidney. The cases with low expression may represent examples of expression in the neoplastic cells or alternatively may reflect contributions from non-neoplastic cells, such as newly formed vascular elements[23]. Favoring the latter possibility is the fact that all four WTs which were examined as short-term explants, and therefore free of non-neoplastic cells, lacked detectable *H19* expression (Fig. 1; WTs 5, 13, 14 and 23). The presence or absence of *H19* RNA in the tumours did not correlate with any obvious histological differences: case 1 (positive for expression) showed blastematous, epithelial and stromal elements as a 30/65/5 percent mixture, while cases 2, 3, 5, 21 and 23 (negative for expression) showed mixtures of 60/20/20, 50/25/25, 15/55/30, 80/5/15 and 0/95/5 percent.

To determine the presence or absence of 11p15.5 LOH, each tumour was typed for DNA polymorphisms at the *TH/IGF2*, *H19* and *HRAS* loci using PCR analysis and genomic Southern blotting (Table 1). An example of PCR

Table 1 11p15.5 alleles, *H19* CpG-methylation and *H19* RNA in WTs

WT Case	*TH*	*IGF2*				*H19*				*RAS*		*H19* me-CpG	*H19* RNA
	TNR	Apa	CA	Cfo	Aat	Rsa	Avi	Alu	B2	EJ	CGG		
1	NI	R	R	R		R		R		R	NI	+/–	+
2	NI	NI	L	L	L	L				NI	NI		–
3	R	R	R	NI	NI	NI	NI	NI	NI	R	NI	+/+	–
4	NI	NI	NI	R		R				R	NI		–
5	NI	NI	NI	L	L	NI	NI	NI		NI	L	+/+	–
6	NI	NI	NI	NI	NI	NI	NI	NI	NI		NI		–
7	NI	L[a]	NI	L[a]	L[a]	L[a]	L[a]	L[a]	NI		NI		–
8	NI	NI	R	NI	NI	NI	NI	NI	R	R	R	+/+	–
9	NI	L	NI	NI	NI	NI	NI	NI	NI		NI		–
10	R	NI	NI	NI	NI	NI	NI	NI	R		R	+/–	(+)
11	NI	NI	NI	L[a]	L[a]	L[a]	L[a]	L[a]	NI		NI	+/–	+
12	R	NI	R	NI	NI	NI	NI	NI	NI		NI	+/+	–
13	L	NI	NI		NI	L	L	L		L	L	+/+	–
14	R	R	R	NI	NI	NI	NI	NI	NI	NI	NI	+/+	–
15		R[b]	R[b]	R[b]	R[b]	R[b]	R[b]	R[b]		NI	R[b]	+/–	+
16	R	NI	NI	R	R	R	R	R		R	R		(+)
17	R	NI	NI	NI	NI	R	NI	NI		NI	R	+/+	–
18	L[a]	NI		L[a]	L[a]	L[a]	L[a]	L[a]		NI	L[a]		(+)
19	R	R	R	NI	NI	NI	NI	NI		R	R	+/+	–
20	NI	NI	NI	NI	NI	NI	NI	NI		NI	NI		–
21	NI	R	R	NI	NI	NI	NI	NI	NI	R	R	+/+	–
22	R	NI	NI	NI	NI	NI	NI	NI		NI	R	+/+	–
23	R	NI	R	NI	NI	NI	NI	NI		R		+/–	–
24	R	NI	NI	NI	NI	NI	NI	NI	NI	R		+/–	+
25	L[a]	L[a]	NI	L[a]	NI	L[a]	NI	NI				+/+	–

R, retention of heterozygosity; L, loss of heterozygosity; NI, not informative. *H19* me-CpG, +/– indicates monoallelic hypermethylation; +/+ indicates biallelic hypermethylation. *H19* RNA; +, expression at the level of fetal kidney; (+), expression reduced approximately 10-fold from fetal kidney; –, undetectable expression or expression reduced more than 20-fold from fetal kidney.

[a]80–90% loss of allelic signal intensity.

[b]25% loss of allelic signal intensity. For most cases the non-neoplastic kidney was the control tissue; for WTs 3, 14, 21 and 23 *H19* markers were also assessed in peripheral blood and found to be homozygous. The order of markers is centromeric (left) to telomeric (right) (Higgins, M. and Shows, T., personal communication). TNR=tetranucleotide (TCAT) repeat.

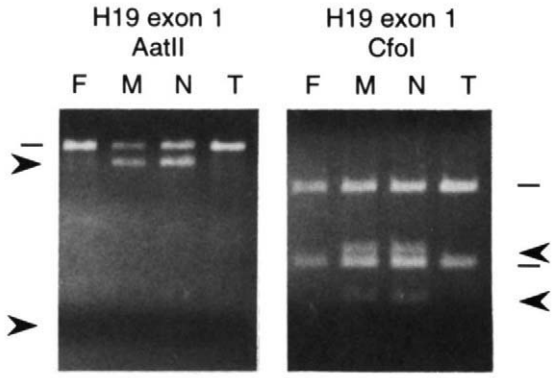


Fig. 2 Loss of the maternal *H19* allele in WT5 detected by PCR analysis of polymorphisms in the first exon. F, leukocyte DNA from the father. M, leukocyte DNA from the mother. N, leukocyte DNA from the patient. T, WT DNA. Bands which derive from the allele which was lost from the tumour are indicated by the arrowheads.

to detect LOH in a WT at two previously undescribed restriction site polymorphisms in the *H19* first exon is shown in Fig. 2. In this case, the tumour showed loss of the active maternal *H19* allele, thereby adding another example to the large published series of such cases. As would be predicted from a stable paternal *H19* imprint in Wilms' tumorigenesis, this tumour showed no expression of *H19* (Fig. 1; WT5). Six other cases, examined as primary biopsies, also showed LOH and no detectable *H19* expression (Table 1). However, WT11 and WT18, which were scored as showing LOH, did contain detectable amounts of *H19* RNA (Table 1 and Fig. 1). These tumours were not entirely devoid of the active *H19* allele, however, since they showed only partial loss of one allelic band for each marker examined (data not shown).

Of greater significance for testing the *H19* hypothesis were the findings in the tumours which retained 11p15.5 heterozygosity. Among these cases, only 3/15 showed H19 expression at a level comparable to fetal kidney, while two showed trace expression and ten cases (67%) showed very low or undetectable expression (Table 1). Among the three positive cases, WT15 showed a small (25–30%) but reproducible reduction in one allelic band on Southern analysis, suggesting that this tumour contained a subclone of cells which had lost the active *H19* allele (data not shown).

Adrenocortical carcinoma (AdCa) in childhood is associated with the Beckwith-Weidemann syndrome (BWS), a feature which it shares with WT, as 11p15.5 LOH appears to be frequent in AdCA[24] and the adrenal gland expresses high levels of *H19* RNA[7,8] (data not shown). *H19* would therefore also appear to be a candidate tumour suppressor gene in AdCA. We found a complete absence of *H19* expression in an AdCa which presented in a neonate (Fig. 1). In this case the fetal adrenal was used as the appropriate control tissue, since this type of congenital tumour most likely derives from fetal adrenal cortex.

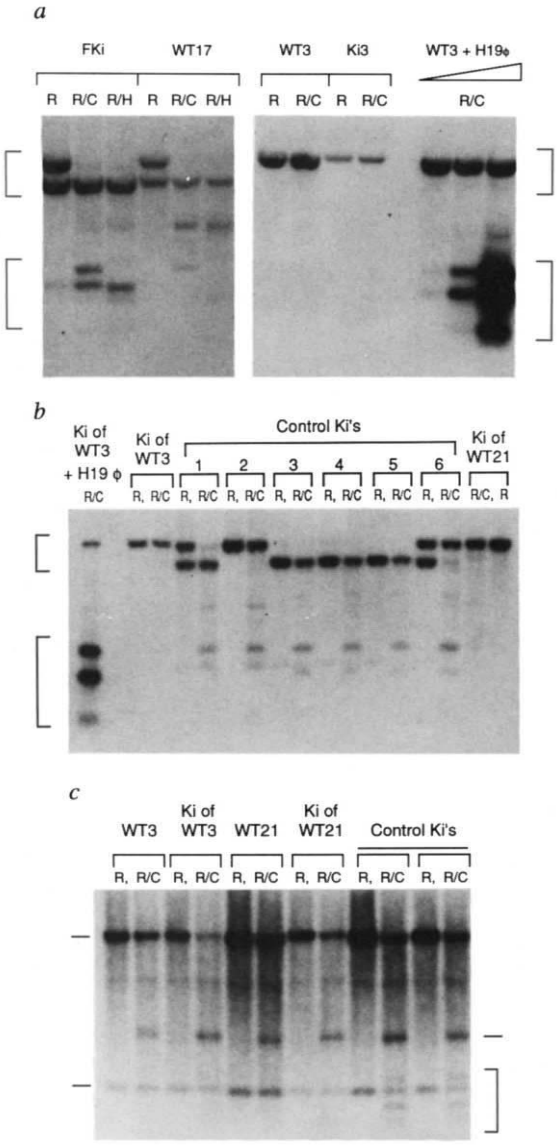
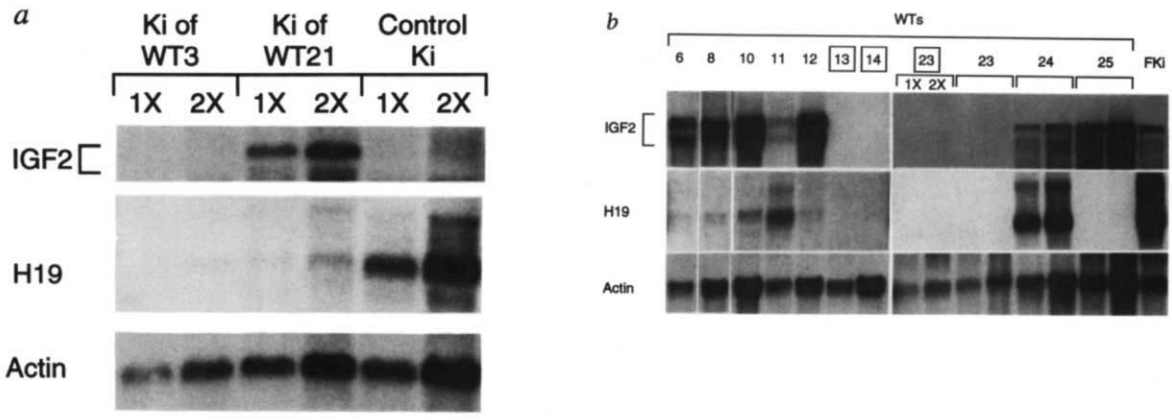


Fig. 3 CpG-methylation of *H19* in WTs and non-neoplastic kidney parenchyma of WT patients and controls. *a*, analysis of methylation in the 3' half of *H19* in a control fetal kidney (FKi), WTs 17 and 3 and the non-neoplastic kidney parenchyma adjacent to WT3 (Ki3). To control for completeness of enzymatic digestion, parallel samples were spiked with increasing amounts of an *H19* phage clone, which is completely converted to the limit-digest fragments (WT3 + H19ϕ). The upper brackets delineate the intact *H19 Rsa*I allelic bands; the lower brackets delineate the *Cfo*I and *Hpa*II limit-digest fragments. *b*, analysis of 3' *H19* DNA in non-neoplastic kidney parenchyma of the patients with WTs 3 and 21 and six control kidneys from children between one month and 3 years of age autopsied for non-neoplastic disorders. *c*, analysis of 5' *H19* DNA. The dashes on the left indicate the 2 kb and 0.4 kb intact *Rsa*I bands; the dash and bracket on the right indicate the *Cfo*I-digested products; a single *Cfo*I site in the minimal promoter was unmethylated, but the other two *Cfo*I sites in the promoter and all three *Cfo*I sites in the first exon[15] were hypermethylated, as shown by marked reduction in limit-digest bands in the patient samples. Densitometry, with normalization to the 0.4 kb *Rsa*I fragments, showed a greater than 90% reduction in intensity of the limit-digest bands in the kidney of the patient with WT3 and an 80% reduction in that of the patient with WT21, relative to the controls. Similar results were obtained with *Hpa*II blots (data not shown). R, *Rsa*I; R/C, *Rsa*I + *Cfo*I; R/H, *Rsa*I + *Hpa*II.



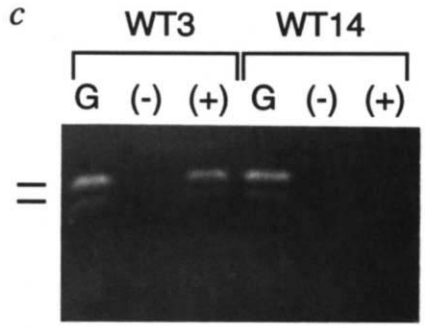


Fig. 4 *H19* and *IGF2* RNA levels in non-neoplastic kidney parenchyma and in WTs and IGF2 allelic expression in WTs. *a*, Non-neoplastic kidney parenchyma; the control kidney was from a 3-year-old child (age-matched with the WT patients) who was autopsied for a non-neoplastic disorder (control Ki 6 in Fig. 3*b*). *b*, WTs. The 6 kb and 4 kb *IGF2* mRNA bands are indicated. *H19* RNA was completely undetectable in the purely epithelial WT23, even on prolonged exposures, and was therefore reduced even from juvenile kidney levels (compare appropriate lanes in *a* and *b*; blots were performed under identical conditions). Tumour explants are indicated by boxes. *c*, reverse PCR of *IGF2* mRNA from WTs 3 and 14. The PCR products were digested with *Apa*I to assess allelic representation. G, genomic PCR products; (+), (–), inclusion or omission of reverse transcriptase. In heterozygous samples, the upper *Apa*I allele is artifactually but reproducibly twice as intense as the lower allele[20]; relaxation of imprinting is therefore complete in WT3 but partial (lower allele predominant) in WT14. Levels of *IGF2* mRNA in WT14 were below the detection threshold of Northern blots but could be detected after 40 cycles of reverse PCR.

Allelic analysis showed LOH at the H19 RsaI marker. We also found that an established AdCA cell line, SW-13, derived from a tumour occuring in an adult, does not express *H19* RNA, while adult adrenal gland continues to express easily detectable *H19* RNA (data not shown). While analysis of additional cases is required, our results in one primary case suggest that imprinting of *H19* can be maintained in AdCA, with loss of the maternal allele resulting in elimination of RNA expression.

Epigenetic inactivation of *H19* in some WTs

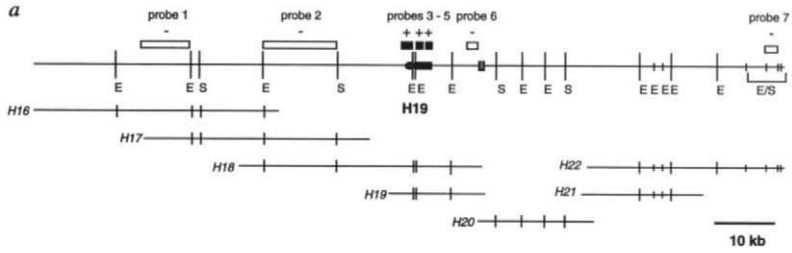
The reduction or elimination of *H19* expression in most WTs could occur either by gross loss of 11p15.5 heterozygosity or by an alternative, possibly epigenetic pathway. As CpG hypermethylation shows a tight correlation with silence of the imprinted H19 allele in normal tissues[15], and as altered DNA methylation can be a feature of cancer cells, one possibility was that the maternal *H19* allele in the non-expressing "retention" cases had become hypermethylated and thereby inactivated. To test this we used a Southern blotting method which we had developed for analysis of *H19* allelic methylation in normal human fetal and adult tissues[15]. We first examined methylation of CpG dinucleotides in the 3' half of the gene, using a hybridization probe spanning exons 3–5. By combining digestion with *Rsa*I, which recognizes a polymorphic site, and either *Cfo*I or *Hpa*II, which are methylation-sensitive and recognize numerous CpGs between the *Rsa*I sites, it is possible to examine the extent of methylation of each allele. In heterozygotes, normal tissues show selective loss of one allelic band on digestion with methylation-sensitive enzymes, while in homozygotes allele-specific methylation can be inferred from a 50% reduction in intensity of the single allelic band after addition of the methylation-sensitive enzyme, with generation of low molecular weight limit-digest bands.

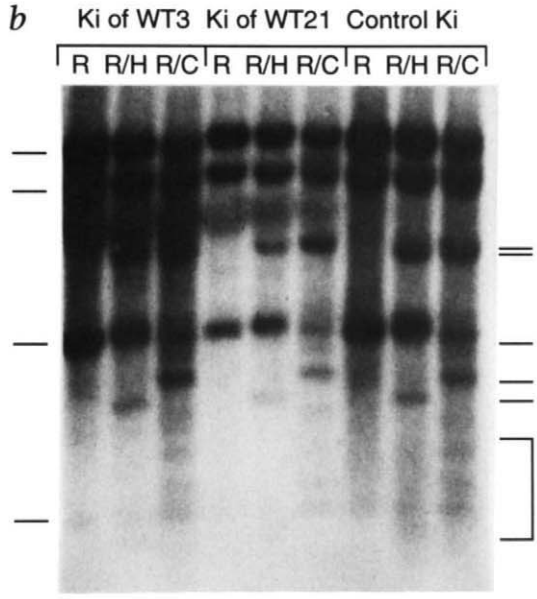
Normal fetal kidney yielded the expected pattern of monoallelic hypermethylation, with the active allele almost completely converted to limit-digest fragments (Fig 3*a*). In contrast, most non-expressing WTs showed either complete or partial biallelic hypermethylation of H19 DNA (Fig. 3*a,c*, Table 1). In two non-expressing cases with 11p15.5 LOH (WT2 and WT5), we observed the expected pattern of hypermethylation of the remaining inactive allele (see case WT2 in ref. 15) and in cases with partial LOH and detectable expression (WTs 11 and 18) we observed hypomethylation of the partially lost allele (data not shown). More importantly, in eight of nine non-expressing cases examined which retained heterozygosity at TH/IGF2 and/or *H-RAS* (but which were homozygous within *H19*) there was complete methylation of every *Cfo*I site within the 3' half of the gene (Fig. 3*a* and Table 1). Several of these cases (WTs 3, 13, 21, 22) were also examined at *Hpa*II sites in the 3' half of the gene and at *Hpa*II and *Cfo*I sites in the first exon and promoter region of the gene: all but one of these sites were fully methylated (Fig. 3*c*, data not shown). The two exceptions were WT17, which showed partial biallelic hypermethylation in both

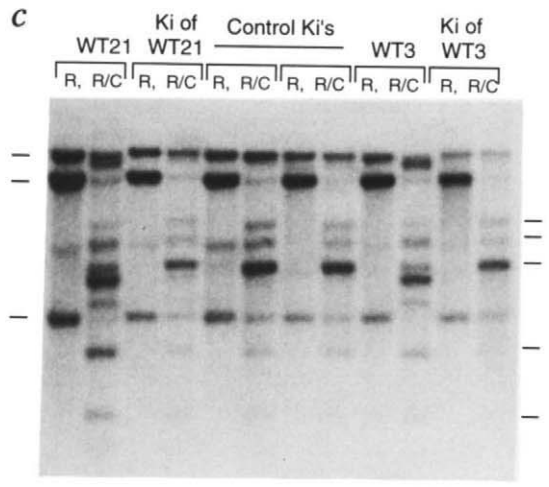
halves of the gene and WT23, which, despite a complete lack of *H19* expression, showed the normal pattern of monoallelic hypermethylation (Fig. 3*a*, data not shown). These data indicate that WTs which lack evidence of gross 11p15.5 LOH nevertheless frequently contain two copies of a hypermethylated and inactive *H19* gene.

Hypermethylation of *H19* in some WT kidneys

In analysing *H19* DNA methylation in the WTs, we were surprised to find that the non-neoplastic kidney parenchyma from two non-expressing "retention" cases also showed biallelic hypermethylation in the 3' and 5' halves of the gene, with nearly complete protection from







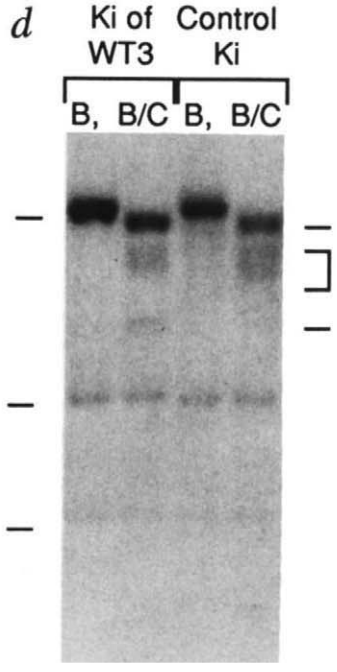


Fig. 5 Regional mapping of DNA hypermethylation in WT kidneys. *a*, Probes in and around the *H19* gene. Probes indicated above the map were applied to Southern blots of DNAs from the non-neoplastic kidney parenchyma of the WT patients and the control juvenile kidneys. The presence or absence of abnormal methylation at *Cfo*I or *Hpa*II sites in the WT patients' kidneys, evaluated by comparison of the band patterns with those generated from the control kidneys, is indicated as (+) or (–), respectively. *b*, Southern blot with probe 1. *c*, probe 6. *d*, probe 7. Dashes on the left indicate intact bands; dashes and brackets on the right indicate *Cfo*I- or *Hpa*II-digested bands. There were no methylation differences between patient and control non-neoplastic kidney tissues with these three probes, but in the *H19* enhancer region assessed by probe 6 the tumour DNAs showed slight *hypo*methylation, with generation of one additional band on CfoI digestion. Mapping of CfoI sites in the cloned DNA showed >14 sites assessed by probe 1, >5 sites by probe 2, >8 sites by probe 6, and >4 sites by probe 7. The region assessed by probe 2 was fully CfoI- and HpaII-methylated in the control kidneys while the regions assessed by each of the other probes were partially unmethylated. The *H19* transcribed region is indicated by the stippled rectangle, the promoter by the oval, and the enhancer by the striped box. R, *Rsa*I; C, *Cfo*I; H, *Hpa*II; E, *Eco*RI; S, *Sal*I; and B, *Bst*XI.

Table 2 Comparison of the frequency of *H19* homozygosity in WT patients and controls

Test group	homozyg.	heterozyg.	% homozyg.	odds ratio	
non-neoplastic	7	21	25	1.0	(reference)
renal cell CA	5	16	24	0.9 (0.2–4.2)	N.S.
glioblastoma	4	20	17	0.6 (0.1–3.1)	N.S.
Wilms' tumour	9	3	75	9.0 (2.6–31.7)	(χ^2=8.5, p<0.01)

The WT group consisted of all patients who retained heterozygosity for at least one 11p15.5 marker and whose tumors were negative or trace-positive for *H19* RNA expression. The anomalous frequency of homozygosity in this group was confirmed by a chi-square test for homogeneity across the four groups (χ^2=14.3, p<.005). The *H19* polymorphisms tended to occur as a consensus haplotype, but there were frequent recombinants (WTs 5, 13, 17, and 25, Table 1). The excess homozygosity in the WT group did not reflect an allelic preference, since "a" and "b" alleles were equally represented (13 chromosomes contained *Rsa*I sites and 11 chromosomes lacked *Rsa*I sites). *H19* allele frequencies did not differ significantly among the four test groups (data not shown). N.S., not significant.

*Cfo*I and *Hpa*II digestion (Fig. 3*a–c*, data not shown). As we had not encountered this pattern in any normal fetal or adult kidneys in which six fetuses and four adults were examined[15], we wondered whether this epigenetic modification might be specific to WT patients. None of six additional control neonatal or juvenile kidneys, from individuals without WTs, showed this abnormality (Fig. 3*b,c*). To test further the specificity of this abnormality, we examined the normal parenchyma of 15 kidneys from adults with a distinct type of neoplasm, renal cell carcinoma, and all of these showed conversion of one *H19* allele to the *Cfo*I limit-digest products (data not shown).

We next assessed *H19* RNA levels in the non-neoplastic kidney tissue of these two patients. Since human *H19* remains transcriptionally active at a reduced level in juvenile kidneys, it was possible to carry out this analysis by northern blotting. When compared to an age-matched control kidney, both WT kidneys showed a reduction in H19 RNA, 4-fold in the kidney associated with WT3 and 10-fold in the kidney associated with WT21 (Fig. 4*a*). The *H19* RNA levels in these two kidneys were also reduced to a similar extent relative to that in the non-neoplastic parenchyma of a kidney from a WT patient (WT2) whose tumour had lost the active *H19* allele by gross 11p15.5 mitotic recombination (data not shown). Significantly, in spite of nearly complete CpG methylation at *Cfo*I and *Hpa*II sites in the body of the gene and its promoter, *H19* expression was not completely lost in the two kidneys which showed the biallelic hypermethylation. Prolonged exposures of the Southern blots hybridized with the first exon probe revealed faint limit-digest bands, suggesting that this residual expression derived from a minor subpopulation of kidney cells (less than 20% by densitometry) in which the gene had not been modified.

To determine whether the hypermethylation was localized or regional, we next hybridized Southern blots containing WT and non-neoplastic kidney DNAs with a series of probes derived from a cosmid and phage contig spanning roughly 100 kb around *H19* (Fig. 5*a*). In contrast to the results obtained with probes for *H19* gene, band patterns obtained with each of four flanking probes gave no indication of abnormal CpG methylation in the non-neoplastic kidney tissue of the patients with WTs 3 and 21 (Fig. 5*a–d*). Thus, within the limits of this intermediate-range mapping, the region of abnormal methylation was confined to *H19*.

The finding that the majority of cells in the non-neoplastic kidney parenchyma contained hypermethylated *H19* DNA suggested that these WT patients had undergone a specific epigenetic alteration at the *H19* locus early in somatic development, resulting in a predisposition to tumorigenesis. However, this was not sufficient to cause bilateral or multifocal tumours. Perhaps this should not be surprising in view of the lower frequency of 11p LOH in bilateral compared to unilateral cases of WT[25]. In terms of the possibility of a "field effect" which might give rise to multiple WT precursor lesions, one of the WT kidneys in which the methylation abnormality was found (case WT3) contained nephrogenic rests, but these were not widespread and constituted less than 5% of the total cortical tissue, and in the second WT kidney with abnormal *H19* methylation (case WT21) only two small and regressed nephrogenic rests were observed in multiple sections. Also of significance in view of the implication of constitutional abnormalities of the 11p15.5 region and deregulation of *IGF2* in BWS[26], neither of the patients showed evidence of organomegaly or other BWS stigmata. Chao *et al.* have reported partial 11p15.5 LOH in non-neoplastic kidney parenchyma of a small subset of WT patients[27] and case WT25 in our series showed this phenomenon. Our data showing the presence of abnormal methylation and partial functional inactivation of *H19* in non-neoplastic tissues in some cases of an additional category of WT patients, those which retain 11p15.5 heterozygosity, indicate that lesions at this locus in WTs are more common than would have been predicted solely from LOH.

Excess H19 homozygosity in WT patients

We were struck by the large number of cases which retained heterozygosity at TH/IGF2 and/or H-RAS but which were homozygous at each of the five RFLP markers within *H19* (Table 1). Of the 12 cases which retained 11p15.5 heterozygosity and which were negative or only trace-positive for *H19* RNA, nine were homozygous at these markers. Since this seemed anomalous based on our previous experience in patients with other types of tumours or non-neoplastic conditions, we tested the data for statistical significance. As indicated by the odds ratio, when compared to a reference group of individuals autopsied for non-neoplastic conditions, WT patients in this category were nine times more likely to be homozygous at the *H19* locus (Table 2). Two other control groups, patients with renal cell carcinomas and patients with malignant gliomas, were also examined and in these groups the odds of homozygosity did not differ significantly from that in the reference group (Table 2).

IGF2 mRNA in WT patients

When the northern blots of the WT and non-neoplastic kidney RNAs were probed for *IGF2* mRNA (Fig. 4*a,b*) several potentially relevant correlations were noted. First, as expected[21,22], most of the WTs expressed abundant *IGF2* transcripts. There was an imperfect tendency towards correlation of high *IGF2* expression with low *H19*

expression (Fig. 4*b*, compare WT11 with WTs 6, 8, 10 and 12 and WT24 with WT25), and there was also a suggestion of this trend in the non-neoplastic kidney tissues (compare the kidney of WT21 with the control kidney). The enhancer competition model for opposite imprinting of *H19* and *IGF2*[16] predicts that, in those tumours which retain 11p15.5 heterozygosity, inactivation of *H19* should result in relaxation of *IGF2* imprinting. Allelic analysis of *IGF2* reverse PCR products from WTs 3 and 14 confirmed this prediction, with biallelic *IGF2* expression in both cases (Fig. 4*c*).

Despite potential enhancer competition, abrogation of *H19* expression was not obligately coupled to increased *IGF2* expression. In three WTs which were examined as short-term explants and which showed no detectable *H19* expression, *IGF2* mRNA was undetectable by northern analysis (Fig. 4*b*, WTs 13, 14 and 23). Since each of these explants consisted entirely of proliferating neoplastic cells with epithelial morphology, and since the predominantly epithelial WT23 also lacked *IGF2* mRNA when examined directly as a tumour biopsy (Fig. 4*b*), we conclude that *IGF2* mRNA is not essential for the proliferation of the epithelial component of WTs *in vivo* and in cell culture.

Discussion

While previous functional, imprinting and mapping data have implicated *H19* as a candidate WT suppressor gene, a proof will require evidence from the molecular analysis of primary tumours. Because *H19* is imprinted, and can therefore be inactivated in a single genetic or epigenetic "hit", the form of this evidence may differ from that obtained for other tumour suppressor genes. In particular, if genetic inactivation through mitotic recombination or epigenetic inactivation through DNA methylation or other modifications are kinetically favored over mutation, as seems likely from evidence in other systems[28,29], then the criterion of intragenic mutation which has been useful for identifying classical tumour suppressor genes may not apply to imprinted ones. Mitotic recombination mapping[30] may be one approach to defining a minimal chromosomal region which must include the 11p15.5 tumour suppressor gene(s). However the absence of cases with sub-regional LOH at 11p15.5 in published series, as well as in this study, suggests that this may require analysis of a large number of cases to find rare tumours with highly localized LOH, if such cases exist.

In this connection, we are intrigued by the significant excess of cases with *H19* homozygosity in the group of patients with non-expressing WTs which retained 11p15.5 heterozygosity at other markers. Some of these cases might represent examples of mitotic recombination occurring early in development (prior to divergence of kidney and hematopoietic cell lineages) and restricted to a local region between *TH/IGF2* and *HRAS* and including *H19*. Alternatively, homozygosity across the highly polymorphic *H19* gene might predispose to a transfer of the paternal imprint to the maternal allele through a "trans-sensing" mechanism[31,32].

The findings of low or absent *H19* expression in most WTs and hypermethylation of this gene in many WTs and in the non-neoplastic kidney tissue of some WT patients are highly suggestive of a tumour suppressor role for *H19* in the pathogenesis of WT, particularly when taken in the context of previous genetic and functional studies. The results also suggest a novel approach to testing the H19 hypothesis: the finding of a specific epigenetic modification at the *H19* locus in WT patients suggests that mapping the centromeric and telomeric limits of the region of abnormal methylation, particularly in the non-neoplastic kidney DNA, where global methylation abnormalities are less likely to exist than in the tumour tissue[33], may identify a minimal modified region in which one or more 11p15.5 WT suppressor gene(s) must lie. Our local mapping data already suggest that this region may in fact be restricted to *H19*, but further analysis might reveal other affected genes.

In terms of the possible involvement of *IGF2* as a dominant oncogene in WTs, while the findings of high expression of *IGF2* mRNA and relaxation of *IGF2* imprinting are certainly consistent with this[14,20], the lack of *IGF2* mRNA in proliferating epithelial cells of WTs which had lost *H19* RNA expression suggests that loss of *H19* expression could function in Wilms' tumorigenesis independently of any persistence of *IGF2* expression. In evaluating the relative contributions of these two genes in the formation of WTs, it also seems significant to us that, while high *IGF2* mRNA expression is characteristic of both WTs and normal renal blastema, low *H19* expression is characteristic only of WTs, including some cases with a blastemal predominance. A further potential difficulty with the *IGF2* hypothesis is the finding in two studies of low or undetectable immunoreactive Igf-2 peptide in WTs[34,35]. Clearly, however, *IGF2* overexpression could play a role at some stage in the growth of WTs. Perhaps it functions early in tumorigenesis to increase the number of potential WT precursor cells, as may occur in the kidney overgrowth associated with BWS[26].

Our expression and methylation data add to the evidence for an important biological role for the enigmatic H*19* gene. An intriguing challenge will be to define the interactions of *H19* RNA with other cellular components. Whatever its mode of action may be, the findings of elimination of *H19* RNA in most WTs and reduction but not elimination of this RNA in the non-neoplastic kidney parenchyma of some WT patients suggest that dosage may be important for its biological activity. While this is speculative, in view of the abundance of *H19* RNA in expressing tissues, findings of a growth-regulatory and tumour suppressor function for the 3' untranslated regions of certain abundant muscle-specific transcripts[36] may indicate some common mechanism in the two systems.

Methodology

Patients, tumours and tumour explants. Among the patients, only cases 24 and 25 had a known family history of WT. The only patient with bilateral WTs was case WT5. Tumours were obtained at surgery and were evaluated histologically by standard methods. Relative amounts of blastemal, stromal and epithelial components were estimated by low power scanning of slides from multiple paraffin blocks. In none of these cases did the proportions of these components vary appreciably in different areas of the tumour. Each of the four explanted tumours showed a purely epithelial "cobblestone" morphology and was harvested for DNA and RNA at the third or fourth passage. Control kidneys were obtained at autopsy. In the WT cases the non-neoplastic kidney samples were obtained at more than 1 cm from the tumours.

Allelic analysis. *TH* TCAT-repeat, PCR primers as reported by Polymeropoulos *et al.*[37]. *IGF2 Apa*I, PCR primers as reported by Tadokoro *et al.*[38] and reverse PCR analysis using nested primers as reported by Ogawa *et al.*[20] and Rainier *et al.*[14]. *IGF2* CA-repeat, PCR primers as described[15]. *H19 Cfo*I and *Aat*II, PCR primers as described for amplification of the first exon[15]. *H19 Rsa*I, *Avi*II and *Alu*I, PCR

primers as previously described for nested PCR of exons 3–5 (ref. 12). The *RsaI* polymorphism was evaluated both by PCR and by Southern analysis of genomic DNA; in each case the two methods gave concordant results. HRAS CGG-repeat, PCR primers as reported by Riggins *et al.*[39]. *H19* B2, a previously undescribed four-allele system detected by Southern analysis of genomic DNA using *RsaI* digestion and hybridization with a 2 kb DNA probe centered 6 kb downstream from the 3' end of the last exon. *HRAS* EJ6.6, *TaqI*/*MspI* polymorphism detected by Southern analysis as described[40].

Northern and Southern blotting. Total RNA, from 2–10 μg, was separated on 1% agarose gels containing formaldehyde and transfered to nylon membranes. The *H19* probes were generated by PCR of the first or third through fifth exons as described[12,15]. The *IGF2* cDNA probe contained the complete coding sequence[41]. The *actin* probe was generated by cDNA PCR using β-actin primers (Clontech). Densitometry was done on lightly exposed autoradiograms using a Macintosh flatbed scanner (ScannerOne) and Image 1.43 software (NIH). Southern blotting and methylation analysis was done as described[15].

Cosmid and phage clones. Cosmid clones were isolated by sequential screenings of a chromosome 11-specific library, provided by L. Selleri and G. Evans. Phage clones were from a human placental genomic library (λFIX, Stratagene).

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