tions and deletions) and point mutations because they did not examine the molecular changes causing the disease in their haemophilia patients. In contrast, our study as well as that of Miyata *et al.*[4] includes only point mutations. As the mechanism causing point mutation seems to be different from that causing indels, the two types of mutation should be treated separately.

The data obtained in this and previous studies support the view that the mutation rate is much higher in males than in females. However, the male to female mutation rate ratio, α_m, seems to be much lower than Miyata *et al.*'s estimate[4]. This is of interest because whether α_m is very large or relatively small has important implications. A very large α_m implies that errors in DNA replication during germ-cell division is the primary source of mutation and that replication-independent mutagenic factors such as methylation and oxygen radicals are not important. In this case, the effect of generation time on the rate of nucleotide substitution can be strong because the number of germ-cell divisions per unit time is larger in short-living organisms than in long-living ones. On the other hand, if α_m is relatively small, say of the order of only 10, then the contribution of replication-independent mutagenic factors may not be negligible. In this case, the generation-time effect[11] may be relatively weak. The exact magnitude of α_m is therefore a very important issue. □

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Relaxation of imprinted genes in human cancer

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GENOMIC imprinting, or parental allele-specific expression of genes, has been demonstrated at the molecular level in insects and mice[1,2] but not in man. Imprinting as a potential mechanism of human disease is suggested by paternal uniparental disomy of 11p15 in Beckwith–Wiedemann syndrome[3] and by maternal uniparental disomy of 15q11-12 in Prader–Willi syndrome[4]. Beckwith–Wiedemann syndrome is characterized by multiorgan overgrowth and predisposition to embryonal tumours such as Wilms' tumour of the kidney[5]. A loss of heterozygosity of 11p15 is also frequently found in a wide variety of tumours, including Wilms' tumour and lung, bladder, ovarian, liver and breast cancers[6–11]; 11p15 also directly suppresses tumour growth *in vitro*[12]. Two genes in this band, *H19* and insulin-like growth factor-II (*IGF2*) undergo reciprocal imprinting in the mouse, with maternal expression of H19 (ref. 13) and paternal expression of IGF2 (ref. 14). Here we find that both of these genes show monoallelic expression in human tissues and, as in mouse, H19 is expressed from the maternal allele and IGF2 from the paternal allele. In contrast, 69% of Wilms' tumours not undergoing loss of heterozygosity at 11p showed biallelic expression of one or both genes, suggesting that relaxation or loss of imprinting could represent a new epigenetic mutational mechanism in carcinogenesis.

We have focused on kidney and Wilms' tumours (WT), because both *H19* and *IGF2* are expressed in developing kidney and because WT occurs 700-fold more frequently in Beckwith–Wiedemann syndrome (BWS) than in the general population[5,15]. DNA from 11 normal kidneys, as well as 17 WTs from these and other patients, were polymorphic for one or more markers. In addition, one rhabdoid tumour and six fetal specimens from various tissues were informative.

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TABLE 1 *H19* and *IGF2* alleles expressed in 34 normal and malignant tissues

Tissue	Patient	Stage	*H19*/*Rsa*I DNA	*H19*/*Rsa*I RNA	*IGF2*/*Apa*I DNA	*IGF2*/*Apa*I RNA	*IGF2*/DR DNA	*IGF2*/DR RNA
Normal kidney	1		b/b				b/c	b/–
Normal kidney	2		b/b		a/b	a_{pat}/–	a/c	–/c_{pat}
Wilms' tumour	2	I			a/b	a/b	a/c	a/c
Normal kidney	3		a/b	–/b_{mat}	a/b	a/–		
Wilms' tumour	3	II	a/b	–/b_{mat}	a/b	a/b		
Normal kidney	4		a/b	a_{mat}/–	b/b			
Wilms' tumour	4	IV	a/b	a_{mat}/–				
Normal kidney	5		a/b	–/b	b/b		b/b	
Wilms' tumour	5	III	a/b	–/b				
Normal kidney	6		a/b	a/–	a/b	–/b_{pat}		
Wilms' tumour	6	III			a/b	a/b		
Normal kidney	7							
Wilms' tumour	7	III			a/b	a/b		
Normal kidney	8		a/b	a/–	a/b			
Wilms' tumour	8	I	a/b	a/–	a/b	a/b		
Normal kidney	9		a/b	a_{mat}/–	a/b	–/b		
Wilms' tumour	9	II			a/b	a/b		
Normal kidney	10		b/b		b/b		b/c	
Wilms' tumour	10	I					b/c	b/c
Normal kidney	11		a/b	–/b				
Wilms' tumour	11	IV	a/b	a/b				
Wilms' tumour	12	II/III	b/b				a/c	a/–
Wilms' tumour	13	II	a/b	a_{mat}/–			b/c	b_{pat}/–
Wilms' tumour	14	I	a/a		a/b	–/b	b/b	
Wilms' tumour	15	I	a/b	a/b	a/b	a/b	a/c	a/c
Wilms' tumour	16	II			b/b		a/b	a/b
Wilms' tumour	17	I			a/b	a/b		
Rhabdoid tumour	18		b/b		a/b	a/b	b/c	
Fetal liver	19		b/b		a/b	–/b	b/c	–/c
Fetal brain	19		b/b		a/b		b/c	–/c
Fetal kidney	19		b/b		a/b	–/b		
Fetal kidney	20		a/b	a/–				
Fetal heart	20		a/b	a/–				
Fetal lung	20		a/b	a/–				

Seventeen normal tissues and 17 cancers polymorphic for *H19*, *IGF2*, or both, and not showing loss of DNA heterozygosity, were examined for monoallelic or biallelic expression, as described in the legend to Fig. 1. Notation: a/b, both alleles present; a/–, monoallelic expression of *a* allele; a_{mat}/–, monoallelic expression of maternal *a* allele; –/b_{pat}, monoallelic expression of paternal *b* allele.

Monoallelic expression of H19 in fetal tissues has been described[16], although the parent of origin was unknown. Consistent with that study, H19 showed monoallelic expression in 15 informative tissues, including seven normal kidneys that had been resected from informative children, five WTs not showing loss of heterozygosity (LOH), and three fetal tissues (Table 1). To determine the parental allele of origin, blood specimens from

one or both parents were also examined in 12 of these cases. In all six cases in which parental origin could be determined, the expressed allele was maternal (Fig. 2*a*; Table 1). IGF2 also showed monoallelic expression in ten informative tissues (four normal kidneys, three WTs, three fetal tissues; Table 1). The allele of origin could be ascertained in three cases and was paternal in all (Fig. 2*b*; Table 1). Patient 13 directly demonstrated reciprocal imprinting of *H19* and *IGF2* in the same tissues (Fig. 2*a*, *b*). Thus, *H19* and *IGF2* displayed imprinting with inactivation of paternal and maternal alleles, respectively.

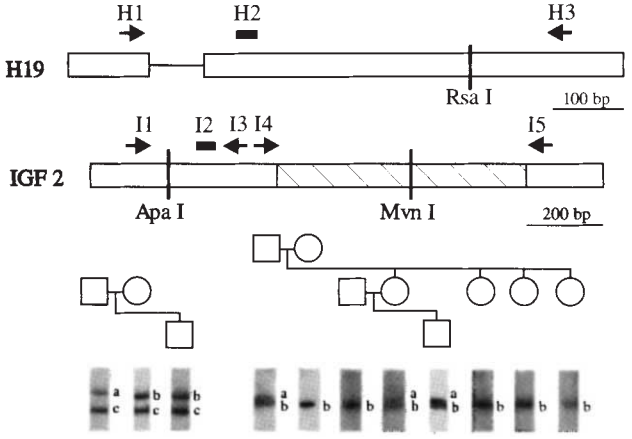

FIG. 1 Analysis of transcribed *H19* and *IGF2* polymorphisms. Maps of *H19* and *IGF2*, and three pedigrees exhibiting mendelian segregation of a polymorphic *IGF2* dinucleotide repeat (DR). Exons are denoted by open rectangles, restriction endonuclease cleavage sites by vertical lines, the DR by a hatched rectangle, probes by horizontal bars, and polymerase chain reaction (PCR) primers by arrows.

METHODS. To assess allele-specific expression of H19, we took advantage of a transcribed *Rsa*I polymorphism by reverse-transcribing RNA and amplifying cDNA by PCR as described[16], but using new primers that permit a single round rather than nested PCR. *IGF2* was analysed using two transcribed polymorphisms, a previously reported *Apa*I variant[36], and a newly described dinucleotide repeat in the 3′-untranslated portion of the gene. Poly(A)$^+$ RNA was isolated (FastTrack, Invitrogen) and reverse-transcribed (AMV reverse transcriptase; Seikagaku) using primer H3 (5′-TGGAATGCTTGAAGGCTGCT-3′) for the *H19*/*Rsa*I polymorphism, primer I3 (5′-CCTCCTTTGGTCTTACTGGG-3′) for the *IGF2*/*Apa*I polymorphism, or primer I5 (5′-AGAATCTTAGCGGGACTTTGGCCT-3′) for the *IGF2*/DR polymorphism. *H19* cDNA was amplified by PCR[37] using primers H1 (5′-TACAACCACTGCACTACCTG-3′) and H3 in 10 mM Tris, pH 8.3, 25 mM KCl, 1.5 mM $MgCl_2$, 5 mM NH_4Cl, using a 3-step cycle (94 °C for 30 s, 70 °C for 30 s, and 72 °C for 1 min) for 35 cycles, with a final 10-min incubation at 72 °C. PCR products were ethanol-precipitated, digested with *Rsa*I, electrophoresed on a 10% polyacrylamide–15% glycerol gel, and ethidium-bromide stained or electroblotted and hybridized[38] with end-labelled probe H2 (5′-CGCTGCTGTTCCGATGGTGT-3′). To detect the *IGF2*/*Apa*I polymorphism, cDNA was amplified with primers I1 (5′-CTTGGACTTTGAGTCAAATTGG-3′) and I3 as described[36]. PCR products were ethanol-precipitated, digested with *Apa*I, electrophoresed on a 10% polyacrylamide–15% glycerol gel, and then stained with ethidium bromide or electroblotted and hybridized with end-labelled probe I2 (5′-CCTCTGACTGCTCTGTGATT-3′). To detect the *IGF2*/DR polymorphism, cDNA was amplified with primers I4 (5′-ACTTTATGCATCCCCGCAGCTACA-3′) and I5. PCR was done in the same buffer as that described for the *H19*/*Rsa*I polymorphism, but using a two-step cycle (94 °C for 30 s, 72 °C for 1 min) for 35 cycles, with a final 10-min incubation at 72 °C. As the DR is relatively large, the polymorphism was detectable by cleaving the PCR product at a central *Mvn*I site. Each half of the DR was visualized separately by end-labelling either primer I4 or I5 before PCR, although the left-sided variation occurred more frequently and was used in all the experiments described here. Digests were then electrophoresed on an 8% polyacrylamide–urea gel. *H19* DNA and RNA products were easily distinguished because of the presence of an intervening intron. Duplicate poly(A)$^+$ RNA samples were incubated in the presence (+RT) and absence (−RT) of reverse transcriptase, and only those samples showing no amplification in the absence of reverse transcriptase were studied. As an additional control, reverse transcription was done with a tailed primer[39] to ensure amplification of RNA only; there was no amplification of DNA in control experiments.

To assess imprinting in cancers, 42 WTs were screened for those not exhibiting LOH of 11p15. Surprisingly, of 16 informative tumours retaining heterozygosity, 11 (69%) expressed both maternal and paternal alleles of *H19*, *IGF2*, or both (Table 1; Fig. 2*c*). The frequency of biallelic expression was greater for *IGF2* (77%) than for *H19* (29%), although this difference was not statistically significant ($P = 0.052$). Biallelic expression of *H19* and *IGF2* was concordant in one case (patient 15; Table 1) but was discordant in two (patients 3 and 8; Table 1). Thus, relaxation of an imprint seems to be necessary but not sufficient

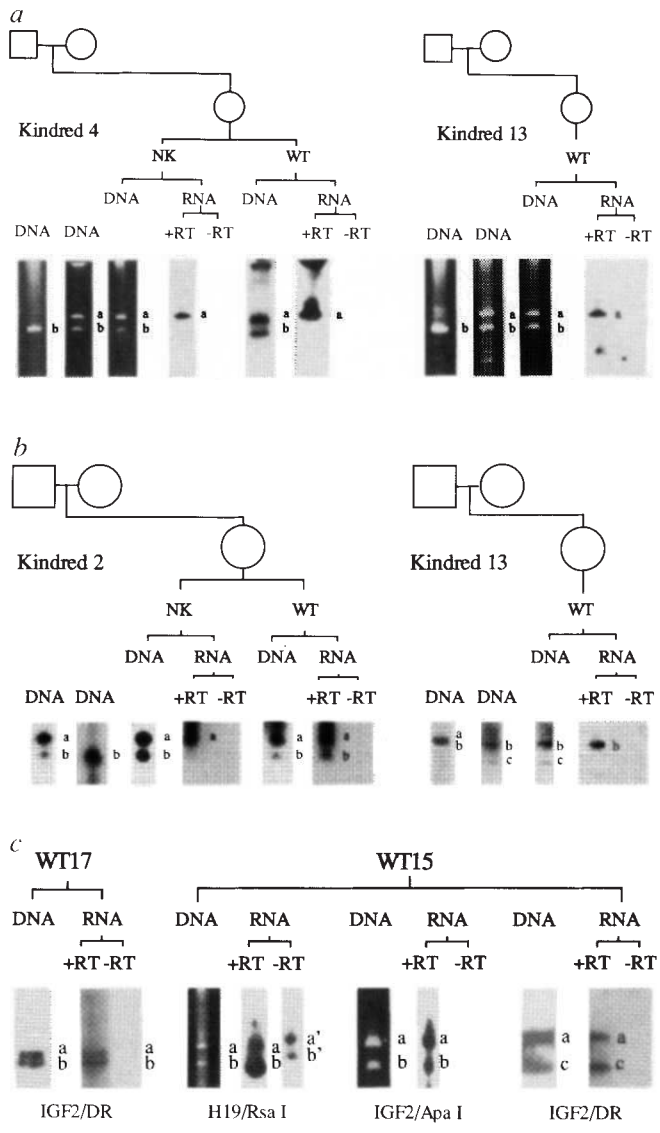

FIG. 2 Imprinting of *H19* and *IGF2* genes. *a*, Maternal monoallelic expression of *H19* in normal kidney (NK) and Wilms' tumour (WT). Kindreds 4 and 13 were analysed using the *Rsa*I polymorphism (alleles a and b) as described in the legend to Fig. 1. Both NK and WT of patient 4 and WT of patient 13 show monoallelic expression of the maternal allele. *b*, Paternal monoallelic expression of IGF2 in normal kidney. Kindred 2 was analysed using the *Apa*I polymorphism, and kindred 13 using the dinucleotide repeat (DR) polymorphism, as described in the legend to Fig. 1. *c*, Biallelic expression of *H19* and *IGF2* in Wilms' tumours. WT17 was analysed using the *IGF2*/DR polymorphism and shows biallelic expression. WT15 was informative for all three polymorphisms. Both *H19* and *IGF2* show biallelic expression, as does the WT from patient 2 (see *b*). This is a qualitative assay for the presence of one or two alleles, and quantitative inferences should not be drawn because of, for example, the presence of heterodimers in PCR products. A single DNA-contaminated RNA sample from patient 15 was deliberately included to illustrate the larger-sized fragments (a′, b′) resulting from amplification of genomic sequences.

for biallelic expression. These changes were not limited to WT, as a rhabdoid tumour also expressed both *IGF2* alleles.

These experiments lead to two principal conclusions. First, some human genes, like those of other species, undergo allele-specific imprinting. In particular, *IGF2* and *H19* are reciprocally imprinted, with expression of the maternal *H19* and paternal *IGF2* alleles. These data thus lend support to an imprinting model for two other probably distinct but similarly paired genes on 15q11-12 that cause Prader-Willi[4] and Angelman[17] syndromes, which also involve abnormal (but in those cases decreased) growth. Indeed, genomic imprinting may have evolved because of differing growth demands of maternally and paternally derived gametes[18]. Interestingly, bands 11p15 and 15q11-12 map to adjacent regions of chromosome 7 in the mouse[19], which also undergoes imprinting, with overgrowth of paternally disomic chimaeric mice[20]. Second, the majority of informative non-LOH WTs showed biallelic expression of *IGF2*, *H19*, or both. That these changes play a causal role in malignancy is suggested by (1) their high frequency, which is 2-5-fold greater than that of LOH[21] and WT1 mutations[22] in WT; (2) their early onset, as half were of the earliest stage (Table 1); and (3) both genes are included within the paternally disomic region in BWS[3], and uniparental disomy is associated with a >50% incidence of cancer[23].

We therefore propose that loss of imprinting (LOI) in neoplasia represents the functional equivalent of uniparental disomy in BWS. As transgenic mice lacking a functional paternal *IGF2* gene undergo growth retardation[14], *IGF2* has been proposed as a candidate gene for BWS[24-26]. Overexpression of IGF2 seen in most WTs[27] could be caused by this alteration. It is important to note, however, that *H19* also showed LOI. *H19* is abundantly transcribed in a wide variety of murine tissues but is of cryptic function and may not encode a protein[28]. *IGF2* and *H19* map to a region of less than 90 kilobases (kb), and thus a regulatory domain lying between them may act as an allele-specific switch controlling the exclusive expression of *IGF2* or *H19* (ref. 29). Thus, biallelic expression of *H19* and *IGF2* in WTs could be caused by a disturbance in this switch, permitting but not requiring expression of both alleles.

As demonstrated in colorectal cancer, multiple genes are involved in multistep neoplastic progression[30]. We do not believe that the relaxed imprinting seen in these experiments involves the tumour suppressor locus detected by 11p15 LOH[6-11] because we have mapped this activity to a region distinct from the *H19/IGF2* region[12], and cytogenetic data also indicate that more than one region is involved[25]. A candidate mechanism for this alteration is DNA hypomethylation, which has been reported in a wide variety of premalignant and malignant tumours[31-33], but for which no gene has yet been shown to be specifically activated in cancer. Furthermore, 1% of normal children under one year old harbour WT precursor lesions that spontaneously regress[34,35]. It is difficult to imagine how a process of such high frequency could occur by conventional mutational mechanisms. Regardless of the precise role that this process plays in neoplasia, these data provide evidence for a novel epigenetic mutational mechanism in cancer, namely relaxation of genomic imprinting. □

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Relaxation of insulin-like growth factor II gene imprinting implicated in Wilms' tumour

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GENOMIC imprinting has been implicated in the onset of several embryonal tumours but the mechanism is not well understood[1-3]. Maternal chromosome 11p15 loss of heterozygosity[4] and paternal chromosome 11 isodisomy[5,6] suggest that imprinted genes are involved in the onset of Wilms' tumour and the Beckwith–Wiedemann syndrome. The insulin-like growth factor II (*IGF2*) gene located at 11p15.5 has been put forward as a candidate gene as it is maternally imprinted (paternally expressed) in the mouse[7], and is expressed at high levels in Wilms' tumours[8,9]. We report here that the *IGF2* gene is expressed from the paternal allele in human fetal tissue, but that in Wilms' tumour expression can occur biallelically. These results provide, to our knowledge, the first evidence that relaxation of imprinting may play a role in the onset of disease and suggest a new genetic mechanism involved in the development of cancer.

The allele specificity of *IGF2* expression in human fetal kidneys was determined using an *Apa*I/restriction enzyme fragment length polymorphism (RFLP)[10] in the 3′ untranslated portion of the *IGF2* gene (Fig. 1). DNA and RNA from six fetal kidneys (12-week gestation) were amplified using polymerase chain reaction (PCR) and the products digested with *Hin*fI/*Apa*I. In all fetal kidneys *IGF2* RNA was monoallelically expressed; two representative cases are shown in Fig. 1. In three additional cases, fetal tissue and maternal blood were used to determine the parental origin of *IGF2* expression, and Fig. 2

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