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Constitutional RB1-Gene Mutations in Patients with Isolated Unilateral Retinoblastoma

Dietmar R. Lohmann,[1] Martin Gerick,[1] Birgit Brandt,[1] Ulrich Oelschläger,[2] Birgit Lorenz,[3] Eberhard Passarge,[1] and Bernhard Horsthemke[1]

[1]Institut für Humangenetik and [2]Zentrum für Augenheilkunde, Universitätsklinikum Essen, Essen; and [3]Abteilung für Kinderophthalmologie, Strabismologie und Ophthalmogenetik der Universität Regensburg, Regensburg

Summary

In most patients with isolated unilateral retinoblastoma, tumor development is initiated by somatic inactivation of both alleles of the RB1 gene. However, some of these patients can transmit retinoblastoma predisposition to their offspring. To determine the frequency and nature of constitutional RB1-gene mutations in patients with isolated unilateral retinoblastoma, we analyzed DNA from peripheral blood and from tumor tissue. The analysis of tumors from 54 (71%) of 76 informative patients showed loss of constitutional heterozygosity (LOH) at intragenic loci. Three of 13 uninformative patients had constitutional deletions. For 39 randomly selected tumors, SSCP, heteroduplex analysis, sequencing, and Southern blot analysis were used to identify mutations. Mutations were detected in 21 (91%) of 23 tumors with LOH. In 6 (38%) of 16 tumors without LOH, one mutation was detected, and in 9 (56%) of the tumors without LOH, both mutations were found. Thus, a total of 45 mutations were identified in tumors of 36 patients. Thirty-nine of the mutations—including 34 small mutations, 2 large structural alterations, and hypermethylation in 3 tumors—were not detected in the corresponding peripheral blood DNA. In 6 (17%) of the 36 patients, a mutation was detected in constitutional DNA, and 1 of these mutations is known to be associated with reduced expressivity. The presence of a constitutional mutation was not associated with an early age at treatment. In 1 patient, somatic mosaicism was demonstrated by molecular analysis of DNA and RNA from peripheral blood. In 2 patients without a detectable mutation in peripheral blood, mosaicism was suggested because 1 of the patients showed multifocal tumors and the other later developed bilateral retinoblastoma. In conclusion, our results emphasize that the manifestation and transmissibility of retinoblastoma depend on the nature of the first mutation, its time in development, and the number and types of cells that are affected.

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Address for correspondence and reprints: Dr. Dietmar R. Lohmann, Institut für Humangenetik, Hufelandstrasse 55, D-45122 Essen, Germany. E-mail: dr.lohmann@uni-essen.de

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Introduction

Inactivation of both alleles of the retinoblastoma-susceptibility gene (RB1) is the crucial event in the development of retinoblastoma, a malignant tumor that originates from embryonal retinal cells (Knudson 1971; Cavenee et al. 1983; Friend et al. 1986). Mutations in one allele of this gene lead to a predisposition for retinoblastoma, which is transmitted as an autosomal dominant trait if the mutation is present in germ-line cells. Tumor development is initiated by inactivation of the second RB1 allele. Since mutations in the second allele can occur independently in several cells, multiple tumor foci arise in most individuals who have inherited a predisposing RB1 mutation. In early childhood, the risk for the development of new tumors decreases, since the population of cells with a potential for tumor formation declines rapidly (Knudson 1971).

All patients with familial, bilateral, or unilateral multifocal retinoblastoma are regarded as carriers of an RB1 germ-line mutation (Vogel 1979). In addition, survivors of isolated unilateral retinoblastoma may have affected children. Estimates of the proportion of hereditary retinoblastoma in patients with isolated unilateral tumors, on the basis of surveys of the occurrence of retinoblastoma in offspring, have resulted in different figures. Most widely cited are those reported by Vogel (1979). Assuming a penetrance of 90% in children with a germ-line mutation inherited from a unilaterally affected parent, he estimated that 10%–12% of isolated unilateral cases are caused by germ-cell mutations. In a recent report, Draper et al. (1992) used the method of maximum likelihood to analyze data from a large population-based group of patients. By following up on selected groups of patients, they estimated a 1.7% probability that a patient with unilateral retinoblastoma and no family history of retinoblastoma in fact carries a germ-line mutation, when a penetrance of 90% is assumed. However, it has been noted that, in children, the penetrance and the expressivity of an RB1 mutation apparently are increased with increased expressivity in the parents (Briard-Guillemot et al. 1974; Matsunaga 1976). Allowing for a lower penetrance in the offspring of unilateral patients, Draper et al. (1992) estimated a

2.3% probability that a patient with isolated unilateral retinoblastoma carries a germ-line mutation.

Early diagnosis of retinoblastoma is important for the prognosis of both vision and survival. Consequently, repeated ophthalmological examinations are necessary for infant relatives of patients with retinoblastoma (Musarella and Gallie 1987). In hereditary retinoblastoma, knowledge of the individual germ-line mutation allows for accurate risk prediction for relatives (Dunn et al. 1989; Yandell et al. 1989). Mutation screening of peripheral blood DNA can detect oncogenic mutations, for most patients with bilateral isolated retinoblastoma or with familial retinoblastoma (Lohmann et al. 1996). A comparison of the costs of molecular versus conventional screening approaches has indicated that mutational analysis will help to reduce health-care expenses (Noorani et al. 1996), and, therefore, DNA testing offers economic advantages, in addition to individual benefits to families.

With the exception of cytogenetic deletions involving 13q14, few constitutional mutations have been reported in patients with isolated unilateral retinoblastoma. By screening peripheral blood DNA, Blanquet et al. (1995) identified bona fide oncogenic mutations in 4 (7.1%) of 56 patients with unifocal isolated retinoblastoma. However, in the same study, mutations were identified in only 46 (26%) of 176 patients with hereditary retinoblastoma. This low rate of detection indicates that mutations also may have been missed in patients with the unifocal disease. In general, since current methods do not detect all mutations, results obtained by screening of constitutional DNA should not be used to estimate the proportion of carriers of mutations, among patients with isolated unilateral retinoblastoma, and such results are of limited value for accurate risk prediction for relatives. It has been inferred from the two-step inactivation mechanism that, if one of the two oncogenic RB1 mutations identified in the tumor also is present in constitutional cells, a patient has hereditary retinoblastoma, whereas the absence of both oncogenic mutations proves that the patient has the nonhereditary disease (Dunn et al. 1989; Yandell et al. 1989). Consequently, mutational analysis is required in both tumor and constitutional cells. However, since only a few patients have been studied previously by use of this approach (Yandell et al. 1989; Hogg et al. 1992; Shimizu et al. 1994), no reliable data exist for the frequency and the nature of constitutional mutations in patients with isolated unilateral retinoblastoma.

Here we report the results of a mutational analysis of tumors from 39 patients with isolated unilateral retinoblastomas. RB1 mutations were identified in 36 tumors. In 6 patients with no signs of multifocal disease, an RB1 mutation also was detected in peripheral blood DNA. In 1 patient, somatic mosaicism was demonstrated by use of molecular analysis. Somatic mosaicism also was suggested in 2 of the patients without a detectable mutation in peripheral blood, because 1 patient showed two spatially distinct tumor foci in one eye and the other later developed a new tumor in the fellow eye. Thus, owing to the presence of mosaicism in some patients, analysis of DNA from tumor cells and from constitutional cells is not sufficient to confirm or to exclude unambiguously hereditary retinoblastoma in patients with isolated unilateral disease. If somatic mosaicism is not rare in patients with isolated retinoblastoma, current concepts of genetic counseling and risk prediction have to be revised accordingly.

Patients, Material, and Methods

Patients and Tumors

Vital samples of retinoblastoma and of peripheral blood were obtained from 89 patients with isolated unilateral tumors. The diagnosis was established by use of current histopathologic criteria. In almost all the patients, enucleation had to be performed because the tumors involved more than one-half of the retina. In a few patients, enucleation was performed because a smaller tumor was located in the fovea. Two of these patients showed distinct multifocal tumor growth, and, for both patients, the largest tumor was examined. Tumor and blood samples were stored at −70°C and −20°C, respectively, until DNA was extracted by use of standard protocols (Kunkel et al. 1977). DNA was extracted from formalin-fixed, paraffin-embedded samples, as described elsewhere (Wright and Manos 1990).

PCR-Mediated Genotyping of DNA Polymorphisms

To determine loss of constitutional heterozygosity (LOH) in tumors, short-tandem-repeat (STR) loci RBi2, located in intron 2 of the RB1 gene (Toguchida et al. 1993), and RB1.20, located in intron 20 of the RB1 gene (Yandell and Dryja 1989), were amplified by PCR, as described in a previous study (Brandt et al. 1992), and were analyzed on an ABI 373 sequencer, by use of the Genescan software (Applied Biosystems). In patients without LOH in the tumor, loci D13S262 (Genome Database [GDB] G00-199-226) and D13S284 (GDB G00-200-238), both of which are linked closely to the RB1 gene, also were investigated. In patients with LOH, the STR polymorphisms D13S115 (mapped to 13q11-q12.1; GDB G00-182-248) and D13S193 (mapped to 13q31-q32; GDB G00-189-227) were analyzed, in addition to the intragenic loci. When available, parents were genotyped, to trace the parental origin of the allele lost in tumor cells.

PCR Amplification, Screening for Small Mutations, and Sequencing

PCR was performed by use of the primers and conditions described in previous studies (Lohmann et al.

1994*a*; Lohmann et al. 1996). An additional primer (RB1lm [5′-CCCTCGCCCAAGAACCCAGAATC-3′]) was used to improve the amplification efficiency of the promoter and the exon 1 region. Nonisotopic SSCP was performed as described in a previous study (Lohmann et al. 1996). In addition, tumors without LOH at both intragenic and linked loci were analyzed by heteroduplex analysis (Ganguly et al. 1993; Lohmann et al. 1996). Exons 1, 5, and 14 and the promoter also were screened for mutations, by use of sequencing. PCR products were purified in microcon 100 filtration units (Amicon) and were used as templates for cycle-sequencing reactions (ABI Prism Ready Reaction Kit with polymerase FS; Applied Biosystems). Sequence analysis was performed on a 310 Genetic Analyzer (Applied Biosystems). Sequence analysis or, if applicable, restriction-enzyme digestion of PCR products was used to identify mutation carriers, among parents and siblings of patients with a constitutional mutation.

Reverse-Transcription (RT) PCR and the Cloning and Typing of PCR Products

To examine the consequences of a potential splice-site mutation identified in one patient, whole-blood RNA was isolated by use of the method of Chomczynski and Sacchi (1987) and was reverse-transcribed primed by random oligos (GeneAmp RNA PCR Kit; Perkin Elmer). For PCR amplification and sequencing, primers RBc9se (5′-GGACTTGTAACATCTAATGG-3′), RBc11se (5′-CCTTGATGAAGAGGTGAATG-3′), RBc13se (5′-CTGCACAGTGAATCCAAAAG-3′), and RBc16as (5′-GCCATTACAACCTCAAGAGC-3′) were used. For sequence analysis of individual products, DNA was size fractionated and excised from agarose gels, eluted through 0.47-μm filter units (Millipore), and amplified by PCR. To confirm the somatic mosaicism suggested by analysis of RNA, exon 13 was amplified from peripheral blood DNA, with primers RBi13se and RBi13as (Lohmann et al. 1994*a*). The products were cloned and transfected, by use of a pGEM-T kit (Promega), by following of the recommended protocols. Individual colonies were picked directly as templates for PCR with exon 13 primers, and SSCP was used to identify wild-type from mutant inserts.

Southern Blot Analysis

The methylation status of the CpG island at the 5′ end of the RB1 gene was tested by use of the methylation-sensitive enzymes *Bss*HII and *Sac*II, as described in a previous study (Greger et al. 1994). Genotyping of RFLP loci within the RB1 gene was performed as described in a previous study (Greger et al. 1988). To identify structural alterations of the RB1 gene, in tumors, Southern blots of DNA samples digested with *Eco*RV were hybridized with a battery of probes that hybridize to five fragments of distinguishable size that cover different parts of the RB1 gene (B. Brandt, B. Horsthemke, and D. R. Lohmann, unpublished data).

Statistical Analysis

To test findings reported by Kato et al. (1993; 1994), the distribution of the age at the time of operation was analyzed by use of the same procedures. The proportion of patients who had not yet had an operation was plotted as a function of time, by application of the Kaplan-Meier method, and the curves were compared by use of the log-rank test (JMP software, version 3.1.6, for the Macintosh; SAS Institute). The same method was used to compare patients with and those without RB1 mutations in peripheral blood.

Results

Testing for LOH in Tumors

Constitutional heterozygosity of polymorphic loci within the RB1 gene was observed in 76 (85%) of 89 patients (fig. 1). In tumors from 22 (29%) of the 76 informative patients, heterozygosity was retained. Further investigation at D13S262 or at D13S284, in 19 of these patients, also showed no allele loss in 16 of 17 patients that were informative at these closely linked loci. The tumor enucleated from a 7-mo-old patient showed allele loss at D13S262, whereas heterozygosity was retained at the intragenic locus RBi2. In this tumor, a homozygous deletion was identified by the Southern blot genotyping of two intragenic RFLPs. This indicated that amplification of nontumorous DNA, which almost inevitably contaminates samples of primary tumors, had resulted in misleading PCR-based genotype data, for the locus encompassed by the deletion (Szabo and King 1995). This also showed that both oncogenic mutations detected in this tumor are of somatic origin. In all other

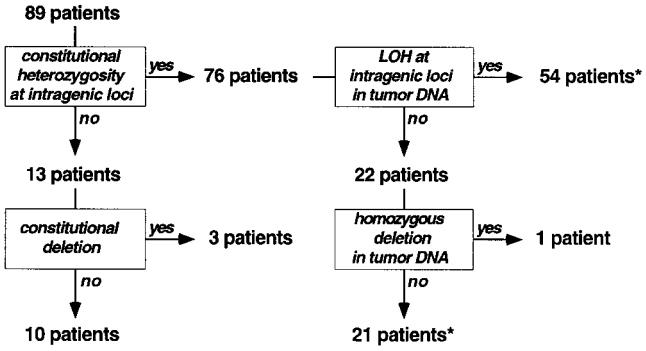

Figure 1 Flow chart showing the results of genotyping of intragenic polymorphisms and examination for deletions in peripheral blood DNA and tumor DNA, from 89 patients with isolated unilateral retinoblastoma. The groups of patients from which tumors were selected randomly for comprehensive mutation analysis are indicated by an asterisk (*).

tumors without allele loss, no homozygous deletion was detected by Southern blot analysis.

Tumors from 54 (71%) of the 76 informative patients showed LOH within the RB1 gene. The parental origin of alleles lost was maternal in 14 tumors and paternal in 9 tumors. A subset of tumors with LOH also was investigated at D13S115 (13q11-q12.1) and at D13S193 (13q31-q32). The centromeric locus showed LOH in tumors of only 9 (39%) of 23 informative patients, whereas at the telomeric locus LOH was detected in tumors of 15 (79%) of 19 informative patients. In all 4 tumors without telomeric allele loss, heterozygosity also was retained at the centromeric locus.

Identification of Constitutional Deletions

Patients without constitutional heterozygosity at intragenic loci were examined for deletions involving the RB1 gene. Constitutional deletions were identified in 3 of 13 uninformative patients. In 2 patients, enucleated at age 22 mo and age 11 mo, cytogenetic analysis of peripheral blood lymphocytes showed deletions of 13q (karyotpes 46,XY,del13[q13-q14.3] and 46,XX,del13[q14-q22], respectively). No chromosomal aberration was detected in the parents, and, in 1 of the 2 patients, segregation analysis showed that the deletion had occurred on the paternal chromosome. None of the deletions extended into 13q32, and, in line with previous reports (Brown et al. 1993, 1995), no malformation was present in either patient. Another patient, who was treated at the age of 27 mo, showed an apparently normal karyotype, but the absence of paternal alleles at RBi2 and RB1.20 was identified by segregation analysis. Since the alleles at both D13S262 and D13S284 were inherited from both parents, he must have a de novo deletion involving the paternal RB1 gene.

Detection of Small Mutations in Tumors and in Constitutional DNA

Tumors from 39 patients were randomly selected for a comprehensive mutational analysis of the RB1 gene. All tumors were screened for mutations, by SSCP. In addition, in tumors without LOH ($n = 16$), heteroduplex analysis was performed. All exons in all tumors were investigated; that is, examination of a sample was not suspended once two oncogenic mutations were found (Cowell et al. 1994). A total of 40 small mutations were identified in the retinoblastomas of 32 patients (table 1). For 6 patients (samples M2205, M2408, M2920, G1142, M6301, and M6680), direct sequencing of PCR products from peripheral blood DNA demonstrated the presence of 1 of the mutations previously identified in the corresponding tumor. For example, exon 23 PCR products from the analysis of tumor sample M2205 showed an aberrant SSCP pattern, and sequencing revealed a heterozygous C→T transition, at position 2359 of the open reading frame (c2359C→T), that changes codon 787 from CGA (arginine) to TGA (stop). In accordance with allele loss at intragenic, centromeric, and telomeric loci in this tumor, no wild-type sequence was present at the site of the base substitution. We also identified this mutation in peripheral blood DNA from the patient (fig. 2). The alleles that were lost in the tumor are of maternal origin, and, therefore, the mutation is in phase with the paternal haplotype. The same paternal haplotype is present in the patient's brother. Exon 23 PCR products from the analysis of the peripheral blood DNA of the father and of the brother show a homozygous wild-type *Taq*I restriction pattern. These findings argue for a new germinal origin or an early embryonal origin of the mutation identified in the patient. However, the possibility of germinal mosaicism in the father is not excluded.

In one patient only, a constitutional mutation was inherited from an unaffected carrier parent. A heterozygous A→T transition (c411A→T; E137D), identified in tumor sample M2408, also was found in the constitutional DNA of the patient and of her mother and her sister. The same type of missense mutation previously had been reported to be present in the constitutional DNA of a patient with bilateral retinoblastoma (Blanquet et al. 1993). Segregation analysis indicated that the mutation is in phase with the haplotype that was inherited from the grandfather. However, since the grandfather did not consent to DNA testing, we cannot determine whether this mutation occurred de novo.

In tumor sample M2920, a G→A transition was detected at the last nucleotide of exon 13 (c1332G→A) (fig. 3*A*). In correspondence with LOH at both the intragenic and the telomeric loci in this tumor, sequence analysis showed no wild-type G at the site of mutation. In peripheral blood DNA, both the mutant and the wild-type nucleotides were found at the site of the mutation. This mutation reduces the similarity of the intron 13 splice-donor sequence to the consensus sequence of eukaryotic splice sites, and, most probably, exon 13 is skipped in the mutant transcript (Shapiro and Senapathy 1987; Talerico and Berget 1990). Since a deletion of this exon does not alter the reading frame, the mutant transcript is not suppressed in constitutional cells (Dunn et al. 1989; Kato et al. 1994), and, therefore, it is possible to verify the functional consequence of this mutation, by RT-PCR analysis of RNA from peripheral blood. Gel electrophoresis of RT-PCR products showed a DNA band of normal length and an additional, evidently less intense, smaller band (fig. 3*B*). The individual bands were excised and sequenced. The larger fragment showed a regular transcript sequence, whereas exon 13 was skipped in the smaller product (fig. 3*C* and *D*). It is important to note that the last nucleotide of exon 13, in the fragment that was spliced regularly, only showed the wild-type G (fig. 3*C*). Therefore, the difference in quantity between normal and mutant RT-PCR products

Table 1

Results of Mutation Analysis of Tumors

Sample	Mutation Type(s)[a]	Site	Sequence[b]	Putative Consequence[c]	Detectable in Blood DNA	Patient's Age at (mo)	
						Operation	Study
G902	Transition Loss of paternal allele	Intron 12 ...	IVS12-2A→G ...	Splice acceptor ...	No ...	67.1	152.5
G909	Loss of maternal allele	...	...	...	...	29.0[d]	114.6
G1142	Transition (CpG)	Exon 20	c1982C→T	R661W	Yes	10.9	90.9
G1166	2-bp deletion Loss of maternal allele	Exon 3 ...	c369delAT ...	PT at codon 129 ...	No ...	17.2	95.6
M461	Transition (CpG) LOH	Exon 14 ...	c1333C→T ...	R445X ...	No ...	6.0	65.3
M734	Hypermethylation LOH	Promoter ...	... ...	Silencing ...	No ...	29.0	86.7
M1324	Transition (CpG) Transition (CpG)	Exon 17 Exon 17	c1654C→T c1666C→T	R552X R556X	No No	14.3	68.0
M1353	Transition (CpG) Loss of maternal allele	Intron 12 ...	IVS12+1G→A ...	Splice donor ...	No ...	3.0	56.5
M1821	100-bp insertion Loss of paternal allele	Exon 20 ...	c2065ins100 ...	PT at codon 700 ...	No ...	17.0	67.8
M1886	Transition (CpG) Transition	Intron 12 Exon 21	IVS12+1G→A c2158C→T	Splice donor K720X	No No	26.3	76.9
M1990	Transversion LOH	Exon 4 ...	c409G→T ...	E137X ...	No ...	25.8	75.4
M2087	Hypermethylation LOH	Promoter ...	... ...	Silencing ...	No ...	15.4	64.5
M2205	Transition (CpG) Loss of maternal allele	Exon 23 ...	c2359C→T ...	R787X ...	Yes ...	18.3	...[e]
M2408	Transversion	Exon 4	c411A→T	E137D	Yes	60.0	107.3
M2920	Transition Loss of paternal allele	Exon 13 ...	c1332G→A ...	Splice donor ...	Yes[f] ...	49.9	94.1
M3293	Transition (CpG) Transition (CpG)	Exon 10 Exon 18	c958C→T c1735C→T	R320X R579X	No No	10.1	52.6
M3297	Transition Transition (CpG)	Exon 2 Exon 17	c184C→T c1654C→T	Q62X R552X	No No	16.9	59.3
M3619	Transition Loss of paternal allele	Intron 19 ...	IVS19+1G→A ...	Splice donor ...	No ...	20.8[d]	60.5
M3865	Transversion LOH	Exon 4 ...	c409G→T ...	E137X ...	No ...	22.1	60.2
M4008	10-bp deletion Hypermethylation	Exon 18 Promoter	c1735del10 ...	PT at codon 607 Silencing	No No	10.3	47.5
M4042	Transition (CpG) 2-bp deletion	Exon 14 Exon 16	c1363C→T c1447delCA	R455X PT at codon 491	No No	7.9	44.8
M4372	Transition (CpG) 1-bp deletion	Exon 10 Exon 20	c958C→T c2084delT	R320X PT at codon 704	No No	15.9	50.8
M4561	No mutation detected	...	...	...	...	18.0	46.9
M4955	5-bp deletion	Exon 3	c336del5	PT at codon 128	No	27.3	58.7
M4957	Transition (CpG) Loss of maternal allele	Exon 10 ...	c958C→T ...	R320X ...	No ...	10.5	41.4
M4972	Hemizygous deletion	...	...	...	No	11.0	41.4
M4976	LOH	...	...	...	...	24.0	54.5
M5266	Transition (CpG) LOH	Exon 17 ...	c1666C→T ...	R556X ...	No ...	27.7	56.4
M5450	Transition (CpG) Loss of maternal allele	Exon 8 ...	c751C→T ...	R251X ...	No ...	59.8	87.2
M5500	Transition (CpG) LOH	Exon 10 ...	c958C→T ...	R320X ...	No ...	15.4	42.7
M5682	1-bp insertion 4-bp deletion	Exon 17 Exon 18	c1585insT c1723del4	PT at codon 554 PT at codon 609	No No	56.4	82.8

(*continued*)

Table 1 (continued)

Sample	Mutation Type(s)[a]	Site	Sequence[b]	Putative Consequence[c]	Detectable in Blood DNA	Patient's Age at (mo)	
						Operation	Study
M5702	Transversion	Intron 3	IVS3+1G→T	Splice donor	No	12.5	38.9
	Loss of maternal allele	...	...	...	...		
M5714	1-bp insertion	Exon 19	c1816insT	PT at codon 652	No	32.9	59.4
	Loss of maternal allele	...	...	...	...		
M5812	1-bp deletion	Exon 2	c227delT	PT at codon 76	No	12.7	38.0
	Loss of maternal allele	...	...	...	...		
M6301	Transition (CpG)	Exon 10	c958C→T	R320X	Yes	36.2	59.6
M6306	Transition (CpG)	Exon 11	c1072C→T	R358X	No	2.3[g]	25.6
	Loss of paternal allele	...	...	...	...		
M6336	Hemizygous deletion	...	...	...	No	23.0	45.5
M6418	Transversion	Exon 16	c1494T→A	Y498X	No	22.7	44.9
	Loss of maternal allele	...	...	...	...		
M6680	Transition	Intron 12	IVS12-1G→A	Splice acceptor	Yes	2.4	25.6
	8-bp insertion	Exon 22	c2244ins8	PT at codon 756	No		

[a] A second mutation was not detected in samples G909, G1142, M2408, M4955, M4972, M4976, M6301, and M6336.
[b] Notation according to the Ad Hoc Committee on Mutation Nomenclature (1996).
[c] PT = premature termination.
[d] Patient had multifocal unilateral retinoblastoma.
[e] Patient died at the age of 28.7 mo.
[f] Allelic imbalance identified in blood DNA indicates that the mutation is present in a mosaic state.
[g] Patient developed a new tumor in the other eye at the age of 10 mo.

was not caused by residual correct splicing of mutant transcripts. To examine if this difference was caused by an imbalance of wild-type and mutant alleles in the patient, the exon 13 sequence was PCR amplified from peripheral blood DNA and was cloned into pGEM-T. Of 60 individual inserts typed by SSCP, only 11 (18%) showed the mutant pattern, thus suggesting that this patient is a somatic mosaic for this mutation.

Mosaicism also was suggested to be present in two patients without a detectable mutation in peripheral blood. One patient showed two distinct tumor foci, and the two mutations identified in the larger tumor (sample M3619) were not detected in peripheral blood DNA. In tumor sample M6306, which was obtained from a patient without an indication of multifocal disease, a C→T transition (c1072C→T; R358X) was identified, and, in accordance with the finding of allele loss at intragenic, linked, and centromeric loci in this tumor, no wild-type sequence was present at the site of mutation. This mutation was not detected by sequence analysis of peripheral blood DNA from this patient. While this study was in progress, the patient developed a new retinoblastoma, thus indicating that cells with a predisposing mutation are present in the retina of the fellow eye. In order to obtain molecular evidence of mosaicism in this patient, other tissues were investigated for both of the mutations identified in the enucleated tumor. DNA was obtained from cells exfoliated from buccal mucous membranes and from palpebral conjuctiva. Both samples were heterozygous at loci with allele loss in the tumor, and restriction-enzyme digestion of exon 11 PCR products did not give any evidence of the presence of the nonsense mutation (fig. 4).

Hypermethylation of the RB1 Promoter

The methylation status at the 5′ end of the RB1 gene was determined in all tumors selected for comprehensive mutational analysis. Tumors from three patients (samples M734, M2087, and M4008) showed *Bss*HII+*Sac*I and *Sac*II+*Sac*I restriction patterns indicative of hypermethylation (table 1). Sequence analysis showed no mutation in the promoter and the exon 1 region of these tumors. No hypermethylation of the RB1 promoter region was detected in peripheral blood DNA from these patients.

Detection of Large Deletions

Southern blot hybridization was performed to complete the mutation detection in the tumors selected for comprehensive analysis. A hybridization probe from within intron 17 identified an abnormal *Eco*RV junction fragment in DNA from tumor sample M4972. In another tumor (sample M6336), densitometric evaluation showed a hemizygous deletion that affected the same region within the RB1 gene. Both alterations were not detected in constitutional DNA.

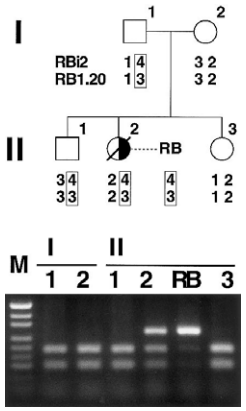

Figure 2 *Top,* Pedigree of family Sch (including the patient from which sample M2205 was taken), with the genotypes at intragenic polymorphic loci. *Bottom,* Results of restriction-enzyme digestion of exon 23 PCR products. The older brother (II-1) has inherited the same paternal haplotype that is retained in the retinoblastoma sample ("RB") obtained from the patient (II-2) with unilateral retinoblastoma. Almost no 98-bp and 171-bp fragments, which result from *Taq*I digestion of wild-type exon 23 PCR products, were detectable in the tumor. Both the father (I-1) and the patient's brother (II-1) show a wild-type restriction pattern. The DNA size marker ("M") shows fragments of 501 bp/489 bp, 404 bp, 331 bp, 242 bp, 190 bp, 147 bp, 111 bp/110 bp, and 67 bp (*Msp*I digestion of pUC19 DNA; Fermentas).

Statistical Analysis

Recently, Kato et al. (1993) reported that, in hereditary cases, the age at operation for 11 patients with tumors without LOH was lower than that for 12 patients with tumors showing LOH. We observed a similar trend in our set of isolated unilateral patients, but, in spite of the large number of tumors investigated, the difference was too small to reach statistical significance (fig. 5*A*). In another study of retinoblastomas from 13 patients with isolated unilateral disease, Kato et al. (1995) found that tumors with the loss of a maternal allele were operated on significantly later than those showing the loss of a paternal allele. We obtained data on the parental origin of alleles lost, for 23 patients with tumors, but we found no association between the parental origin of RB1 alleles and the age at operation (fig. 5*B*). We also compared the age at operation for patients with a constitutional mutation with that for patients in which neither of the two mutations identified in the tumor was detectable in peripheral blood DNA, but we found no significant difference (fig. 5*C*).

Discussion

To define the frequency and nature of constitutional mutations in isolated unilateral retinoblastoma, we analyzed tumors and corresponding peripheral blood DNA, sampled from 89 patients. Several classes of mutation that cause inactivation of RB1 alleles can be detected as LOH in tumors (Cavenee et al. 1983). To identify somatic mutations of this kind, all samples were investigated at several polymorphic loci on chromosome 13. We found LOH in tumors from 54 (71%) of 76 informative patients, which is consistent with previous reports (Zhu et al. 1992; Kato et al. 1993). In 14 (61%) of 23 patients with intragenic allele loss, heterozygosity was retained at a centromeric locus. Although PCR-mediated genotyping does not allow one to distinguish between homozygosity and hemizygosity, these data confirm that nondisjunction is not the foremost mechanism responsible for LOH in retinoblastoma (Zhu et al. 1992).

Recently, Kato et al. (1995) reported that tumors with loss of the maternally derived RB1 allele are operated on later than tumors with loss of the paternal allele. On further investigation of this finding, they identified a gene, located near the RB1 gene, that shows exclusive maternal expression (Kato et al. 1996). They hypothesized that this gene or other, unidentified imprinted genes in the same region may be associated with delayed occurrence of retinoblastoma with maternal allele loss (Kato et al. 1996). However, this hypothesis is challenged by our data, because we did not observe any difference in the age at operation between patients with tumors with maternal allele loss versus those with tumors with paternal allele loss.

Heterozygosity was retained at intragenic loci, in the tumors of 22 informative patients. Szabo and King (1995) noted that PCR may not detect homozygous deletions in primary tumors, because DNA from nontumorous cells inevitably is included in these samples. They predicted that, in tumors with homozygous deletions, heterozygosity appears to be retained at intragenic loci but may be lost at markers outside the deleted region. Genotyping of flanking loci showed this paradoxical LOH pattern in one of the tumors investigated in this study, and a homozygous deletion of somatic origin was identified by Southern RFLP analysis, as expected.

Thirteen of 89 patients were uninformative at intragenic polymorphisms. Further analysis showed constitutional cytogenetic deletions in 2 patients and a submicroscopic deletion in another. Studies of the rate of chromosome aberrations have revealed that cytogenetic deletions involving 13q14 occur in 1%–4% of patients with isolated unilateral retinoblastoma (Ejima et al. 1988; Bunin et al. 1989) and, thus, are in accordance with the proportion of 2 (2%) of 89, observed in this study. However, our figure underestimates the frequency of visible chromosomal changes, because our study does not include samples of tumors from patients in whom a deletion involving 13q14 was known prior to the diagnosis of retinoblastoma.

An exhaustive mutational analysis was performed in the retinoblastomas of 39 patients, in order to identify mutations that did not result in LOH. Oncogenic mutations were detected in 21 (91%) of 23 tumors with intragenic LOH. In 6 (38%) of 16 tumors without LOH, one

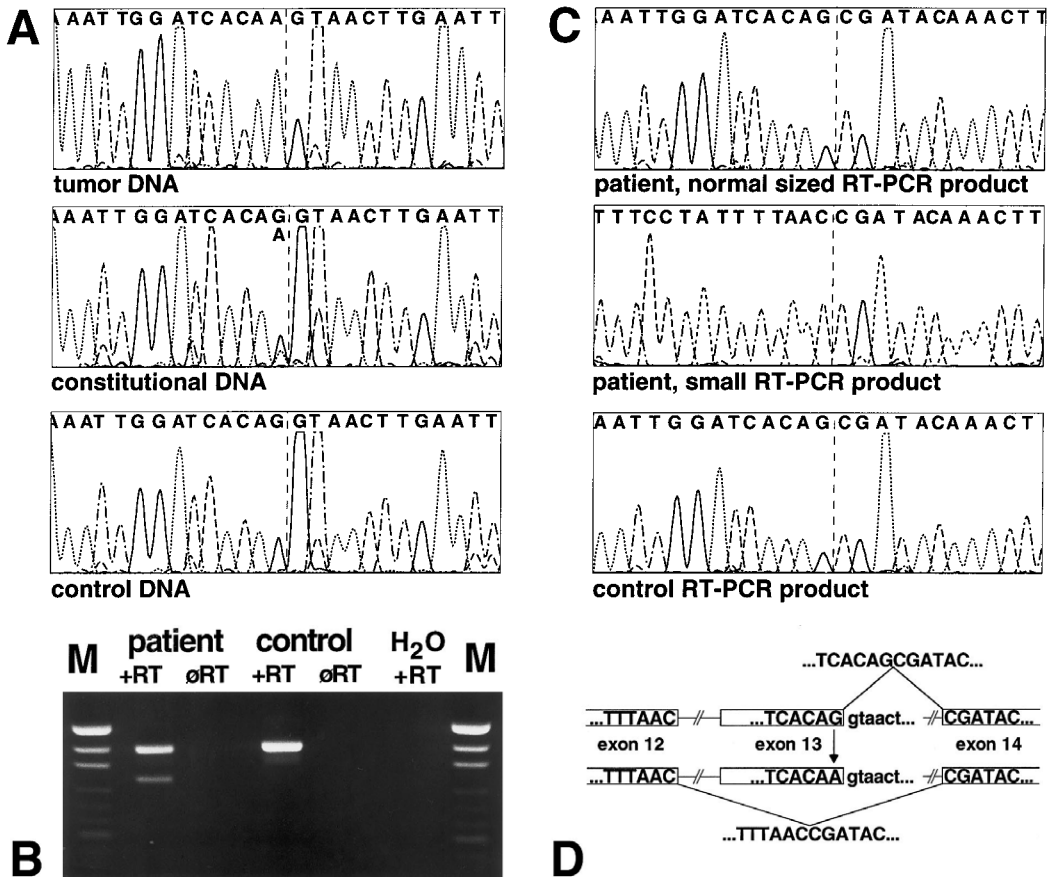

Figure 3 *A,* Results of sequence analysis of exon 13 PCR products from tumor DNA from sample M2920 (*top*), from the corresponding constitutional DNA (*middle*), and from the unaffected brother of the patient ("control DNA") (*bottom*). Tumor DNA shows only the mutant A at the end of exon 13, which is indicated by a dashed line. Both mutant A and wild-type G are detectable in peripheral blood DNA. *B,* Results of RT-PCR of RNA from peripheral blood of the patient. The primers RBc11se and RBc16as, which span exons 12–15 of the RB1 gene, were used. The results show a product of normal size (407 bp) and a less intense, smaller band. RT-PCR of RNA from human brain tissue ("control") only shows a product of wild-type length. The DNA size marker ("M") shows fragments of the lengths listed in figure 2. *C,* Results of sequence analysis of individual excised RT-PCR products. The normal-sized RT-PCR products from the patient (*top*) and the control (*bottom*) show the regular spliced transcript sequence, whereas exon 13 was skipped in the small RT-PCR product from peripheral blood RNA of the patient (*middle*). *D,* Schematic representation showing the mutation identified in tumor sample M2920 and showing the regular and the mutant transcript sequences.

mutation was detected, and, in 9 (56%) tumors without LOH, both mutations were found. In only 1 tumor without LOH, no mutation was determined. Thus, a total of 45 mutations were identified in tumors from 36 patients. Thirty-nine mutations were not detected in the corresponding constitutional DNA and, thus, are of somatic origin. The spectrum of somatic mutations includes 34 small mutations, 2 large structural alterations, and, in 3 tumors, hypermethylation of the 5′ end of the RB1 gene. Corrresponding to previous findings for hereditary retinoblastoma, most of the 7 small deletions and 4 insertions identified here are associated with homocopolymer tracts and direct repeats (Lohmann et al. 1994*b*). All small-length alterations and each of the 18 single-base substitutions in the open reading frame resulted in premature termination codons. In 5 tumors, formation of a regularly spliced transcript was precluded, because of nucleotide changes at invariant splice donor and acceptor sites (Shapiro and Senapathy 1987; Nakai and Sakamoto 1994). Since 15 (65%) of 23 single-base substitutions are transitions at CpG sites, this type of mutation is as predominant in constitutional mutations identified in hereditary retinoblastoma (Lohmann et al. 1996). Whereas the frequent occurrence of transitions at methylated CpG dinucleotides most probably is due to the hypermutability of 5-methylcytosine (Krawczak et al. 1992), the recurrence of a c409G→T transversion (E137X), in tumors from two patients (samples M1990 and M3865) and in constitutional DNA from a patient with bilateral retinoblastoma (Lohmann et al. 1996), might be caused by a two-step dislocation involving transient misalignment with an upstream quasi-repeat sequence containing a monotonic T run (Kunkel and Soni 1988; Ripley 1990; Hogg et al. 1993).

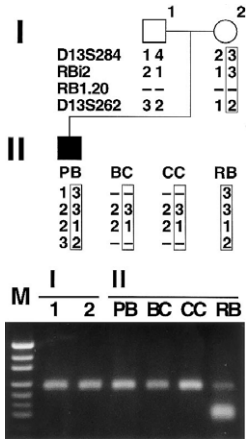

Figure 4 *Top,* Pedigree of family Bie (including the patient from which sample M6306 was taken), with the genotypes at polymorphic loci. *Bottom,* Results of restriction-enzyme digestion of exon 11 PCR products. DNA from peripheral blood (lane PB) and from cells exfoliated from buccal mucous membranes (lane BC) and conjunctiva (lane CC) are heterozygous at intragenic loci. In the enucleated tumor (lane RB), the paternal haplotype is lost. Since the mutation identified in tumor sample M6306 creates a new *Nla*III site in exon 11, PCR products from tumor DNA were digested almost to completion. The 101-bp and 112-bp fragments, which resulted from *Nla*III digestion of mutant products, were not detectable in the diverse samples of constitutional DNA from the patient. The DNA size marker ("M") shows fragments of the lengths listed in figure 2.

In two tumors without LOH at Rbi2 and RB1.20, the identical 6-kb *Eco*RV fragment downstream of exon 17 was affected by a somatic deletion. This fragment is located halfway between the intragenic loci RBi2 and RB1.20, which are located in the 5′ and the 3′ third of this gene, respectively. Hemizygous deletions involving these regions are manifested as LOH, and, therefore, in tumors without LOH, a bias toward the detection of deletions affecting the middle of the RB1 gene was to be expected and does not indicate that this region is prone to somatic deletions (Fung et al. 1987).

To date, aberrant methylation at the 5′ CpG island of the RB1 gene, which is constitutively unmethylated, has been reported in the tumors of 12 (11%) of 106 patients with isolated unilateral retinoblastoma (Greger et al. 1989, 1994; Sakai et al. 1991*b;* this study). Although we performed an exhaustive mutational analysis of tumors with hypermethylation, no structural alterations were found in 2 tumors with LOH, and the tumor without allele loss showed only one heterozygous mutation. Thus, our findings support the hypothesis that hypermethylation is functionally equivalent to an inactivating structural mutation. Aberrant methylation is not specific for the RB1 gene but has been found to be associated with transcriptional silencing of several tumor suppressor genes, in a variety of common human cancers (for an overview, see Herman et al. 1996). To date, no aberrant methylation has been identified in constitutional cells from patients with hypermethylation in tumor cells. It has been shown that *cis*-acting elements are required to prevent de novo methylation at CpG islands of constitutively unmethylated genes (Brandeis et al. 1994; Macleod et al. 1994). Thus, it is conceivable that structural alteration of these sequences can cause constitutional hypermethylation of RB1 alleles if the alterations occur prior to the generation of the normal methylation pattern during early embryonal development.

Most patients investigated in this study had single tumors that were too large to allow us to exclude unambiguously the presence of multifocal disease. However, two patients showed distinct multifocal tumors. In one of these patients, the larger of two tumors was sampled, for DNA analysis, at the time of enucleation. In this sample (M3619), a splice mutation in intron 19 and

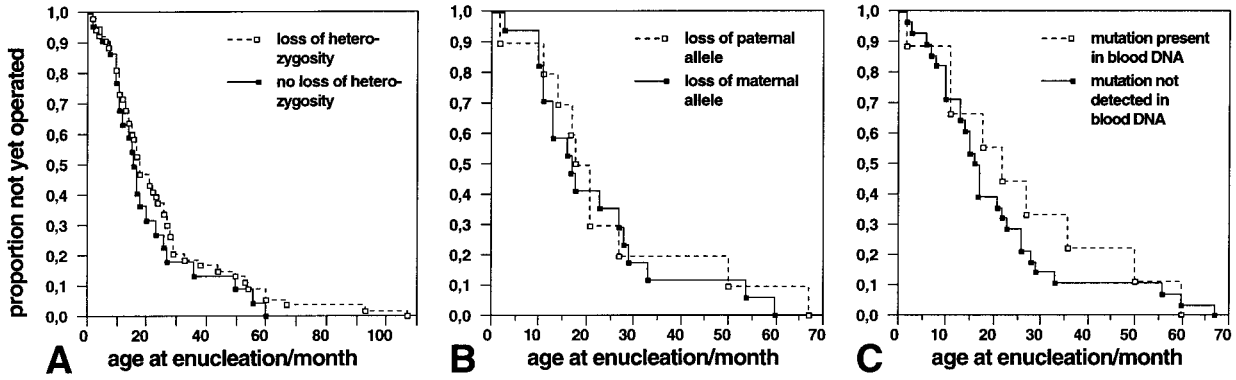

Figure 5 Proportion of patients not yet operated on, plotted as a function of time, by application of the Kaplan-Meier method. *A,* Comparison of results obtained from 53 patients with LOH and from 22 patients without LOH. The difference between the curves is not significant, when compared by use of the log-rank test ($\chi^2 < .92$; df = 1). *B,* Comparison of results obtained from 9 patients with loss of the paternal allele and from 14 patients with loss of the maternal allele, in the tumors (log-rank test: $\chi^2 < .23$; df = 1). *C,* Comparison of results obtained from 9 patients with a constitutional mutation and from 28 patients in which neither of the two mutations identified in the tumor was detectable in peripheral blood DNA (log-rank test: $\chi^2 < .5$; df = 1).

LOH at intragenic and telomeric polymorphic loci, indicative of mitotic recombination, were identified. However, peripheral blood DNA from this patient did not show the splice mutation identified in the tumor. Despite several attempts, PCR analysis of DNA extracted from microdissected archival tissue of the smaller-tumor focus failed. In a recent study of the histopathology of retinoblastoma, a meticulous examination of histological sections showed that most patients with isolated unilateral disease have, in fact, multifocal tumors (Moll et al. 1996). The authors assumed that additional foci are due to paracrine growth stimulation by factors released from the original tumor and concluded that multifocal tumors also may occur in nonhereditary patients. However, multifocal disease in patients without a mutation in peripheral blood DNA also may be caused by somatic mosaicism. In one patient, who was enucleated at the age of 2.3 mo, a new tumor focus was identified in the other eye, at the age of 10 mo. To terminate tumor growth, successive sessions of cryocoagulation were required. To our surprise, both mutations identified in the enucleated tumor sample (M6306) were not detected in the constitutional DNA of peripheral blood or in cells of cephalic origin. A similar patient with bilateral retinoblastoma, whose fibroblast DNA did not show the mutations identified in the tumor, also was reported by Shimizu et al. (1994). When it is considered that only a few tumors have been investigated so far, it is intriguing to find such an unexpectedly high rate of cases that are suggestive of somatic mosaicism. The screening of constitutional DNA will fail to identify postzygotic mutations, unless the analysis includes cells that are part of the mutant clone. Therefore, mosaicism may contribute to the lower rates of the detection of mutations in constitutional DNA that are observed for patients with isolated retinoblastoma, as compared with the rates obtained for familial cases (Blanquet et al. 1995).

In 6 (17%) of 36 patients, we identified one of the mutations in the tumor and in constitutional DNA. In addition, 3 (3.4%) of 89 patients showed constitutional mutations that were ascertained by the genotyping of polymorphic loci. None of these patients showed a new tumor, in repeated follow-up investigations. However, one patient (sample M2205) died of metastatic disease 10 mo after the initial diagnosis of unilateral retinoblastoma. In a recent retrospective survey of patients with unilateral retinoblastoma, Abramson et al. (1994) observed new intraocular tumors in only 4 (1.2%) of 338 patients with isolated disease treated by enucleation, and the last new tumor was identified before the patient reached the age of 31 mo. Almost all of our patients were followed beyond this age, and, therefore, it is not likely that any of them will develop a new tumor.

It has been inferred from Knudson's (1971) two-hit hypothesis that patients with an early presentation of unilateral tumors are likely to be carriers of a predisposing mutation (Cowell and Cragg 1996). Starting from this prediction, Cowell and Cragg (1996) investigated peripheral blood DNA of three patients with isolated unilateral retinoblastoma whose tumors were treated before the patients were 12 mo old, and they identified constitutional RB1-gene mutations in two of them. In our series, three patients treated before the age of 12 mo showed constitutional RB1-gene mutations, whereas, in seven patients, neither of the two mutations identified in the tumor was detected in peripheral blood. On the whole, there was no significant difference in the age at operation between patients with a constitutional mutation and those in whom neither of the two mutations identified in the tumor was detected in peripheral blood leukocytes.

All but one of the constitutional mutations were determined to have occurred de novo, because the mutations were not detected in peripheral blood DNA from the patients' parents and siblings. A missense mutation, in exon 4 of the RB1 gene (c411A→T; E137D), that was identified in both the tumor and the constitutional cells of sample M2408 also was found in the patient's mother and sibling. Segregation analysis indicated that the mutation is in phase with the grandpaternal haplotype. Since the grandfather did not consent to DNA testing, we cannot show whether this base substitution was inherited or whether it occurred de novo. An identical mutation was reported previously in a patient with bilateral retinoblastoma but was not identified in >300 patients tested thus far (Blanquet et al. 1993, 1995; Lohmann et al. 1996; this study). However, the putative consequence of this mutation, a substitution of glutamic acid for aspartic acid, at position 137, is unusual in view of the spectrum of missense mutations in the RB1 gene, because the substituted amino acids have very similar properties. The sequence environment of this mutation gives no clue as to whether splicing may be affected, but mutations may have unexpected effects on splicing (Nakai and Sakamoto 1994). Thus, at present, we cannot decide if this alteration is a predisposing mutation or a neutral variant.

Most mutations identified in patients with bilateral retinoblastoma result in premature-termination codons (Lohmann et al. 1996), and, on average, patients with these mutations show more than three tumors per eye (Munier et al. 1994; Lohmann et al. 1996). The actual number of tumors developing in a patient depends on the number of random second mutations that occur in predisposed cells. Given that the chance of a second event follows a Poisson distribution (Knudson 1971) and that a mean number of six tumors develops in the eyes (Munier et al. 1994; Lohmann et al. 1996), unilateral disease is expected in <10% of carriers of a null allele. Distinct RB1-gene mutations, however, have been identified in rare families with so-called low penetrance and an unusually high proportion of unilateral disease

(Sakai et al. 1991*a;* Yandell et al. 1991; Onadim et al. 1992; Dryja et al. 1993; Lohmann et al. 1994*a;* Cowell et al. 1996). One patient (sample G1142) in this study carries a de novo missense mutation (R661W), which was found to be associated with low penetrance in several unrelated families (Yandell et al. 1991; Onadim et al. 1992; Lohmann et al. 1994*a*). An increased proportion of unilateral disease also has been noted in patients with interstitial deletions involving 13q14 (Matsunaga 1980). In view of these genotype-phenotype correlations, unilateral presentation in one patient (sample G1142) and in the two patients with cytogenetic deletions identified here was to be expected.

In one patient (sample M2920), a mutation that results in the deletion of exon 13 is present in only some peripheral blood leukocytes. Mutations resulting in the identical in-frame deletion have been reported in previous studies of patients with bilateral retinoblastoma (Kato et al. 1994; Lohmann et al. 1996). Somatic mosaicism can be an important cause of phenotypic variation in the expression of genetic traits (Hall 1988). Mosaic deletions involving 13q14 have been observed in some patients with retinoblastoma (Ribeiro et al. 1988), and the results of a large study suggest that unilateral cases are affected preferentially (Bunin et al. 1989). A milder clinical presentation in patients with mosaic mutations also has been reported in type 1 and in type 2 neurofibromatosis (Bourn et al. 1994; Colman et al. 1996). Carlson and Desnick (1979) used mutational mosaicism as a developmental model to analyze the origin, onset, and transmissibility of retinoblastoma. They predicted that the variability of penetrance and expressivity, in familial cases, is due to multiple allelism and, thus, anticipated the finding of distinct mutations in families with low-penetrance retinoblastoma. They also suggested that mutational mosaicism can explain most of the variable expressivity observed in patients with the isolated unilateral disease and the anticipation-like phenomena in offspring (Herrmann 1976). In accordance with this model, reduced expressivity in patients who are mosaic for mutations predisposing to tumor formation reflects that fewer cells can be the target for a second hit.

Molecular analysis of mosaicism often is intricate, and, therefore, it is difficult to assess its rate in retinoblastoma. However, several lines of evidence indicate that the frequency of somatic mosaicism, in patients with isolated retinoblastoma, is not negligible. In a karyotype analysis of 123 patients with unilateral retinoblastoma, two of six chromosomal abnormalities affecting 13q were mosaic deletions (Bunin et al. 1989). So far, molecular evidence of somatic mosaicism for intragenic mutations was presented in 2 patients (Greger et al. 1990; this study). In 3 additional patients, the presence of a mutant sector was suggested by the occurrence of additional tumor foci in ipsilateral or fellow eyes (Shimizu et al. 1994; this study). Since the mutant clone also may be present in germinal tissue, the model of mutational mosaicism can reconcile the apparently high rate of constitutional mutations identified in this study with the significantly lower rate of affected children observed among survivors of unilateral disease (Draper et al. 1992).

The significance of mosaicism for genetic counseling has been outlined previously (Carlson and Desnick 1979; Hall 1988). Possible mosaicism in some patients also complicates molecular risk assessment for isolated retinoblastoma. As the presence of a mutant sector in retinal tissue cannot be excluded, follow-up investigations are required for all patients, regardless of their constitutional genotypes. However, the identification of the two oncogenic mutations, in tumor samples, is a prerequisite for the predictive testing of relatives. If these mutations are not detected in constitutional tissues, a postzygotic origin has to be assumed, and, consequently, siblings are not at risk. Nevertheless, a mutant clone may be present in germinal tissues, and, therefore, the children of these patients have to be tested for the two mutations. If a patient shows constitutional heterozygosity for an oncogenic mutation, siblings need to be investigated, even if this mutation is not identified in either parent. Children of these patients are at a high risk, although the segregation ratio may be skewed because of germinal mosaicism. In conclusion, our results emphasize that the manifestation and transmissibility of retinoblastoma depend on the nature of the first mutation, its time in development, and the number and types of cells that are affected (Hall 1988).

Acknowledgments

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