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EDITORIAL REVIEW

Genetics of Wilms' tumor: A blend of aberrant development and genomic imprinting

Wilms' tumor or nephroblastoma (WT), one of the most common childhood solid tumors (1:10,000, 8% of childhood tumors), is probably the one that best deserves the designation of embryonal tumor. Wilms' tumors are composed of three major elements in variable proportions: compact areas of blastemal cells, tubular structures of various sizes and fibrous or mixoid stroma containing elongated stellate-shaped large round cells. These components are reminiscent of normal human nephrogenesis, known to be initiated by the ingrowth of the ureteral bud into the metanephrogenic mesenchyma which then condenses and forms the different portions of the nephron. These tumors thus show a remarkable mimicry of the normal nephrogenic processes, although in an extravagant mode leading to incompletely differentiated structures with as many dead ends as a labyrinth [1]. These structures are accompanied, in a minority of cases, by heterologous ectopic tissues of mesodermal origin including bone, cartilage and skeletal muscle [2].

The first insights into the genetics of Wilms' tumor came with proposals for another embryonal tumor, retinoblastoma. In the early 70s, Knudson formulated his now famous two-hits hypothesis based on observations of unilateral and bilateral cases of this tumor [3]. The nature of events underlying sporadic versus hereditary cases was thus postulated. In both instances two events were required, the first one being present as a germline defect on one allele in hereditary cases while occurring as a somatic event in sporadic cases. The second event, assumed to hit the second allele in a somatic cell was required for the tumor to develop, in hereditary as well as in sporadic cases. This was indeed demonstrated later on by Cavenee and coworkers, who showed that somatic chromosomal events leading to the loss of constitutional heterozygosity (loss of alleles or LOH) in somatic cells were responsible for the loss of the second allele [4]. The loss of both functional alleles was amply confirmed at the molecular level with the cloning of the RB gene involved in retinoblastoma and further insights into its mode of action. This shed light on a major clue to the understanding of tumorigenesis: whatever rare hereditary cases can be, the search for genes for hereditary predisposition would obviously be helpful for uncovering genetic events also involved in sporadic cases, which are by far the most frequent.

In spite of strong beliefs that a similar story should be able to account for nephroblastoma, the genetics of Wilms' tumor, with hereditary cases representing only a few percent, appears to be far more complicated. Indeed, cases with a constitutional deletion of the chromosomal band 11p13 [5] together with somatic losses of the 11p region in some tumors [6–9] had led to the assumption that a tumor suppressor gene in 11p13, similar in many ways to the RB gene, had to be the culprit. This was indeed confirmed by the cloning of the first Wilms' tumor gene (WT1) in 11p13 [10, 11]. But, in contrast to retinoblastoma, there was also accumulating evidence for the existence of at least two other genes, one in 11p15 (WT2) and the other (WT3) as yet unmapped. Moreover, hereditary and somatic events occurred in an unusual, non-mendelian manner, which strongly suggested that one (or more) of the three genes could undergo genomic imprinting. An ever growing number of studies have now addressed the nature of these events, the number of different loci involved in predisposition and tumor progression, the recessive or dominant mode of action of mutations in identified genes, their role in embryonic and adult development, the nature of epigenetic events underlying genomic imprinting, and the role they play both in urogenital development and in tumorigenesis. The aim of this review is thus to focus on the main streams of research, with particular reference to genetic events both in genetic susceptibility to tumor and tumor development as well as to the emerging role of epigenetic phenomena such as imprinting, chromatin compaction, timing of replication and some more speculative trans-sensing effects. Thus the genetics of WT provide strong arguments against well-established dogma, including the two-hits hypothesis of Knudson, the recessive nature of mutations of tumor suppressor genes, and the expected presence of a tumor suppressor (antioncogene) rather than a growth factor (oncogene) at the sites revealed by losses of alleles [12–15].

WT is seen most commonly as a sporadic event in an otherwise healthy child, though it may occur in association with congenital abnormalities. Overall, about 5% of children with WT present with developmental anomalies of the genitalia including cryptorchidism, hypospadias and other genital anomalies as well as anomalies of the urinary tract, such as duplex ureter and horseshoe kidney. This is reminiscent of the close association during embryogenesis between the development of the reproductive and urinary system, both arising from the mesonephros. Although familial cases are rare and represent only 1 to 2% of the cases, the frequency of bilateral cases (7%) suggests that some of them, as well as an unknown proportion of unilateral cases, represent either *de novo* germline mutations, or somatic events occurring early during embryogenesis. A few percent of cases are associated with WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities, mental retardation), Denys-Drash syndrome (DDS), partial or complete manifestations of the Wiedemann-Beckwith syndrome (WBS) whether or not associated with hemihypertrophy. A few other genetic defects including Perlman syndrome and hemihypertrophy carry an increased risk of developing WT. Thus the locus and the type of gene alteration involved

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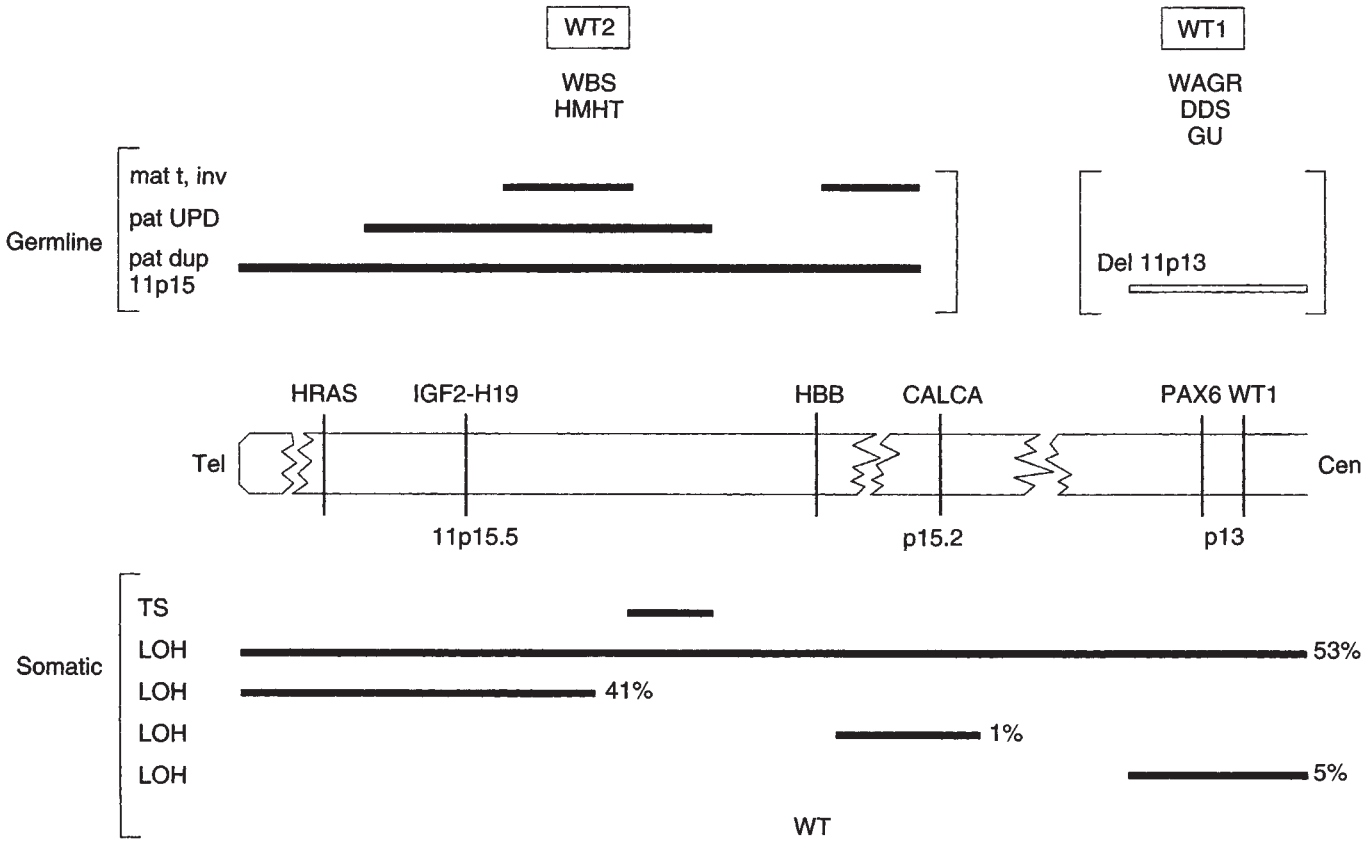

Fig. 1. *Diagrammatic representation of the short arm of chromosome 11.* **Top.** Germline alterations leading to WAGR, DDS, or GU (genitourinary abnormalities) at the WT1 locus in 11p13 or to WBS, hemihypertrophy (HMHT) at the WT2 locus in 11p15.5. **Bottom.** Loss of heterozygosity studies in tumors delineating three different regions involved in WT. Using all available markers, LOH can be detected in 57% of informative samples. Of these, 53% of cases show a loss of alleles encompassing both WT1 and WT2, whereas LOH is limited to WT1 or WT2 in 41% and 5% of cases, respectively. The preferential loss of maternal allele was observed in tumors with LOH either limited to WT2 or encompassing WT1 and WT2. The number of tumors with LOH limited to WT1 is too small to be informative in that respect. TS = Tumor suppressor sequence. Another region located between WT1 and WT2 is lost in 1% of the tumors analyzed. If confirmed, this subregion would correspond to the second cluster of breakpoints in WBS. IGF2 and H19 are located 400 kb distal to the first cluster of breakpoints "mat t, inv".

may be different but the genetic bases for some of these predispositions are still unclear [16–19].

For the sake of clarity we shall first consider the different possible germline anomalies affecting three different loci, WT1 in 11p13, WT2 in 11p15 and WT3 unmapped. Subsequently, we shall examine the different somatic alterations that can be observed as the second event following either one of the hereditary events. The search for the third gene WT3 or any other gene may obviously benefit from both approaches.

The WT1 locus at 11p13

Characterization of the WT1 gene

The 11p13 region associated with WAGR and Denys-Drash syndromes, has been intensively studied by several groups who have provided numerous markers precisely mapped in the intervals of breakpoints of deletions of more than fifty patients with complete or partial WAGR syndrome and have established the first long range map of 16 megabases [20]. This region harbors several genes including the WT1 gene, involved in kidney and genitourinary development and in Wilms' tumor, and the PAX6 gene, involved in aniridia (Fig. 1). The WT1 gene, a 50 kb/10 exons-long gene, was cloned by positional cloning [10, 11]. Evidence for the WT1 gene as the Wilms' tumor gene was provided by analysis of patients with germline intragenic deletions and point mutations [21–28]. Two alternative splice sites give rise to four different mRNA [29] (Fig. 2). The most abundant form contains both the 17 amino acid insertion and a three amino acid insertion (KTS=lys-thr-ser) in exons 5 and 9, respectively. This gene encodes a 3.2 kb mRNA transcribed into four isoforms (49 to 54 kDa) with four zinc fingers, in the C terminal region, similar to those observed in some transcription factors, including SP1 and the EGR1 and EGR2 genes (early growth response 1 and 2) which appear to be involved in cellular growth signals. Using antibodies raised against the entire zinc finger domain, the protein product was localized within the nucleus [30]. The highest level of mRNA expression is in the epithelial podocyte layer of the glomerulus but WT1 is also expressed in cells of hematopoietic origin and in other mesodermally derived tissues which experience a mesenchymal to

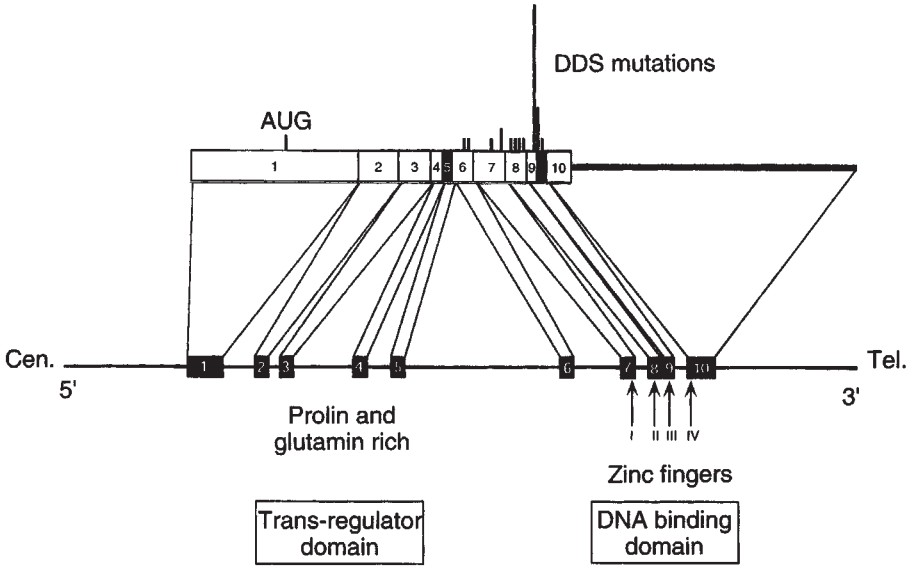

Fig. 2. *Diagrammatic representation of the WT1 gene with DDS mutations.* Germline mutations observed in DDS patients are represented by vertical bars. A hot spot with 69% of mutations in exon 9 is shown. The two alternative splice sites in exon 5 and exon 9 are shown in black boxes.

epithelial transition. These include the gonads, spleen, brain, and mesothelium [31–35]. The introduction of a homozygous mutation into the murine WT1 by gene targeting in embryonic stem cells demonstrated the involvement of WT1 in early kidney, gonad, and heart development [36].

The WT1 gene product is a regulator of transcription and binds *in vitro* to specific G-rich DNA sequences including the EGR1 consensus sequence: 5′-GCGGGGGCG-3′ [37, 38]. It has been shown that the three zinc fingers from Zif268, homologous to the three C terminal zinc fingers of WT1, are required for binding to the DNA consensus sequence [39, 40]. Thus WT1 could be a repressor of growth stimulating factor(s). WT1 could also act as an activator of tumor suppressor genes. Moreover, the four alternatively spliced forms expressed in various tissues have different target specificity to synthetic oligonucleotides and most likely bind to different target genes in the genome [38, 41]. The significance of these different forms is still elusive. It may be that the ratio participates in the modulation of WT1 in the different tissues and at different stages of development. Although it has been shown that WT1 can act as a transcriptional repressor on two growth factors, IGF2 (insulin-like growth factor II) and PDGFA (platelet-derived growth factor A chain), it may be that either the different forms or their relative proportions or a different concentration of WT1 may have different effects [39, 42–44]. Transfection of each of four wild-type WT1 isoforms was shown to suppress the growth of a WT cell line devoid of exon 2 sequences. This mutation resulted in a protein with altered transactivational properties, thus representing a distinct mechanism for inactivating WT1 in WT [45]. Many of these properties of the WT1 gene are reminiscent of those of another transcription factor, Krüppel, which may explain some of the intriguing features of WT1 mutants [46].

The promoters that drive expression of the fetal transcripts of the IGF2 gene are GC-rich and contain several potential high-affinity binding sites for WT1. This raised the possibility that the IGF2 gene may be one of the targets of WT1 transcriptional repression. Using transient transfection assays in HepG2 cells, Drummond and coworkers [39] demonstrated that WT1 interacts directly with the IGF2 promoter. Moreover, the potent repressor effect of WT1 on IGF2 transcription is dependent on the presence of multiple high and low-affinity binding sites flanking the P3 site of the main fetal IGF2 transcriptional initiation. Functional loss of WT1 transcriptional repressor activity may result in continued synthesis of large amounts of IGF2 in kidney blastemal cells and unrestrained autocrine growth stimulation both in kidney blastemal cells and in tumor cells. WT1 may function in a similar fashion to p53 and Rb [47, 48]. Moreover, since interaction between these two proteins p53 and WT1 has recently been observed, they may act in a synergistic manner [49].

Germline mutations of the WT1 gene have dominant effects on kidney development

De novo constitutional overlapping deletions of the 11p13 region including WT1 are characteristic features of WAGR syndrome. *De novo* constitutional deletions or point mutations have been reported in WT patients with or without genitourinary abnormalities, but without aniridia [27, 50]. One case of father-to-son transmission has been described [25]. This patient with hypospadias and bilateral cryptorchidism which may make him unfertile, developed a WT at the age of 3 while his father had been successfully treated for WT. It is salient to point out that the father had no genital phenotype although he presented with Wilms' tumor which was cured. A third case of transmitted WT1 mutation has been reported [51]. In this report an unaffected father of a DDS mutation child himself carries the same mutation in two independently taken blood samples. Thus here we have a case of non-penetrance of the DDS phenotype and another case of WT1 mutation transmitted through the germline. Although the exclusion of 11p linkage in four WT pedigrees suggests that mutations at a different locus (WT3) can predispose to familial WT [52–54], the inheritance of the WT1 mutation together with the only one instance of transmission of a WAGR associated deletion from a mother with aniridia to her son are the first examples of the involvement of the 11p13 locus in familial WT [55]. Thus a point mutation or a deletion of WT1 can indeed be

genetically inherited. However, although familial transmission is rarely observed, these mutations most likely account for a minority of familial cases or patients with *de novo* germline mutations, probably about 10% [12]. The low frequency of familial WT1 cases may well be explained by reduced fertility in WT1 mutation carriers. Females may also be more subtly affected. 46 XX DDS patients do in some cases have gonadal anomalies as described by Pelletier et al [56, 57].

De novo constitutional mutations of WT1 have been found in approximately 30 patients with Denys-Drash syndrome (DDS) [42, 51, 56, 58, 59]. DDS patients who develop WT often have bilateral tumors and present earlier than sporadic WT patients. These children suffer from severe abnormalities of the podocyte layer of the glomerulus, a condition that leads to early renal failure. These WT1 mutations mainly consist in amino acid changes in either one of the four zinc finger domains of WT1, with a hot spot (18 of 28 cases, 64%) in exon 9 corresponding to zinc finger III, but also in two cases, in a chain termination mutation, leading to alteration or complete or partial loss of the DNA binding domain, but sparing the N terminal portion involved in gene regulation (Fig. 2). Most DDS mutations lead to the inability to produce one isoform of WT1, the so-called +KTS form, thus supporting the importance of differential splicing [42, 60].

The underlying differences between WAGR patients and DDS patients, with their more severe gonadal dysgenesis and the characteristic nephropathy, are still poorly understood. This cannot be totally accounted for by differences in the nature of the mutation, a deletion (WAGR) and a point mutation affecting the DNA binding domain (DDS), since there are now two examples reported in the literature of DDS patients with 11p13 deletion and aniridia [22, 61]. That the majority of individuals with WAGR syndrome do not develop nephropathy suggests that the renal system is not as sensitive as the genital system to a reduction in WT1 levels during embryogenesis. The WT1 mutations observed in DDS are dominant in their effects on urogenital development. However, because of the incomplete penetrance (only 2/3 of WAGR patients develop a tumor), the dominant effect of these mutations in tumorigenesis is more difficult to establish. Several hypotheses can be proposed. Firstly, they may compromise or abolish binding of the WT1 zinc finger domain to its normal target DNA binding sites, perhaps blocking the binding of the wild-type allele gene product, resulting in a dominant negative mutation. Secondly, they may confer the ability to recognize novel but inappropriate DNA binding sites (dominant mutation). The altered peptide products could behave either in a dominant negative fashion by sequestering a polypeptide required for WT1 function or in a gain-of-function manner by activating or repressing genes not normally under WT1 control [12].

Homozygous mutation of WT1 introduced into mouse embryonic stem cells by gene targeting resulted in embryonic lethality. The mutant embryos exhibited a failure of kidney and gonad development. At day 11 of gestation, the cells of the metanephric blastema underwent apoptosis, the ureteric bud failed to grow out of the Wolffian duct, and the inductive events that lead to formation of the metanephric kidney did not occur. Moreover the mutation caused abnormal development of the mesothelium, heart, and lungs. These observations now clearly demonstrate the role of this gene in early genital development, although the animal model used does not entirely reproduce the features associated with human WT1 mutations [36].

WT1 and urogenital development

The frequency of genital anomalies in WAGR patients with del 11p13, >50% of boys, led to the proposal that a gene different from the WT gene may map to the same region. There have been several attempts to map this putative gene within 11p13 using overlapping deletions in patients with different manifestations, but obviously the WT1 gene itself is implicated. This is strongly supported by: (1) the observed expression pattern of WT1 in the developing gonads mainly over the germinal epithelium and the sex cords (34); (2) the observation of WT1 mutations in two WT patients with cryptorchidism and hypospadias [25], and (3) WT1 point mutations in DDS patients who invariably present with gonadal dysgenesis and predisposition to WT or gonadoblastoma. These DDS mutations either result in an amino acid change in the DNA-binding domain or even the complete loss of this domain [58]. High levels of WT1 mRNA expression are observed in the developing gonads. In mature gonads, WT1 expression is restricted to the Sertoli cells of the testis and granulosa and epithelial cells of the ovaries. The possibility that WT1 has a transcriptional relationship with müllerian inhibiting substance, or with any of the genes controlling testosterone biosynthesis and action, or with the Y chromosome gene, SRY, which initiates male sexual development, needs to be evaluated [62]. One particularly fascinating WAGR associated deletion observed in a family with a balanced translocation resulted in isolated aniridia in a 46XXdel11p13 girl and in ambiguous genitalia, WT, aniridia and mesangial sclerosis in a once removed cousin 46XYdel11p13 [61]. This difference in penetrance can be explained in terms either of developmental plasticity in the genitourinary system or of genetic background effects. More likely this is a reflection of the presence of the Y chromosome in the child with the more severe phenotype as in the majority of DDS patients. Although the DDS phenotype is usually more severe than the WAGR phenotype, there is an obvious overlap between them. Furthermore, germline hemizygous inactivation of WT1 may result in apparently non-WT associated genital anomalies. We are testing this hypothesis in XY patients with gonadal dysgenesis but without mutation in the SRY gene (Henry et al, unpublished data). Nonetheless, the germline and somatic mutations found in the WT1 gene provide a direct link between development and oncogenesis. Although there is accumulating evidence that WT1 is indeed a tumor suppressor gene and that both copies can be lost in approximately 10% of the tumors, this not the rule and other genes are obviously involved [12, 63].

The WT2 locus at 11p15

The Wiedemann-Beckwith syndrome

That the 11p15 region carries a second gene for predisposition to or progression of WT was proposed on the basis of (1) a subset of Wilms' tumors with non-random maternal losses of alleles limited to 11p15, not involving 11p13 [64–66]; (2) the description of patients with the Wiedemann-Beckwith syndrome (WBS) carrying constitutional chromosomal duplications overlapping the 11p15.5 region (Fig. 1). This syndrome is characterized by a number of congenital disorders including neonatal gigantism, exomphalos, macroglossia, visceromegaly, hemihypertrophy and neonatal hypoglycemia. About one tenth of patients with this overgrowth syndrome develop tumors, including WT, adrenocortical carcinoma, rhabdomyosarcoma and hepatoblastoma. To

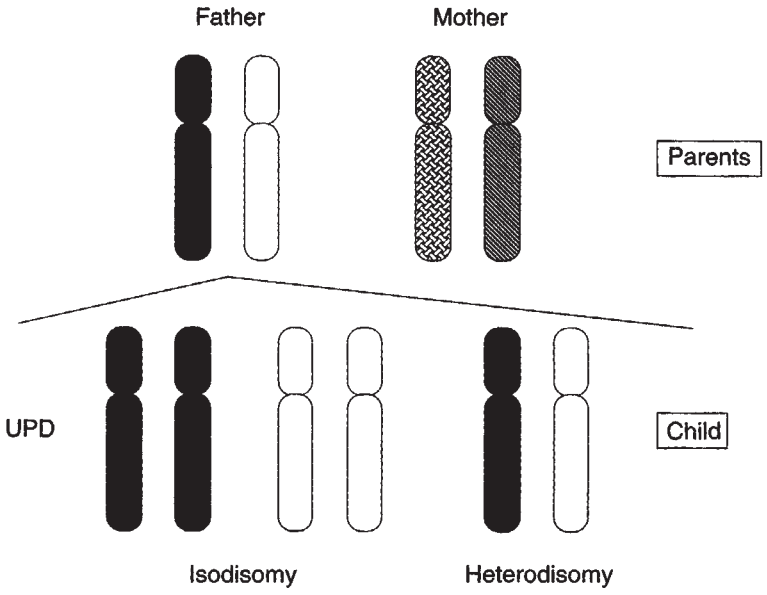

Fig. 3. *Uniparental paternal disomy.* Isodisomy corresponds to the presence of two identical copies and heterodisomy to that of both homologs from the same parent. Heterodisomy results from non-disjunction during the first mitosis of meiosis while isodisomy can occur either following non-disjunction during the second mitosis of meiosis, or at a post-zygotic stage. Partial isodisomy can result from a mitotic recombination and, depending on the stage of development and the fate of embryonic tissues lead to mosaicism.

date, less than twenty cases with dup11p15 have been reported. In every case where the parental origin of the duplicated region could be identified it was of paternal origin [13]. More recently, eight WBS patients with apparently balanced reciprocal translocations having in common a breakpoint in 11p15.5 have also been reported [67, 68]. Again a similar bias in parental origin was observed, since in all eight patients the rearranged chromosome 11 was of maternal origin. Familial cases, which represent about 15% of all cases, showed linkage to markers in the 11p15.5 region [69, 70], namely, HRAS (c-H-ras), INS (insulin) and IGF2. A parental bias in familial transmission of the defect was observed with a threefold excess of carrier mothers. Although there are rare cases of paternal transmission, affected offsprings tend to display less severe symptoms. This can have two explanations: the lower penetrance when transmitted by a father, and the reduced fecundity of affected males. This may be the consequence of an altered developmental process affecting gonads and external genitalia, mostly in male patients, and resulting in cryptorchidism and hypospadias [13, 71].

Imprinting

The biases in parent of origin observed in the different forms of WBS and associated tumors strongly suggested that the gene or genes were submitted to imprinting. This also applies to various human hereditary diseases, where imprinting conveys a unique pattern of inheritance [72, 73], and to several embryonal tumors, in which a parental bias in allele loss has led to the proposal that some tumor-suppressor genes may undergo genomic imprinting [74, 75]. Genomic imprinting can be defined as the differential modification(s) of the maternal and paternal genetic contributions to the zygote that results in the differential expression of parental alleles during embryonic and adult development. Genes subject to imprinting are therefore monoallelically expressed.

The loss of maternal 11p alleles limited to tumor cells is reminiscent of uniparental paternal disomy which can be observed in a whole zygote. Uniparental disomy (UPD) refers to the presence in a zygote of two copies of all or part of a chromosome from the same parent, with a lack of the corresponding chromosomal region from the other parent. Several mechanisms occurring during meiosis I or II or at a post-zygotic stage can result in UPD (Fig. 3). Using crosses between mice carrying different translocations, Beechey and Cattanach (1987) have obtained offspring carrying maternal or paternal uniparental disomies [76–78]. To date, fifteen different chromosomal regions on the mouse genome show no complementation by the other parent chromosome. Although the actual number of imprinted genes is not known, each of these fifteen regions must carry at least one imprinted gene. To date, four murine genes have been found to be imprinted. Two of them, H19 and IGF2R (insulin-like growth factor II receptor) are expressed exclusively from the maternal allele while the other two, IGF2 and SNRNP (small nuclear ribonucleoprotein polypeptide N) are found to be expressed only from the paternal allele. Interestingly, the human H19 and IGF2 genes map to 11p15 are also imprinted in humans.

Both WBS and a possibly related condition in mice are associated with paternal UPD. Chimeric mouse embryos containing cells paternally disomic for the distal part of chromosome 7 are abnormally large [79]. The distal end of mouse chromosome 7 is homologous to human chromosome 11p15.5. Using 11p15 polymorphic markers, comparison of the genotypes of WBS children with the genotypes of their parents showed that approximately 20% of sporadic WBS cases present with paternal isodisomy with complete or partial lack of a maternal allele [64, 80–82]. The common region of isodisomy found in WBS patients is depicted in Figure 1. Moreover, most WBS patients with UPD are mosaics [83] and, UPD limited to kidney was observed in a proportion (4 of 64) of Wilms' tumor patients [84] (Fig. 4). Thus UPD may not be detectable in a blood sample from a patient but be present only in some tissues and account for the extreme variability of the syndrome. Alternatively, UPD may be present only in kidney and account for bilaterality or multifocality in an otherwise apparently healthy sporadic, non-hereditary case. Moreover, patients with UPD are at increased risk of developing a tumor, 64% versus 10% for all WBS (Table 1). Detection of UPD, which was demon-

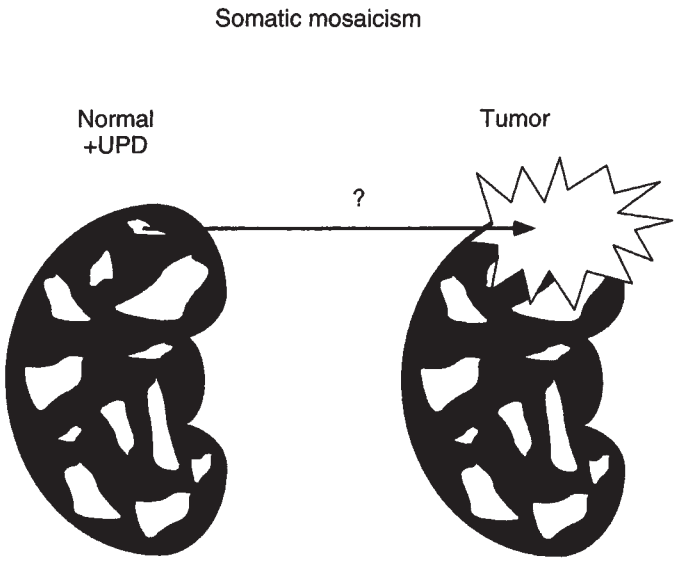

Fig. 4. *Mosaicism for uniparental disomy in kidney giving rise to a tumor.* UPD can be considered as a loss of alleles. Cells with UPD are therefore more prone to develop a tumor. However, since not every patient (64%) with UPD develops a tumor, another event, which has not been defined yet, must occur.

Table 1. WBS, uniparental disomy and risk of tumor

	Risk of tumor
• All WBS	→ 8.5% (17/200)
• Dup 11p15	→ 0–7.5% (1/15)
• UPD	→ 64% (7/11)

strated here for the first time in a cancer predisposing syndrome, therefore represents a new important test for predicting the risk of cancer in these patients.

A defect in genomic imprinting can occur constitutionally, leading to growth abnormalities and predisposition to Wilms' tumor. Recently, relaxation of IGF2 imprinting in four of six fibroblasts cultures from WBS patients has been detected [85]. In one child with generalized overgrowth, IGF2 was transcribed from both alleles in her kidney, peripheral blood leukocytes and Wilms' tumor [86]. In contrast, kidney samples from nine children with normal growth profiles showed monoallelic transcription of IGF2 [87]. This is consistent with constitutional relaxation of IGF2 imprinting arising from a germline mutation or as a very early event during embryogenesis.

A third gene, WT3, involved in familial cases

A third region, yet unknown, harbors a gene for familial predisposition to WT. Linkage analysis yielded significant lod scores for exclusion of the 11p13 region, the WT1 gene itself (1 family), and the 11p15 region [54, 69, 70]. However, the small number of informative families is still insufficient for demonstration of linkage to a specific region of the genome. In these families, the absence of characteristic clinical features led to the proposal that most familial cases without partial or complete overgrowth syndrome (WBS) and/or without genitourinary abnormalities (WT1) carry a mutation at the WT3 locus. However, due to incomplete penetrance of these developmental abnormalities, the exact proportion of familial cases carrying a mutation of this putative WT3 gene remains unknown. As previously mentioned, genetic transmission of a WT1 mutation has already been reported in two cases [25, 55]. Because of the frequent association of genitourinary abnormalities and the poor prognosis of WT in the past decades these must be rare today. However, there has been considerable progress in the treatment of WT recently and, with long-term survival close to 90%, it would not be surprising if a greater number of these individuals, provided they are not infertile, have offspring with the mutant gene and who would therefore be at risk of developing a tumor.

Segregation analysis performed in familial cases showed that the risk of being affected is not significantly different when the transmitting parent is a man rather than a woman [88]. These results, which are clearly in contrast with the familial cases of WBS, do not provide evidence for genomic imprinting of the WT3 gene.

Cytogenetic and molecular analysis of the tumors

One of the most perplexing aspects of the genetics of predisposition to cancer is that germline mutations or deletions may have a dominant mode of action resulting in developmental anomalies whereas, in agreement with the retinoblastoma model, a recessive mode of action has been assumed for somatic mutations leading to tumors. This implies that inactivation of both alleles is required to initiate tumorigenesis. However, as discussed below, and although demonstrated in 10% of WT this may not always apply in the case of WT. This seriously questions the nature, dominant or recessive, and the role of the mutations occurring at the somatic level as well as the function(s) of the gene itself as an antioncogene.

In WT as in many other tumor types the tumorigenic process is characterized not only by loss, but also by equally frequent gain of genetic material including duplications the significance of which is still elusive. Cytogenetic analyses of Wilms' tumors allowed characterization of the different regions involved. A large number of tumor karyotypes were performed, and a review of 107 cases showed that numerical and structural rearrangements appeared to be non-randomly distributed. Chromosomes 1, 11, 12, and 16 are the chromosomes most often involved in the rearrangements (Table 2).

LOH studies have been used to identify regions frequently deleted with or without reduplication of the remaining chromosome, thus pointing to areas that may harbor possible tumor suppressor genes. The involvement of these chromosomal regions, 1p, 11p and 16q, was amply confirmed and refined by LOH analyses in a large number of tumors using molecular markers. LOH was observed in 20% of tumors studied for the short arm of chromosome 1, in 55% to 57% of tumors studied for the short arm of chromosome 11, and in 20% of tumors studied for the long arm of chromosome 16. The most striking features of LOH studies on 11p was the preferential loss of maternal alleles (52/53) in sporadic cases as well as in WAGR syndrome or familial cases [89–91; Henry, unpublished data].

At least two different regions in 11p

Cytogenetic analysis and molecular analysis looking for allele loss revealed that two different regions on chromosome 11, 11p13

Table 2. Numerical and structural somatic chromosomal rearrangements in 107 Wilms' tumors

Chromosomes	Number of rearrangements	% Of rearrangements	Specific aberrations
1	35	33	Dup 1q21-qter
2	9	8	
3	19	18	Total trisomy
4	7	6	
5	5	5	
6	21	20	Total trisomy
7	25	23	Total trisomy, Dup7q
8	20	19	Total trisomy
9	16	15	Total trisomy
10	18	17	Total trisomy
11	37	35	Del 11p13
12	39	36	Total trisomy
13	11	10	
14	11	10	
15	4	4	
16	32	30	Total monosomy Del 16q
17	17	16	Monosomy, trisomy
18	16	15	Total trisomy
19	11	10	
20	10	9	
21	5	5	
22	14	13	
X	8	7	
Y	5	5	
Total	107 tumors	100 tumors	

Chromosomes 1, 11, 12, and 16 are the chromosomes most often involved in the rearrangements. The q12 region to qter of chromosome 1 and chromosome 12 in its entire length are duplicated in 33% and 36% of tumors, respectively. Other chromosomes were shown to be duplicated in WT, but either less than 15% of tumors were involved or the rearrangement, such as the 7q duplication (23%) was common to a large number of different types of tumors and therefore not specifically associated with tumor progression. The p13 region of chromosome 11 and the long arm of chromosome 16 are deleted in 35% and 30% of the tumors, respectively (Austruy et al, manuscript in preparation). Cytogenetic studies in tumor cells therefore delineated two different regions, namely 11p and 16q, frequently deleted and therefore harboring at least three possible tumor suppressor genes. Surprisingly, 1p deletions were not found in this analysis. This is in contrast with the description of losses of alleles for region 1p36 reported in WT (Mannens personal communication). This may reflect chromosomal rearrangements undetected by cytogenetic analyses, that is, microdeletions or mitotic recombination.

and 11p15, can show LOH but not necessarily in complete agreement with Knudson's two-hits hypothesis. More than half of the tumors, 57%, showed LOH (Henry, unpublished data). Considering all tumors informative for 11p13 and 11p15 markers, we showed that WT1 is specifically involved in 5% of cases, WT2 is specifically involved in 41% of cases, and WT1 and WT2 are both involved in 54% of cases. Demonstration of LOH limited to 11p15 thus suggested that a mutation in a gene mapping in 11p15 may also be involved (Henry, unpublished data). More direct evidence for the involvement of WT2 comes from the observation of three WAGR cases who retained their unique WT1 allele at 11p13 but showed LOH limited to region 11p15, suggesting successive involvement of WT1 and WT2 [92, 93]. However, since a high proportion of LOH encompasses 11p13 and 11p15, this may suggest that these two loci must be involved sequentially [21] and interact (Fig. 1). These figures demonstrate the major role played by somatic rearrangements of WT2 in the pathogenesis of WT, whether or not WT1 is involved. The WAGR cases are a separate matter in some ways since here LOH for the germline mutation would most likely be cell lethal. Thus, for the functional copies of WT1 to be lost an independent second hit is required. This has been seen in several cases, but may not always occur. It is possible that in some of these cases UPD for the WT2 region can interact with loss of one copy of WT1 and so lead to a sort of hybrid tumor mechanism. What has not been clearly described in any cases is WT1 mutation in patients with UPD.

Although only a small fraction of sporadic WT exhibit homozygous deletions of the WT1 gene, some of them intragenic, the WT1 in 11p13 therefore conforms to the definition of a tumor suppressor gene [10, 21, 25, 27, 50, 94–98]. Except for one case [59], in every case of DDS in which tumor material was examined and compared with constitutional DNA, a reduction to homozygosity for the germline mutation was observed [56]. However, dominant mutations of WT1 may lead to tumorigenesis without requiring the loss of the second normal allele, as suggested by a sporadic WT that had a WT1 mutation deleting zinc finger 3, yet retained the normal WT1 allele [21]. Recently, Park et al reported a case of adult human mesothelioma that contains a homozygous point mutation within WT1 [32]. This mutation within the putative transactivation domain, converts the protein from a transcriptional repressor of its target sequence to a transcriptional activator. Little et al also described a sporadic WT with heterozygosity in the tumor for the WT1 mutation demonstrated. Therefore there are several reported cases where WT1 mutation does not become homozygous in the tumor [23]. Alternatively, as recently shown, more subtle mutations of WT1 may be present in some cases [25]. Analysis of the remaining allele in a WT from a WAGR patient revealed the deletion of a single nucleotide in exon 7 [97]. This mutation likely played a role in tumor formation, as it prevents translation of the DNA binding zinc finger domain that is essential for the function of the WT1 polypeptide as a transcriptional regulator. Another patient carried a heterozygous mutation while the other allele was wild-type and expressed at normal levels [99]. It was suggested that the mutant WT1 protein acted either in a dominant mode or as a dominant negative that interfered with the function of the wild-type protein. In cotransfection experiments, the mutant form of WT1 was able to cooperate with the adenovirus E1A oncoprotein to transform primary baby rat kidney cells, while the wild-type WT1 form exerted no such effect [99]. This is not the first example of a dominant mode of action for a genuine tumor suppressor: there are now several examples of P53 somatic mutations with analogous properties [48]. In most tumor samples, however, no deletion or mutations of WT1 were found. RNA transcripts of this gene could still be detected in many cases at levels comparable to those in the developing kidney.

Moreover, further experiments showed that sequences with features of tumor suppressor gene mapped to 11p15. Dowdy and coworkers showed that a sequence mapping to 11p15 was responsible for suppression of tumorigenicity of a WT cell line (G401) [100]. Koi et al [101] showed that a sequence mapping between HBB and D11S12, thus different from WT2 and WT1, had a tumor suppressor effect on a rhabdomyosarcoma cell line (Fig. 1). Genetic predisposition to rhabdomyosarcoma is often associated

with susceptibility to other tumor types in the context of either WBS or SBLA (Li-Fraumeni syndrome).

The unexpected, relatively rare implication of WT1, may thus be explained by the existence of at least three other loci, TS, WT2 and WT3 postulated to play a role in an unknown proportion of WTs.

Genomic imprinting, development and tumorigenesis

Although the molecular bases for genomic imprinting are still a matter of debate, strong *a priori* considerations designate DNA methylation as a good candidate. Firstly, DNA methylation is a transmissible epigenetic modification that has profound effects on gene expression [102, 103]. Secondly, DNA is known to be differentially methylated in paternal versus maternal gametes, and, thirdly, DNA methylation is reversible in that it can be maintained during several cell cycles and can be erased in somatic cells as well as in germ cells [104–108]. The simplest explanation for an imprinting process would be the establishment during gametogenesis of allelic methylation differences at critical CpGs (imprinting boxes) over imprintable loci, with persistence of subsequent effects on allelic gene expression during critical stages of development and differenciation [109]. These differences in methylation patterns are indeed observed and often accompagnied by differences in DNA compaction as shown by sensitivity to several DNAses [110–113]. The role of DNA methylation in genomic imprinting was recently demonstrated by experiments in mice homozygous for a DNA methyl-transferase mutation. These embryos die at embryonic day 11 with their DNA substantially demethylated. The expression of three imprinted genes Igf-2, Igf-2r and H19 in mutant mice that are deficient in DNA methyl-transferase activity was examined. Expression of all three genes was affected in mutant embryos: the normally silent paternal allele of H19 gene was activated, whereas the normally active paternal allele of the Igf-2 gene and the active maternal allele of the Igf-2r gene were repressed. These results demonstrate that the normal level of DNA methylation is required for controlling differential expression of the paternal and maternal alleles of imprinted genes [114]. Moreover, it has recently been shown that imprinted regions are characterized by asynchronous replication [115]. Reasonable theoretical arguments can also be made for more complicated models, those which involve spread of gene inactivation along the chromosome from hypothetical distant imprinting controlling elements, perhaps in a process analogous to heterochromatinization or X-inactivation. This could account for the as yet unexplained spreading over large distances of the breakpoints of WBS associated translocations (Fig. 1).

Several studies have been carried out to determine which of the three loci, WT1 WT2 or WT3, is responsible for the imprinting phenomenon. There is now growing evidence that the WT1 gene is not imprinted. Firstly, in two families in which malsegregation of a balanced translocation led to the birth of children with deletion of band 11p13 [55, 116]. Secondly, two reports demonstrate the biallelic expression of WT1 which is equally transcribed from both alleles in most WT and in the surrounding normal kidney [117, 118]. Thirdly, the corresponding region on mouse chromosome 2 is not imprinted. Segregation analysis performed in familial cases did not provide evidence for genomic imprinting of the WT3 gene [88]. In contrast, as already mentioned for WBS, there is now accumulating evidence that WT2 is the best candidate for imprinting.

Preferential LOH of a specific parental allele in several childhood tumors may provide indirect evidence for genomic imprinting in cancer [74]. However, as already demonstrated for bilateral retinoblastoma, since new germline mutations on the RB gene preferentially occur in paternal gametes, the loss of the maternal allele in the child's tumor only reflects this hypermutability of male gametes. Hypermutability of the region 11p13 in paternal gametes has also been demonstrated [119]. Other arguments are thus needed to conclude that preferential maternal LOH reflects genomic imprinting. The involvement of genomic imprinting in expression of some genes can be demonstrated by: (1) differences in the patterns of methylation of a gene or a genomic region for the paternally inherited chromosome versus the maternally inherited one; (2) the existence of uniparental disomy for a region known to be involved in the same disorder; (3) monoallelic gene expression with only the paternal or the maternal allele being expressed; (4) differences in the timing of replication for the same region; and (5) differences in chromatid compaction of the two alleles of one gene. Two genes localized in the 11p15 region, namely H19 and IGF2, known to be imprinted in opposite directions, are therefore good candidates to account for genomic imprinting in WBS and associated tumors.

The H19 gene in 11p15 is paternally imprinted

The H19 gene codes for a spliced and polyadenylated RNA which is highly expressed in a large variety of fetal tissues at a stage when cells are differentiating [120–123]. H19 transcripts may also play a role in the process of cytotrophoblast differentiation [124]. Gene expression was shown to be down-regulated to nearly zero in the postnatal period in all tissues except skeletal muscle [125]. Transcription of H19 remains detectable in differentiated myoblast cultures, and at a level somewhat lower than that of corresponding fetal organs, in several adult mouse and human organs [121, 122, 125–128]. Because of the apparent lack of evolutionarily conserved open reading frames, the protein coding potential of H19 RNA is uncertain. It has therefore been proposed that this gene may act at the level of its RNA, perhaps through the formation of a ribonucleotide particle [129]. While one report has suggested that the human H19 gene might give rise to a polypeptide *in vivo* in adult muscle, this data have not yet been confirmed [127].

Parental imprinting of the H19 gene was demonstrated in several ways.

(1) Specific sites in the CpG island promoter and 5′ portion of the gene are only methylated on the paternal allele [110].

(2) H19 is included in the region of paternal disomy associated with Wiedemann-Beckwith syndrome [64, 83] (Fig. 1).

(3) Monoallelic expression has been demonstrated using different approaches. Using a DNA polymorphism detectable at the RNA level, Zhang and Tycko demonstrated that, as in the murine tissues, only the maternal allele is expressed in human tissues [118]. This was confirmed by experiments on either androgenetic tissue, such as a complete hydatidiform mole, or gynogenetic tissue showing expression of H19 only on gynogenetic tissue [124]. More recently, a tissue-specific somatic allele switching correlating with demethylation of the imprinted allele has been characterized in the cerebellum and an apparent erasure of imprinting with biallelic expression in the lung has been reported in only one adult case [113].

(4) H19 is included in the 11p15.5 region of about 1 to 2 Mb in

which the two homologous alleles replicate asynchronously. The paternal allele always replicates earlier. This phenomenon of asynchronous replication has also been demonstrated for other regions known to be imprinted in the mouse as well as in the human chromosome [115].

(5) The active maternal allele in mouse embryos disomic for the maternal allele (Mat Di7) is more sensitive and accessible to nucleases. The differences in chromatid compaction between the two alleles is certainly associated with the pattern of methylation and confirms that imprinting only occurs on the paternal allele of the H19 gene [110].

The IGF2 gene in 11p15 is maternally imprinted and expressed in WT

Several lines of evidence from expression studies in mice and humans suggest that the fetal mitogen IGF2 seems justifiably implicated in the pathogenesis of WT. IGF2 is overexpressed in all WT examined to date, including tumors from WBS patients [130–133]. As an alternative hypothesis it has recently been proposed that the IGF2 gene products can behave not only as a growth promoter, but also as a tumor suppressor gene [134]. The IGF2 gene precisely maps within the region involved in WBS, in 11p15.5. Studies of WT transplanted in nude mice have shown that the tumor cells express the IGF1 receptor and that antibodies that block this receptor inhibit tumor growth [135]. This is not suprising since the IGF1 receptor binds IGF2. These data strongly suggest that IGF2 may function as an autocrine growth factor in WT. IGF2 is abundantly expressed in kidney blastemal cells, but not in differentiated epithelial cells, whereas a reciprocal pattern of expression has been described for WT1 [33, 34]. As previously mentioned, the WT1 gene has been shown to interact with several sequences in the P3 promoter region of IGF2 [39].

The suggestion that IGF2 might be responsible for the overgrowth in WBS is supported by the finding that only the paternal allele of the IGF2 gene is transcribed in most tissues in the mouse and that pups with an inactive IGF2 paternal allele do not reach full size [136]. Furthermore, abnormally large chimeric mice resulted from a duplication of a chromosomal segment containing the paternal IGF2 allele. De Chiara, Efstratiadis and Robertson found that the normally proportioned dwarfism seen in mice lacking IGF2 activity persisted throughout their adult life [137]. Previous explanations for the role of the IGF2 gene in WBS have incriminated gene dosage effects in which duplication of the active paternal allele for IGF2 leads to overexpression. An alternative model involving trans-sensing effects was proposed which also assumes imprinting of the human IGF2 gene but does not require increased copies of the paternal allele [138]. This hypothesis might therefore account for some but not all sporadic cases of WBS for which a biparental contribution has been observed [139]. It may be that the familial cases where no translocation is involved may be the most exciting for throwing light on the mechanism of imprinting, since the simplest hypothesis would be that these cases are point mutations which alter the imprinting mechanism. They may or may not lie in an expressed portion of the genome, but if we can find them, they should throw light on how imprinting is mediated and what interferes with the mechanism.

Parental imprinting of the IGF2 gene was demonstrated in several ways.

(1) Differences in the pattern of methylation between the two alleles has been demonstrated for the IGF2 gene. Surprisingly, for the region of the gene analyzed, hypomethylation was observed for the non-expressed maternal allele in the various tissues tested [111, 140]. The same phenomenon is observed in the IGF2R gene. Indeed, in this gene the promoter is hypomethylated on the expressed maternal allele, while an intronic region is hypermethylated on the same allele. While the differential methylation pattern in the promoter is progressively established after fertilization, the intronic one is established earlier, during gametogenesis and rather than the promoter itself it may therefore represent an important key signal for imprinting [109, 112].

(2) IGF2 is included in the region of disomy characterized in the Wiedemann-Beckwith syndrome [64, 83] (Fig. 1).

(3) Monoallelic expression of IGF2 was established with the same type of experiments as for H19. IGF2 is only expressed in androgenic tissues [124]. The specific expression of the paternal allele was confirmed with studies using a DNA polymorphism detectable at the RNA level [141, 142].

(4) IGF2 and H19 map on the same 200 kb fragment which belongs to the same 1 to 2 Mb unit showing asynchronous replication [115, 143].

The physical linkage of the H19 and IGF2 genes in both mice and humans suggests that they constitute a functional imprinting domain, although one in which the imprint can be imposed in either direction and for which the paternal region replicates earlier. Both genes share a very similar pattern of expression suggesting the possibility that they may share transcriptional regulatory elements. Both genes are initially activated in extraembryonic tissues by day 4.5, and in a very similar set of tissues in the embryo proper at 8.5 days. After birth, the expression of both genes is repressed in most tissues. One interesting exception to their co-expression occurs in the postnatal choroid plexus and leptomeninges, the only tissues which are known to express both alleles of IGF2, while neither expresses H19 [143]. The preservation of physical linkage and imprinting of these genes in mammals implies that their imprinting has important selective advantages to mammalian survival [144].

These two genes, H19 and IGF2 and their specific parental imprint, may thus account for the pattern of inheritance observed, the variable expressivity, the specific loss of alleles and the loss of imprint [145]. However, these genes map 400 kb away from a cluster of breakpoints observed in the cytogenetic cases of WBS, suggesting that other genes could be involved [146]. Indeed, although mapping to a different subregion, a sequence with properties of a tumor suppressor (rhabdomyosarcoma cell line) has recently been isolated [101]. Furthermore, neither reduplication of the active IGF2 paternal allele nor relaxation of IGF2 imprinting is sufficient for tumorigenesis, thus indicating that other mutation(s) must occur.

Since imprinting is expected to start in the germline, a heritable molecular tag is needed. This tag should be able to regulate gene expression and be removable during each cycle of germ cell development. DNA methylation may be one of the molecular markers [147]. IGF2 and H19 are closely linked in mice and humans, but are imprinted reciprocally. It has been proposed that expression of the two reciprocally imprinted genes H19 and IGF2 is functionally and/or mechanistically related and that the imprinting of a single chromosomal site might control the activity of both genes [148]. It is suggested that the observed methylation and condensed chromatin of the inactive paternal H19 promoter may be the controlling event. Imprinting of H19 and IGF2 might be

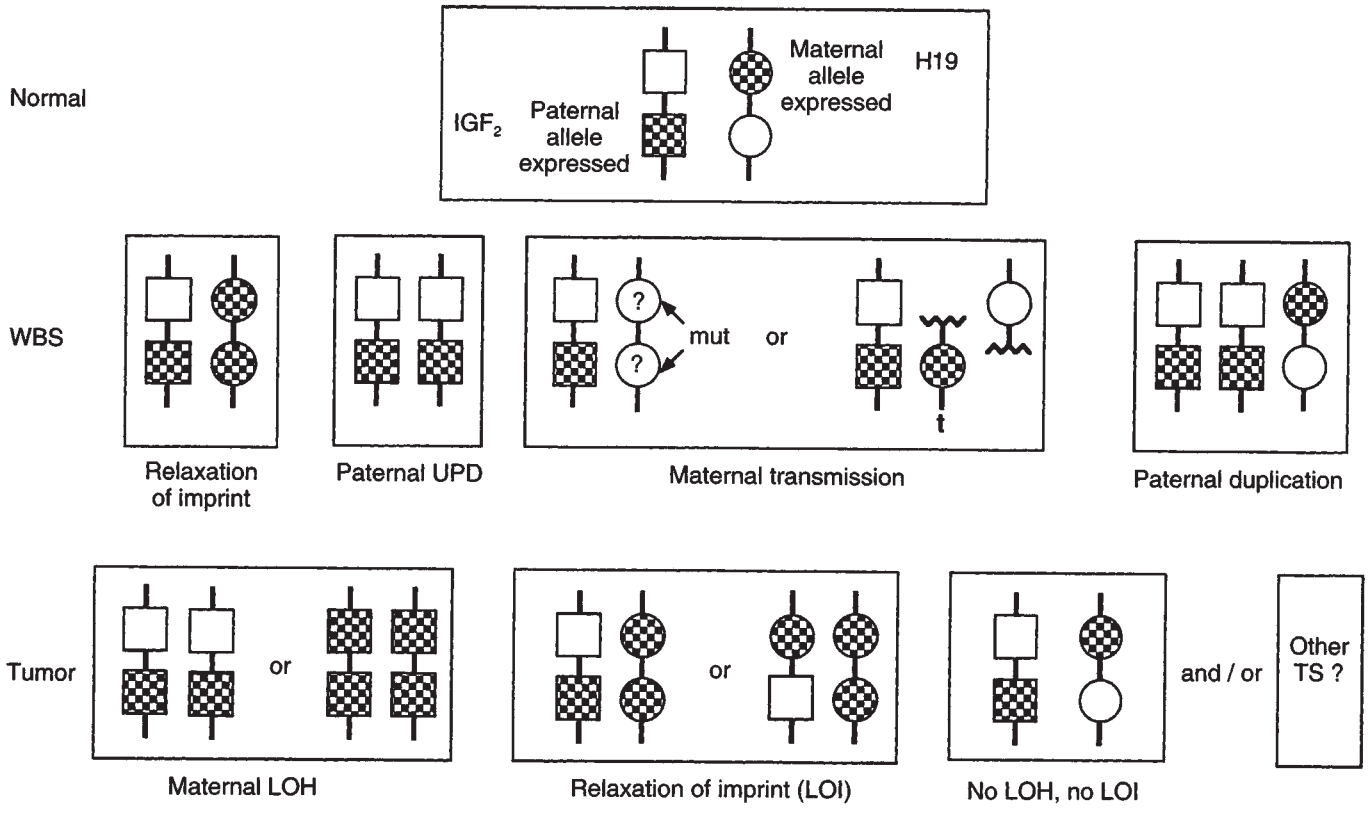

Fig. 5. *WBS, imprinting and tumorigenesis.* A model proposing two genes imprinted in opposite direction to account for the different situations observed. A good candidate for the maternally expressed gene is represented by H19 while the paternally expressed gene could be IGF2. In addition a tumor suppressor (TS) gene different from H19 and IGF2 could be involved in a proportion of WT.

linked mechanistically if the IGF2 and H19 promoters compete for the two enhancers downstream of H19 [110, 148, 149]. The enhancers may activate IGF2 expression, but only if the H19 gene is methylated and inactive, as on the paternal chromosome [148].

The fate of parental imprints in mutant mice with impaired methylation is striking: the imprints on three genes including IGF2 and H19 are lost, resulting in either repression (IGF2) or expression of both parental copies (H19) and the death of mutant embryos. In agreement with previous proposals, the paternal IGF2/H19 domain becomes functionally maternal [114]. This confirms the view that DNA methylation is a key event in the imprinting process. Embryos with the maternal disomy for chromosome 7 have excess H19 and no IGF2 (just like the methyltransferase mutant embryos) and are lethal [110]. However, the observation that both alleles of the two genes are expressed in androgenetic mononuclear trophoblasts also suggests that a biparental contribution may be required for expression of the reciprocal IGF2/H19 imprint [150]. In addition, that imprinted regions replicate asynchronously and that, whatever the imprinting pattern, the paternal chromosome replicates earlier adds further complexity to this puzzling syndrome [115].

Several recent papers report biallelic or monoallelic expression of IGF2, but whether or not H19 was simultaneously expressed was not investigated systematically [82, 86, 87, 141, 142, 150–152].

Relaxation of imprint in tumors

The biased LOH at 11p15 in WT might reflect parental imprinting of one or more growth-regulatory genes, perhaps including H19 and IGF2. How then would monoallelic expression still fit the Knudson's two-hits model? It was first argued that imprinting of one allele, resulting in its inactivation, represents the first hit, and LOH with loss of the active allele would therefore represent the second hit. This implied two things: (1) the gene had to be a tumor suppressor; (2) the allele inactivated by imprinting had to be the paternal one. This should lead, as for germline mutations, to an earlier age of onset for tumors with LOH. However, this did not prove to be the case. Even more puzzling was the association of overgrowth with two paternal copies in WBS patients, in at least two situations, dup11p15.5 and paternal UPD for 11p15. This finding suggested that the gene involved in WBS is a growth factor expressed by the paternal allele. This apparent paradox (tumor suppressor gene or growth factor?) could only be resolved if two different genes were involved, a growth factor expressed by the paternal allele and a maternally expressed tumor suppressor gene (Fig. 5). More recent evidence suggests an alternative hypothesis: that loss of imprinting is associated with aberrant gene activation in cancer. The determination of the parental origin of the allele expressed in tumors without LOH clearly demonstrates that in 69% of cases there is relaxation of the imprint for IGF2 or H19 (20%) or both (one case) [74, 86, 152]. Another study on a Wilms' tumor with a loss of the maternal allele in 11p15 and a reduplication of the paternal allele showed that one or both paternal copies of H19 is/are expressed [93; Jeanpierre, unpublished data 1993]. Thus loss or relaxation of imprint (LOI or ROI) is not restricted to tumors

retaining paternal and maternal H19 alleles and can occur in conjunction with maternal 11p15 LOH. A similar type of phenomenon has also been described in the switching of expression in an adult cerebellar tissue [113]. Regardless of the precise role played by this process in neoplasia, these data provide evidence for a novel epigenetic mutational mechanism in cancer, namely relaxation of genomic imprinting. However, this mechanism questions the relevance of Knudson's two-hits hypothesis and conflicts with the dogma according to which LOH unmasks a mutation in a tumor suppressor gene. The consequences of activation of the maternal allele of a growth factor such as IGF2 can be easily explained in terms of excess cellular growth. In contrast, the hypothesis that H19 might be a tumor suppressor gene is apparently incompatible with the observations that the paternal allele can be activated not only in a tumor without LOH [152], but also in a tumor with maternal LOH (Jeanpierre, personal communication). Although overexpression of H19 in transgenic mice results in late embryonic lethality, suggesting that the level of the gene product is strictly controlled, the function of H19 remains unknown [153].

How many independent events in the different forms?

Whether single mutational events at these loci are sufficient for tumor formation or whether there are interactions between the mutant gene products of these loci remains to be determined. Data from 511 cases of WT in France including 12 familial cases and 8 pedigrees from the literature, were analyzed to test three modifications of Knudson's classical bimutational theory based on genomic imprinting in Wilms' tumor carcinogenesis [88]. In order to determine the number of independent events for WT development and to look for a differential role of paternal and maternal alleles, analysis of age at diagnosis and segregation analysis were performed. For familial cases, the linear regression was consistent with Knudson's classical bimutational theory in which the first mutation is germinal and the second mutation is somatic. In familial cases, no effect of the sex of the transmitting parent on either age at diagnosis or segregation ratio was observed. Unexpectedly, in isolated unilateral cases which are considered to be mainly non-hereditary, the linear regression could be explained by only one rare event. However, the possibility of two non-independent events remains. This could be explained by models of genomic imprinting which assume two non-independent events, or only one rare genetic event. Surprisingly, in bilateral cases a bimodality for age at diagnosis was observed, suggesting a mixture of hereditary (40%) and non-hereditary cases (60%). This result seriously questions the classical assumption that all bilateral cases would be hereditary. The rejection of the two-hits model was not due to familial cases, but to bilateral cases as well as isolated unilateral cases. Since there are different predisposing factors to WT, the different ages of onset may be found in hereditary cases, but the predisposition in the two categories may be different, for example, due to WT1 mutation in one group, but part of the WT2-WBS spectrum or the WT3 group in the other cases. It is quite reasonable to suppose that the second hit may arise at different rates in association with different predisposing genes. These genetic findings support the hypothesis that WT arises from a variety of etiological pathways.

Histopathology of WT

Islands of cells resembling metanephric blastema may persist during postnatal life in both normal and dysplastic kidneys. It has been suggested that these so-called nephrogenic rests may represent a precursor lesion. Indeed, nephroblastomatosis, which is present in only 1% of infant postmortem examinations, can be found in 40% of kidneys from children with unilateral WT and in almost 100% of bilateral cases. Based on histologic features, Beckwith, Kiviat and Bonadio suggested that Wilms' tumors can be divided into two categories namely tumors associated with perilobar nephrogenic rests (PLNR) and those associated with intralobar nephrogenic rests (ILNR) [154].

Tumors with heterologous stromal elements (striated muscle, cartilage, and bone) and a prominent stromal component are associated with the WAGR syndrome, and the presence of ILNR are therefore able to mimic the whole range of nephrogenesis. Those that do not have heterologous elements and are composed primarily of blastema with epithelial differentiation, are associated with the WBS and PLNR, and mimic later stages of nephrogenesis. Intralobar WT is associated with an early age of diagnosis of presumed genetic Wilms' tumor and is almost universal in the tumors associated with aniridia, Denys-Drash syndrome and genital anomalies. Perilobar WT has the typical age of diagnosis of sporadic tumors and is associated with WBS and hemihypertrophy. This might suggest that these two pathogenic groupings represent WT with different biologic characteristics and possibly different mechanisms, the ILNR representing earlier events than the perilobar ones, each probably reflecting the timing of the original tumorigenic insult. The first clue as to the genetic origin of nephrogenic rests and their relationship to the associated Wilms' tumors was recently provided by Park et al [155]. WT1 mutations identical to the mutations found in the tumors were identified in two cases. One case of ILNR was associated with a sporadic WT while one case of PLNR was associated with signs of WBS. This indicates that these two lesions, WT and nephrogenic rests, share a clonal origin although topographically distinct [155].

Pritchard-Jones and Fleming have shown that WT1 expression in the tumors remained very high in contrast to the low levels normally seen in childhood, thus providing molecular evidence for the abnormal persistence of cells with fetal characteristics [156]. However, in view of the continuing role of the WT1 gene in the differentiation processes of nephrogenesis, one might expect tumor cells involving this gene to be blocked at an early stage of the mesenchymal-epithelial transition (condensed blastema) or to be totally unable to enter this pathway and divert to the stromal pathway or show heterologous differentiation. However, in order to explain the ability of WT cells to differentiate beyond the blastemal stage, we must propose either that the WT1 mutation causes an alteration rather than a loss of protein function which allows some epithelial differentiation. Alternatively, the WT1 protein may be not absolutely critical in the multistep process of nephron formation and some degree of bypass of a differentiation block can be achieved. The latter hypothesis is supported by four cases that were classified as typical triphasic WT and showed homozygous WT1 deletions [11, 24, 94, 157].

Kikuchi and coworkers compared genomic alteration and histopathology [157]. They found three cases, out of 25 unilateral tumors, of genomic deletions of both alleles of WT1. The three

tumors that showed genomic deletions were histologically classified as triphasic nephroblastic WT, and one of them was associated with intralobar nephroblastomatosis and a rhabdomyomatous component. In another report, Gerald and coworkers compared the expression of WT1 in WT by Northern blot analysis and quantitative RNA slot blot analysis with clinical, histologic and molecular features [135]. They found a significant difference between the mean level of relative WT1 RNA for the two groups, although exceptions to the general trend existed for both categories. The levels of WT1 mRNA were lower in the group of tumors with heterologous elements and stromal predominance [157]. However, although none of the tumors analyzed showed a transcript with an altered size, point mutation affecting the protein product and its function cannot be ascertained by these experiments.

If IGF2 plays a causal role in WT onset, then it is possible that the cellular distribution of IGF2 expression could differ from the normal situation. In some tumors, the pattern of IGF2 synthesis was strikingly different to the pattern found in the normal fetal kidney. In other tumors, the difference was more subtle but nevertheless distinct from the expression pattern in fetal kidney, thus providing evidence for the role of IGF2 in the onset of WT [131–133]. IGF2 mRNA expression, detected by *in situ* hybridization, should be confined mainly to the stem cells and terminal differentiation of either normal or neoplastic cells would be coupled with a decrease or loss of its expression [158]. In the triphasic type, hybridization signals were localized mainly to blastematous cells and not to cells with epithelial differentiation. In the blastemal predominant type which does not display differentiation, all tumor cells showed IGF2 mRNA expression. However, in the monomorphous tubular type, signals were found even over the tumor cells with epithelial differentiation. This suggests that IGF2 could potentially regulate epithelial cell growth or differentiation in an autocrine fashion. In this case, unregulated expression of IGF2 could lead to autonomous cellular growth or to decreased sensitivity to factors that induce differentiation. One of these factors is probably WT1. The regional distribution of IGF2 mRNA in the kidney was largely complementary to that of N-*myc*, that is, predominantly over the blastemal cells with a relative lack of hybridization over the epithelial structures. N-*myc* expression was primarily detected over epithelially differentiating mesenchyme. The apparent coexpression of the IGF2 and N-*myc* genes in immature kidneys largely occurs in distinct cell types [159]. High levels of N-*myc* RNA were present in the blastemal elements in the Wilms' tumors [160]. The precise role of each gene and their relationships in the complex processes of differentiation and tumorigenesis remain to be clarified.

Conclusion

Although there are still a few percent of WT cases which ultimately will resist any therapy, considerable progress in the treatment of WT has been achieved in the past few years. This was paralleled by important discoveries in the understanding of the complex genetics of this tumor and the close relationship with kidney and urogenital development. Obviously, the next step should provide tools not only for recognition of the tumors with poor prognosis, but also for prevention of tumors in those rare conditions that predisposes to WT such as uniparental disomies, and ultimately, although more speculative, in sporadic cases. Neither the involvement of a germline mutation in WT1, WT2 or WT3 nor somatic mutations at either locus revealed by LOH studies have linked a particular histological type to a particular chromosomal locus. It is not surprising that such a simple correlation has not been established when so few studies have examined only a few of the large number of parameters involved. Further genetic and histologic data are needed to assess the relationship between alteration of the WT1 gene and histopathological features. This will require complete histological and molecular analysis of large series of genetically well-characterized WT. Until then the histological diversity observed in WT will remain an enigma.

As the first example of a tumor associated with developmental anomalies and involving the fascinating phenomenon of genomic imprinting, the WT story now represents a model of unexpected complexity, with several genes involved and possibly interacting. Step by step, this model reveals the subtlety of unforeseen epigenetic phenomena such as genomic imprinting, postzygotic uniparental disomies leading to a vast spectrum of mosaicism, relaxation of imprint, and an ever higher order of gene action with alternatively spliced forms of a same gene, uncovering new modes of fine gene regulation.

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