Screening of *RB1* Alterations in Brazilian Patients With Retinoblastoma and Relatives With Retinoma: Phenotypic and Genotypic Associations

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Purpose. To identify constitutional alterations of the retinoblastoma 1 gene (*RB1*) in two cohorts of Brazilian patients with retinoblastoma and to analyze genotype–phenotype associations.

Methods. Molecular screening was carried out by direct sequencing of the 27 *RB1* exons and flanking regions in blood DNA of 71 patients with retinoblastoma and 4 relatives with retinoma, and with multiplex ligation-dependent probe amplification (MLPA) in 21 patients. The presumed impact of nucleotide substitutions on the structure of the retinoblastoma protein (pRB) was predicted by Polymorphism Phenotyping-2 (PolyPhen-2). Kaplan-Meier and log-rank test were used for estimating 60-month survival rates.

Results. One hundred two nucleotide substitutions were detected, 92 substitutions in 59 patients with retinoblastoma and 10 substitutions in 4 individuals with retinoma. Eight substitutions were novel. The majority of substitutions were intronic (86.2%). More than one substitution was present in 37.3% of patients. Twenty-one duplications and 11 deletions were found in 12 patients; some of which with both types of alterations. Duplications/deletions were found in four patients lacking constitutional alterations when analyzed by sequencing, and in eight patients carrying one or more polymorphic intronic substitutions. The global 60-month survival rate in patients was 91.8% (Confidence Interval$_{95\%}$ = 85.0 − 99.1). Significant, lower survival rates were found in extraocular presentation (81.0%) versus intraocular tumors ($P = 0.014$), first enucleation after 1 month following diagnosis (80.9%) versus earlier first enucleation ($P = 0.020$), and relapse (100.0%) versus absence of relapse ($P = 0.0005$).

Conclusions. Fifteen substitutions (4 intronic and 11 exonic) were identified as probably or likely pathogenic. Four of these 11 exonic substitutions were novel. Survival rates, however, were not affected by presence of these probably or likely pathogenic alterations, most of which not found in patients with retinoblastoma from other Latin American countries. These differences might be related to the different ethnic composition of the Latin American cohorts.

Keywords: retinoblastoma, gene *RB1*, molecular screening

Objetivo. Identificar alterações constitucionais no gene retinoblastoma 1 (*RB1*) em duas coortes de pacientes brasileiros com retinoblastoma e analisar associações genótipo – fenótipo.

Métodos. Foi realizada uma triagem molecular, por sequenciamento direto dos 27 exons do gene *RB1* e suas respectivas regiões flanqueadoras a partir de DNA extraído de sangue de 71 pacientes com retinoblastoma e de 4 familiares com retinoma, e com a técnica de MLPA (multiplex ligation-dependent probe amplification) em 21 pacientes. O provável efeito das substituições nucleotídicas na estrutura da proteína retinoblastoma (pRB) foi estimada pelo PolyPhen-2. As probabilidades de sobrevida em 60 meses foram estimadas pelo método de Kaplan-Meier e testadas por meio do log-rank test.

Resultados. 102 substituições nucleotídicas foram detectadas, 92 em 59 pacientes, e 10 em indivíduos com retinoma. Oito substituições foram descritas pela primeira vez. A maioria das substituições foi encontrada em introns (86.2%). A presença de mais de uma substituição foi encontrada em 37,3% dos pacientes. Vinte e uma duplicações e 11 deleções foram identificadas em 12 pacientes; alguns dos quais com ambos os tipos de alterações. Foram encontradas duplicações/deleções em 4 pacientes que não apresentaram alterações constitucionais quando analisados por sequenciamento e em 8 pacientes portadores de uma ou mais substituições intrônicas polimórficas. A probabilidade global de sobrevida em 60 meses nos pacientes foi de 91.8% ($IC_{95\%} = 85.0 - 99.1$). Probabilidades de sobrevida significativamente mais baixas foram encontradas em pacientes com apresentação extraocular (81.0%) vs. tumores intraoculares ($P = 0.014$); com enucleação ocular inicial um mês após diagnóstico (80.9%) vs. enucleação inicial mais precoce ($P = 0.020$), e recaída (100.0%) vs. ausência de recaída ($P = 0.0005$).

Conclusão. Quinze substituições (4 intrônicas e 11 exônicas) foram identificadas como prováveis ou potencialmente patogênicas. Quatro dessas substituições exônicas foram descritas pela primeira vez. As probabilidades de sobrevida, porém, não foram afetadas pela presença das alterações prováveis ou potencialmente patogênicas, a maioria delas ausentes em pacientes de outros países de América Latina com retinoblastoma. Essas diferenças devem ser provavelmente relacionadas à diferente composição étnica das coortes Latino-americanas.

Retinoblastoma (RB) is the most common intraocular malignancy in childhood, occurring in approximately 1 of 15,000 to 25,000 live births (MIM# 180200), consequently to mutations that render both alleles of the tumor suppressor *RB1* gene functionless. Somatic mutations affecting both *RB1* alleles in retinal cells are responsible for nonhereditary RB.[1] Conversely, in the hereditary form, a mutation in one *RB1* allele is transmitted as an autosomal dominant trait associated with RB predisposition with high penetrance. In these patients, in whom the inherited mutation is constitutional, RB arises due to a second mutation in retinoblasts, while other tissues are also susceptible to developing tumors. Cumulative incidence rates of secondary tumors were 20% at 10 years from diagnosis, 50% at 20 years, and 90% at 30 years in irradiated patients, while in nonirradiated patients, they accounted for 10% at 10 years, 30% at 20 years, and 68% at 32 years.[2] Identification of constitutional *RB1* mutations is, thus, crucial for genetic counseling and for estimating risk in relatives of affected individuals.[3]

A wide spectrum of alterations may occur along *RB1* exons, introns, and regulatory regions.[4] Several methods like DNA sequencing, fluorescence in situ hybridization (FISH), heteroduplex DNA analyses, single strand conformational polymorphism (SSCP), and multiplex ligation-dependent probe amplification (MLPA) have been used for detecting them, but none have been capable of screening the complete *RB1* gene due to the large size of intronic regions. Moreover, several reports are restricted to few cases, which do not allow for adequate analyses of genotypic–phenotypic associations.

Limited data are presently available on the incidence of *RB1* alterations in Brazil,[5–8] a country with marked regional disparities, different ethnic profiles, and diverse migration inflows from countries where mutational screenings have been carried out. Here, we report our findings of *RB1* screening in a sample of patients with RB from Brazil in association with clinical data and mortality.

Methods

Seventy five individuals were analyzed, comprising 71 unrelated patients with RB and four related individuals with retinoma, referred to the Instituto Nacional de Câncer (Rio de Janeiro, Brazil). One patient (PT130) with a previous history of RB and prenatal mutational screening was a known carrier of a constitutional p.R455X mutation.

Clinical diagnosis was carried out following current ophthalmologic and histopathologic criteria. Staging followed the International Classification of Retinoblastoma for intraocular RB[9] and the Chantada international system for extraocular RB.[10]

Self-identification was used for characterizing the ethnic composition of the patient's cohort. Family data and sample collections were obtained with informed consents from patients or parents following the guidelines of the Helsinki Declaration. All procedures carried out at the Instituto Nacional de Câncer were approved by the local ethics committee.

RB1 Screening

Blood DNA was isolated from peripheral blood leukocytes from patients and individuals with retinoma. *RB1* exons and flanking regions were PCR amplified with primer pairs shown in Table 1, in PCR reaction mixes[8] and a touchdown protocol[11] with temperatures between 50°C to 60°C, sequenced and aligned as previously reported,[5,8,12] and compared with reference sequence data (Genbank L11910.1) and *RB1* gene mutation database.[13]

Multiplex ligation-dependent probe amplification (MLPA) was carried out in 21 patients using P047 RB1 probes purchased from MRC-Holland (Amsterdam, The Netherlands). Procedures were carried out according to the manufacturer's recommendations.[14] Amplification products were run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

In Silico and Statistical Analyses

Polymorphic variants were identified in the Single Nucleotide Polymorphism database (dbSNP).[15] The putative impact of amino acid substitutions on the structure and function of RB protein (pRB) was assessed with Polymorphism Phenotyping-2 (PolyPhen-2).[16] MLPA data were analyzed with Genemapper 3.7 (Applied Biosystems).

A descriptive data analysis was performed to evaluate the consistency and quality of information and to describe the study population. Subsequently, the nonparametric method of Kaplan-Meier was used to estimate 60-month survival rate[17] and the log-rank test to assess statistical differences between different strata of the same variables (sex, age at diagnosis, ethnic group, lag time [interval between first signs and diagnosis], leukocoria, laterality, tumor localization, staging, ocular survival, enucleation, interval between diagnosis and first enucleation, relapse, interval between diagnosis and relapse, family history of RB or retinoma, *RB1* alteration and type). These analyses were carried out with the www.r-project.org open access software R.[18]

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Table 1. Primers Used in This Study

Amplicon Number	Primer Position	Primer Sequence (5′→3′)	Fragment Size in bp and *RB1* Position	Reference
Amplicon 1	1 F	GGTTTTTCTCAGGGGACGTT	454 (1925-2378)	8
	1 R	AACCCAGAATCCTGTCACCA		
Amplicon 2	2 F	TTTGGAATGACCATGAAAAAGA	464 (5226-5689)	This work
	2 R	AAATTTCCTCTGGGTAATGGAA		
Amplicon 3	3 F	ACAAACATTTATTTTGTATGCTGAA	388 (39330-39720)	This work
	3 R	CTCCATGAGAGAATGGCAGTT		
Amplicon 4	4 F	ACAAATTTTTAAGGTTACTGATTTAC	237 (41876-42112)	12
	4 R	CCAGAATCTAATTGTGAACAATGAC		
Amplicon 5	5 F	AACTACTATGACTTCTAAATTACG	221 (44607-44827)	12
	5 R	CTTAATTTATGAAGTAGCCTGCTA		
Amplicon 6	6 F	CTGGAAAACTTTCTTTCAGTGATA	210 (45734-45943)	5
	6 R	GGAATTTAGTCCAAAGGAATGCC		
Amplicon 7	7 F	ATACAAAGATCTGAATCTCTAACT	226 (56800-57025)	5
	7 R	CTAGACATTCAATAAGCAACTGC		
Amplicon 8	8 F	GAATGTTACCAAGATTATTTTTGACC	376 (59548-59917)	This work
	8 R	TGCTACTGCAAAAGAGTTAGCAC		
Amplicon 9	9 F	GTTCAAGAGTCAAGAGATTAGATT	209 (61661-61869)	5
	9 R	CAATTATCCTCCCTCCACAGTCTCA		
Amplicon 10	10 F	GCCTCTGTGTGCTGAGAGATGTA	277 (64264-64540)	5
	10 R	AATGATATCTAAAGGTCACTAAGC		
Amplicon 11	11 F	GATTTTATGAGACAACAGAAGCA	244 (65264-65507)	5
	11 R	ATCTGAAACACTATAAAGCCATG		
Amplicon 12	12 F	AGAGACAAGTGGGAGGCAGTG	327 (70120-70446)	5
	12 R	GATAACTACATGTTAGATAGGAG		
Amplicon 13	13 F	TGGAAGTGTTTCCACATTTTT	304 (73636-73939)	This work
	13 R	CGAACTGGAAAGATGCTGCT		
Amplicon 14	14 F	ATTGTGATTTTCTAAAATAGCAGG	766 (76377-77142)	This work*
	15–16 R	AAGAAACACACCACATTTTAACT		
Amplicon 15	17 F	AGCTCAAGGGTTAATATTTCATAA	431 (78032-78462)	This work†
	17 R	GGTGCTCGATTAAAGCTCCA		
Amplicon 16	18 F	ATGTACCTGGGAAAATTATGCTT	336 (149939-150274)	This work†
	18 R	TTTGGGTCATGTACCTTTTATTACT		
Amplicon 17	19 F	ATCTGTGATTCTTAGCCAACTTG	250 (153154-153403)	5
	19 R	AGTCAGCCTAGTTTCAGAGTC		
Amplicon 18	20 F	CTGGGGGAAAGAAAAGAGTGG	328 (156544-156871)	5
	20 R	GAGGAGAGAAGGTGAAGTGCT		
Amplicon 19	21 F	GAACAAAACCATGTAATAAAATTCT	219 (160676-160894)	5
	21 R	ACCTATGTTATGTTATGGATATGG		
Amplicon 20	22 F	ACTGTTCTTCCTCAGACATTCA	431 (161982-162412)	This work*
	23 R	TCCCCCTCTCATTCTTTACTAC		
Amplicon 21	24 F	TCATCTCTGCAAAATTGTATATGG	210 (170309-170525)	5
	24 R	TATGCAATATGCCTGGATGAGG		
Amplicon 22	25 F	TTGCTAACTATGAAACACTGGC	880 (173647-174526)	This work*
	26 R	ATGCATAAACAAACCTGCCAACT		
Amplicon 23	27 F	TGCAAGGTCCTGAGCGCCAT	238 (176894-177131)	5
	27 R	GAGAGACAATGAATCCAGAGGTG		

Positions refer to the normal *RB1* sequence (GenBank L11910).

* Combinations of previously reported primers.[5]

† Forward primer previously reported[5]; reverse primer designed in this report.

Results

Patient Characteristics and Clinical Findings

Supplementary Table S1 lists characteristics of patients with RB and individuals with retinoma, data on pathology, disease progression, and *RB1* screening.

The majority of patients were referred from centers located in the Southeastern (81.6%) and Southern (18.3%) regions of Brazil, comprising 40.8% Caucasians, 18.3% mixed-race (Caucasian-black), 11.2% black, and 1.4% of Asian descent. The age-adjusted incidence rate (per million) of retinoblastoma in children and adolescents of less than or equal to 19 years of age was estimated as 6.63 in the southeast versus 4.29 in the south and an overall estimate of 4.99 for Brazil.[19] The proportion of males and females with RB was 46.4% and 53.2%, respectively with a median age of 12 months for patients diagnosed at 24 months or less, and 36 months for patients diagnosed after 24 months. Bilateral RB was present in 42.2% of patients and unilateral RB in 56.3%, while laterality was not reported in one patient (1.4%). Extraocular localization occurred in 29.5%. Median age at diagnosis of patients with intraocular RB was 10 months for bilateral RB and 33 months for unilateral RB, while median age at diagnosis of extraocular RB was 23 months for bilateral RB and 31 months for unilateral RB.

Median lag time was 5 months, varying from 0 to 36. Leukocoria was present in 71.8% of patients. Enucleation was

carried out in 88.6% of patients; in 56.3% of them less than 1 month after diagnosis. Relapse occurred in 26.7% of patients, up to 1 year after diagnosis (44.4%).

RB1 Screening

Primer designs and combinations resulted in larger amplified fragments than previously reported, allowing for the identification of *RB1* alterations in 23 amplicons comprising all 27 *RB1* exons, 46 flanking regions, and 4 complete introns (14, 15, 22, and 25). A total of 102 constitutional alterations comprising single nucleotide substitutions were identified, 92 substitutions in 59 patients with RB, and 10 substitutions in 4 individuals with retinoma. The majority of substitutions occurred in introns, 82.3% outside splicing sites and 3.9% at splicing sites. These alterations accounted for 31 different substitutions, 8 of which not previously reported (Table 2). Four novel intronic substitutions were likely to be nonpathogenetic and polymorphic variants.

Thirty three substitutions were identified in 25 patients with bilateral RB, 58 in 29 patients with unilateral RB, and one in a single patient with unknown laterality. In 4 individuals with retinoma, 10 intronic substitutions were identified, 9 outside splicing sites, and 1 in 1 splicing site. Twenty two RB patients (30.9%) carried more than one substitution, accounting for 18.3% with two substitutions, 5.6% with three, 5.6% with four, and 1.4% with five.

Three individuals with retinoma (RT113, RT116, and RT122) shared the same alterations of their respective relatives with RB, but RT122 carried two additional variants (IVS14+231A > T and IVS16+5G > A) not present in her affected child (PT122) or in any other patient. One substitution in individual RT116 (IVS3+37A > G) was also present in her mother who showed a normal fundoscopy. Finally, a fourth individual with retinoma (RT120) carried two other substitutions (IVS3+45C > T and IVS14+235G > C) not present in her affected brother (PT120).

MLPA Analysis

Twenty one duplications and 11 deletions were found in 12/21 patients; some of them showing these two types of alterations (Supplementary Table S1). Some of them were found in four patients lacking constitutional alterations when analyzed by *RB1* sequencing and in eight patients carrying one or more polymorphic intronic substitutions. Duplications and deletions were observed in exons 1, 5, 7, and 13, while the most common duplication occurred in exon 16, which was observed in 6 cases.

Survival Rates

Table 3 lists all variables, family history, and presence of *RB1* alterations respective to vital status. The global, 60-month survival rate of RB patients was 91.8 (Confidence Interval$_{95\%}$ = 85.0 – 99.1). Variables showing significant statistical differences ($P \leq 0.05$) between groups were: tumor localization, interval between diagnosis, and first enucleation and occurrence of relapse (Table 4). The Figure shows survival curves for these variables.

Table 2. List of *RB1* Substitutions Identified in This Study, Probable Effect and SNP Identification

SNPs	Effect	Genomic Position	ID (rs) dbSNP
Intronic substitutions	Probably nonpathogenic	**g.39408A>G (IVS2-33A>G)**	N/A
		g.5625T>C (IVS2+75T>C)	rs3092865
		g.39598A>G (IVS3+37A>G)	rs118072861
		g.39606T>C (IVS3+45T>C)	rs520342
		g.42068G>A (IVS4+23G>A)	N/A
		g.42068G>T (IVS4+23G>T)	rs198617
		g.64497G>A (IVS10+58G>A)	rs3092883
		g.73724A>G (IVS12-29A>G)	rs3092886
		g.76526G>A (IVS14+40G>A)	N/A
		g.76737A>G (IVS14-152A>G)	N/A
		g.76717A>T (IVS14+231A>T)	rs3092893
		g.76721G>C (IVS14+235G>C)	rs2070752
		g.156616A>G (IVS19-77A>G)	rs198580
		g.174351T>A (IVS25-10T>A)	rs77317605
		g.173882T>C (IVS25+33T>C)	rs3020646
	Probably pathogenic	g.70241G>A (IVS11-1G>A)	N/A
		g.77082G>A (IVS16+5G>A)	N/A
		g.162368G>A (IVS23+1G>A)	N/A
		g.162368G>T (IVS23+1G>T)	N/A
Exonic substitutions	Synonymous	g.70254C>T (p.N380N)	N/A
	Missense probably pathogenic	g.59789A>G (p.D286G)	N/A
		g.78133A>T (p.I517F)	N/A
		g.78158C>G (p.A525G)	rs4151539
		g.156713C>T (p.R661W)	rs137853294
		g.173752G>A (p.D856N)	N/A
	Nonsense likely pathogenic	**g.56954G>T (p.E237X)**	N/A
		g.64348C>T (p.R320X)	rs121913300
		g.65386C>T (p.R358X)	rs121913301
		g.76460C>T (p.R455X)	rs121913302
		g.156836C>T (p.Q702X)	N/A
		g.173716A>T (p.K844X)	N/A

N/A, not available in dbSNP.
Novel alterations are shown in bold font.

Table 3. Variables Respective to Vital Status of Patients

Variables	Vital Status				Total	
	Live		Dead			
	n	%	*n*	%	*n*	%
Global	66	92.9	5	7.0	71	100.0
Sex						
Male	31	94.9	2	6.0	33	46.4
Female	35	92.1	3	7.8	38	53.5
Age at diagnosis						
24 mo or less	32	94.1	2	5.8	34	47.8
More than 24 mo	34	91.8	3	8.1	37	52.1
Ethnic group						
Black	9	81.8	2	18.1	11	15.4
Nonblack	54	94.7	3	5.2	57	80.2
Not informed	3	100.0	0	0.0	3	4.2
Lag time (interval between first signs and diagnosis)						
1 mo or less	13	100.0	0	0.0	13	18.3
More than 1 mo	51	91.0	5	8.9	56	78.8
Not informed	2	100.0	0	0.0	2	2.8
Leukocoria						
Present	47	92.1	4	7.8	51	71.8
Absent	10	100.0	0	0.0	10	14.0
Not informed	9	90.0	1	10.0	10	14.0
Laterality						
Unilateral	38	95.0	2	5.0	40	56.3
Bilateral	27	90.0	3	10.0	30	42.2
Not informed	1	100.0	0	0.0	1	1.4
Tumor localization						
Intraocular	48	97.9	1	2.0	49	69.0
Extraocular	17	80.9	4	19.0	21	29.5
Not informed	1	100.0	0	0.0	1	1.4
Enucleation						
Without enucleation	2	100.0	0	0.0	2	2.8
1 eye	44	95.6	2	4.3	46	64.7
Both eyes	14	82.3	3	17.6	17	23.9
Not informed	6	100.0	0	0.0	6	8.4
Interval between diagnosis and first enucleation						
1 mo or less	39	97.5	1	2.5	40	56.3
More than 1 mo	15	83.3	3	16.6	18	25.3
Not informed	4	80.0	1	20	5	7.0
Relapse						
With relapse	14	73.6	5	26.3	19	26.7
Without relapse	41	100.0	0	0.0	41	57.7
Not informed	11	100.0	0	0.0	11	15.4
Interval between diagnosis and relapse						
1 y or less	6	75.0	2	25.0	8	44.4
More than 1 y	7	87.5	1	12.5	8	44.4
Not informed	1	50.0	1	50.0	2	11.1
Family history of RB or retinoma						
Present	14	93.3	1	6.6	15	21.1
Absent	45	91.8	4	8.1	49	69.0
Not informed	7	100.0	0	0.0	7	9.8
Constitutional alteration						
Present	54	91.5	5	8.4	59	83.0
Absent	12	100.0	0	0.0	12	16.9

Table 3. Continued

Variables	Vital Status: Live *n*	Live %	Dead *n*	Dead %	Total *n*	Total %
Number of constitutional alterations						
1	31	91.1	3	8.8	34	57.6
More than 1	23	92.0	2	8.0	25	42.3
Substitution type						
Likely nonpathogenic	29	90.6	3	9.3	32	54.2
Likely pathogenic	25	92.5	2	7.4	27	45.7

Discussion

Genotype–Phenotype Associations

RB1 alterations resulting in pRB dysfunction might affect the regulation of protein-encoding genes as well as RNA polymerase pol I and pol III transcription. pRB appears to be the major player in a regulatory circuit in the late G1 phase, and is involved in regulating an elusive switch point between cell cycle, differentiation and apoptosis, probably cooperating with another major tumor suppressor, p53.[20] Alterations of different pRB regions involved in interactions with other proteins might thus result in dysfunctional effects leading to tumor development because the A and B domains of pRB are required for repressor activity, forming a repressor motif interacting with E2F, with a dominant inhibitory effect on transcription. The pRB pocket was originally defined as the binding site for oncoproteins from DNA tumor viruses like adenovirus E1a.[21]

Exonic Substitutions, Probably Pathogenic

Nonsense substitutions are likely to be pathogenic, leading pre-mRNA to nonsense-mediated decay.[3] In our sample, they were restricted to RB patients; six different types were found in eight patients (Supplementary Table S1, Table 2); three of them (p.R320X, p.R358X, and p.R455X) being "recurrent"[2] and two novel (p.E237X and p.K844X). In p.R320X, p.R358X, and p.R455X, premature stop codons resulted from C > T transitions, probably by deamination of 5-methyl-cytosine at CpG dinucleotides unlike the C > T transition originating p.Q702X, while p.E237X and p.K844X resulted from transversions (G > T and A > T, respectively). The novel p.E237X and the two recurrent p.R320X and p.R358X affected the pRB N-terminus contrary to p.R455X and p.Q702X that affected the A/B pocket, while p.K844X affected the pRB C-terminus.[22] To present, alterations at residues 237 or 844 have not been reported.[13]

Missense substitutions were also restricted to RB patients. p.D286G was located in the N-terminus of pRB, a crucial region for functional integrity, embryonic development, tumor suppression activity,[23] suppression of p84N5-induced apoptosis,[24] and association to proteins involved in DNA replication.[25] PolyPhen-2 analysis predicted p.D286G as probably damaging.

The novel, missense substitution p.I517F (g78133A > T), a unique alteration in the A/B pocket in a single patient (PT25), was considered to be possibly damaging by PolyPhen-2 analysis. Another missense substitution, p.A525G (g.78158C > G), in the A/B pocket, was present in a single patient (PT115) in association with three other, two affecting intronic regions outside splicing sites and another one at a splicing site; this last one being the known deleterious mutation IVS23+1G > T associated to bilateral RB.[3] p.A525G is presently listed as resulting from g.78158C > G or g.78156del and also associated to bilateral RB.[13] PolyPhen-2 analysis predicted this alteration as possibly damaging.

The recurrent substitution p.R661W (g.156713C > T), a unique alteration in a single patient with bilateral RB (PT13), has been found to decrease E2F1 binding in vitro, resulting in partial pRB activity.[26,27] It has been associated to unilateral RB[3] and reduced penetrance,[28,29] and its recurrence is probably due to a C > T transition at the arginine codon containing a CpG dinucleotide. This alteration affects the A/B pocket and was predicted as probably damaging by PolyPhen-2 analysis. Finally, the novel missense substitution p.D856N (g.173752G > A), found in association with three intronic substitutions outside splicing sites in a single patient (PT126), affected the pRB C-terminus, at a site where mutations have not been reported.[13] The C-terminal region interacts with the c-Abl tyrosine kinase[30] and with p53 regulated by MDM2.[31] Phosphorylation of the pRB C-terminal region by Cdk4/6 was found to initiate successive intramolecular interactions between the C-terminal region and the central pocket, displacing histone deacetylase from the pocket and blocking active transcriptional repression by pRB.[32] PolyPhen-2 analysis predicted this alteration as possibly damaging.

Exonic Substitution, Probably Nonpathogenic or of Unknown Significance

Only one synonymous substitution (p.N380N) was found as a unique variant in PT19, a patient with unilateral RB. This alteration is probably nonpathogenic or of unknown significance.

Intronic Substitutions, Pathogenic or Probably Pathogenic

Three patients (PT4, PT76, and PT115) were carriers of probably pathogenic substitutions at splicing sites (IVS23+1G > A, IVS11-1G > A, and IVS23+1G > T, respectively). Another substitution (IVS16+5G > A) with a known deleterious effect[13] was found in one individual with retinoma (RT122), but not in her affected son (PT122; Supplementary Table S1, Table 2).

Intronic Substitutions, Probably Nonpathogenic or of Unknown Significance

Analyses intronic substitutions outside splicing sites showed four novel alterations (IVS2-33A > G, IVS4+23G > A, IVS14+40G > A, and IVS14-152A > G) (Table 2). Eleven other intronic substitutions outside splicing sites found in this study have been reported as polymorphisms.[15,33]

Table 4. Sixty-Month Survival Rates

Variables	60-Month Survival Rates		
	$P(S_t)$	Confidence $Interval_{95\%}$	*P* $Value_{Log\text{-}rank}$
Global	91.8	(85.0–99.1)	
Sex			
Male	90.5	(78.4–100.0)	$P = 0.767$
Female	91.7	(83.0–100.0)	
Age at diagnosis			
24 mo or less	100.0	–	$P = 0.779$
More than 24 mo	89.6	(81.1–98.9)	
Ethnic group			
Black	80.8	(60.0–100.0)	$P = 0.118$
Nonblack	93.9	(87.2–100.0)	
Lag time (interval between first signs and diagnosis)			
1 mo or less	100.0	–	$P = 0.271$
More than 1 mo	89.6	(81.1–98.9)	
Leukocoria			
Present	90.6	(82.1–100.0)	$P = 0.424$
Absent	100.0	–	
Laterality			
Unilateral	94.7	(87.7–100.0)	$P = 0.355$
Bilateral	86.6	(72.7–100.0)	
Tumor localization			
Intraocular	96.5	(90.1–100.0)	$P = 0.0146$
Extraocular	81.0	(65.8–99.6)	
Enucleation			
Without enucleation	100.0	–	$P = 0.13$
1 eye	95.3	(89.2–100.0)	
Both eyes	75.3	(53.0–100.0)	
Interval between diagnosis and first enucleation			
1 mo or less	96.5	(90.1–100.0)	$P = 0.020$
More than 1 mo	80.9	(63.4–100.0)	
Relapse			
With relapse	68.8	(48.3–97.9)	$P = 0.0005$
Without relapse	100.0	–	
Interval between diagnosis and relapse			
1 y or less	75.0	(50.3–100.0)	$P = 0.946$
More than 1 y	59.3	(25.8–100.0)	
Family history of RB or retinoma			
Present	91.6	(77.2–100.0)	$P = 0.784$
Absent	91.4	(83.7–99.9)	
Constitutional alteration			
Present	89.3	(80.8–98.8)	$P = 0.295$
Absent	100.0	–	
Number of constitutional alterations			
1	89.1	(77.9–100.0)	$P = 0.575$
More than 1	88.5	(74.8–100.0)	
Substitution type			
Likely nonpathogenic	90.7	(81.1–100.0)	$P = 0.567$
Likely pathogenic	85.7	(69.2–100.0)	

MLPA Analysis

Duplications and deletions were found in 12 patients who did not carry any apparent constitutional alteration or were carriers of known polymorphic substitutions when analyzed by direct DNA sequencing. In fact, changes in exon copy have been found to account for approximately 15% of all *RB1* mutations and 5% of mosaic mutations,[34] a finding that makes MLPA a complementary procedure for diagnosing molecular alterations associated to retinoblastoma[35] in view of its better

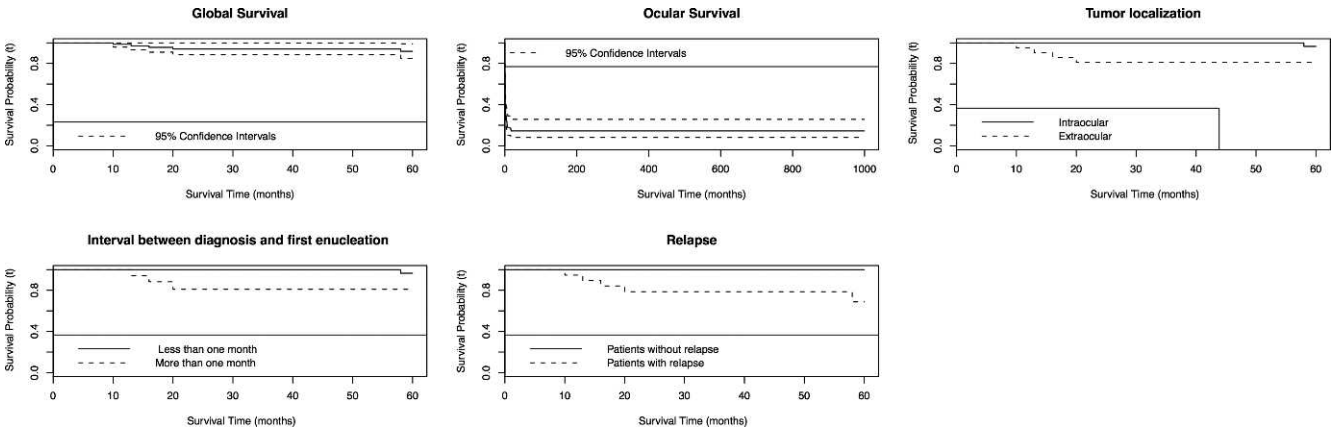

Figure. Survival rates of patients with retinoblastoma (global and respective to variables significantly affecting clinical outcome).

resolution for identifying *RB1* deletions or duplications than direct DNA sequencing. MLPA is especially valuable for diagnosing complete *RB1* deletions that would otherwise be undetected by DNA sequencing.

RB1 Screening of Other Latin American Patients With Retinoblastoma

Analysis of 10 Argentine families with sporadic bilateral retinoblastoma, carried out with Southern blotting combined with endonuclease digestions and heteroduplex analyses identified two large *RB1* deletions and four small mutations resulting in a truncated pRB protein: one 4 bp insertion in exon 7, one C > T transition in exon 18, one CT deletion in exon 19, and one 1 bp deletion in exon 20.[36] Another report of other 21 Argentine families analyzed by "exon by exon" PCR-heteroduplex and sequencing identified four substitutions resulting in stop codons. One of them was the recurrent g.65386C > T (p.R358X) in exon 11 herein reported (Table 2), while one in exon 18 affected codon 579 and another, in exon 23 affected codon 787. This study also reported one missense mutation in exon 13 affecting codon 433 as likely pathogenic.[37] Further studies of the same group in 40 patients with four polymorphic intragenic markers, FISH and heteroduplex/sequence analyses identified three complete *RB1* deletions, and two small 1 bp deletions.[38]

A survey of 19 Mexican cases by cytogenetic, SSCP, and sequencing analyses reported three pathogenic, frame shift mutations in three patients affecting exon 8 (with one insertion resulting in a stop codon), exon 18 (with a single nucleotide deletion), and exon 20 (with a single nucleotide deletion resulting in a stop codon).[39] Another study, by sequencing analysis carried out in Ecuador,[40] identified a g.162190T > C substitution (IVS22 −14T > C) in blood and tumor DNA of one patient resulting in altered splicing.[13] Finally, a study of 19 Colombian and 14 Cuban patients, carried out with sequencing and fluorescent probes[41] identified 12 mutations in each group. In the Cuban cohort, nucleotide substitutions in exons 8, 10, and 17 resulted in stop codons (p.R251X, p.E323X, and p.R556X, respectively), while another stop codon in exon 8 resulted from one dinucleotide insertion. Three missense substitutions were identified in exons 14, 17, and 19 (p.G449E, p.W563C, and p.V654L, respectively), the first two considered as probably pathogenic and the last one as benign by PolyPhen-2. Five other substitutions affected introns 6, 13, 22, and 24 resulting in altered splicing sites and in the appearance of a new acceptor site. In the Colombian cohort, seven substitutions affecting exons 7, 10, 12, 14, 21, and 23 resulted in stop codons (p.S230X, p.R320X, p.Y321X, p.G383X, p.R445X, p.Y709X, and p.R787X). One of these alterations, in exon 10 (g.64348C > T; p.R320X), was also found in this report (Table 2). Five frame shift alterations in exons 11, 13, 19, 20, and 23 due to insertions or deletions, also resulted in stop codons.[41]

Interestingly, the *RB1* mutational spectrum found in other Latin American cohorts differed from one herein reported. Only two exonic, likely pathogenic substitutions herein reported (p.R320X and p.R358X) were found in other surveys. Small insertions/deletions resulting in frame shift mutations were not identified in this study. These differences might be related to the different ethnic composition of the Latin American cohorts.

Penetrance of *RB1* Deleterious Alterations

Familial *RB1* alterations might show variable penetrance and expressivity. Low penetrance occurs when a constitutional carrier does not develop RB, while reduced expressivity may result in unilateral tumors or retinomas.[42] Loss of pRB expression has been found in retinomas with low level genomic instability and high expression of senescence-associated proteins.[43] Low penetrance has been postulated to occur by alternative, in frame translation start sites in low penetrance alleles[44] or consequently to mutations at the pRB C-terminus.[45] The molecular basis of low penetrance might also result from nontruncating alterations reducing expression or partially inactivating pRB.[42]

The IV16+5G > A substitution has been identified as a causative deleterious alteration associated with RB[13] but it was present in one individual who developed retinoma (R122), probably due to reduced penetrance.

The novel mutations p.K844X and p.D856N affected exon 25, in a region encoding the pRB C-terminus where mutations have been associated with low penetrance RB. This was the case of deletion of exons 24 and 25 in a low penetrance RB family[46] and an insertion in exon 27 in a patient with unilateral RB and his unaffected father.[47]

Conversely, some truncating *RB1* alterations (including the recurrent p.R358X found in PT63) are not exclusive of retinoblastoma because they have been found in familial retinoma.[45] These findings indicated that specific, constitutional mutations are not necessarily associated with malignant development while other factors, like stage of cell maturation at the time when the second *RB1* mutations occurs might be involved.[48] Thus, genotypic–phenotypic associations are still incompletely understood as well as factors accounting for incomplete penetrance and variable expressivity. In fact, nonsense, missense, and synonymous mutations might inacti-

vate genes by inducing the splicing machinery to skip exons, while single nucleotide substitutions might influence splicing accuracy or efficiency.[49]

Clinical Progression of RB Patients

The global, 60-month survival rate estimated in this study (92.9%) was higher than a previous estimate of 78% in a survey of 1317 Brazilian patients.[50] This metaanalysis, however, estimated survival data in a nonsystematic manner across a wide spectrum of publications and different institutions, accounting for heterogeneous cohorts of patients. In Brazil, a country with marked regional disparities, this lower survival estimate[50] was expected respective to our patients attended at reference centers.

In this study, significant differences in survival rates were not found between: (1) sex, unlike a previous report showing males with a better overall survival rates, albeit attributable to differences in age and tumor stage between sexes[51]; (2) age at diagnosis, in coincidence with a previous report[52] (our study indicated a median age of 32 months for unilateral RB and 12.5 months for bilateral RB); (3) ethnic group; (4) lag time (a median of 4 months for unilateral and 6.5 months for bilateral RB was found), similar to a previous estimate in Brazilian patients,[53] but longer than the median lag time in North American patients (1.5 month for unilateral and 2.25 months for bilateral RB[54]), showing, as in our patients, a shorter median lag time for unilateral RB, contrary to a report of Chinese patients,[55] with median lag time of 1.0 month for bilateral and 2.0 months for unilateral RB; (5) leukocoria, accounting for better survival when present; (6) laterality, accounting for better survival of unilateral RB; (7) enucleation, associated to better survival if not carried out or affecting only one eye; (8) interval between diagnosis and relapse; (9) family history of RB or retinoma. Patients with family history showed shorter lag time when compared with those with negative family history[56] due to early detection of leukocoria or strabismus; (10) number of constitutional alterations in agreement with lack of additive effects[57]; and (11) presence of constitutional alteration. These clinical findings were associated with a favorable 60-month survival but with poor ocular survival for both unilateral and bilateral tumors.[2] In fact, almost all patients herein analyzed were enucleated by 20 months after diagnosis.

The survival rate of RB patients with extraocular tumors was found be significantly lower than patients with intraocular tumors in agreement with the proposition that extraocular extension is indicative of poor prognosis.[52] Extraocular tumors were also associated with late diagnosis,[53] with a median lag time of 24 weeks, and metastatic RB with a median lag time of 50 weeks.[58] In our patients with extraocular disease, the median age at diagnosis was 13 months higher than those with intraocular disease and the difference of median lag time equaled 5 months between these groups.

Patients suffering enucleation more than 1 month after diagnosis showed a significantly less favorable outcome, probably because they presented more advanced stages of tumor development at the time of enucleation (Supplementary Table S1). This was coincident with the poorer prognosis of patients with relapse respective to those who did not relapse. Cumulative survival curves for all variables showed an evident decrease in survival around 20 months after diagnosis, indicating that all factors accounting for poor prognosis might be simultaneously operating at this time. Cancer control policies may change this scenario by improving the general knowledge of RB symptoms as well as proving prompt diagnosis.

In conclusion, *RB1* screening identified 15 substitutions (4 intronic and 11 exonic) considered as probably or likely pathogenic. Four of these 11 exonic substitutions were novel. Survival rates, however, were not affected by the presence of these probably or likely pathogenic alterations, most of which apparently not present in RB patients of other Latin American countries. These differences might be related to the different ethnic composition of the Latin American cohorts.

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References

1. Lee V, Hungerford JL, Bunce C, Ahmed F, Kingston JE, Plowman PN. Globe conserving treatment of the only eye in bilateral retinoblastoma. *Br J Ophthalmol*. 2003;87:1374–1380.
2. Abramson DH, Ellsworth RM, Kitchin FD, Tung G. Second nonocular tumors in retinoblastoma survivors. Are they radiation-induced? *Ophthalmology*. 1984;91:1351–1355.
3. Richter S, Vandezande K, Chen N, et al. Sensitive and efficient detection of RB1 gene mutations enhances care for families with retinoblastoma. *Am J Hum Genet*. 2003;72:253–269.
4. Lohmann DR, Horsthemke B. No association between the presence of a constitutional RB1 gene mutation and age in 68 patients with isolated unilateral retinoblastoma. *Eur J Cancer*. 1999;35:1035–1036.
5. Braggio E, Bonvicino CR, Vargas FR, Ferman S, Eisenberg AL, Seuanez HN. Identification of three novel RB1 mutations in Brazilian patients with retinoblastoma by "exon by exon" PCR mediated SSCP analysis. *J Clin Pathol*. 2004;57:585–590.
6. de Andrade AF, Barbosa da Hora R, Vargas FR, et al. A molecular study of first and second RB1 mutational hits in retinoblastoma patients. *Cancer Genet Cytogenet*. 2006;167:43–46.
7. Barbosa RH, Vargas FR, Aguiar FC, et al. Hereditary retinoblastoma transmitted by maternal germline mosaicism. *Pediatr Blood Cancer*. 2008;51:598–602.
8. Barbosa RH, Vargas FR, Lucena E, Bonvicino CR, Seuanez HN. Constitutive *RB1* mutation in a child conceived by in vitro fertilization: implications for genetic counseling. *BMC Med Genet*. 2009;10:75.
9. Linn Murphree A. Intraocular retinoblastoma: the case for a new group classification. *Ophthalmol Clin North Am*. 2005;18:41–53. viii.
10. Chantada G, Doz F, Antoneli CB, et al. A proposal for an international retinoblastoma staging system. *Pediatr Blood Cancer*. 2006;47:801–805.
11. Korbie DJ, Mattick JS. Touchdown PCR for increased specificity and sensitivity in PCR amplification. *Nat Protoc*. 2008;3:1452–1456.
12. Lohmann DR, Brandt B, Hopping W, Passarge E, Horsthemke B. Distinct RB1 gene mutations with low penetrance in hereditary retinoblastoma. *Hum Genet*. 1994;94:349–354.

Investigative Ophthalmology & Visual Science

13. Lohmann D, Degen S. Retinoblastoma Genetics. Leiden Open Variation Database. Available at: http://rb1-lovd.d-lohmann.de/home.php?select_db=RB1. Accessed December 11, 2012.
14. Janssen B, Hartmann C, Scholz V, Jauch A, Zschocke J. MLPA analysis for the detection of deletions, duplications and complex rearrangements in the dystrophin gene: potential and pitfalls. *Neurogenetics*. 2005;6:29–35.
15. National Center for Biotechnology Information. dbSNP Short Genetic Variations. Available at: http://www.ncbi.nlm.nih.gov/projects/SNP/. Accessed December 5, 2012.
16. PolyPhen: Prediction of Functional Effect of Human nsSNPs. Available at: http://genetics.bwh.harvard.edu/pph/. Accessed December 3, 2012.
17. Carvalho M, Andreozzi VL, Codeço CT. *Análise de sobrevida: teoria e aplicações em saúde*. Rio de Janeiro: Editora Fiocruz; 2005.
18. Bates D, Chambers J, Dalgaard P, et al. The R Project for Statistical Computing. Available at: http://www.r-project.org. Accessed December 17, 2012.
19. de Camargo B, Santos MO, Rebelo MS, et al. Cancer incidence among children and adolescents in Brazil: first report of 14 population-based cancer registries. *Int J Cancer*. 2010;126:715–720.
20. Herwig S, Strauss M. The retinoblastoma protein: a master regulator of cell cycle, differentiation and apoptosis. *Eur J Biochem*. 1997;246:581–601.
21. Chow KN, Dean DC. Domains A and B in the RB pocket interact to form a transcriptional repressor motif. *Mol Cell Biol*. 1996;16:4862–4868.
22. DiCiommo D, Gallie BL, Bremner R. Retinoblastoma: the disease, gene and protein provide critical leads to understand cancer. *Semin Cancer Biol*. 2000;10:255–269.
23. Riley DJ, Liu CY, Lee WH. Mutations of N-terminal regions render the retinoblastoma protein insufficient for functions in development and tumor suppression. *Mol Cell Biol*. 1997;17:7342–7352.
24. Doostzadeh-Cizeron J, Evans R, Yin S, Goodrich DW. Apoptosis induced by the nuclear death domain protein p84N5 is inhibited by association with RB protein. *Mol Biol Cell*. 1999;10:3251–3261.
25. Sterner JM, Tao Y, Kennett SB, Kim HG, Horowitz JM. The amino terminus of the retinoblastoma (RB) protein associates with a cyclin-dependent kinase-like kinase via RB amino acids required for growth suppression. *Cell Growth Differ*. 1996;7:53–64.
26. Otterson GA, Chen W, Coxon AB, Khleif SN, Kaye FJ. Incomplete penetrance of familial retinoblastoma linked to germ-line mutations that result in partial loss of RB function. *Proc Natl Acad Sci U S A*. 1997;94:12036–12040.
27. Whitaker LL, Su H, Baskaran R, Knudsen ES, Wang JY. Growth suppression by an E2F-binding-defective retinoblastoma protein (RB): contribution from the RB C pocket. *Mol Cell Biol*. 1998;18:4032–4042.
28. Lohmann D, Horsthemke B, Gillessen-Kaesbach G, Stefani FH, Hofler H. Detection of small RB1 gene deletions in retinoblastoma by multiplex PCR and high-resolution gel electrophoresis. *Hum Genet*. 1992;89:49–53.
29. Onadim Z, Hogg A, Baird PN, Cowell JK. Oncogenic point mutations in exon 20 of the RB1 gene in families showing incomplete penetrance and mild expression of the retinoblastoma phenotype. *Proc Natl Acad Sci U S A*. 1992;89:6177–6181.
30. Welch PJ, Wang JYA. C-terminal protein-binding domain in the retinoblastoma protein regulates nuclear c-Abl tyrosine kinase in the cell cycle. *Cell*. 1993;75:779–790.
31. Hsieh JK, Chan FS, O'Connor DJ, Mittnacht S, Zhong S, Lu X. RB regulates the stability and the apoptotic function of p53 via MDM2. *Mol Cell*. 1999;3:181–193.
32. Harbour JW, Luo RX, Dei Santi A, Postigo AA, Dean DC. Cdk phosphorylation triggers sequential intramolecular interactions that progressively block Rb functions as cells move through G1. *Cell*. 1999;98:859–869.
33. Sivakumaran TA, Shen P, Wall DP, Do BH, Kucheria K, Oefner PJ. Conservation of the RB1 gene in human and primates. *Hum Mutat*. 2005;25:396–409.
34. Albrecht P, Ansperger-Rescher B, Schuler A, Zeschnigk M, Gallie B, Lohmann DR. Spectrum of gross deletions and insertions in the RB1 gene in patients with retinoblastoma and association with phenotypic expression. *Hum Mutat*. 2005;26:437–445.
35. Sellner LN, Edkins E, Smith N. Screening for RB1 mutations in tumor tissue using denaturing high performance liquid chromatography, multiplex ligation-dependent probe amplification, and loss of heterozygosity analysis. *Pediatr Dev Pathol*. 2006;9:31–37.
36. Szijan I, Lohmann DR, Parma DL, Brandt B, Horsthemke B. Identification of RB1 germline mutations in Argentinian families with sporadic bilateral retinoblastoma. *J Med Genet*. 1995;32:475–479.
37. Dalamon V, Surace E, Giliberto F, Ferreiro V, Fernandez C, Szijan I. Detection of germline mutations in argentine retinoblastoma patients: low and full penetrance retinoblastoma caused by the same germline truncating mutation. *J Biochem Mol Biol*. 2004;37:246–253.
38. Fernandez C, Repetto K, Dalamon V, Bergonzi F, Ferreiro V, Szijan I. RB1 germ-line deletions in Argentine retinoblastoma patients. *Mol Diagn Ther*. 2007;11:55–61.
39. Rodriguez M, Salcedo M, Gonzalez M, Coral-Vazquez R, Salamanca F, Arenas D. Identification of novel mutations in the RB1 gene in Mexican patients with retinoblastoma. *Cancer Genet Cytogenet*. 2002;138:27–31.
40. Leone PE, Vega ME, Jervis P, Pestana A, Alonso J, Paz-y-Mino C. Two new mutations and three novel polymorphisms in the RB1 gene in Ecuadorian patients. *J Hum Genet*. 2003;48:639–641.
41. Alonso J, Frayle H, Menendez I, et al. Identification of 26 new constitutional RB1 gene mutations in Spanish, Colombian, and Cuban retinoblastoma patients. *Hum Mutat*. 2005;25:99.
42. Harbour JW. Molecular basis of low-penetrance retinoblastoma. *Arch Ophthalmol*. 2001;119:1699–1704.
43. Dimaras H, Khetan V, Halliday W, et al. Loss of RB1 induces non-proliferative retinoma: increasing genomic instability correlates with progression to retinoblastoma. *Hum Mol Genet*. 2008;17:1363–1372.
44. Sanchez-Sanchez F, Ramirez-Castillejo C, Weekes DB, et al. Attenuation of disease phenotype through alternative translation initiation in low-penetrance retinoblastoma. *Hum Mutat*. 2007;28:159–167.
45. Abouzeid H, Schorderet DF, Balmer A, Munier FL. Germline mutations in retinoma patients: relevance to low-penetrance and low-expressivity molecular basis. *Mol Vis*. 2009;15:771–777.
46. Bremner R, Du DC, Connolly-Wilson MJ, et al. Deletion of RB exons 24 and 25 causes low-penetrance retinoblastoma. *Am J Hum Genet*. 1997;61:556–570.
47. Mitter D, Rushlow D, Nowak I, Ansperger-Rescher B, Gallie BL, Lohmann DR. Identification of a mutation in exon 27 of the RB1 gene associated with incomplete penetrance retinoblastoma. *Fam Cancer*. 2009;8:55–58.
48. Gallie BL, Dunn JM, Chan HS, Hamel PA, Phillips RA. The genetics of retinoblastoma. Relevance to the patient. *Pediatr Clin North Am*. 1991;38:299–315.
49. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nat Rev Genet*. 2002;3:285–298.

Investigative Ophthalmology & Visual Science

50. Canturk S, Qaddoumi I, Khetan V, et al. Survival of retinoblastoma in less-developed countries impact of socio-economic and health-related indicators. *Br J Ophthalmol*. 2010;94:1432–1436.
51. Sanders BM, Draper GJ, Kingston JE. Retinoblastoma in Great Britain 1969-80: incidence, treatment, and survival. *Br J Ophthalmol*. 1988;72:576–583.
52. Bouguila H, Malek I, Boujemaa C, et al. Prognosis of retinoblastoma. Report of 50 cases [in French]. *J Fr Ophthalmol*. 2001;24:1053–1056.
53. Erwenne CM, Franco EL. Age and lateness of referral as determinants of extra-ocular retinoblastoma. *Ophthalmic Paediatr Genet*. 1989;10:179–184.
54. Butros LJ, Abramson DH, Dunkel IJ. Delayed diagnosis of retinoblastoma: analysis of degree, cause, and potential consequences. *Pediatrics*. 2002;109:E45.
55. Bai S, Ren R, Li B, et al. Delay in the diagnosis of retinoblastoma in China. *Acta Ophthalmol*. 2011;89:e72–e74.
56. Wallach M, Balmer A, Munier F, et al. Shorter time to diagnosis and improved stage at presentation in Swiss patients with retinoblastoma treated from 1963 to 2004. *Pediatrics*. 2006; 118:e1493–1498.
57. Lohmann DR, Brandt B, Hopping W, Passarge E, Horsthemke B. The spectrum of RB1 germ-line mutations in hereditary retinoblastoma. *Am J Hum Genet*. 1996;58:940–949.
58. Chantada G, Fandino A, Manzitti J, Urrutia L, Schvartzman E. Late diagnosis of retinoblastoma in a developing country. *Arch Dis Child*. 1999;80:171–174.

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