

Testing Genomic Imprinting in Wilms' Tumor

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Abstract

Data from 511 cases of Wilms' tumor 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. Analysis of data of age at diagnosis and segregation analysis were performed to determine the number of independent events for Wilms' tumor development and to search for a differential role of paternal and maternal alleles. Unexpectedly, we show that only one rare event is required for tumor development in isolated unilateral cases which are considered to be mainly nonhereditary. In familial cases, we observe no effect of the sex of the transmitting parent on either age at diagnosis or segregation ratio. We show that this could be explained by models of genomic imprinting which assume two nonindependent events, or only one rare genetic event. In bilateral cases we show a bimodality for age at diagnosis which could be due to a mixture of hereditary and nonhereditary cases. This result completely questions the classical assumption according to which all bilateral cases would be hereditary. These findings support the hypothesis that this childhood cancer arises from a variety of etiological pathways and might be useful to find strategies for further molecular investigations.
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Key Words

Wilms' tumor
Genomic imprinting
Age at diagnosis
Segregation analysis
Modeling

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Introduction

Previous epidemiologic and genetic studies [1–3] support the hypothesis proposed by Knudson that Wilms' tumor is due to a 'two-hit' mechanism. This tumor of the kidney might be the result of two mutational events. In the hereditary form, the first event would be inherited through the germ line, and in the sporadic form it would consist in a somatic event. In both cases, the second event would always be a somatic mutation. Previous molecular studies showed that the mechanism of Wilms' tumor seems to be more complex than the single-locus model of retinoblastoma which is the basic model for childhood cancers. Effectively, at least three predisposing loci are probably involved in Wilms' tumor carcinogenesis [6]: (a) WT1 at 11p13 in patients with a WAGR syndrome (Wilms' tumor, aniridia, genitourinary abnormalities and mental retardation). The WT1 gene was shown to be different from the aniridia gene by using new markers for this region. WT1 was then isolated and the sequence of the cDNA clone predicted a product with four zinc fingers suggesting a role as a transcriptional regulator; (b) WT2 at 11p15.5 in patients with Wilms' tumor in the context of Wiedemann-Beckwith syndrome (WBS; multiorgan developmental abnormalities and predisposition to cancers including Wilms' tumor), and (c) WT3, still unmapped and responsible for a substantial proportion of familial Wilms' tumors since linkage analyses performed on three families segregating for Wilms' tumor [5–7] excluded tight linkage with 11p13 and 11p15.5.

Polymorphic DNA markers revealed somatic loss for 11p markers [8–10] which can be restricted to 11p13 [11] or 11p15 both in sporadic Wilms' tumors [12] and in WAGR patients [13, 14]. A recent compilation by Seizinger et al. [15] and a review from Van Hey-

ningen and Hastie [4] revealed that loss of alleles of 11p markers occurred in approximately one third of Wilms' tumor cases.

Nonrandom retention of paternal alleles (or nonrandom loss of maternal alleles) on chromosome 11 [16–18] has been observed in a very high proportion (35/36) of cases, including a familial case [19] and a WAGR case [14]. This suggests a differential genomic imprinting of maternally and paternally derived alleles. Several findings provide arguments in favor of imprinting for the WT2 gene at 11p15.5 which would account for the unusual patterns of transmission of WBS and for the parental bias in allele loss found in associated tumors [20]. First, rare WBS cases with different cytogenetic abnormalities involving 11p15 show that duplications are always paternally inherited, whereas balanced translocations are always maternally inherited [21–24]. Second, we have recently demonstrated that the excess of transmitting females in familial WBS can partly be explained by a lower penetrance when the deleterious gene is paternally inherited [25]. Third, Henry et al. [26] and Grundy et al. [27] described uniparental paternal disomy in Wilms' tumor cases in the context of WBS or hemihypertrophy [28]. Moreover, human homologs of two murine genes known to display transcriptional imprinting, *Igf2* [29] and *H19* [30], are located at 11p15.5. In contrast, indirectly supporting imprinting at WT2 rather than WT1, two papers recently showed biallelic expression of WT1 [31, 32]. Several modifications of the two-mutational-event theory have been proposed to account for this phenomenon [33–35].

Models

Wilkins [33]. In this model it is suggested that imprinting induced by selective methylation normally renders inactive a transforming gene on the maternally derived chromosome 11. In normal cells, the alleles of a trans-

forming gene (Tr) are suppressed by a diffusible product of the alleles of a regulatory gene (Wg). If one regulatory gene is rendered inactive through a mutational event (the first event which can be inherited), the transforming genes are still suppressed by the remaining regulatory gene. The second event consists in loss of a segment of chromosome 11 (which has been observed in many Wilms' tumors) that includes the Wg and Tr alleles. The remaining Tr is then released from the suppressed state and is expressed at a high level if paternally derived or at a low level or not at all if maternally derived. The expression is thus higher when the first event occurs on the paternally derived chromosome and the second event occurs on the maternally derived one. In agreement with recent knowledge, the Tr gene and the Wg gene would respectively correspond to WT2 and WT1 [4].

Reik and Surani [34]. This model involves only one gene called 'Wilms' gene'. In some normal cells, the maternal Wilms' gene allele is rendered inactive by imprinting. If a mutation inactivates the paternal allele in such cells, it provokes a clonal expansion which increases the number of target mitoses for a second event (loss of the maternal allele).

Scrable et al. [35]. This theory of carcinogenesis, based on a model originally proposed by Sapienza et al. [36], was proposed to explain nonrandom retention of paternal alleles at 11p15.5 in rhabdomyosarcomas but it can be extended to other embryonal tumors. In this model, the first event is an epigenetic one (i.e., genomic imprinting) which inactivates the paternal tumor suppressor allele. If one genetic event occurs on its maternal homologue, it provokes the development of the tumor. Inactivation of the paternal tumor suppressor allele (first event) is a phenomenon which normally occurs with a given but still unknown frequency. Imprinting can be increased by the high expression of a gene in-

involved in generating or maintaining genomic imprinting (IMP gene) unlinked with the tumor suppressor gene. In hereditary cases, the IMP gene is inherited as the predisposing mutation which increases the proportion of cells submitted to genomic imprinting (first event in Wilms' tumor carcinogenesis). This model explains the observed nonrandom loss of maternal alleles on the short arm of chromosome 11 but it also explains exclusion of linkage at 11p markers for familial cases [5-7].

These theories imply a higher risk and an earlier development of the tumor when the first event occurs on the paternal chromosome. However, according to Scrable et al. [2], the sex of the transmitting parent in hereditary cases is not predicted to have an effect since the inherited gene is not the tumor suppressor. In the models of Wilkins [33] and Scrable et al. [35], two genes (WT1 with WT2, and IMP with WT, respectively) are involved, and the events leading to the tumor are independent. In the model of Reik and Surani [34], only one gene is involved and two nonindependent events are necessary.

The purpose of this study is to answer two questions. How many independent events are there in the different forms? Is there a differential expression of the paternal and maternal alleles? This was carried out by studying the distribution of age at diagnosis on the one hand, and by performing segregation analysis in familial cases on the other.

Material

Data were of two kinds. On the one hand we had clinical and familial data on 501 cases from the French Wilms' tumor study: 337 from the Institut Gustave Roussy in Villejuif, 56 from the Institut Curie in Paris and 108 from other centers in France. A complete description of the clinical data is given in another paper [37]. Familial data were obtained either by a self-questionnaire given during a consultation and brought back at the next, by mail or by interviewing

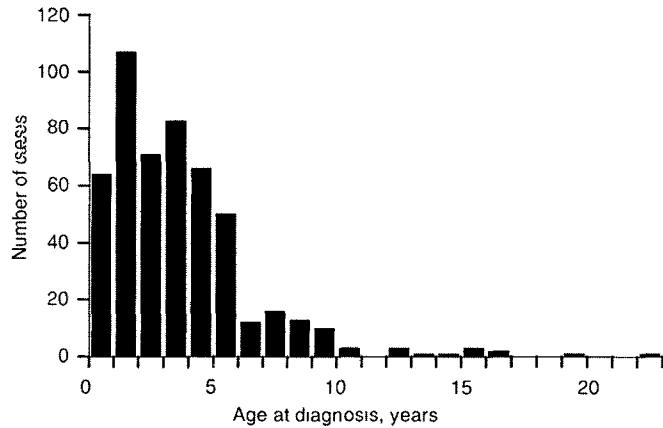


Fig. 1. Distribution of age at diagnosis in our sample.

Table 1. Distribution of French cases according to laterality and family history

	Number of cases	Mean age at diagnosis, months	SD
Isolated unilateral	463	44.1	36.0
Isolated bilateral	32	30.1	17.9
Familial unilateral ¹	12	42.5	24.0

¹ All the familial cases in our data were unilateral.

the parents. These data consist of information about the proband and his or her family members ascending to the grandparents' sibships, allowing us to detect familial cases with distant affected relatives. We added 10 bilateral cases from the Institut Gustave Roussy for whom we only had clinical but not familial data. Since no molecular data were available for this study, patients were categorized as isolated unilateral considered as nonhereditary, isolated bilateral considered as hereditary and partly linked to WT1 [38, 39], and familial cases probably linked to WT3 but also to WT1. Four and three isolated unilateral Wilms' tumor patients were diagnosed as WAGR and WBS, respectively, but these numbers are obviously too small to allow the constitution of separate groups. Even if they were removed from the sample, the remaining cases would still represent a heterogeneous group. Distribution of patients according to laterality and family history, and distribution of age at diagnosis are shown in table 1 and figure 1, respectively. To enlarge our panel

of familial cases we also used some informative published families where the transmitting parent was known: 6 families reported by Matsunaga [2], one family from Huff et al. [6], and another one from Grundy et al. [5], and data of age at diagnosis from Knudson and Strong [1] and Matsunaga [2], which provided a total of 68 familial cases. The family from Grundy et al. [5] was also studied by Matsunaga [2] but we used it once. Note that none of the familial cases were diagnosed as WBS.

Methods

Number of Independent Events

Assuming that the delay between the occurrence of Wilms' tumor and the diagnosis is constant, we used age at diagnosis to determine the number of independent events required for tumor occurrence. The differ-

ent tests were performed using all the patients whose age at diagnosis was known: 507 in our sample and 56 familial cases from the literature.

We first compared age at diagnosis of our cases with respect to laterality and family history by a rank-sum test, since assumptions of the t test were not fulfilled, in order to know whether bilateral cases (32) are diagnosed earlier than familial cases (12) and much earlier than isolated unilateral cases (463) of which a great majority is assumed to be nonhereditary.

It then seemed interesting to test Knudson's two-hit theory on the whole set of data using a test of model which includes biological parameters. Hethcote and Knudson [40] proposed a general two-mutation model for embryonal tumors and applied it to retinoblastoma data from a previous French study [41]. This model takes into account that the number of susceptible cells reaches a peak and then declines during childhood as noted by Moolgavkar and Knudson [42]. In this model, the quantity to determine is $f(t)$, the fraction of cell divisions that have occurred before age t . This model accounts for $m(\infty)$ the mean number of mutations in an affected child, and for $a(\infty)/b(0)$, the mean number of cells in a kidney, where $a(\infty)$ is the mean number of cell divisions in a kidney and $b(0)$ the number of tissue-specific cells initially present. All unilateral familial cases were considered to be in the hereditary group and all unilateral isolated cases were put in the nonhereditary group even if some of the isolated cases result from new germline mutations. The fraction of cases that are still not diagnosed at age t are noted $HB(t)$ for bilateral cases, $HU(t)$ for hereditary unilateral cases, and $N(t)$ for nonhereditary cases. The value of $f(t)$ is estimated from the observed values of $HB(t)$, $HU(t)$, and $N(t)$ by a least-squares procedure in which the patients are weighted equally, the weight factors $WB(t)$, $WU(t)$, and $WN(t)$ being the numbers of undiagnosed cases at age t for hereditary bilateral, familial unilateral and isolated unilateral cases, respectively. For each age t , $f(t)$ is estimated using the optimization procedure Gemini [43] to minimize:

$$Q(t) = WB(t) \left\{ \text{obs } HB(t) - \left[\frac{e^{-m(\infty)f(t)/2} - e^{-m(\infty)/2}}{1 - e^{-m(\infty)/2}} \right]^2 \right\}^2 \\ + WU(t) \left\{ \text{obs } HU(t) - \left[\frac{e^{-m(\infty)f(t)/2} - e^{-m(\infty)/2}}{1 - e^{-m(\infty)/2}} \right]^2 \right\}^2 \\ + WN(t) \left\{ \text{obs } N(t) - 1 + f(t) + \frac{f(t) \ln f(t)}{\ln[a(\infty)/b(0)] - 1} \right\}^2$$

The predicted incidences and undiagnosed fractions at age t are then calculated from the obtained $f(t)$,

and a χ^2 test of the fit to the model is performed. For our data, $m(\infty)$ was chosen as 1, as proposed by Knudson and Strong [1]. The value used for $a(\infty)/b(0)$ was 10^{12} but this choice is not crucial because the term using this value is very small compared to the others and the value of $f(t)$ changes less than 1% when any value between 10^5 and 10^{18} is chosen for $a(\infty)/b(0)$.

We also tested the theory of two independent events by comparing our observations with the expected equations of age at diagnosis established by Knudson [44]: in the hereditary form (bilateral and familial cases): $\log_{10}S = -k_1X$; in the sporadic form: $\log_{10}S = -k_2X^2$, where S is the fraction of cases not yet diagnosed at age X , and k_1 and k_2 are two positive constants. We used polynomial regressions obtained by a BMDP regression program. Since isolated cases are most probably a mixture of hereditary and sporadic forms, we also considered the regression equation: $\log_{10}S = -k_3X - k_4X^2$. Thus we used a general regression program and compared the different models by a nested-model test. This regression analysis in an oversimplification of the biological phenomenon since it does not include any parameter for the number of cells at risk for transformation but it allows us to determine which category of cases is responsible for the rejection of the bimutational theory.

Differential Expression of the Paternal and Maternal Alleles?

This can be tested in familial cases for whom the parental origin of the chromosome where the first event is present was known, i.e., in cases where we suspect the transmission of a susceptibility gene, either WT3 [5-7], or WT1 [19, 38].

To test whether the risk of being affected is higher when the mutated allele is paternally derived as expected following two imprinting theories [33, 34], we performed classical segregation analysis as described by Morton [45] with maximum likelihood (ML) estimation. Figure 2 illustrates pedigree of the kindreds from our study. Two kindreds were not available for this analysis: in one case the questionnaire was incomplete and in another (No. 5) it was not possible to determine who the transmitting parent was. There were two probands in four families (No. 3-6). Our informative kindreds were consequently reduced to 6. By adding 8 informative pedigrees from the literature we obtained 37 sibships which were analyzed by taking into account the way they had been detected.

Single selection for the proband's sibship:

$$L_1 = \binom{s-1}{r-1} \times p^{r-1} \times (1-p)^{s-r},$$

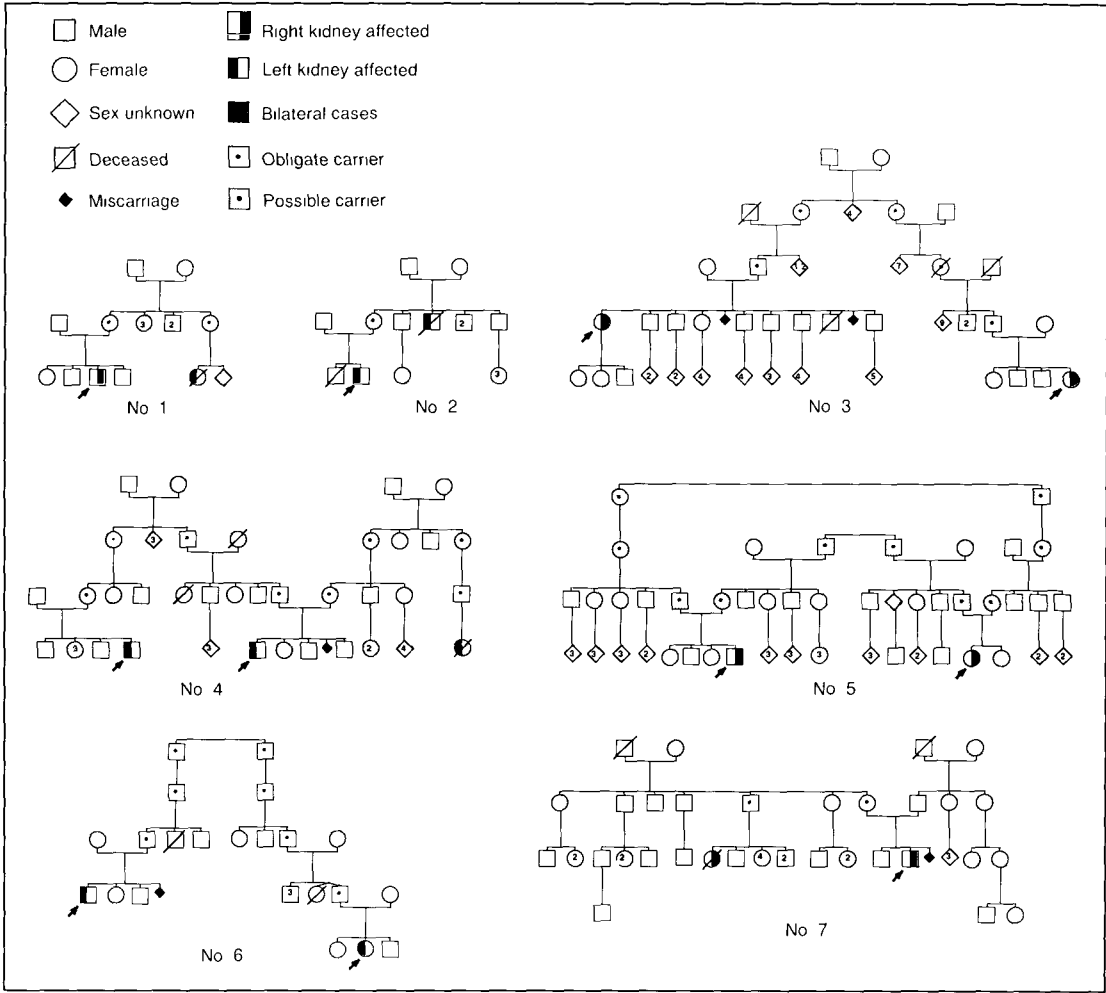


Fig. 2. Pedigrees of the familial Wilms' tumor cases in our sample.

where s is the size of the sibship, r the number of affected individuals and p the segregation ratio.

Truncate selection for other sibships of pedigrees selected through at least one affected individual:

$$L_2 = \frac{\binom{s}{r} \times p^r \times (1-p)^{s-r}}{(1-p)^s}$$

Complete selection for sibships selected through an affected or carrier parent:

$$L_3 = \binom{s}{r} \times p^r \times (1-p)^{s-r}$$

We compared the estimated segregation ratio p among individuals born to gene carrier women to that among individuals born to gene carrier men by a homogeneity test using the likelihood ratio: if L_f is the ML of p when the transmitting parent is the father, L_m the ML when the transmitting parent is the mother, and L the ML for all sibships, then minus twice the natural logarithm of the ratio $L/(L_f \times L_m)$ asymptotically follows a χ^2 distribution with 1 d.f.

Since Wilms' tumor always occurs during childhood, the probability that a gene carrier is affected depends on his or her age. This is why it would be interesting to take the age of each individual of the sibship

into account but the data from the literature concerning age of the individuals were not sufficient to perform this analysis.

The models of Wilkins [33] and Reik and Surani [34] predict an earlier diagnosis in the cases of paternal transmission. We tested this hypothesis by comparing age at diagnosis according to the sex of the transmitting parent, using a classical t test.

Results

Number of Independent Events

The comparison of age at diagnosis with respect to laterality and family history shows a significantly earlier diagnosis for bilateral cases than for isolated unilateral ones ($p < 0.001$ by rank sum test) which is in agreement with results of other authors [1–3, 46–48]. Familial cases are diagnosed significantly earlier than isolated unilateral ones ($p = 0.005$ by rank sum test) and significantly later than bilateral cases ($p < 0.001$). The three classes are thus significantly different for age at diagnosis.

Observed and predicted distributions of the cases by age at diagnosis according to the model of Hethcote and Knudson [40] are shown in table 2 and figure 3. For the test of fit to the model, low frequency classes are combined so that the expected incidence is at least 5. All bilateral cases diagnosed at 3 years or later were pooled, and similarly all familial cases diagnosed at 6 years or later, and all isolated unilateral cases diagnosed at 10 years or later. The deviation between observed and expected values is significant ($\chi^2 = 21.06$, d.f. = $21 - 15 - 3 = 3$, $p < 0.001$). To test the sensitivity of the method to $m(\infty)$, we performed another analysis using 3 for $m(\infty)$ but the results remained significant ($\chi^2 = 26.93$, d.f. = 2, $p < 0.001$). The two-hit model of embryonal carcinogenesis is thus rejected but we cannot determine which class of cases is responsible for this rejection.

Table 3 presents the results of the nested-model tests. According to Knudson's theory and in the hereditary form, only one somatic event is required for Wilms' tumor. We thus expect a linear regression for the familial and bilateral cases. In familial cases, comparison between residuals (in fact between F ratios) in the regressions shows that, as expected, the best model is linear ($Y = -0.19 X$, fig. 4). In bilateral cases however, we obtained a quadratic regression ($Y = -0.06 X^2$) which is an unexpected finding. It is possible that this quadratic regression is due to a heterogeneity among bilateral cases. In fact, distribution of

Table 2. Observed and expected distributions of Wilms' tumor cases by age at diagnosis

Age, years:	<1	1	2
<i>Bilateral cases (n = 41)</i>			
Incidence (O)	13	7	4
HB(t) (O)	1	m	0.683
HB(t) (E)	1	0.676	0.332
Incidence (E)	13.28	14.1	6.11
<i>Familial unilateral cases (n = 64)</i>			
Incidence (O)	9	17	10
HU(t) (O)	1	0.859	0.594
HU(t) (E)	1	0.822	0.577
Incidence (E)	11.39	15.68	9.54
<i>Isolated unilateral cases (n = 463)</i>			
Incidence (O)	55	100	66
N(t) (O)	1	0.881	0.665
N(t) (E)	1	0.877	0.674
Incidence (E)	56.95	93.99	65.75
Calculated f(t)		0.1257	0.3325

HB(t) = Fraction of bilateral cases not yet diagnosed at age t; HU(t) = fraction of hereditary unilateral cases not yet diagnosed at age t; N(t) = fraction of non-hereditary cases not yet diagnosed at age t.

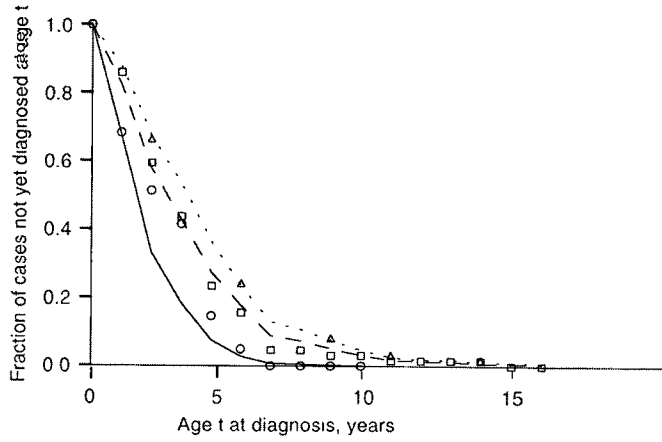


Fig. 3. Observed data and predicted curves for the undiagnosed fractions of bilateral (O, —), familial unilateral (□, ---), and isolated unilateral (△, ·····) cases.

	3	4	5	6	7	8	9	10	11	12	13	14	≥ 15
11	4	2	0	0	0	0	0	0	0	0	0	0	0
	0.415	0.146	0.049	0	0	0	0	0	0	0	0	0	0
	0.183	0.075	0.028	0.008	0.005	0.003	0.001	0	0	0	0	0	0
	4.43	1.93	0.82	0.12	0.08	0.08	0.05	0	0	0	0	0	0
13	5	7	0	1	0	1	0	0	0	0	1	0	0
	0.438	0.234	0.156	0.047	0.047	0.031	0.031	0.016	0.016	0.016	0.016	0	0
	0.428	0.274	0.167	0.089	0.073	0.051	0.031	0.017	0.013	0.013	0.008	0.007	0.006
	9.86	6.85	4.99	1.02	1.41	1.28	0.9	0.26	0	0.32	0.06	0.13	0.31
69	61	47	12	15	13	10	3	0	3	1	1	7	
	0.523	0.374	0.242	0.14	0.114	0.082	0.054	0.032	0.026	0.026	0.019	0.017	0.015
	0.532	0.365	0.234	0.129	0.107	0.075	0.047	0.025	0.019	0.019	0.012	0.011	0.009
	77.32	60.65	48.61	10.19	14.82	13.96	10.19	2.78	0	3.24	0.46	0.93	4.16
	0.4749	0.6392	0.7668	0.8685	0.8897	0.9206	0.9479	0.9691	0.9749	0.9749	0.9816	0.9832	0.9852

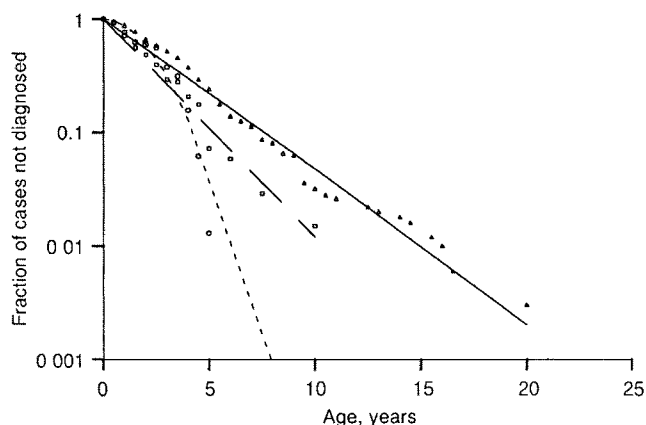


Fig. 4. Semilogarithmic plot of the data and estimated curves for bilateral (○, ·····), familial unilateral (□, ---), and isolated unilateral (△, —) cases with Wilms' tumor.

age at diagnosis for bilateral cases (fig. 5) seems to be bimodal. We tested this hypothesis using the Skumix method [49] which maximizes the likelihood of several kinds of distributions (one normal, two normal, one skewed or two skewed distributions). The models were then compared by a nested-model test and, effectively, the best model is a mixture of two skewed distributions corresponding to respectively 40% (11.23 ± 4.73 months) and 60% (43.00 ± 10.13 months) of the bilateral cases. A possible explanation for this bimodal distribution is that early-onset bilateral cases (diagnosed before 2 years of age) are hereditary whereas late-onset ones are not. Under this hypothesis, similar shapes should be observed for distribution and curves of age at diagnosis between early-onset bilateral cases and familial cases on the one hand, and between late-onset bilateral cases and isolated unilateral ones on the other. It therefore seemed interesting to compare age at diagnosis between those cases. Both comparisons lead to significant differences ($p < 0.001$ and $p < 0.01$, respectively). We also performed a regression analysis for both categories of bilateral cases. For late-

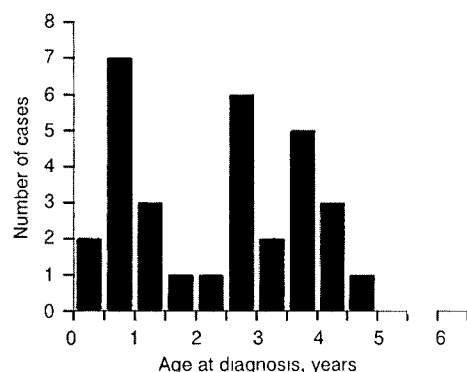


Fig. 5. Distribution of age at diagnosis for bilateral Wilms' tumor cases.

onset bilateral cases, we obtained a quadratic regression ($Y = -0.004 X^2$, fig. 6) which was expected, but we also obtained a quadratic regression for early-onset bilateral cases ($Y = -0.920 + 0.07 X - 0.001 X^2$).

In the isolated unilateral cases, we expected a quadratic model under the assump-

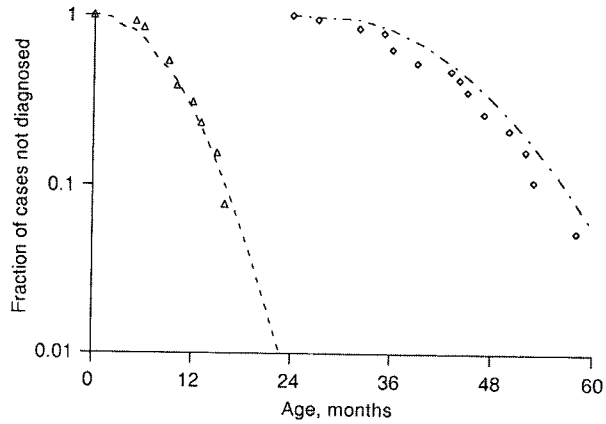


Fig. 6. Semilogarithmic plot of the data and estimated curves for early-onset (Δ , ---) and late-onset (\diamond , -.-) cases of patients with bilateral Wilms' tumor.

Table 3. Comparison of regression models by the nested model test

	Models	Residual	d.f.	SMS	F ratio
Isolated bilateral cases	$y = -0.5027x$	0.50267	9	0.05582	80.14
	$y = -0.0556x^2$	0.08653	9	0.00961	508.78
	$y = -0.0441x + 0.0661x^2$	0.07496	8	0.00937	261.61
Familial cases	$y = -0.1921x$	0.14790	12	0.01232	863.67
	$y = -0.0242x^2$	1.38844	12	0.11570	81.28
	$y = -0.1879x + 0.0006x^2$	0.14719	11	0.01338	397.80
Isolated unilateral cases	$y = -0.1314x$	0.27227	29	0.00939	5,236.10
	$y = -0.0088x^2$	5.39147	29	0.18591	236.91
	$y = -0.1412x + 0.0007x^2$	0.24491	28	0.00875	2,812.29

$y = \log$ (percent of not yet diagnosed cases); $x = \text{age}$ in years; SMS = sum of mean squares = residual/d.f.

tion of two independent events. Comparison of the F ratio between the expected quadratic form and the linear one (table 2) shows that the best model is linear. If we consider that isolated unilateral cases include a few hereditary cases and add a quadratic term to the linear form, the F ratio is still lower. This

leads us to conclude that, unexpectedly, the linear model ($Y = -0.13 X$; fig. 3) best explains the data. These findings show that the rejection of the two-hit model obtained above is not due to familial cases but to bilateral cases as well as isolated unilateral cases.

Table 4. Segregation analysis according to the sex of the transmitting parent

	P	SD ¹	-2 ln (likelihood)	
Among all cases	0.10	0.03	55.14	} 0.00 } NS
Paternal transmission	0.10	0.04	29.25	
Maternal transmission	0.11	0.04	25.89	

¹ SD of P.

Role of the Transmitting Parent in Familial Cases

The results of the segregation analysis are shown in table 4. The estimated segregation ratio is 0.10 (SE = 0.04) when the transmitting parent is a man and 0.11 (SE = 0.04) when the transmitting parent is a woman. These risks are not significantly different ($\chi^2 < 0.01$).

Comparison of age at diagnosis according to the sex of the transmitting parent shows that Wilms' tumor tends to be diagnosed earlier when the carrier parent is a male (32.00 months, SE = 23.62) than when the carrier parent is a female (40.54 months, SE = 30.21) but this difference is not significant ($p = 0.42$).

Discussion

Two different approaches were used to test the classical theory of two independent events in Wilms' tumor carcinogenesis. The first approach rests on a realistic model because it accounts for biological reality but, since it consists in fitting the whole set of data to the model, it is not possible to determine which category permits rejection of the model. The second approach, a classical regression analysis, assumes a simplifying model but isolated unilateral cases, where two events are assumed to occur, can be dealt with separately from familial and bilateral cases, where only one event is expected.

For familial cases, the linear regression is consistent with the classical bimutational theory where the first mutation is germinal and the second somatic. Segregation analysis and analysis of age at diagnosis with respect to the sex of the transmitting parent did not give any evidence for a differential expression of paternal and maternal alleles. Note that the segregation ratio ($p = 0.10$) may be overestimated because there were many affected individuals in the kindreds from the literature. Matsunaga [2] selected 'interesting' families from several authors and Huff et al. [6] and Grundy et al. [5] chose pedigrees informative for linkage analysis. However, this selection should not affect our conclusion concerning segregation ratio according to the sex of the transmitting parent since the bias should not be different in families where the mutation was transmitted by the mother or the father. These results do not provide any evidence for genomic imprinting of the inherited gene in familial cases.

For isolated unilateral cases, we could not test imprinting theories by using age at diagnosis and risk with respect to the parental origin of the chromosome where the first event occurred since we had no molecular data. However, we could search for the number of independent events using age at diagnosis on a large sample of cases. The linear regression could be explained by only one mutational event but in such a case one would not expect a later age at diagnosis than for inherited

cases. Since the analysis of age at diagnosis only points out rare events, there could also be two nonindependent events with a high probability of a second event when the first event has occurred.

For bilateral cases, the unexpected quadratic regression can be explained by the heterogeneity of cases due to a bimodality of age at diagnosis. Knudson and Strong [1] had already noticed that bilateral Wilms' tumor patients and Wilms' tumor patients with hemihypertrophy or genitourinary anomalies unexpectedly had an average age at onset and distribution suggestive of a two-event phenomenon. They suggested that, in some sporadic cases, a possible 'metabolic derangement caused by environmental forces during embryogenesis could mimic a germinal mutation and produce tumors with a high incidence of bilaterality and single-event distribution, but without the risk of affected offspring'. The exact nature of this 'derangement' still remains to be explained. Bond [46] has also observed a heterogeneity among bilateral cases according to their focality and to simultaneity of the tumors. This could be due to a mixture of hereditary cases with earlier onset and nonhereditary bilateral cases but, in such a case, one would not expect a quadratic regression for early-onset cases. A possible explanation of our results could be that each distribution corresponds to a mixture of hereditary cases, due to real germline mutations, and nonhereditary cases due to very early somatic mutations. Two different genes could be involved with a higher probability of mutation for the gene responsible for the early-onset bilateral cases. These genes could either be tumor suppressor or imprinting genes [50]. These unexpected findings suggest genetic heterogeneity among bilateral cases and that the proportion of bilateral cases which are nonhereditary is much higher than is usually assumed.

Table 5 summarizes the three models of Wilms' tumor carcinogenesis based on genomic imprinting, with their expected consequences on age at diagnosis, the risk when there is paternal transmission, and the results of our analyses. As can be seen, most expectations under Wilkins' model are not verified which leads to rejection of this model. In contrast, our data could be explained by the two other models.

On the one hand, the model of Reik and Surani [34] would fit the rejection of the independent occurrence of two somatic events in isolated unilateral cases. Indeed the involved loci (most probably WT2 in 11p15.5) could be submitted to genomic imprinting which would explain the linear regression observed in isolated unilateral cases. This would be corroborated by the nonrandom loss of maternal alleles on chromosome 11 observed in isolated cases [16, 17]. The first event would thus be a rare somatic mutation which would provoke a clonal expansion and increase the risk for the second event if it occurred on a paternally derived gene. One should be able to test this hypothesis using molecular data. One could for instance search for genomic changes in cells surrounding the tumor. Indeed, these cells should come from the clonal expansion due to the first mutational event and the parental origin of this event could then be known. Postfertilization paternal uniparental disomy (UDP) has been observed in several WBS cases [27, 51]. Thus mosaicism for UDP could account for this kind of event and be responsible for an increased risk of developing a tumor [51]. Alternatively, a test could then be performed in order to know whether the risk is higher when the first mutation occurs on the paternally derived chromosome as predicted by Reik and Surani [34]. The hereditary form of Wilms' tumor would be due to another gene, most probably WT3 and in some cases WT1 [38] since none of the

Table 5. Observed and expected findings according to genomic-imprinting theories

	Wilkins [33]	Reik and Surani [34]	Scrable et al. [35]	Results
Genes	regulatory Wg (WT1) transforming Tr (WT2)	tumor suppressor WT	imprinting IMP tumor suppressor WT	- -
Events	primary inactivation of one Wg secondary inactivation of the other Wg	primary inactivation of one paternal WT leading to clonal expansion secondary inactivation of the other WT	primary imprinting on paternal WT: inactivation secondary inactivation of the other WT	- -
Number of events	2	2	2	-
Regression				
Familial cases	linear	linear	linear	linear
Bilateral cases	linear	linear	linear	quadratic
Nonhereditary cases	quadratic	linear	linear or quadratic ²	linear
Transmitted gene in familial cases	WT1	WT	IMP	-
Risk when paternal transmission ¹	increased	increased	not increased	not increased
Diagnosis when paternal transmission ¹	earlier	earlier	not earlier	not earlier

¹ In hereditary cases.

² Depends on the proportion of cells submitted to genomic imprinting in a normal individual: linear if imprinting is frequent and quadratic if it is rare.

cases analysed in this study presented a feature of the WBS and since linkage to 11p13 and 11p15.5 was rejected in two families used for this study [5, 6]. WT3 should not be imprinted and should now be localized by further molecular investigations.

On the other hand, we can also apply the model proposed by Scrable et al. [35]. The phenomenon of genomic imprinting should be frequent enough in normal individuals so that the only rare event in Wilms' tumor car-

cinogenesis would be the somatic mutation on the tumor suppressor gene (WT1 at 11p13 or WT2 at 11p15.5). This would explain the linear regression obtained for isolated unilateral cases. In hereditary cases, the gene inherited as the susceptibility gene (WT3) would be identical to IMP and would not be a tumor suppressor gene. It would be involved in generating and maintaining genomic imprinting and would increase the number of cells submitted to genomic imprinting and, conse-

quently, the number of cells at risk for the second event at the tumor suppressor gene. Since the inherited gene (IMP) would not be submitted to genomic imprinting, the sex of the transmitting parent would have no effect, which would explain our results. This would be corroborated by the exclusion of linkage at 11p loci [5–7], and by the somatic rearrangement at 11p loci in hereditary (familial cases and WAGR) Wilms' tumor [5, 13]. The presence of two human homologues of murine imprinted genes, IGF2 [29] and H19 [30] at 11p15.5, suggests that IMP could be involved in imprinting at these loci but our results do not provide information on the exact role of these two genes in Wilms' tumor carcinogenesis. Molecular verification of this model will require evaluation of the rate of genomic im-

printing in the tissues involved. When the susceptibility gene (WT3) is identified and cloned, it should be possible to test its role on genomic imprinting.

Since no molecular data were available for this study, we are aware that we did not deal with homogeneous molecular groups, but our findings clearly indicate that Wilms' tumors arise from a variety of complex mechanisms. It is now necessary to test these hypotheses using molecular data.

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