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Genetics

Oncogenic point mutations in exon 20 of the *RB1* gene in families showing incomplete penetrance and mild expression of the retinoblastoma phenotype

(single-strand conformation polymorphism/tumor-suppressor gene)

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ABSTRACT **The retinoblastoma-predisposition gene, *RB1*, segregates as an autosomal dominant trait with high (90%) penetrance. Certain families, however, show an unusual low-penetrance phenotype with many individuals being unaffected, unilaterally affected, or with evidence of spontaneously regressed tumors. We have used single-strand conformation polymorphism analysis and PCR sequencing to study two such families. Mutations were found in exon 20 of *RB1* in both cases. In one family a C → T transition in codon 661 converts an arginine (CGG) to a tryptophan (TGG) codon. In this family, incomplete penetrance and mild phenotypic expression were observed in virtually all patients, possibly indicating that single amino acid changes may modify protein structure/function such that tumorigenesis is not inevitable. In the second family the mutation in codon 675 is a G → T transversion that converts a glutamine (GAA) to a stop (TAA) codon. However, this mutation also occurs near a potential cryptic splice acceptor site, raising the possibility of alternative splicing resulting in a less severely disrupted protein.**

Retinoblastoma (RB) is an intraocular eye tumor with an incidence of 1 in 15,000–25,000 (1). It occurs predominantly in children under the age of 2 years and is rare over the age of 5 years. Approximately 15% of all RB patients have a prior family history and the tumor phenotype segregates as an autosomal dominant trait in most cases (1). In ≈10% of families, unaffected individuals can be identified who can transmit the mutant gene (1). This phenomenon is referred to as "incomplete penetrance."

Knudson (2) demonstrated that, in cases of hereditary RB, a single, additional, random genetic event was required for tumor development, and he provided one explanation of the phenomenon of incomplete penetrance by suggesting that these patients formed part of a Poisson distribution where, by chance, the second random mutation did not occur. These patients, however, still carry germ-line mutations and their children have a 50% chance of inheriting the predisposing mutation. Knudson's "two-hit" hypothesis also predicted that mutant gene carriers would develop mostly multiple, bilateral tumors with an earlier age of onset when compared with sporadic cases, which would be mostly unilateral, unifocal, and present later in life. However, the distribution of cases of incomplete penetrance is not entirely random and families have been reported where the majority of gene carriers are either unaffected or only unilaterally affected (3–6). These we refer to as "low-penetrance" families. Another feature of RB is that it sometimes apparently regresses spontaneously, leaving characteristic scars on the retina (4, 5). An alternative suggestion is that these scars represent a more benign form of the disease, retinoma (7). Identification of unaffected mutant gene carriers has, until recently, required that they have affected children, but use of classical linkage analysis and restriction fragment length polymorphisms (RFLPs) from within the *RB1* gene (8, 9) has allowed unequivocal identification of cases of incomplete penetrance in RB families (5, 6). In addition, patients with typical retinal scarring and a strong family history also carry a mutant *RB1* gene (5). This heterogeneity is difficult to interpret in terms of the Knudson two-hit hypothesis, especially when the majority of gene carriers are only unilaterally affected. We suggest that there are alleles of the *RB1* gene that are only partially defective and, as a consequence, give rise to a mild or incompletely penetrant phenotype. With the cloning of *RB1* (10), it should now be possible to determine the nature of mutations in low-penetrance families to establish whether they affect the gene in a distinct way or simply reflect random mutation events throughout the gene.

RB1 has a complex structure with 27 exons varying in size from 31 to 1873 base pairs (bp) (11) and two very large [>33-kilobase (kb)] introns. The sequences of intron regions immediately adjacent to each exon have been determined (11), allowing amplification of individual exons by the polymerase chain reaction (PCR). This approach has already been used to identify mutations in RB tumors (12). Such a screening program can proceed more rapidly with analysis of each exon of *RB1* by single-strand conformation polymorphism (SSCP) analysis (13). This procedure depends on the sequence dependence of the migration of a single-stranded DNA molecule in a nondenaturing polyacrylamide gel. Hence, mutations affecting a DNA sequence will lead to a conformational change affecting mobility and produce novel bands on gels.

We have used standard linkage analysis in RB families to identify cases of incomplete penetrance and have undertaken an exon-by-exon SSCP analysis combined with PCR sequencing to identify the specific mutation responsible for mild phenotypes in two low-penetrance families.

MATERIALS AND METHODS

PCR Amplification. Two primers were used to amplify a 350-bp fragment including the entire 146 bp of exon 20 and flanking intron sequences. The 5′ primer (no. 9438), 5′-TTCTCTGGGGGAAAGAAAAGAGTGG-3′, was located in intron 19 and the 3′ primer (no. 14928), 5′-AGTTAACAAG-TAAGTAGGGAGGAGA-3′, was located in intron 20. For direct sequencing from PCR products either the biotinylated version of primer 9438 (no. 18322) or a biotinylated internal primer, 5′-CATGATTTGAAAAAAATCTACTTG-3′ (no.

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Abbreviations: RB, retinoblastoma; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism.

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17295), was used with primer 14928. The 17295/14928 primer pair amplifies a 269-bp fragment. This particular primer set was also used to amplify exon 20 for *Msp* I digestion in family RBF29.

PCR was carried out essentially as described by Hogg *et al.* (14) with 30 cycles of denaturation at 94°C for 20 sec, annealing at 60°C (for the 9438/14928 primer pair) or 52°C (for the 17295/14928 primer pair) for 20 sec, and extension at 72°C for 60 sec.

SSCP and Sequencing. Detailed methods for SSCP and direct sequencing from PCR products have been described (14). For SSCP, exon 20 was amplified using primers 9438 and 14928 in a PCR mixture with 1 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; Amersham; 1 Ci = 37 GBq) added and a nonradioactive dCTP concentration of only 0.02 mM. Denatured samples were electrophoresed in nondenaturing 6% (wt/vol) polyacrylamide/10% (vol/vol) glycerol gels. For direct sequencing, primers 18322/14928 or 17295/14928 were used. Primers 18322 and 17295 were biotinylated at the 5′ end to allow immobilization of single-stranded DNA on streptavidin-coated magnetic Dynabeads (Dynal; Merseyside, UK), which were used to separate the DNA strands (14). Both single strands were sequenced by using a Sequenase kit (United States Biochemical) according to the manufacturer's instructions.

RESULTS

Included in our SSCP analysis of a large number of patients with hereditary RB were two families that showed an unusual pattern of inheritance in that many individuals had "mild" forms of the disease or were unaffected gene carriers. The pedigrees of these two "low-penetrance" families, RBF29 and RBF18, are given in Fig. 1. In both cases, abnormal banding patterns were seen in the SSCP gel for exon 20.

RBF29. Linkage analysis for family RBF29 (Fig. 1) was reported previously (5). In this family there are six affected individuals (I.1, I.3, II.2, II.5, III.3, III.4), as well as four unaffected gene carriers (II.4, II.7, III.1, III.2; arrows in Fig. 1) who were identified by using the RS2.0 polymorphism (5).

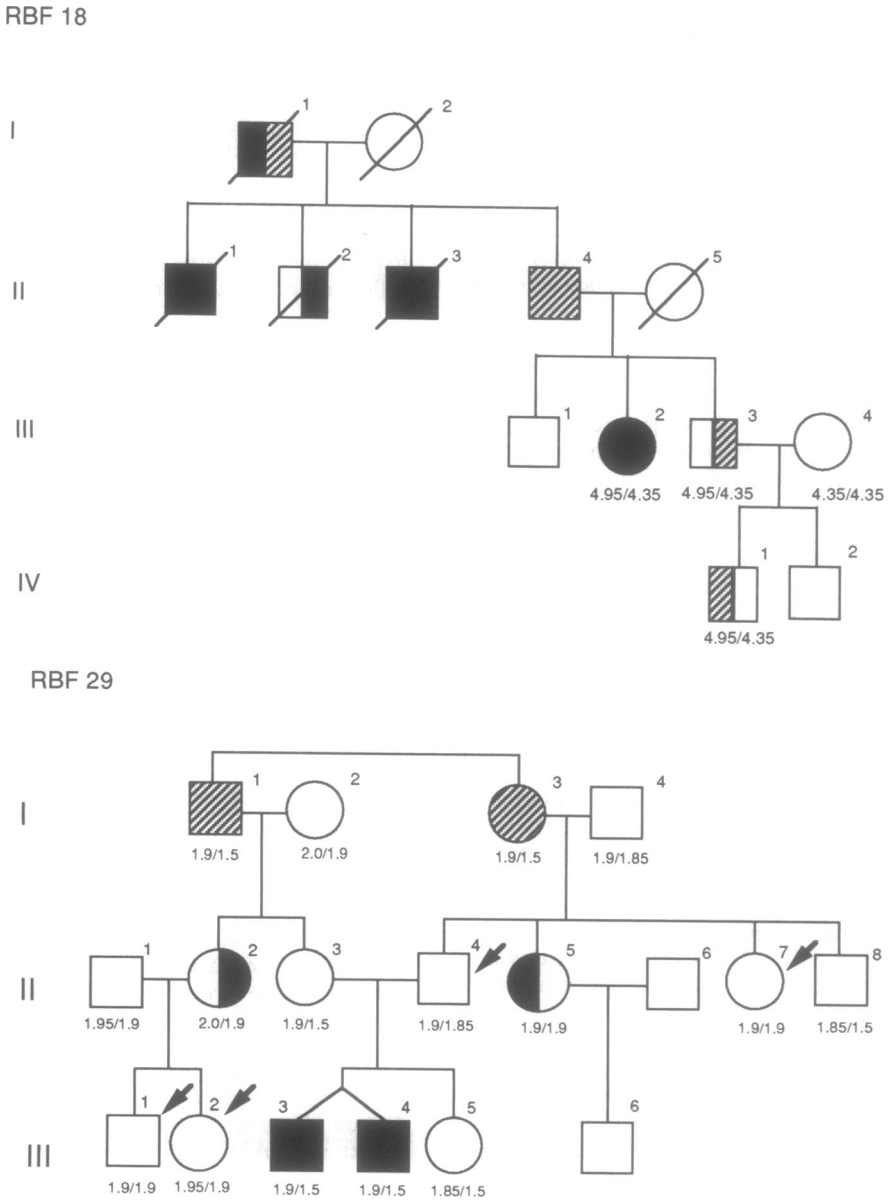

FIG. 1. Family pedigrees for RBF18 and RBF29. Arrows indicate asymptomatic gene carriers. For individuals whose DNA was available for analysis, the numbers below the symbols indicate the sizes (kb) of the allelic fragments (R0.6 polymorphism in family RBF18 and the RS2.0 polymorphism in family RBF29). Individuals with regressed tumors are indicated by hatching.

The parents (II.3, II.4) of the bilaterally affected twins (III.3, III.4) are first cousins and each has a unilaterally affected sister (II.2, II.5). The father of the twins (II.4) is an unaffected gene carrier as are individuals II.7, III.1, and III.2. After the birth of (nonidentical) affected twins, the eyes of the grandparents, I.1 and I.3, were examined and spontaneously regressed tumors were identified in both.

Our preliminary screening program involved exon-by-exon analysis of the *RB1* gene using SSCP in conjunction with PCR sequencing. Extra bands were identified in exon 20 in the DNA of individual II.4 from RBF29 in SSCP gels (Fig. 2) and not in any of the other (20 out of 27) exons analyzed. In the SSCP gel shown in Fig. 2 the 350-bp fragment encompassing exon 20 amplified from II.4 exhibited an additional lower band when compared with samples from other patients. Sequence analysis of exon 20 from II.4 in RBF29 (Fig. 3) revealed a heterozygous C → T transition in the coding strand, 21 bases from the 5′ end of exon 20. This mutation converts codon 661 from an arginine (CGG) to a tryptophan (TGG) codon. The same mutation was identified in the DNA of all the affected members and unaffected gene carriers in RBF29, but not in the unaffected members of the family. The mutation in RBF29 occurs within an *Hpa* II/*Msp* I restriction site in exon 20 (CCGG → CTGG), so that the presence of an undigested 269-bp fragment indicates a mutant gene carrier (Fig. 4). The undigested 269-bp band was observed in the DNA of all affected members and unaffected gene carriers, whereas only the two smaller normal bands were seen in individuals from this family identified not to be gene carriers (Fig. 4). DNA from III.5 was obtained for prenatal screening from chorionic villus (CV) tissue and from cord blood samples (B) taken from the same individual at birth. Exon 20 from III.5 was also sequenced and found to be free of the 661 mutation, confirming our original prediction made with linkage analysis (15). *Msp* I digestions of exon 20 DNAs from 34 unrelated RB patients and 38 unrelated healthy individuals did not show this mutation.

RBF18. Family RBF18, first reported by Hine (16), shows eight affected individuals in the family (Fig. 1), three of whom (II.2, III.3, IV.1) had unilateral disease. Four generations of males (I.1, II.4, III.3, IV.1) have a mild form of the disease. Although I.1 had one eye removed for RB, the tumor in his other eye regressed naturally. II.4 has spontaneously regressed tumors in both eyes and III.3 and IV.1 have unilateral regressed tumors. II.1 and II.3, however, died at the age of 1 year 9 months and 3.5 years, respectively, as a result of orbital extension (16) of their tumors. I.1 and II.2 died of second tumors at the age of 61 and 40, respectively. Only DNA from individuals III.2, III.3, III.4, and IV.1 was available to us. The RB predisposition is linked to the 4.95-kb allele in the R0.6 polymorphism (6).

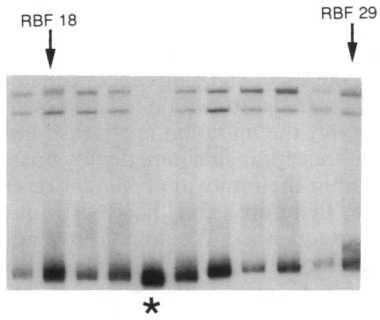

FIG. 2. SSCP analysis of exon 20 from a number of RB patients and controls as well as members from families RBF18 and RBF29 (lanes marked as such). DNA was amplified by using primers 9438 and 14928. A 350-bp PCR product was generated and electrophoresed in a nondenaturing 6% polyacrylamide/10% glycerol gel at 30 W at room temperature for 6 hr. Lane RBF18 (individual III.3) shows an additional upper band, and lane RBF29 (individual II.4) exhibits an additional lower band (near the bottom of the gel), compared with samples from other patients and from controls. An undenatured sample was included in the fifth lane to indicate the position of double-stranded DNA (*).

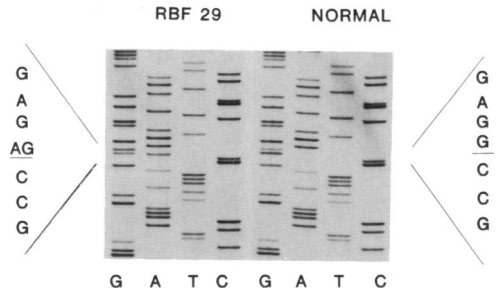

FIG. 3. Sequence from the biotinylated (5′) strand of exon 20 showing the heterozygous G → A transition in RBF29 (*Left*) compared with the same sequence from a normal individual (*Right*).

SSCP analysis of DNA from III.3 from RBF18 showed an additional upper band (Fig. 2) when compared with other samples. Sequence analysis of exon 20 from III.3 (Fig. 5) revealed a heterozygous G → T transversion in the coding strand, 63 bases from the 5′ end of exon 20. This mutation converts codon 675 from a glutamine (GAA) to a stop (TAA) codon. However, 3 bp downstream of this mutation lies a TAG sequence (Fig. 6), which is compatible with a consensus splice acceptor sequence (17). The G → T transversion removes an AG dinucleotide (converting it to AT) that ordinarily would have prevented the downstream TAG sequence from becoming a splice acceptor site. In addition, this transversion increases the pyrimidine/purine ratio in the region immediately preceding the TAG site, thereby enhancing its potential to be a cryptic splice site (18). A branch-point sequence exists (Fig. 6) upstream of this cryptic site, which, although not as good as the real branch point in intron 19, would nevertheless be adequate if this site were activated. In this case, the reading frame would be intact but the first 23 amino acids encoded by exon 20, codons 654–676, would be lost. Such a deletion would disrupt the leucine zipper motif in exon 20, removing 3 of the 4 leucines. The same mutation was identified in the DNA of III.2 and IV.1, who were known to carry the RB-predisposition gene. III.4, who is an unaffected normal individual, has the normal sequence. This G → T mutation does not alter any known restriction enzyme site.

DISCUSSION

There have been few reports of mutations within the *RB1* gene in patients with RB, and those mutations that have been

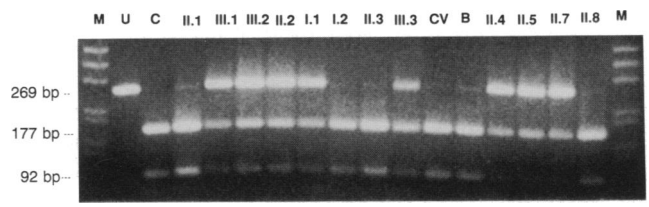

FIG. 4. Restriction enzyme analysis of the mutation in exon 20 from family RBF29. DNA was amplified by using primers 17295 and 14928 to generate a 269-bp fragment. *Msp* I digestion of this fragment results in two fragments, 177 bp and 92 bp long (lanes C, II.1, I.2, II.3, CV, B, and II.8). Individuals carrying the G → A mutation, which destroys the *Msp* I site, display the uncut 269-bp fragment (lanes III.1, III.2, II.2, I.1, III.3, II.4, II.5, and II.7). Lanes: M, 1-kb marker (GIBCO/BRL); U, uncut control 269-bp fragment; C, control sample known to be homozygous for the 177-bp and 92-bp fragments; CV, chorionic villus DNA from II.3; B, cord blood DNA from III.5.

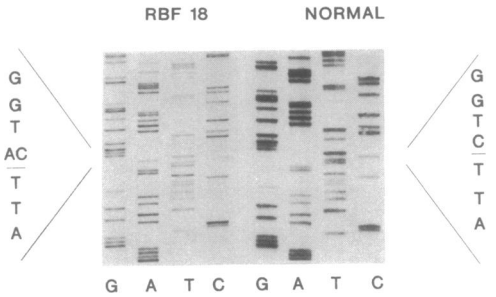

FIG. 5. Sequence from the biotinylated (5′) strand of exon 20 showing the heterozygous C → A transversion in RBF18 (*Left*) compared with the same sequence from a normal individual (*Right*).

described seem to be randomly distributed throughout the gene (12, 19). The majority of these mutations in bilaterally, multifocally affected individuals cause major disruptions of the gene and its consequent processing, resulting in nonproduction of the RB protein (20). The exact role of *RB1* in tumorigenesis is still not fully understood, but its product, RB1, seems to be part of a signaling pathway controlling cell proliferation (21). The RB1 protein interacts with the transforming oncoproteins of DNA tumor viruses (22–24). The domains of RB1 that bind these proteins are encoded by exons 13–17 and 18–22, amino acids 393–572 and 646–772, respectively (25, 26). It has been suggested that, with appropriate folding of RB1, a "pocket" is created that facilitates binding to the viral transforming proteins and endogenous cellular proteins (27). The observation that *RB1* mutations exist that produce proteins which fail to bind viral proteins or to associate with endogenous cellular proteins has led to the suggestion that this pocket contributes to the growth-regulatory function of RB1 (27–30).

Recently, mutations in the promoter region of *RB1* were detected in two families with low-penetrance phenotypes (31). A plausible explanation for this is that promoter mutations may result in reduced levels of RB1. In many cells the production of sufficient RB1 protects them against tumorigenesis, but occasionally a cell produces insufficient RB1, thereby escaping its normal growth control. Since promoter mutations were not found in a large number of other families

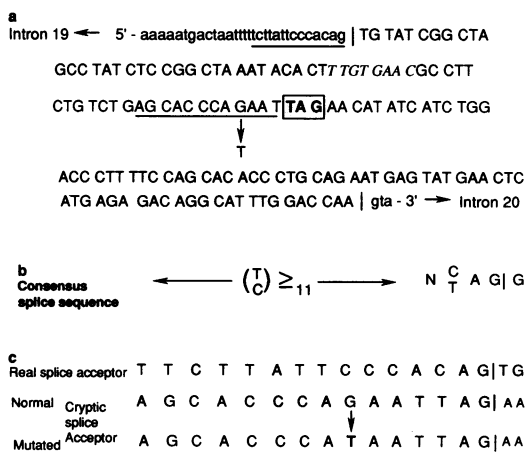

FIG. 6. Nucleotide sequence of exon 20 (uppercase) and its flanking intron sequences (lowercase) are shown in *a*. The normal splice acceptor in intron 19 is underlined, as is the cryptic splice site in the exon sequence containing the G → T transversion. The absolutely required TAG sequence of the splice acceptor (described in *b*) is boxed. A potential branch site (TTGTGAAC) located 24 bp upstream of cryptic splice site is shown in italics. In *c*, the real splice acceptor site from intron 19 is compared with the cryptic splice acceptor with and without the G → T mutation.

exhibiting a similar phenotype, one would assume that alternative possibilities exist. Subtle changes in the RB1 amino acid sequence, for example, may reduce only its functional efficiency, and only when a threshold level of activity is not maintained do tumors develop. The mutation in family RBF29 is possibly one such example with only a single amino acid change. Why, then, did the twins in this family develop multifocal disease? In 70% of tumors the initial mutation is duplicated (32, 33) in tumor precursor cells. Duplication of a "weak" mutation might still result only in a mild phenotype but if, as in 30% of tumors, the second mutation is more serious the combination could result in multifocal tumor formation. Such independent somatic mutations have been identified in tumors from bilaterally affected RB patients (19). The tumors from RBF29 family members, however, were successfully treated and so not available for analysis. Whether subtle changes anywhere in the gene would result in a mild phenotype or whether specific regions, such as exon 20, are more important is not clear. The only other amino acid substitution reported in RB tumors was in exon 18, and that patient was bilaterally affected (12).

It is interesting that the other family in our study showing a low-penetrance phenotype also has a mutation in exon 20. At first sight it appears that the mutation in RBF18 results in the generation of a stop codon, which could not be described as a mild mutation since the protein would be missing 254 amino acids at the C-terminal end. However, the mutation also alters the DNA sequence, potentially generating a cryptic splice acceptor site in that region. Under normal circumstances, in the presence of cis competition with the normal site, cryptic sites are never used. A change in the local sequence environment, however, can change the splicing pattern (34). In a cis-competition assay for splice-site selection, Reed and Maniatis (34) showed that sequences located downstream from intron 1 in the human β-globin gene splice acceptor site, for example, can have a profound effect on the efficient use of the adjacent splice site. Moreover, they found that the interaction between factor(s) present in a splicing extract and the splice sites is affected by exon sequences, which may play a key role in distinguishing between normal splice sites and cryptic splice sites located throughout pre-mRNAs. A mutation in the exon sequence, therefore, might improve the chances of recognition and/or the affinities of splicing components for the cryptic site, thus giving rise to a stronger and more stable splice complex. It is possible that under some circumstances, or in a specific cell type, the cryptic site is used either exclusively or in combination with the real site. However, many factors affect splicing and, without a functional assay, it is difficult to predict the outcome. For example, a G → T transversion in exon 22 of the *RB1* gene in the small-cell lung cancer cell line NC1-H69C simultaneously created a stop codon and a novel splice donor site (20). However, the mutation must have also influenced the normal splice acceptor site immediately upstream of exon 22, as it resulted in the removal of the entire exon.

It is possible, therefore, that the RBF18 mutation could have similar consequences. We do not have access to RNA from this family to investigate this possibility. It is also difficult to assess functional properties of protein(s) thus produced. There have been reports of shorter RB proteins, resulting from in-frame deletions of exons 20–22, with impaired biochemical properties (27, 28, 30, 35, 36). Sheffner *et al.* (37) reported that a 4-amino acid deletion resulting from a splice acceptor-site mutation in exon 20 in a cervical carcinoma cell line produced defective RB1. It is not clear, however, what the functional consequences of such protein(s) would be in a developing retinal cell.

The *RB1* gene seems to have a variety of functions, depending on the stage of development and cell type. The effect of naturally occurring *RB1* mutations on this expanding

repertoire of RB1 activities has not been investigated, but it is conceivable that particular mutations—for example, amino acid substitutions in particular regions of the gene—rather than abolishing the function of RB1, modify it so that it works less efficiently. One consequence of this modification might be that only occasionally is there insufficient RB1 to prevent tumorigenesis. Alternatively, once initiated the transformed phenotype might be overcome by subsequent adequate production of RB1—for example, through alternative splicing where stop codons are involved—resulting in regressed/benign tumors. To clarify this situation, more families of the low-penetrance phenotype need to be analyzed for their mutations.

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