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Frequency of Somatic and Germ-Line Mosaicism in Retinoblastoma: Implications for Genetic Counseling

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Summary

Although mosaicism can have important implications for genetic counseling of families with hereditary disorders, information regarding the incidence of mosaicism is available for only a few genetic diseases. Here we describe an evaluation of 156 families with retinoblastoma; the initial oncogenic mutation in the retinoblastoma gene had been identified in these families. In 15 (~10%) families, we were able to document mosaicism for the initial mutation in the retinoblastoma gene, either in the proband or in one of the proband's parents. The true incidence of mosaicism in this group of 156 families is probably higher than our findings indicate; in some additional families beyond the 15 we identified, mosaicism was likely but could not be proven, because somatic or germ-line DNA from key family members was unavailable. Germ-line DNA from two mosaic fathers was analyzed: in one of these, the mutation was detected in both sperm and leukocyte DNA; in the other, the mutation was detected only in sperm DNA. Our data suggest that mosaicism is more common than is generally appreciated, especially in disorders such as retinoblastoma, in which a high proportion of cases represent new mutations. The possibility of mosaicism should always be considered during the genetic counseling of newly identified families with retinoblastoma. As demonstrated here, genetic tests of germ-line DNA can provide valuable information that is not available through analysis of somatic (leukocyte) DNA.

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Introduction

Retinoblastoma is a malignant tumor of the retina that arises predominantly in children <7 years of age. It affects 1/23,000–1/16,000 live births (McLean 1996). Retinoblastoma has served as a prototype for hereditary cancer, in part because the genetics of the disease is explained by the action of a single gene, the retinoblastoma gene (*RB1* [MIM 180200]), within chromosome band 13ql4. Retinoblastomas arise only from retinal cells that have lost the function of both allelic copies of *RB1*. The initial mutation, which affects one *RB1* allele, can be in a patient's germ line, in which case the patient is classified as having hereditary retinoblastoma. Patients with hereditary retinoblastoma can transmit the initial mutation and the corresponding predisposition to retinoblastoma as a dominant Mendelian trait. Alternatively, the initial mutation can arise in retinal cells or their embryonic precursors and not involve the germ line. This is the genetic basis for nonhereditary retinoblastoma. Regardless of whether the initial mutation is inherited or arises somatically, the retinal cells that carry it can become malignant only if they also lose the remaining normal copy of *RB1*. This loss of the second functional *RB1* allele can be due to a separate somatic mutation (~30% of tumors [Kato et al. 1993]), hypermethylation (a small percentage of tumors [Greger et al. 1994; Ohtani-Fujita et al. 1997]), or loss of heterozygosity at syntenic loci, including *RB1*, over a large segment of 13q (~70% of tumors). The loss of heterozygosity occurs through mitotic recombination, mitotic nondisjunction, or a large deletion (Cavenee et al. 1983; Godbout et al. 1983; Dryja et al. 1984). The rate of loss of the second allele, through mutation or other mechanisms, is high enough among fetal and infantile retinal cells to ensure that at least one retinoblastoma develops in >90% of patients with an inherited initial mutation. Nonpenetrant (i.e., unaffected) carriers are either individuals who possess an initial mutation but in whom, fortuitously, no sensitive retinal cell loses the remaining wild-type *RB1* allele, or individuals who have an initial *RB1* mutation with partial function (Sakai et al. 1991*a*; Onadim

et al. 1992; Dryja et al. 1993; Kratzke et al. 1994; Lohmann et al. 1994; Cowell et al. 1996; Sterner et al. 1996; Bremner et al. 1997; Schubert et al. 1997).

Studies of the proportion of affected offspring of retinoblastoma patients who survive to adulthood have prompted geneticists to place all multifocal cases (either bilateral or unilateral) into the hereditary retinoblastoma category (Vogel 1979). Most multifocal cases are not associated with a family history of retinoblastoma, and these are assumed to represent new germ-line mutations. Unilateral (unifocal) cases associated with a positive family history are also considered to be hereditary retinoblastoma. Unilateral cases that are not associated with a previous family history of retinoblastoma can be in either the nonhereditary or the hereditary category (Briard-Guillemot et al. 1974; Vogel 1979). The proportion of unilateral, simplex cases that involve an initial mutation in the germ line has been estimated to be ~12% (Vogel 1979).

These categorizations underemphasize the possibility of mosaicism for the initial mutation either in the first affected individual in a family or in one of the parents of such a proband. Mosaicism can occur when a mutation in *RB1* arises at some point during embryogenesis; the point at which the mutation occurs during embryogenesis determines the degrees to which the various tissues carry the defect. Whereas it is usually assumed that the initial *RB1* mutation is present either in all cells (the "hereditary" type of retinoblastoma) or in only the retinal cell that immediately precedes the progenitor tumor cell (the "nonhereditary" type), a mosaic individual may have the initial mutation gene in some but not all cells, distributed among many tissue types. Significant errors can result when mosaicism is not considered during genetic counseling (Hall 1988; Paller et al. 1994). Mosaicism and its effect on genetic counseling have been described for a number of autosomal dominant and X-linked disorders, including neurofibromatosis (Lázaro et al. 1994) and Duchenne muscular dystrophy (Bakker et al. 1987, 1989; Passos-Bueno et al. 1990). However, very little information is available regarding the incidence of mosaicism in retinoblastoma and the degree to which genetic counseling should therefore be modified to account for it. We set out to determine how often mosaicism could be documented in a group of families that included one or more individuals affected with retinoblastoma.

Methods

This research was performed in accordance with the Declaration of Helsinki and was approved by the Human Studies Committees of the Massachusetts Eye and Ear Infirmary and Harvard Medical School. Cases were derived from a clinical laboratory (the Ophthalmic Genetics Laboratory at the Massachusetts Eye and Ear Infirmary) that provides genetic testing for retinoblastoma. Blood samples were obtained from retinoblastoma patients and their relatives at the Massachusetts Eye and Ear Infirmary and at other institutions. In many cases, tumor samples were obtained from affected eyes that had been enucleated as part of the treatment of the patients. Semen samples were obtained from unaffected fathers in families 139 and 262 only.

Blood samples were transported at room temperature, and tumor and semen samples were transported on dry ice. For semen samples, half of each sample was purified over a discontinuous Percoll column, and the other half was not (McClure et al. 1989; Ord et al. 1990). Purification over a Percoll column was designed to remove nonmotile cells—specifically, nonsperm cells. Each semen and purified-sperm sample was mixed with an equal amount of test yolk buffer medium (Irvine Scientific) prior to freezing and transport. DNA was purified from leukocyte nuclei, unfixed tumor fragments, semen, or purified sperm by standard methods that included treatment with proteinase K, phenol-chloroform extraction, and ethanol precipitation. DNA was stored in 10 mM Tris, pH 7.7, 1 mM EDTA, at 1°–10°C, for $\leqslant$15 years before analysis.

Five intragenic polymorphisms were analyzed to determine the transmission of alleles within families and to determine whether, in each tumor, an *RB1* allele was lost. These polymorphisms were a *Bam*HI RFLP in intron 1 (Bookstein et al. 1988), a *Sac*I RFLP in intron 2 (Sakai et al. 1991*b;* Rothberg et al. 1997), an *Xba*I RFLP in intron 17 (McGee et al. 1990), a VNTR in intron 17 (Wiggs et al. 1988), a tetranucleotide-repeat polymorphism in intron 20 (Yandell and Dryja 1989), and a *Tth111*I RFLP in intron 24 (Vaughn et al. 1990). The haplotypes deduced from the analysis of these polymorphisms were labeled with letters specific for each family. In family 26, additional RFLP markers on chromosome 13 were examined to verify the designated paternity (Squire et al. 1986; Scheffer et al. 1989).

Southern blot analysis with cDNA and genomic probes derived from the retinoblastoma locus was used to screen for gene deletions or rearrangements (Wiggs et al. 1988). Point mutations and small deletions or insertions that are beyond the resolution of Southern blot techniques were identified by means of exon-by-exon SSCP and direct genomic-sequencing techniques (Yandell et al. 1989; Hogg et al. 1992; Shimizu et al. 1994). The mutation in family 452 was discovered by direct genomic sequencing, after the size and copy number of all exons were found to be normal by multiplex PCR. Mutations are specified in this article in accordance with the numbering scheme of the *RB1* sequence in the Genome Database (http://www.gdb.org; accession number L11910) (Toguchida et al. 1993). For all tumors that

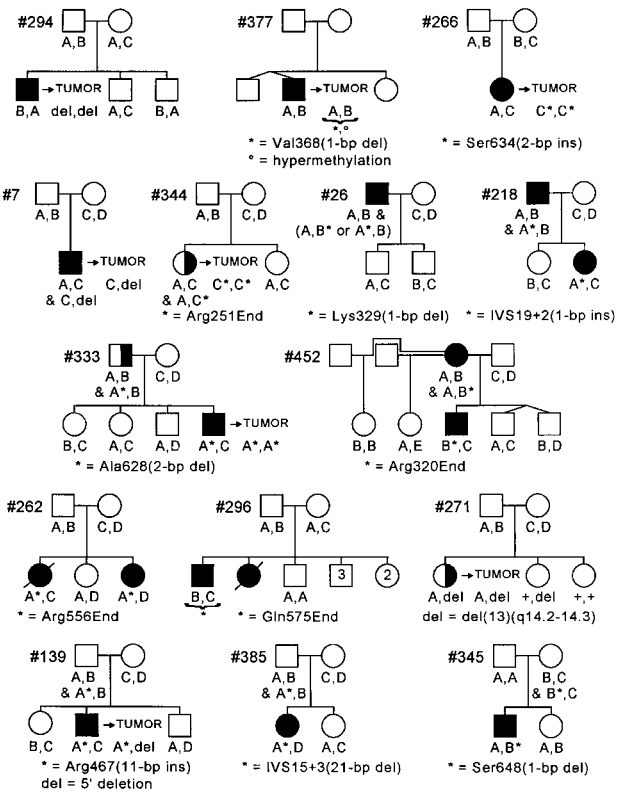

Figure 1 Pedigrees of families with one member in whom mosaicism could be documented. The family identification number appears at the upper left of each pedigree. A blackened symbol indicates an individual affected with bilateral retinoblastoma; a half-blackened symbol indicates an individual affected with unilateral retinoblastoma. The letters below each individual indicate the haplotypes at the *RB1* locus in leukocyte DNA; letters under the word "TUMOR" indicate the haplotypes that were found in tumor DNA. Alleles at intragenic polymorphisms were used to deduce haplotypes; in each family, the haplotypes were labeled alphabetically, beginning with those found in the father (unless the father was not analyzed). No haplotype letters appear below individuals who were not analyzed. An asterisk (*) or a small circle (°) indicates an allele with a mutation; the mutations to which these symbols refer are noted below each pedigree. The abbreviation "del" designates a deletion of the entire *RB1* gene, as revealed by Southern blotting, unless otherwise indicated. A plus sign (+) (family 271) indicates a normal chromosome 13, determined by karyotyping; no DNA analysis was performed on these individuals. Each pedigree is described in the text.

had loss of heterozygosity, the intensities of *RB1* gene fragments on Southern blots were evaluated, to determine whether the initial mutation was hemizygous (i.e., the second allele was deleted) or homozygous (i.e., the tumor was isodisomic for the initial mutation).

In some cases, quantitation of the proportion of normal DNA to mutant DNA in blood and sperm samples was performed. Two quantitation methods were used. The first method involved visual comparison of the intensities of the normal and mutant SSCP bands, in the sample of interest, to a scale that was constructed by mixing known quantities of DNA from samples with and from samples without the mutation. The second method was performed with computer software (IMAGE WORKS [MacIntosh version], PDI) that was used to measure the intensities of bands from digitized images that were obtained by means of a scanner.

Results

We reviewed records of 405 families who had undergone DNA analysis of *RB1* because at least one family member was affected with retinoblastoma. In 156 families, an initial mutation had been identified. In 80 of the 156 families, the first affected individual had unilateral retinoblastoma (11 from multiplex families), and in 76 families, the first affected individual had bilateral retinoblastoma (8 from multiplex families). In most cases the initial mutation was unambiguously recognized, either because it was detected in leukocyte DNA of an affected individual or because it was found to be homozygous in tumor DNA. In a few cases, the initial mutation was known only as one of two separate heterozygous mutations, identified in tumor DNA, that presumably represent heteroalleles; in each of these cases, neither mutation was in the leukocyte DNA from the patient, so we could not determine which of the two was the initial mutation. However, this ambiguity did not affect the assessment of mosaicism in any of these cases. We included eight isolate cases in which the tumors appeared to be due to homozygous hypermethylation of the promoter region of *RB1*. In reports of these and other such cases, mosaicism for aberrant hypermethylation of *RB1* has never been found, and hypermethylation of *RB1* has never been transmitted through the germ line (Ohtani-Fujita et al. 1997). We did not include five families with low-penetrance retinoblastoma, because the families were so large that the founder could not be identified (three of these families had the missense mutation Arg661Trp [Onadim et al. 1992; Lohmann et al. 1994], one had a mutation in the promoter region [Sakai et al. 1991*a*], and one had a deletion of exon 4 [Dryja et al. 1993]).

Of the 156 families with an identified initial mutation, a mosaic member could be documented in 15 (~10%). In six families, the mosaic member was an unaffected parent, and in nine families, the mosaic member was the first affected individual (seven affected bilaterally and two affected unilaterally). Nine mosaic individuals were males and five were females (in family 296, the sex of the mosaic member, who was either the father or the mother, was undetermined). Figure 1 displays schematic pedigrees of these cases and illustrates the transmission of *RB1* alleles. The cases are summarized below, in groups with similar clinical characteristics.

Bilateral Simplex Retinoblastoma without Initial Mutation Detected in Leukocyte DNA (Families 294, 377, and 266)

In family 294, unaffected parents gave birth to a child who was initially diagnosed at age 18 mo with unilateral retinoblastoma. The affected eye was enucleated. Southern blot analysis of tumor DNA revealed a homozygous deletion of the entire *RB1* gene. Analysis of leukocyte DNA revealed that the affected child had inherited nondeleted alleles from both the father and the mother; this finding suggested nonhereditary retinoblastoma. At a follow-up eye examination, when the child was 37 mo of age, a tumor was found in the fellow eye; this tumor was successfully treated without enucleation.

In family 377, unaffected parents gave birth to a child who developed bilateral retinoblastoma. Analysis of the child's tumor DNA revealed both erroneous hypermethylation of the promoter region (a somatically arising, epigenetic defect that inactivates *RB1* [Ohtani-Fujita et al. 1993; Greger et al. 1994; Ohtani-Fujita et al. 1997]) and the frameshift mutation Va1368(1bp del) (65417delT). Both gene defects were heterozygous in the tumor and were presumably allelic. Analysis of the patient's leukocyte DNA revealed neither gene defect.

The index patient in family 266 also had bilateral retinoblastoma and no family history of the disease. Analysis of the child's tumor DNA revealed the homozygous frameshift mutation Ser634(2bp ins) (153295insTC). This mutation was not detected in the patient's leukocyte DNA.

Cases of Retinoblastoma with an Initial Mutation Is Detected in Only a Fraction of Leukocytes (Families 7, 344, 26, 218, 333, and 452)

In family 7, unaffected parents gave birth to a child who was affected with bilateral retinoblastoma. Analysis of the child's tumor DNA revealed a deleted paternal allele at the *RB1* locus; no defect in the maternal allele has yet been identified. Analysis of leukocyte DNA revealed an undeleted paternal allele, but the concentration was markedly lower than that of the maternal allele.

In family 344, analysis of tumor DNA from a child affected with unilateral retinoblastoma revealed a homozygous nonsense mutation, designated Arg251End (59683C→T). Analysis of the child's leukocyte DNA demonstrated the presence of the mutation, but the ratio of mutant to wild-type sequence was less than the 50:50 ratio expected for a heterozygote.

In family 26, two unaffected children inherited different alleles from a bilaterally affected father. Mutation analysis revealed a frameshift mutation, Lys329(1-bp del) (64377delA), in the father's leukocyte DNA, but at a level that was less than the 50% expected for a heterozygote. This mutation could not be detected in the DNA of either child. Results of analysis of four additional polymorphic sites on chromosome 13 were consistent with the designated paternity.

The bilaterally affected father in family 218 had the mutation IVS19+2(1-bp ins) (153355insG), which was detected in his leukocytes at a level that was less than the 50% expected for a heterozygote. He had passed the mutation to his second daughter, who also developed bilateral retinoblastoma.

In family 333, a father affected with unilateral retinoblastoma had four children, one of whom developed bilateral retinoblastoma. Mutation analysis revealed a frameshift mutation, Ala628(2-bp del) (153276delCA), that was heterozygous in the affected child's leukocytes and homozygous in the child's tumor. Examination of the father's leukocyte DNA revealed the same mutation; however, the ratio of mutant to wild-type sequence was less than the 50:50 ratio expected for a heterozygote. Analysis of intragenic DNA polymorphisms indicated that the father had passed the same gene homologue to two unaffected, noncarrier children and to the affected, carrier child.

In family 452, a mother affected with bilateral retinoblastoma had passed the same haplotype to three children, only one of whom developed bilateral retinoblastoma. Leukocytes from the affected child carried the heterozygous nonsense mutation Arg320End (64348C→T). The mother also displayed the mutation in her blood, but the ratio of mutant to wild-type sequence was less than the 50:50 ratio expected for a heterozygote (fig. 2). The unaffected children had no evidence of the mutation in their leukocyte DNA, yet two of them had inherited the same gene homologue, as determined by analysis of intragenic RFLPs.

Affected Siblings Inherit the Same Mutation from an Unaffected Parent, and the Mutation Is Not Detected in the Parent's Leukocyte DNA (Families 262, 296, and 271)

In family 262, unaffected parents gave birth to three daughters, one of whom had retinoblastoma that involved the pineal gland and both eyes (trilateral retinoblastoma [Bader et al. 1982]), and another of whom had bilateral retinoblastoma. Both affected children heterozygously carried the nonsense mutation Arg556End (78250C→T) in their leukocyte DNA. RFLP analysis showed that both affected daughters had inherited the same paternal haplotype but different maternal haplotypes; their unaffected sibling had also inherited the same paternal haplotype, but she did not carry the nonsense mutation. The mutation could not be detected in the leukocyte DNA from either parent, but it was detected in the father's purified sperm DNA, at a level that in-

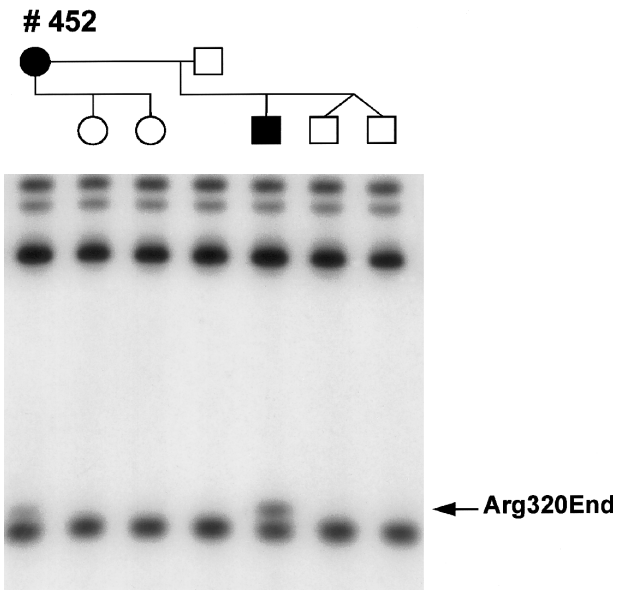

Figure 2 SSCP analysis of the nonsense mutation Arg320End, in family 452. The results of analysis of leukocyte DNA from each individual appear directly below that person's symbol. Numerous bands are present because the amplified fragment was digested with an endonuclease before SSCP analysis. The mutant fragment is indicated with an arrow at the lower right. The intensity of the mutant fragment in the mother (blackened circle) is less than in her affected son (blackened square); this reduction in intensity was also observed in quantitative sequencing (data not shown).

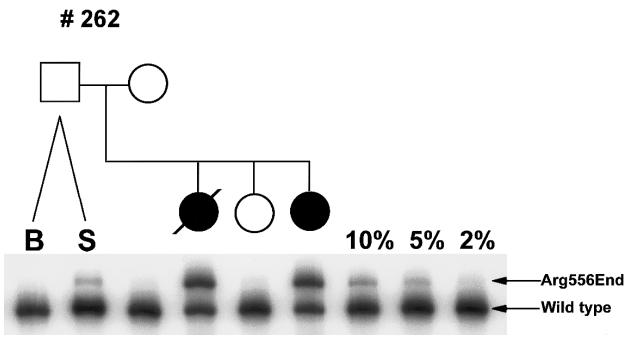

Figure 3 SSCP analysis of the nonsense mutation Arg556End, in family 262. The results of analysis of blood (leukocyte) DNA from each individual are located directly below that person's symbol, except for the father (unblackened square, upper left), under whose symbol are lanes B and S, which show results from leukocyte and purified sperm DNA, respectively. The lanes under the terms "10%," "5%," and "2%" show results from mixtures of wild-type and mutant DNA (derived from the mother and the younger affected daughter, respectively) that would contain the indicated proportions of mutant and wild-type alleles. On the basis of these scaling lanes, we estimate the proportion of mutant sperm to be ~5%.

dicated that ~5% of the sperm carried the mutation (fig. 3).

In family 296, unaffected parents gave birth to two children affected with bilateral retinoblastoma (one since deceased), as well as six unaffected children. The mutation, Gln575End (150025C→T), was detected in the blood of one of the affected children; blood from the deceased child was not available. The mutation was not detected in the blood of either parent.

In family 271, unaffected parents gave birth to three children. The first child had unilateral retinoblastoma, the second had mental retardation and facial dysmorphism, and the third was without retinoblastoma, mental retardation, or dysmorphism. Karyotype analysis revealed a deletion of 13q14 in the first two children (46,XX,del[13][q14.2q14.3]); the third child had a normal karyotype. Analysis of leukocyte DNA, from the parents and from the child affected with retinoblastoma, revealed that the mutant chromosome was maternal in origin but that the mother did not have it in a sufficient proportion of cells to reduce the intensity of either of the allelic fragments at a microsatellite polymorphism within *RB1*. Chromosome analysis of the mother was not performed.

An Affected Child Inherits a Mutation from an Unaffected Parent, and the Mutation Is Detected in Only a Fraction of the Parent's Leukocytes (Families 139, 385, and 345)

In family 139, unaffected parents gave birth to three children, one of whom developed bilateral retinoblastoma. Analysis of a tumor from the affected child revealed two presumably allelic mutations. One was a deletion that extended off the 5′ end of the gene and eliminated exon 1; the second was a frameshift, Arg467(11-bp ins), that resulted from a duplication of 11 bp in exon 15 (76900insATTATCCATTC). The frameshift mutation was present in the affected child's leukocyte DNA, but the deletion of exon 1 was not; this indicates that the frameshift was the initial mutation. The Arg467(11-bp ins) mutation was also detectable in the blood of the father, but the ratio of mutant to wild-type DNA was less than the 50:50 ratio expected for a heterozygote carrier (fig. 4). Additional evidence that the father was a germ-line mosaic came from the observation, based on intragenic RFLPs, that the father had passed the same haplotype both to the affected child and to an unaffected child who did not display the mutation. Analysis of the father's semen revealed the frameshift mutation in a proportion (20%–30%) that was similar to that found in leukocyte DNA. The same proportion of mutant DNA was found both in whole semen and in purified sperm.

In family 385, analysis of leukocyte DNA from a child affected with bilateral retinoblastoma indicated a 21-bp deletion that began at the third base of intron 15

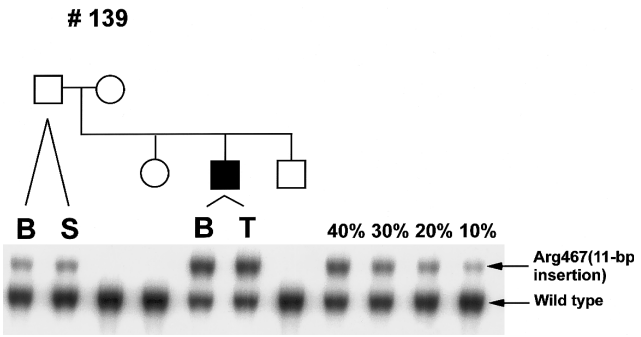

Figure 4 Analysis of the frameshift mutation Arg467(11-bp ins) in family 139. SSCP results of analysis of leukocyte DNA appear directly below the symbol for each individual, except for lanes under the father (unblackened square) and the affected son (blackened square), where B indicates leukocyte DNA; S, semen DNA; and T, tumor DNA. The affected son displays approximately equal amounts of mutant and normal DNA in blood and tumor. Unaffected family members display only the wild-type sequence, with the exception of the father, who displays the mutant band in both blood and semen, but at a reduced intensity. The lanes under the terms "40%," "30%," "20%," and "10%" show results from mixtures of wild-type and mutant DNA (derived from the unaffected daughter's leukocytes and the affected son's tumor, respectively) that would contain the indicated proportions of mutant and wild-type alleles. On the basis of these lanes, we estimate the proportion of mutant sperm in the father's semen to be ~20%–30%.

(76923de121bp). The unaffected father also exhibited the mutation in leukocyte DNA, but the ratio of mutant to wild-type sequence was less than the 50:50 ratio expected for a heterozygote carrier. RFLP analysis, performed with intragenic DNA polymorphisms, indicated that the father had passed the same haplotype both to the affected child and to an unaffected child who did not carry the mutation.

In family 345, a bilaterally affected child heterozygously carried a frameshift mutation, Ser648(1-bp del) (153335delT). The unaffected mother also carried this mutation, but the ratio of mutant to wild-type sequence was less than the 50:50 ratio expected for a heterozygote carrier. Intragenic RFLPs demonstrated that the mother had passed the same haplotype both to the affected child and to an unaffected child who did not carry the mutation.

Discussion

We confined our search for mosaicism to families in which the initial mutation in *RB1* had been identified. This was done because, in most cases, mosaicism can be unsuspected, or impossible to prove, unless the responsible gene defect in a family is known and pertinent family members are available for analysis. In fact, prior to DNA analysis, mosaicism could have been suspected in only 2 of the 15 families with a documented mosaic member. These were families 262 and 296, in which unaffected parents had more than one affected child. Even in these two families, it was only after the initial mutation was discovered and analyzed that the possibility that one parent was a nonpenetrant, homogeneous carrier was ruled out.

In 15 (~10%) of the 156 families in our survey, mosaicism was documented in a family member. In addition, there were many families with one member who was likely, but could not be proved, to be mosaic. As demonstrated in family 262, absence of an initial mutation in leukocyte DNA does not rule out germ-line mosaicism; in this situation, mosaicism would be recognized only if the mosaic parent had another child who inherited the mutation or if germ-line DNA were available for analysis. In some cases, mosaicism could not be proved because key family members were unavailable, unwilling to be analyzed, or deceased. Hence, the actual proportion of retinoblastoma families with a mosaic member is likely to be higher than the 10% reported here.

As a group, the mosaic individuals identified in the present study exhibited an expected reduced penetrance and expressivity. Six (40%) of the 15 mosaics did not have retinoblastoma, and 2 (13%) developed unilateral retinoblastoma. This contrasts with published values for the penetrance and severity of hereditary retinoblastoma (Vogel 1979) that indicate that only ~10% of carriers are unaffected, ~30% are unilaterally affected, and the remainder are bilaterally affected ($\chi^2 = 16.9$; df = 2; $P < .001$). Carlson and Desnick (1979) proposed that mutational mosaicism was the explanation for variable expressivity in retinoblastoma patients. According to this model, reduced expressivity in patients who are mosaics for a retinoblastoma-predisposing mutation reflects the fact that fewer cells are targets for a second mutation. Mosaicism may also explain the anticipation that has been reported in some families with retinoblastoma (Schappert-Kimmijser et al. 1966); the larger proportion of bilateral retinoblastoma compared with unilateral retinoblastoma in the second generation of two-generation pedigrees may reflect a high frequency of mosaicism and, consequently, lower penetrance in the affected founder in the first generation. Mosaicism is an established cause of phenotypic variation in the expression of other genetic diseases (Hall 1988). For example, a milder clinical presentation has been reported in mosaic individuals who have mutations in the genes for type 1 or type 2 neurofibromatosis (Bourn et al. 1994; Colman et al. 1996).

A review of the literature reveals a number of previous reports of mosaicism in retinoblastoma; most of these reports describe one or only a few cases (Munier et al. 1988; Greger et al. 1990; Shimizu et al. 1994; Blanquet et al. 1995; Lohmann et al. 1997). A survey (Munier et

al. 1988) of published case reports of cytogenetically detectable chromosome 13 deletions in patients with retinoblastoma found that 25 (20%) of 126 reported individuals were mosaics. Of course, a literature review provides only an approximate value for the incidence of mosaicism because of the inherent biases in reporting such cases. However, this tabulation of mosaics with cytogenetically detectable abnormalities is noteworthy because it corroborates our impression, based mainly on cases with mutations that are beyond the resolution of cytogenetic techniques, that mosaicism among families with retinoblastoma is not rare.

The incidence of mosaicism should be high in diseases, such as retinoblastoma, in which (1) a large proportion of all cases represent new mutations, (2) substantial somatic mosaicism can occur in a parent with a normal phenotype, and (3) there is no strong paternal age effect (Wijsman 1991). However, data from which one can estimate the actual incidence of mosaicism have been obtained for very few diseases. Duchenne muscular dystrophy, another disease with a high new-mutation rate (Vogel and Rathenberg 1975), is notable in this regard. In a study of 41 families with an identified mutation, a mosaic member could be identified in 7 (17%) (Bakker et al. 1989). Another study used serum creatinine kinase levels to infer that ~12% of females with no prior history of Duchenne dystrophy, but with two or more carrier or affected offspring, are mosaics (Passos-Bueno et al. 1990).

Our experience indicates that the high frequency of mosaicism is unrecognized by many genetic counselors and clinicians who care for families with retinoblastoma. When genetic counseling is provided to patients and family members, mosaicism is often not emphasized—partly because of the uncertainties in recurrence risks that mosaicism produces and partly because of the difficulty of explaining mosaicism clearly to patients who are unfamiliar with the principles of genetics. Because of the substantial incidence of mosaicism reported here, the possibility of mosaicism should be entertained whenever a family with an isolated case of retinoblastoma is identified. Specific revisions to the traditional ways in which patients with retinoblastoma are categorized (Vogel 1979), cared for, and counseled are presented below.

1. *In a unilateral, simplex case of retinoblastoma, absence of a detected mutation in leukocytes does not reliably predict that no tumors will arise in the fellow eye in the future*. It has been customary for children with unilateral, simplex retinoblastoma to be examined frequently for tumors that might arise in the fellow eye. During the first 3-5 years of life, when retinoblastomas are most prone to arise, many children are not sufficiently cooperative to allow thorough examination of their retinas. At many retinoblastoma clinics, eye examinations of young children are performed under general anesthesia. After the identification of *RB1* (Friend et al. 1986; Toguchida et al. 1993) and the subsequent availability of molecular genetic analysis of patients (Wiggs et al. 1988; Dunn et al. 1989; Yandell et al. 1989; Hogg et al. 1992; Shimizu et al. 1994; Blanquet et al. 1995; Lohmann et al. 1996), it was hoped that patients who had unilateral simplex retinoblastoma, without a germ-line mutation, could be identified and spared repeated exposures to general anesthesia (Wiggs and Dryja 1988; Noorani et al. 1996). The cases reported here (families 294, 377, and 266) and elsewhere (Lohmann et al. 1997) indicate that, even when an initial mutation is identified in the tumor from the first affected eye and analysis of leukocyte DNA does not detect the mutation, children are still at risk for development of a tumor in the fellow eye. Our three examples are derived from 60 originally unilateral simplex cases in which an identified initial mutation was absent from leukocyte DNA. On the basis of these cases, the estimated risk for developing a tumor in the fellow eye is ~5%. This figure makes it advisable to continue to closely monitor the eyes of children with unilateral retinoblastoma, regardless of the results of leukocyte DNA analysis.

2. *Some bilateral simplex retinoblastoma patients and unilateral, multifocal simplex retinoblastoma patients have a recurrence risk among their offspring that is much less than 45%–50%*. Previous studies that combine data from many cases have found that ~45%–50% of the offspring of patients with bilateral simplex retinoblastoma developed retinoblastoma. These data prompted the conclusions that all bilaterally affected patients are germ-line carriers and that the occasional nonpenetrant-carrier offspring explain the transmission ratio of slightly less than 50% (Vogel 1979). Here, however, we describe mosaic, bilaterally affected patients who have an initial mutation that either is present in only a fraction of leukocytes (families 7, 26, 218, and 452) or is not detectable in leukocytes at all (families 294, 377, and 266). It is likely that the germ line in these individuals is either mosaic or free of the mutation. Evidence for a reduced abundance of mutant germ cells comes from three mosaics who have reproduced (families 26, 218, and 452). Of the nine offspring in these three families, only two were carriers, and only these two were affected; haplotype analysis indicated that three others (one in family 26 and two in family 452) inherited the haplotype associated with the mutation but did not inherit the mutation itself.

These cases require modification of the dictum that all bilaterally affected patients are germ-line carriers. It is still uncertain whether, in bilaterally affected mosaics, the proportion of mutant to wild-type DNA in leukocytes is a useful predictor of the proportion of mutant

germ cells. Whenever possible (e.g., in male mosaics), it is best to base the recurrence risk estimate on an analysis of germ-line DNA. In this regard, it should be noted that the germ line may not be stable. In patients with chromosomal mosaicism for 13q deletions that cause retinoblastoma, the percentage of lymphocytes that have the cytogenetic abnormality changes over time (Orye et al. 1982; Motegi and Minoda 1984; Ribeiro et al. 1988). The possibility that the proportion of spermatozoa that carry an *RB1* mutation also changes over time warrants further investigation. For the purpose of recurrence risk estimation, it is advisable for now to perform germ-line analysis as close as possible to the time period during which a couple wants to conceive a child.

3. *It is possible—but not yet proven—that every bilateral or unilateral retinoblastoma patient in whom an initial mutation is identified in tumor cells, but not in leukocytes, has a recurrence risk of zero*. Every germline mosaic reported here and elsewhere (Munier et al. 1988; Greger et al. 1990) had an initial mutation that was detectable in leukocyte DNA. Whether the converse is true—whether the germ line is never involved in affected individuals without an identified initial mutation in leukocyte DNA—remains an open question. Of our three (all bilaterally) affected patients with an identified initial mutation that was not detected in leukocyte DNA, none has reproduced, and, to our knowledge, no comparable individuals who have reproduced have yet been reported by others. Two of the three patients we report are males, but they are too young to provide germ-line DNA for analysis. Future identification, evaluation, and follow-up of many such patients are required to determine whether they can be considered to have no recurrence risk. Besides the importance for genetic counseling, this might have implications regarding the pattern of human embryonic development. Perhaps the retinal anlage is separated from the precursors of leukocytes and germ cells early in embryogenesis.

4. *If unaffected parents with no previous family history of retinoblastoma have an affected child, DNA-based estimates of the recurrence risk for future children should include, whenever possible, an analysis of paternal germ-line DNA*. After reviewing data published prior to the availability of mutation-detection techniques, Vogel (1979) estimated the recurrence risk to a child born with simplex retinoblastoma at ~6%, if the retinoblastoma was bilateral, and at ~1%, if the retinoblastoma was unilateral. These recurrence risks were postulated to stem from the possibility that one parent was an asymptomatic carrier or a mosaic. The results of the present study indicate that a major portion of the recurrence risk in these situations comes from germ-line mosaicism in one of the parents.

In simplex cases in which an initial mutation is identified, recurrence risk among siblings of the affected individual is negligible if the affected individual is mosaic. On the other hand, whenever the index case is found to carry a new germ-line mutation homogeneously, the recurrence risk is elevated. Measurement of that risk requires evaluation of the parents, who may be homogeneous carriers or germ-line mosaics. As demonstrated in family 262, reliable estimates of recurrence risks may require analysis of germ-line DNA. Since ~85% of new germ-line mutations in *RB1* arise in the paternally derived allele (Dryja et al. 1997), and since germ-line DNA is noninvasively procurable from fathers, evaluation for germ-line mosaicism, in most cases, will focus on fathers.

5. *The risk that a patient with unilateral simplex retinoblastoma will have mutant germ cells may be much higher than 12%; however, some will be mosaics, and <50% of their germ cells will be mutant*. Patients with unilateral simplex retinoblastoma are traditionally considered to be either noncarriers, with no risk of having affected children, or carriers, with a 50% recurrence risk for each offspring (Vogel 1979). The calculated relative proportions of each type (88% noncarriers, 12% carriers) were based on retrospective surveys of the offspring of patients in this category; these surveys have shown, among these patients' offspring, a retinoblastoma recurrence risk of 5%–6%. In light of our observations and those of Lohmann et al. (1997), these values must be revised. The 156 families in our study included 80 in which the first affected individual had unilateral retinoblastoma. The initial mutation was detected in leukocyte DNA from 23 individuals, including 2 who were proven to be mosaics (families 333 and 344), in whom a mutation was detected in a fraction of leukocytes. On the basis of an analysis of leukocyte DNA from 36 unilateral simplex retinoblastoma patients with an identified initial mutation, Lohmann et al. (1997) found 6 in whom the initial mutation was detected in leukocytes, and in 1 of these, somatic mosaicism could be documented. These numbers provide a rough estimate of the proportion (~25%) of newly diagnosed, unilateral simplex retinoblastoma patients who are either homogeneous carriers or mosaics. Ascertainment biases make this number very approximate. For example, there is a bias against including cases in which no tumor DNA is available for analysis, since identification of an initial mutation is less likely in these cases. However, the incidence of mosaicism among unilateral simplex cases, as among bilateral simplex cases, is undoubtedly substantial. Mosaicism in retinoblastoma must be given serious consideration, both for scheduling follow-up eye examinations for young children and for genetic counseling.

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