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Concordance between Parental Origin of Chromosome 13q Loss and Chromosome 6p Duplication in Sporadic Retinoblastoma

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Summary

Two hypotheses are capable of explaining nonrandom loss of one parent's alleles at tumor suppressor loci in sporadic cases of several pediatric cancers, including retinoblastoma—namely, preferential germ-line mutation or chromosome imprinting. We have examined 74 cases of sporadic retinoblastoma for tumors in which at least two genetic events—loss of heterozygosity for chromosome 13q markers and formation of an isochromosome 6p—have occurred. Sixteen cases were found to contain both events. In 13 of 16 such tumors, the chromosomes 13q that were lost and chromosomes 6p that were duplicated are derived from the same parent. These data may be explained within the framework of the genome imprinting model but are not predicted by preferential germ-line mutation.

Introduction

Sporadic cases of some pediatric tumors exhibit preferential loss of maternal alleles at loci linked to the putative tumor suppressor gene. These include markers on chromosome 11p in Wilms tumor (Reeve et al. 1984; Schroeder et al. 1987; Grundy et al. 1988; Williams et al. 1989) and embryonal rhabdomyosarcoma (Scrable et al. 1989) and on chromosome 13q in osteosarcoma (Toguchida et al. 1989) and bilateral retinoblastoma (Dryja et al. 1989; Leach et al. 1989; Zhu et al. 1989). These observations may be explained by preferential germ-line mutation of the father's tumor suppressor gene (Dryja et al. 1989; Zhu et al. 1989) or by genome imprinting (Leach et al. 1989; Reik and Surani 1989; Scrable et al. 1989; Sapienza 1991).

The strongest evidence in favor of genome imprinting is derived from the study of sporadic and familial cases of Wilms tumor (Sapienza 1991). The genome imprinting model predicts that sporadic forms of this disease will show nonrandom loss of maternal alleles of one or more Wilms tumor suppressor genes located at chromosome 11p13 and/or 11p15 and also predicts that familial forms of the disease will not show genetic linkage to either of these locations. Both of these predictions are fulfilled by experiment (Reeve et al. 1984; Schroeder et al. 1987; Grundy et al. 1988; Huff et al. 1988). The strongest case in favor of the preferential paternal mutation model is represented by sporadic retinoblastoma (Dryja et al. 1989; Zhu et al. 1989). Bilateral cases of this disease exhibit preferential loss of maternal alleles on chromosome 13q in tumor tissue (Dryja et al. 1989; Leach et al. 1989; Zhu et al. 1989), while unilateral cases show little (Leach et al. 1989) or no (Dryja et al. 1989; Zhu et al. 1989) bias with regard to which parental chromosome 13q alleles remain in

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the tumor. In addition, all reported familial forms of retinoblastoma cosegregate with markers linked to RB-1 at chromosome 13q14 (e.g., see Cavenee et al. 1986; Scheffer et al. 1989).

Despite such strong arguments in favor of the preferential germ-line–mutation model in sporadic retinoblastoma, there is evidence in favor of a role for genome imprinting in some fraction of cases (Leach et al. 1989; Blanquet et al. 1991). Because both models predict the same experimental results for bilateral sporadic cases (namely, nonrandom elimination of one parent's tumor suppressor allele in tumor tissue), it has not been possible to distinguish which of the two models is more likely to be true in any particular set of patients.

In an attempt to circumvent this inherent problem, we have analyzed 74 sporadic retinoblastomas for those in which two genetic events have occurred: loss of alleles on chromosome 13q and duplication (see Material and Methods) of alleles at chromosome 6p. This latter event is associated with karyotypic changes (i.e., formation of chromosome 6p+ or isochromosome 6p) that occur in approximately 25%–40% of retinoblastomas (Squire et al. 1985; Turleau et al. 1985; Potluri et al. 1986; Horsthemke et al. 1989). Our reason for attempting this analysis is that the models make different predictions about the parental origin of the genetic changes when two (or more) events are considered, if relevant loci at both chromosomal locations are affected by the process of genome imprinting (Sapienza 1991, 1992; Bartolomei and Tilghman 1992). We chose to examine an event affecting chromosome 6p because both genetic and biochemical analyses of the homologous region of the mouse genome (chromosome 17) provide strong evidence for more than one imprinted locus in this region (Barlow et al. 1991; Cattanach 1991; Forejt and Gregorova 1992).

Because the formation of an isochromosome 6p is thought to be a somatic event and perhaps related to tumor progression (Horsthemke et al. 1989), the germ-line–mutation model does not predict a relationship between the event that occurred on chromosome 13q and which parent's chromosome 6p is involved in isochromosome formation; that is, the second event is independent of the first with respect to parent of origin. The genome imprinting model, on the other hand, assumes that the original cell that gave rise to the tumor bore a genome imprint. Because the imprinting process affects loci on many different chromosomes (reviewed by Cattanach 1991; Sapienza and Hall, in press), this hypothesis predicts that genetic events involving two unlinked loci will be related with respect to parent of origin, if both loci are imprinted (Sapienza 1991; Bartolomei and Tilghman 1992). If alleles at the two affected loci are imprinted in the same way, one will observe perfect concordance between the two events. If they are imprinted in the opposite way, one will observe perfect discordance between the two events. In neither case will the parent of origin of the second event occur at random with respect to the first.

We find that the two genetic events assayed in sporadic retinoblastoma are not independent with respect to parent of origin. These data are not predicted by the preferential germ-line–mutation model.

Material and Methods

DNA Isolation

Genomic DNA was isolated from fresh retinoblastoma tumor tissue, xenografts, and peripheral blood lymphocytes of patients and their parents or from lymphoblastoid cell lines, as described by Sambrook et al. (1989).

Southern Hybridization and PCR Assays

In cases where sufficient DNA was available, tumors were analyzed for 6p-allele duplication and 13q-allele loss by blot hybridization (Sambrook et al. 1989). Five to ten micrograms of DNA were digested with *Taq*I and fractionated on 0.7% agarose gels. The DNAs were transferred to nylon membranes (Magna Graph, MSI) in 20 × SSC, and the blots were then baked at 80°C for 2 h. The probes used for 6p analysis and for 13q analysis of the 16 patients discussed in this report are presented in table 1. Probes were labeled with [^{32}P]dCTP by random-primer labeling (Feinberg and Vogelstein 1983). In those cases in which only small amounts of DNA were available, relative copy numbers of alleles at the tumor-necrosis-factor (TNF) locus (Nedospasov et al. 1991) and D6S265 and and D6S273 (Weissenbach et al. 1992) loci on chromosome 6p were determined by PCR. For the TNF locus, an extensive description of reaction conditions is given by Nedospasov et al. (1991). For D6S265 and D6S273, 30 amplification cycles (95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, for each cycle, 1.5 mM $MgCl_2$) were performed. We note that it is difficult to determine *absolute* copy number of alleles by PCR methods because unequal amplification of alleles is sometimes observed. Our conclusions are therefore based on comparisons of *relative* copy number of alleles between normal and tumor DNAs from the same individual, so that any hypothetical amplification differences on the basis of allele size

Table 1

Chromosome 13q LOH and 6p Duplication in Sporadic Retinoblastomas

Tumor	No. (probe) of 13q Loci Showing Loss of Alleles	No. (probe) of 6p Loci Showing Duplication of Alleles
RB383	1 (p68)	5 (pRTV1, J819, pCH6, pHLA1.1, TNF)
RB412	2 (p68, p9D11)	3 (pRTV1, CRI-L171, CRI-L320)
RB409	2 (ED1, ESD-enzyme)	4 (pHLA1.1, pCH6, CRI-L320, TNF)
151	3 (p123 M1.8, RB1.20, p35R0.6)	1 (TNF)
280	1 (RB1.20)	2 (TNF, D6S273)
RB577	1 (p7F12)	2 (pHLA1.1, pRTV1)
312	1 (RB1-PCR)	2 (TNF, D6S265)
104	1 (p123 M1.8)	1 (TNF)
RBL-14	2 (ESD, 1E8)	2 (pHLA1.1, pRTV1)
RBL-32	2 (RB-PCR 5', 1E8)	2 (pHLA1.1, pRTV1)
235	2 (p68AS2, RB1.20)	1 (TNF)
237	1 (RB1.20)	3 (TNF, D6S265, D6S273)
314	3 (p88PR0.6, p68AS2.0, RB1.20)	1 (TNF)
270	3 (p88PR0.6, RB1.20, p35R0.6)	2 (TNF, D6S265)
RB447	1 (p7F12)	3 (pCH6, CRI-L320, TNF)
1S	1 (RB1-PCR)	1 (TNF)

or allele sequence should apply equally to both samples. We observed no tendency for either the larger or the smaller allele to be identified as "duplicated" in our samples. The results obtained by these methods can only be invalidated by preferential amplification of alleles on the chromosome *not* involved in formation of the isochromosome, such that the opposite chromosome is identified as duplicated. For example, highly selective amplification (by an overall factor of nine) of maternal alleles, when the tumor actually carried an isochromosome 6p of paternal origin, could result in identification of the incorrect parent's chromosome as being involved. However, in the 11 tumors for which multiple markers were informative (table 1) for determining parental origin of the additional copies of chromosome 6p markers (all 11 of these tumors included at least the TNF PCR marker, and 3 of the tumors included at least two markers examined by blot hybridization), there were no disagreements in the identification of parental origin of alleles obtained with any informative marker. Because the number of tumors observed to contain "duplicated" 6p alleles by our assays is very close to the number that would be predicted on the basis of cytogenetic studies, it also seems unlikely that we could have systematically excluded some tumors with an isochromosome 6p in their karyotype.

Definition of 6p Amplification

The intensity of the autoradiographic signals of polymorphic alleles in blot hybridization and PCR experiments was determined by densitometry using an Ultroscan XL Laser densitometer (LKB). The signal intensities of the tumor alleles were normalized to the signal intensities of the alleles from lymphocytes. Several exposures of each autoradiogram were scanned to ensure that the signals were in the linear response range. Comparison of data from blot hybridization and PCR analyses with cytogenetic data for the same tumors demonstrated that copy number may be accurately determined by these methods. Accurate determination of the presence of an isochromosome 6p by similar methods has been reported elsewhere (Horsthemke et al. 1989). As a control for detection of allele amplification by PCR of alleles at the TNF locus, we established a calibration curve through a mixing experiment (fig. 1). The DNAs of a heterozygous child and his homozygous parent were mixed in different proportions to obtain allele ratios of 2:1 and 3:1 (corresponding to karyotypes +6p and isochromosome 6p, respectively). This calibration revealed that signal intensity does not increase in direct proportion to an increase in number of copies of the allele (fig. 1). We considered an allele as amplified if its signal was at least 1.6 times more intense than the signal of the other allele. This factor represents the lower limit of measurements on control samples with an allele ratio of 3:1. Repetitive calibrations (seven independent experiments; see fig. 1) showed that there was no overlap between densitometric values determined for allele ratios of 2:1 or 3:1 as compared with allele ratios of 1:1. However, we did observe overlap between

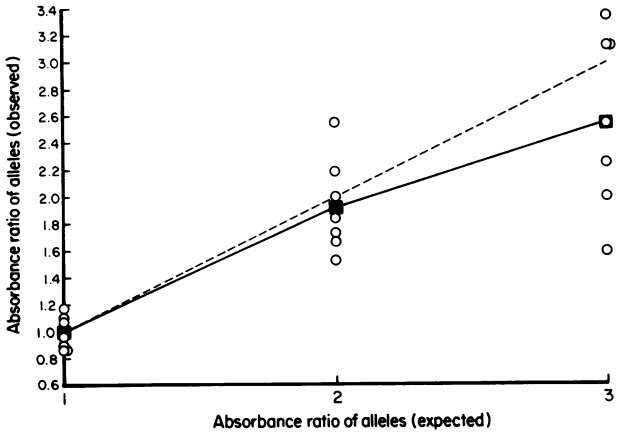

Figure 1 Results of densitometry of PCR of alleles at the TNF locus dinucleotide repeat in mixing experiments (see Material and Methods). Blackened squares are mean values of seven separate and independent determinations (unblackened circles) observed for each allele ratio. Dashed line represents theoretical, expected relationship. Solid line has been drawn through the mean of observed values.

densitometric values for allele ratios of 2:1 as compared with 3:1. Consequently, we are not able to unequivocally state whether the tumors analyzed solely by this method correspond to karyotypes of +6p or isochromosome 6p. It is also not possible to determine by this method whether a tumor is mosaic for an isochromosome 6p. For these reasons, we refer to these events as "duplication."

Fisher's Test

The formula for calculating the probability, P, of obtaining any set of values in a two-by-two matrix of the form

		Event 2		
		Variable 1	Variable 2	Total
Event 1	Variable 1	a	b	n1
	Variable 2	c	d	n2
	Total	n3	n4	N

is

$$P = \frac{n1! \times n2! \times n3! \times n4!}{N! \times a! \times b! \times c! \times d!}.$$

This formula calculates the probability that two events are unrelated with respect to the variables measured (Fisher 1934; Langley 1971). The more familiar χ^2 statistic cannot be used because of our relatively small sample size (Langley 1971) and our lack of rigorous criteria for assigning expected values to each compartment. The validity of Fisher's test is not restricted by assumptions concerning expected values (Langley 1971).

Definition of Cases

Fifteen of the cases in tables 1, 4, and 5 and 73 of the cases in tables 2 and 3 are true sporadic cases, and none of the patients has any affected relatives. Patient 1S has a cousin with bilateral retinoblastoma, but neither the father of patient 1S nor his sister (mother of the affected cousin) is affected. In addition, the tumor from patient 1S lost the paternal RB-1 allele, indicating that any predisposing mutation in this family (if it is truly a retinoblastoma family) is not genetically linked to RB-1 because the affected individuals cannot have inherited the same tumor-specific chromosome 13q haplotype (although both affected individuals may have different RB-1 mutations). This family more closely resembles "family 190" described by Dryja et al. (1993). In that family, although the informative affected individual does have an RB-1 mutation, it has occurred (apparently) independently of the other mutation present in this family. The existence of a limited number of such families may be explained on the basis of chance (Dryja et al. 1993). Even if patient 1S is excluded from the calculation of P by Fisher's method, $P = .019$ rather than $P = .013$. In either case, the hypothesis that the two genetic events are unrelated with respect to parent of origin is rejected.

Results

Our sample set consists of 74 cases of sporadic retinoblastoma. Among these 74 cases, there were 26 patients with bilateral disease and 48 patients with unilateral disease. These proportions of bilateral versus unilateral disease are in agreement with published reports (Knudson 1971; Francois et al. 1975). Parental origin of chromosome 13q alleles lost and chromosome 6p alleles duplicated was determined by a combination of blot hybridization and PCR techniques using polymorphic DNA markers (e.g., see figs. 1–3, table 1, and Material and Methods).

The overall results for loss of heterozygosity (LOH) at chromosome 13q with respect to parental origin are shown in table 2. Fifty cases were informative, and 39 (78%) of these exhibited LOH, while 11 did not. We were able to determine which parent's alleles were eliminated in all 39 cases. In agreement with two previous

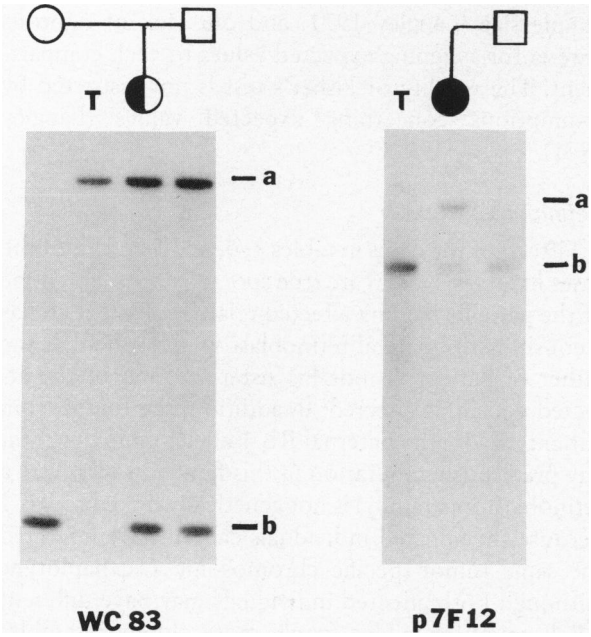

Figure 2 Determination of parental origin of 13q LOH in retinoblastomas by blot hybridization. Blots were hybridized with probes wc83 and p7F12. T = tumor. "a" and "b" indicate bands corresponding to polymorphic alleles. In both cases the maternal alleles are lost.

reports (Dryja et al. 1989; Zhu et al. 1989), we observed significant bias in retention of paternal alleles in tumors from bilateral but not from unilateral patients.

Results for duplication of alleles at chromosome 6p are given in table 3. Sixty-six cases were informative, and 29 of the patients showed a significant increase in copy number of alleles at 6p loci. Five of these cases were initially selected on the basis of karyotypic analy-

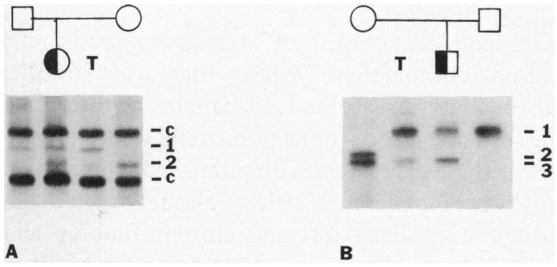

Figure 3 Determination of parental origin of duplicated 6p alleles in retinoblastomas. *A*, Blot hybridization of *Taq*I-digested DNA samples from mother's, father's, and patient's lymphocytes and tumor, with chromosome 6p probe CRI-L320. "c" indicates constant bands. "1" and "2" indicate bands corresponding to polymorphic alleles. The paternal allele is duplicated in the tumor. *B*, PCR of TNF locus dinucleotide repeat. PCR products were separated on a 5% denaturing polyacrylamide gel. "1," "2," and "3" indicate bands corresponding to polymorphic alleles. The paternal allele is duplicated in the tumor.

Table 2

Loss of 13q Alleles in 74 Cases of Sporadic Bilateral and Unilateral Retinoblastomas

Allele Lost	No.
Bilateral:	
Maternal	11
Paternal	3
Heterozygous	2
Not informative[a]	10
Unilateral:	
Maternal	12
Paternal	13
Heterozygous	9
Not informative[a]	14
Total:	
Maternal	23
Paternal	16
Heterozygous	11
Not informative[a]	24

NOTE.—13q LOH for some samples has been reported elsewhere (Dryja et al. 1989; Leach et al. 1989; Zhu et al. 1989). We have repeated the analyses in all reported cases in which sufficient DNA was available and found no discrepancies between our results and those reported elsewhere.

[a] Or insufficient DNA for analysis.

Table 3

Duplication of 6p Alleles in 74 Cases of Sporadic Bilateral and Unilateral Retinoblastomas

Allele Duplicated	No.
Bilateral:	
Maternal	7
Paternal	4
None detected	13
Not informative	2
Unilateral:	
Maternal	9
Paternal	9
None detected	24
Not informative	6
Total:	
Maternal	16
Paternal	13
None detected	37
Not informative	8

Table 4

Parental Origin of Chromosome 13q LOH and 6p Duplication in 16 Sporadic Retinoblastomas

Tumor (type[a])	13q	6p
RB447 (U)	M	M
RBL-32 (U)	M	M
151 (U)	M	M
RB409 (B)	M	M
104 (B)	M	M
312 (B)	M	M
RBL-14 (U)	M	M
270 (U)	M	M
280 (B)	M	P
RB383 (U)	M	P
237 (U)	M	P
RB412 (U)	P	P
314 (U)	P	P
1S (B)	P	P
RB577 (U)	P	P
235 (U)	P	P

NOTE.—M = maternal; and P = paternal.

[a] U = unilateral retinoblastoma; and B = bilateral retinoblastoma.

ses, indicating the presence of an isochromosome 6p (and all of these showed 6p duplication by our assay criteria). The observed frequency of 6p duplication in unselected cases was 39%, which is in good agreement with previously published karyotypic analyses of retinoblastoma tumors (Squire et al. 1985; Turleau et al. 1985; Potluri et al. 1986). We observed no significant difference between duplication of maternally versus paternally derived 6p alleles in either bilateral or unilateral cases.

The data sets for LOH at chromosome 13q and amplification of chromosome 6p are, individually, unremarkable. However, the purpose of our study was to determine whether the two genetic events assayed are unrelated. On the basis of chance, one predicts that 78% × 39% × 50 (number of cases in which we had information on both events), or 16 tumors, will have suffered both genetic events. We observed 17 cases in which both events occurred. Of these, we could unequivocally determine the parental origin of both events in 16 cases.

Each tumor may fall into one of four categories: both events paternal, both events maternal, chromosome 13q LOH maternal and chromosome 6p duplication paternal, or chromosome 13q LOH paternal and chromosome 6p duplication maternal. Data for each of the 16 informative cases are shown in table 4, and the observed distribution of the tumors among the four categories is shown in table 5.

If the two genetic events (i.e., LOH for chromosome 13q and amplification of chromosome 6p) are unrelated with respect to the measured variables (e.g., parent of origin), one may precisely calculate the probability of obtaining the observed distribution by applying Fisher's test (Fisher 1934; Langley 1971). The null hypothesis tested is that two events (i.e., the genetic changes on two different chromosomes) are unrelated with respect to the variables measured (i.e., parent of origin). The calculated probability (see Material and Methods) of obtaining the data in table 5 is $P = .013$. We therefore reject the hypothesis that the two genetic events are unrelated with respect to parent of origin.

Discussion

The observed concordance between the parental origin of genetic events occurring on two different chromosomes is not predicted by the preferential germline–mutation model. Even if one were to concede the possibility that mutation of an Rb-1 allele occurred in the germ line of one of the parents (generally the father), there seems to be no explanation, within this model, for the tumor cell–specific selection of one parent's chromosome 6p as the substrate for the formation of the (presumed) isochromosome.

Simple chromosome imprinting models are capable, in part, of explaining the data; that is, if portions of the paternal genome on both chromosomes 13q and 6p were inactivated by imprinting, then one would expect elimination of the active (maternal) Rb-1 allele in tumor tissue and amplification of the active (maternal), rather than the inactive (paternal), growth-promoting/tumor-progression gene on chromosome 6p. We do not intend to imply a mechanistic relationship between genome imprinting, LOH for chromosome 13q markers, and

Table 5

Distribution of Sporadic Retinoblastoma Cases among Event/Parental Origin Categories

	6p DUPLICATION	
	Maternal	Paternal
13q loss:		
Maternal	8	3
Paternal	0	5

NOTE.—Probability of obtaining this distribution is .013.

isochromosome formation. Our explanation for the parental origin interdependence of the two events is based on the imprinting of alleles on both chromosomes but also on the random occurrence (with respect to which parent's chromosome) of genetic events resulting in chromosome loss or duplication. We assume that only events that result in a change in cellular phenotype will be selected as a tumor; that is, neither the elimination of an already inactive RB-1 allele nor the duplication of an inactive tumor-progression gene would result in a selectable change in cellular phenotype. Such events may occur with equal frequency, but they would not be observed as tumors.

Another hypothesis, one derived from recent evidence from tumors of the colon (Aaltonen et al. 1993; Ionov et al. 1993; Thibodeau et al. 1993), is the possibility that either parent has a "mutator" phenotype. If we assume that most mutations result in the inactivation of a gene, then the prediction of this model is that the "mutator" parent's RB-1 allele will remain in the tumor and the "nonmutator" parent's chromosome 6p will be duplicated. This hypothesis is also consistent with the data we have obtained; that is, there will be concordance of parental origin between *loss* of alleles on 13q and duplication of alleles on 6p. However, it should be noted that tumors from patients with unilateral disease, as well as tumors from patients with bilateral disease, exhibit such concordance. If we consider only the patients with unilateral sporadic disease from table 4 and apply Fisher's test, the probability that the two events are unrelated with respect to parental origin is .045. Because patients with unilateral sporadic disease are generally thought to be somatic mosaics for RB-1 mutations (and 6p duplication is clearly a somatic event in the tumors we have analyzed; see Material and Methods), whatever mutator activity might be responsible for these changes must operate postfertilization and in a parental origin–specific manner. In other words, invoking the existence of a mutator phenotype does not free one from the requirement for parental origin specificity.

If an imprinting model applies, then our observation that there are five tumors in which both genetic events occur on the paternal chromosomes (tables 4 and 5) rather than the maternal chromosomes must be ascribed to chance, to "reverse imprinting," or to some unknown process. Because we observe five tumors in the paternal 13q/paternal 6p category and no tumors in the paternal 13q/maternal 6p category, the probability of observing this distribution by chance (i.e., the probability that the parental origin of the 6p event is unrelated, in light of the parental origin of the 13q event) is $(.5)^5 = .03$. It therefore seems unlikely that this result has been obtained by chance.

A reverse-imprinting explanation requires the existence of genetic variants at loci responsible for imprinting. While such variants have been described in the mouse (Forejt and Gregorova 1992; Sapienza et al. 1992), there is no conclusive evidence for their existence in the human, although the genetic behavior of some human diseases may be the result of such variants (Naumova and Sapienza 1994 [in this issue]; Sapienza and Hall, in press). The testing of these and other models awaits the biochemical isolation of genes responsible for parent-of-origin–dependent differences in the expression of imprinted loci.

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