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Molecular Etiology of Low-Penetrance Retinoblastoma in Two Pedigrees

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Summary

In one family with low-penetrance retinoblastoma, a germ-line deletion is shared by affected and unaffected, obligate carriers. The deletion encompasses exon 4 of the retinoblastoma gene and corresponds to a mutant protein without residues 127–166. In a second family, RFLP analysis shows that two distant relatives have independently derived mutations. These families, together with others reported elsewhere, indicate that attributes of alleles at the retinoblastoma locus specify penetrance.

Introduction

Hereditary retinoblastoma generally behaves as an autosomal dominant trait, with almost 50% of the offspring of an affected individual also developing the retinal cancer. The trait is due to any of a number of *null* alleles at the retinoblastoma locus within chromosome band 13q14. The tumors themselves arise from retinal cells that have lost the function of the homologous, wild-type allele through any of a variety of somatic mechanisms (e.g., point mutation, mitotic recombination, chromosomal nondisjunction, or inappropriate methylation) (Cavenee et al. 1983; Godbout et al. 1983; Dryja et al. 1984; Greger et al. 1989; Sakai et al. 1991*b*). Although the exact quantities are unknown, both the frequency of somatic loss of the remaining normal allele and the number of retinal cells that are able to proliferate in response to its absence are sufficient to ensure that about 90% of individuals with a germ-line *null* allele will develop at least one retinoblastoma. The actual number of tumors, (i.e., the number of sensitive retinal cells that lose the homologous allele) in a patient is thought to be determined by a stochastic process, since the proportions of carriers with either zero, one, two, three, etc., tumors follow a Poisson distribution (Knudson 1971).

This dogma explaining hereditary retinoblastoma as an accidental, one-step process (in addition to the inherited germ-line mutation) is tested by the existence of infrequent families with "low-penetrance" retinoblastoma. The first account of this phenomenon was 33 years ago in the *Journal,* when Macklin described a series of large pedigrees with relatively few and distantly related affected members (Macklin 1960). Other examples have subsequently been reported (Strong et al. 1981; Connolly et al. 1983; Onadim et al. 1990, 1991, 1992; Sakai et al. 1991*a;* Munier et al. 1992). In most cases, the basis for low-penetrance retinoblastoma has been obscure until recently. Speculative explanations for the low penetrance include the possibility that the incidence of somatic mutation, recombination, and nondisjunction is extremely low in some families, encouraging the retention of the remaining normal allele in all retinal cells in carriers of a germ-line mutation. Another possibility is that additional genetic events beyond the homozygous inactivation of the retinoblastoma gene are required to produce a retinoblastoma in some individuals. Alternatively, in some families there may be either a second retinoblastoma locus operating by a different mechanism to predispose weakly to this cancer or perhaps one or more "modulator" genes that reduce the risk in lucky carriers (Matsunaga 1978, 1980).

Here we describe two kindreds with low-penetrance retinoblastoma. Analysis of one of these supports the

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concept of "weak" alleles at the retinoblastoma locus as causes for this entity, since the affected members and the obligate, asymptomatic carriers share a deletion that encompasses a single exon of the retinoblastoma gene leading to an in-frame loss of 40 codons. The second family's low-penetrance retinoblastoma has a conceptually different origin. RFLP analysis shows that two distantly related, affected individuals do not share a germ-line mutation. We present calculations supporting the prediction that additional families exist in which two affected relatives do not share a germ-line mutation. It appears that the genetics of low-penetrance retinoblastoma, like that of nonhereditary retinoblastoma and "normal penetrance" hereditary retinoblastoma, is explained by defects of the retinoblastoma gene rather than by loci elsewhere in the genome or by other mechanisms.

Methods

Families were ascertained through the ophthalmological practices of the authors. DNA was purified from blood and tumor samples and was screened for deletions by using Southern blot techniques and probes derived from the cloned retinoblastoma locus. Previously reported intragenic DNA polymorphisms distributed along the length of the 180-kb transcriptional unit (Bookstein et al. 1988; Wiggs et al. 1988; Yandell and Dryja 1989) were also analyzed according to standard methods.

The sequence analysis of exon 23 of the retinoblastoma gene has been reported elsewhere (Yandell et al. 1989). To determine the sequence of the deletion breakpoints in family 327, the junction was directly sequenced after amplification by PCR using the following primers: sense, 5′-TGCAGCAGTTGACCTAGATGAG-3′; and antisense, 5′-GAACTGCTGGGTTGTGTCAA-3′. For the amplification, we used 100–500 ng of genomic DNA as a template in a solution of 20 mM Tris pH 8.4, with 50 mM KCl, 1.0 mM $MgCl_2$, 0.02 mM of each of four dNTPs, and 0.5 units of *Taq* DNA polymerase. Thirty-five cycles of PCR were performed with an annealing temperature of 52°C and a denaturing temperature of 93°C.

Results

Two pedigrees, identified as 327 and 190, form the basis for this study and will be presented separately.

Pedigree 327—Low-Penetrance Retinoblastoma Caused by a Deletion Encompassing Exon 4

The propositus of this family is a 20-mo-old male who was diagnosed with unilateral retinoblastoma at 11 mo of age (member IV-1 in fig. 1). Southern blot analysis of DNA derived from this patient's tumor revealed two abnormalities, both of which were heterozygous. One abnormality was a novel *Taq*I restriction fragment in the vicinity of exons 18–27 that was seen with a 3.8-kb, *Eco*RI, cDNA fragment as a probe (Friend et al. 1986; McGee et al. 1989). Subsequent direct genomic sequencing of exon 23 revealed a C-to-T transition in codon 787, causing the codon to change its specificity from arginine (CGA) to stop (TGA) (data not shown). We designate this mutation as "Arg787-End." This abnormality has been described elsewhere as a new germ-line mutation in an unrelated case of retinoblastoma (Yandell et al. 1989).

The second abnormality found in the tumor DNA was a set of novel restriction fragments with the enzymes *Taq*I, *Xba*I, *Sac*I, or *Pst*I identified with probes derived from the region around exons 3–5. A comparison of the sizes of the novel restriction fragments with the normal restriction map in this region suggested a deletion of approximately 4–5 kb beginning a few hundred bases 3′ of exon 3. The suspected deletion junction was amplified from the tumor DNA by PCR using as primers a sense oligomer derived from exon 3 and an antisense oligomer from exon 5. Although the wild-type genomic fragment bounded by these primer sequences is 5,211 bp, the primers amplified a 656-bp fragment from the tumor DNA, but not from normal individuals. A comparison of the sequence of this mutant fragment with the wild-type sequence in this region revealed a deletion of 4,555 bp with both breakpoints having the sequence AACT (fig. 2). One copy of this 4-bp sequence was retained, and one was removed by the deletion. The 5′ deletion breakpoint was 414 bp downstream of exon 3, and the 3′ breakpoint was 137 bp upstream of exon 5. This deletion should eliminate only exon 4. Removal of this 120-bp exon from the mRNA transcript would be predicted to delete codons 127–166.

Of these two mutations, the Arg787End was not present in leukocyte DNA from this patient, whereas the deletion of exon 4 was present heterozygously in the patient's leukocyte DNA. Analysis of 20 of the patient's relatives revealed 9 who carried this deletion, as indicated in figure 1. One of these carriers (III-3) had a unilateral, regressed retinoblastoma diagnosed in adulthood only after his identification as a carrier by DNA analysis. Seven of the identified carriers (I-11, II-2, II-4, II-7, III-1, III-5, and III-10), as well as some obligate carriers whose DNA was not available for analysis (e.g.,

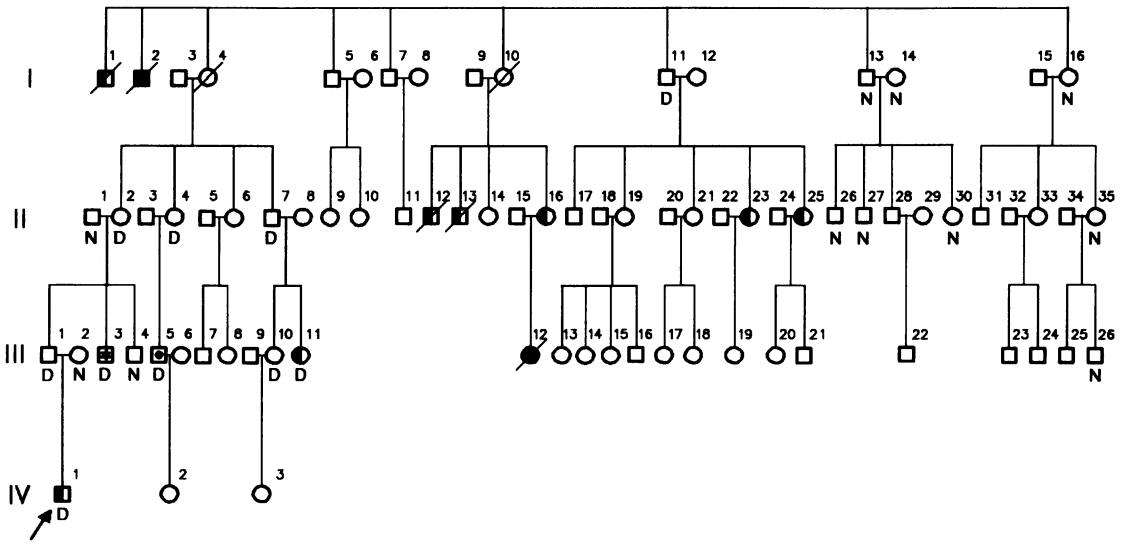

Figure 1 Structure of pedigree 327. Half-blackened symbols denote unilateral retinoblastoma, and completely blackened symbols denote bilateral retinoblastoma. The asterisk (*) within the square denotes regressed retinoblastoma, and the dot within the square denotes cutaneous melanoma. "D" and "N" signify, respectively, that an individual does carry or does not carry a deletion of exon 4, by analysis of leukocyte DNA. The arrow indicates the proband.

I-4 and I-10), did not develop retinoblastoma. One identified carrier without retinoblastoma (III-5) developed cutaneous melanoma.

Pedigree 190—"Pseudo-Low-Penetrance" Retinoblastoma Caused by Two Independent Germ-Line Mutations in Distant Relatives

The two affected members in this kindred, III-1 and IV-9, are first cousins, once removed (fig. 3). We were unable to procure blood samples from individuals II-1, II-2, II-3, and III-1. Blood samples were obtained from the affected individual IV-9, her two siblings, her parents, and her maternal grandmother. Analysis of these samples with five intragenic RFLPs within the retinoblastoma gene is summarized in figure 3. The affected patient IV-9 lacks a paternally derived allele. Since the intensity of bands hybridizing to probes from the retinoblastoma gene was reduced on Southern blots by approximately one-half compared with that in normal individuals (fig. 4), we concluded that patient IV-9 carries a deletion of the paternally derived allele. RFLP analysis demonstrates that the remaining allele in this patient is derived from the maternal grandmother, II-4. These results conclusively indicate that patient IV-9 has a deletion of the retinoblastoma gene and shares no allele at this locus with her affected cousin.

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                                        39975
                                          :
5'-deletion breakpoint:   5'-GTTTTAACTAGAGGATATTATAACTAAATGCTTTTTTTCAGATTAT-3'
                            ************************  * * * * *   ***
     junction fragment:   5'-GTTTTAACTAGAGGATATTATAACTTTGTTTTATAATGCTATATAT-3'
                            *  * *  ** *  ************************
3'-deletion breakpoint:   5'-TAAATTGGGAAAATCTACTTGAACTTTGTTTTATAATGCTATATAT-3'
                                          :
                                        44531
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Figure 2 Sequences of deletion breakpoints in family 327. The underlined bases AACT are present at both the 5′ and 3′ breakpoints. One copy of this tetramer is retained and one is deleted from the mutant allele. The asterisks (*) indicate identical bases between two sequences. The numbers above and below denote the nucleotide positions within the retinoblastoma gene, based on the complete genomic sequence of the gene (J. Toguchida, personal communication; GenBank accession no. L11910).

Discussion

The genetics of at least seven families with low-penetrance retinoblastoma have been described elsewhere, and in each case a defect in the retinoblastoma gene has been implicated. Strong et al. (1981) described a low-penetrance family that segregated an insertional translocation involving 13q14. The unaffected carriers had a balanced karyotype or partial trisomy; the affected individuals had the deleted chromosome 13q but not the balancing insertion on chromosome 3p12. Connolly et al. (1983) showed that the locus conferring predisposition to low-penetrance retinoblastoma in a large Canadian pedigree was closely linked to the esterase D gene within 13q14. Since there is close linkage between the esterase D and the retinoblastoma loci (Sparkes et al. 1980; Bowcock et al. 1988; Haines et al. 1988), it is likely that the affected and unaffected carriers in this

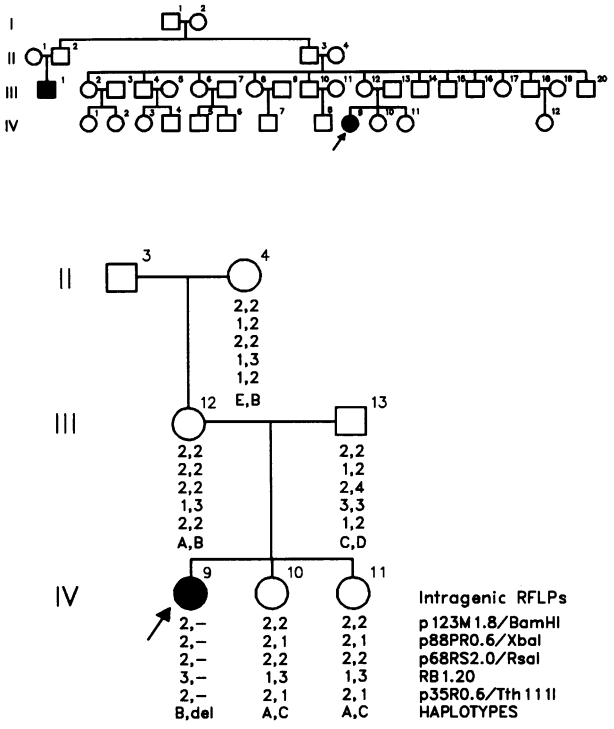

Figure 3 *Above,* Structure of pedigree 190. Blackened symbols denote bilateral retinoblastoma. The arrow indicates the proband. *Below,* Results of analysis of a branch of pedigree 190, with intragenic RFLPs. Probe/enzyme combinations are described in other publications (Bookstein et al. 1988; Wiggs et al. 1988; Yandell and Dryja 1989) and are listed according to their position, 5′ to 3′, within the retinoblastoma gene (McGee et al. 1989). Beneath each set of RFLP alleles are the haplotypes (*uppercase letters*) deduced by the segregation of RFLP alleles in the family. Del = deletion. The arrow indicates the proband.

kindred share a mutant allele at the retinoblastoma locus. Other low-penetrance pedigrees demonstrating cosegregation of predisposition to retinoblastoma with intragenic RFLPs have subsequently been reported (Onadim et al. 1990, 1991; Munier et al. 1992). Sakai et al. (1991*a*) described two low-penetrance families with point mutations in the promoter region of the gene that substantially reduced but presumably did not completely eliminate transcription. Finally, Onadim et al. (1992) described two low-penetrance families, one with a germ-line missense mutation (Arg661Trp) and the other with a change that might interfere with the RNA splicing of exon 20 (Gln675End). Both of these mutant alleles were assumed to code forms of the retinoblastoma protein with poor but nevertheless partial tumor-suppressive function.

In each of these families the retinoblastoma locus is implicated either by linkage or by the direct identification of a mutation. This is also the case in the two families described in the present paper. In one of the families (family 327), the allele shared by all affected members and obligate carriers is a 4,555-bp deletion that encompasses exon 4 of the retinoblastoma gene. Absence of this exon from the transcript would delete 40 amino acids near the amino terminus of the protein. This region is not within the E2F binding domain, the region that likely specifies one of the important tumor-suppressive functions of the protein (Cao et al. 1992; Helin et al. 1992; Kaelin et al. 1992; Shirodkar et al. 1992; Weintraub et al. 1992). It is reasonable to propose that the mutant protein encoded by this allele has weak tumor-suppressive function. This property is probably common to the other low-penetrance alleles that have been sequenced. These results indicate that low penetrance in hereditary retinoblastoma is a property conferred by the mutant allele segregating in a family and is not due to a second retinoblastoma locus or to hypothetical host resistance genes (Matsunaga 1978).

Pedigree 327 is the first low-penetrance pedigree to be described in which a tumor from an affected family member has been available for analysis. The tumor from the propositus retained heterozygosity, while the allele presumably without the germ-line deletion has a nonsense mutation within exon 23 that eliminates the carboxy-terminal 140 amino acids (of a total of 928

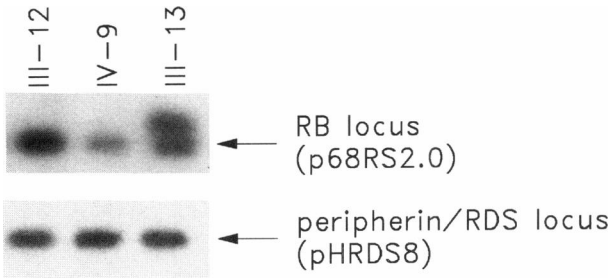

Figure 4 Southern blot analysis of *Rsa*I-digested leukocyte DNA from patients III-12, IV-9, and III-13 in pedigree 190. The top set of bands are those hybridizing with the probe p68RS2.0, a genomic fragment derived from intron 17 of the retinoblastoma gene that recognizes a VNTR-type polymorphism (Wiggs et al. 1988). The intensity of the hybridizing band is less in IV-9 than in III-12 and is comparable to that of one of the allelic hybridizing bands of III-13. The same blot was reprobed with a cDNA fragment derived from the peripherin/RDS locus assigned to human chromosome 6p (Travis et al. 1991). The intensity of hybridizing bands to this control probe (bottom set of bands) is roughly equivalent in the three lanes, indicating approximately equal loading of DNA. These results are similar to those obtained with other probes derived from the retinoblastoma gene and are consistent with a deletion of the retinoblastoma gene in patient IV-9.

encoded by the open reading frame). The absence of homozygosity for a low-penetrance allele is in accord with a previously published conjecture (Sakai et al. 1991*a*) that retinal cells homozygous for a low-penetrance germ-line mutation have sufficient tumor-suppressive capability to proceed with normal retinal development. On the other hand, retinal cells with a *null* mutation of the homologous allele would be effectively hemizygous for the germ-line mutation. Such cells should possess less tumor-suppressive activity than do homozygous cells, perhaps less than is sufficient to prevent a retinoblastoma (assuming that there is no compensatory overexpression of the remaining allele). A testable prediction of this conjecture regarding tumors in low-penetrance retinoblastoma is that homozygosity for the germ-line mutation will not be observed. Indeed, the requirement for a *null* allele as the second "hit" might be the primary factor reducing the penetrance of retinoblastoma, since the somatic recombination or nondisjunction that instigates approximately 70% of retinoblastomas overall would be ineffectual in producing a tumor. If this line of reasoning is valid, then measurements of the tumor-suppressive function of proteins encoded by low-penetrance alleles might allow an estimation of the threshold activity of the retinoblastoma protein that distinguishes benign from malignant cells. In fact, it might be possible to define a low-penetrance allele as one that will not cause a retinoblastoma if homozygous, but only if the homologous allele is made *null* by a second mutation.

An alternative explanation of the lack of homozygosity in tumors from families with low-penetrance retinoblastoma is that the chromosomal mechanisms resulting in the development of homozygosity in somatic cells are poorly operative in some kindreds—and that this deficiency demands a second mutation of the homologous wild-type allele for a tumor to develop. This hypothesis would be in accord with the host-resistance model of variable penetrance, proposed in the *Journal* in 1978 by Matsunaga (1978). However, if it were true, then one would expect that the mutation spectrum of low-penetrance, germ-line alleles would parallel that found in "normal penetrance" retinoblastoma. This is evidently not the case, since short, in-frame deletions or missense mutations are rarely found in retinoblastomas overall. Instead, the mutations found in most retinoblastomas are usually large deletions, nonsense errors, or splice-site errors that obviously result in the absence or complete inactivation of the encoded protein (Friend et al. 1986; Lee et al. 1988; Dunn et al. 1989; Yandell et al. 1989; Hogg et al. 1992).

The spurious low-penetrance in pedigree 190 has a different basis, although still related to defects in the retinoblastoma gene. Two distant relatives have separate mutations of the retinoblastoma gene. In her landmark paper describing low-penetrance retinoblastoma, Macklin (1960) considered the likelihood of such an occurrence to be exceedingly small. She wrote: "It is possible that in a rare instance two persons supposed to have derivatives of the same mutant gene from some common ancestor, are, in fact, instances of independent mutations. That this should occur as frequently as it appears to have done in this group of families seems to be most improbable" (Macklin 1960, p. 38). In fact, it may not be so improbable. In seven of the low-penetrance pedigrees reported by Macklin, only two distantly related, affected relatives were identified of scores who were traced. In one such family with only two affected members, 395 members were ascertained, while in another family, with four affected sibships, over 4,000 members were included in the analysis! Table 1 shows the results of calculating, using the Poisson approximation to the binomial distribution, the chance of having two or more affected relatives with retinoblastoma due to separate initial mutations in a family of a given size. The likelihoods appear small; however, if one arbitrarily divides the population of the United States (approximately 250,000,000 people) into families of each respective size, one can calculate, again using the Poisson approximation, the most likely number of families with two or more independently affected individuals (table 1).

Inspection of the table shows, for example, that if one divides the United States into families of 20 members each (the approximate size of pedigree 190), then there should be approximately six families with two cases of retinoblastoma caused by independently derived initial mutations (new germ line or somatic). Among kindreds of 300 members, there should be almost 100 with two or more independent cases of retinoblastoma. The actual number of such families available for ascertainment is probably much larger, for the following reasons: First, the estimates in table 1 make the assumption that the population of the United States is discretely divided into kindreds of each family size. However, the actual division of a population into family groups is not so precise. Overlaps between kindreds increase the recombinatorial complexity and thereby increase the estimate of families with two independent cases of retinoblastoma. Second, for these estimations the current population of the United States was used as a base. However, in tracing a pedigree, ancestors or relatives outside the United States and living long ago would be counted. Third, when one "traces" a pedigree

Table 1

Likelihood of Two Independently Affected Cases of Retinoblastoma in a Family, as a Function of Family Size

Size of Family	Probability of Two or More Independent Cases of Retinoblastoma	Most Likely No. of Such Families in the United States
5	3.12×10^{-8}	1
10	1.25×10^{-7}	3
15	2.81×10^{-7}	4
20	5.00×10^{-7}	6
30	1.12×10^{-6}	9
40	2.00×10^{-6}	12
50	3.12×10^{-6}	15
60	4.49×10^{-6}	18
70	6.11×10^{-6}	21
80	7.98×10^{-6}	24
90	1.01×10^{-5}	28
100	1.25×10^{-5}	31
150	2.80×10^{-5}	46
200	4.97×10^{-5}	62
250	7.75×10^{-5}	77
300	1.11×10^{-4}	92

NOTE.—This table lists the likelihood of two independently affected cases of retinoblastoma in a family (middle column) as a function of family size (left column). The right column gives an estimate of the number of such families in the United States, assuming that one divides the population (250,000,000) into "kindreds" of each respective family size. Since the overall incidence of retinoblastoma is approximately 1:18,000 (Devesa 1975), and since both hereditary (germ-line initial mutation) and nonhereditary (somatic initial mutation) cases would be scored, the incidence of independent cases of the disease is approximately 1:20,000, if one excludes the 10% of new cases who inherit a mutant allele from an affected parent. The Poisson approximation to the binomial distribution was used (Rosner 1986), i.e., $P(0) = e^{-np}$ and $P(k+1) = [np/(k+1)]/P(k)$, giving the probability of $k+1$ independent cases of retinoblastoma, with n being the number of family members and p set at 1/20,000, the likelihood of independently developing retinoblastoma. For the middle column, the value $1 - P(0) - P(1)$ is given. For the right column, the same formula was used, but with n equal to the number of families in the United States of each family size and p set at the value in the middle column.

by questioning one affected branch, there is an inherent bias toward ascertaining branches of the pedigree with affected relatives and ignoring branches in which the disease did not occur. Hence, the actual family size surveyed in the memories of the subjects is probably larger than what is reported to the interviewer. Because of these factors, the most likely number of families with two or more independent cases is probably much higher in practice than what is listed in table 1.

The results from two families with low-penetrance retinoblastoma presented here, together with those reported earlier, strongly support the idea that all cases of familial retinoblastoma, be they low penetrance or more typical penetrance, are due to mutations at the same locus. There is as yet no molecular evidence for variation in penetrance due to host resistance genes or other extraneous factors. Rather, it appears that penetrance is predetermined by the nature of a mutation at the retinoblastoma locus, with a low-penetrance allele generally being one encoding either little functional protein or a protein with minimal but not zero tumor-suppressive activity. Finally, because of the relatively high mutation rate at the retinoblastoma locus, there should be numerous instances, in any large human population, of extended kindreds with two or more independently derived cases of retinoblastoma. These families mimic those with low-penetrance retinoblastoma and should be distinguished from them for genetic counseling purposes.

Acknowledgments

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