



Ten novel *RB1* gene mutations in patients with retinoblastoma

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Purpose: To study phenotype-genotype correlations in 65 retinoblastoma patients, who were seen between March 2004 and January 2006 and to report undescribed *retinoblastoma 1* (*RB1*) mutations identified in ten additional patients in whom mutations were detected before 2004.

Methods: Complete ophthalmic examinations were performed in all patients and their parents. DNA was extracted from peripheral blood leukocytes, and the *RB1* gene was screened by denaturing high-performance liquid chromatography and direct sequencing of the promoter and all the exons.

Results: Seven cases were familial, 30 were sporadic bilateral, and 28 were sporadic unilateral. Screening of the *RB1* gene resulted in the identification of four mutations in the familial cases (57%), 22 in the sporadic bilateral cases (73%), and three in the sporadic unilateral cases (10.7%). Twenty-two mutations, were single-base substitutions (76%). Of these mutations, 68% were of the nonsense type (15 cases). Ten patients with bilateral retinoblastoma in whom ten mutations were detected in a non-systematic approach between 1995 and 1998 were added to our recent series. In total, ten novel mutations were identified, including four single base substitutions, four small deletions and two small duplications. These are g.39445G>A, g.41924A>G, g.56851A>G, g.156795T>G, g.41983delT, g.44699_44706delAGCAGTTC, g.73788_73789delAA, g.78253delA, g.2157dupC, and g.2179_2183dupGGACC. Two patients had dysmorphic features associated with 13q14 large deletions.

Conclusions: The detection rates of 73% in the sporadic bilateral cases and of 10.7% in the sporadic unilateral cases in our series are in accordance with recently published literature. Our pattern of mutations confirms the predominantly gene-inactivating mutations, i.e. single-base non-sense mutations and splice site mutations.

RB (OMIM 180200) is the most frequent intraocular tumor of childhood and is caused by mutations in the *RB1* gene. The predisposition to develop RB is inherited as an autosomal dominant trait but mutations in both alleles are necessary to cause the disease [1]. Mutations of *RB1* gene are highly heterogeneous and spread in promoter and exons 1-25 [2-6]. Previous reports described a wide range of detection rate, from 20 to 80%, according to the case selection and the screening technique [2,3,6]. While more than 900 *RB1* mutations (see Retinoblastoma Genetics and [7]) the rate of mutation detection remains relatively low. This is attributable to the large size of the *RB1* gene, to the significant mutational heterogeneity of the disease, and to limitations of currently available screening techniques. Furthermore, unusual mutation location can also impede on mutation detection. Single base substitutions represent the most frequent mutations and among them, nonsense mutations predominate [3,4,6]. Studies on genotype-phenotype correlation concluded there was an association between nonsense or frameshift mutations and severity of the disease defined as bilateral multifocal RB [3-5]. Meanwhile, variable expression of RB is well-known with description of unaffected carriers, unilateral RB or benign retinoma [8,9]. Thus, low-penetrant phenotype was associated with the

p.R661W mutation of the *RB1* gene [10], with 4-kb deletion spanning exons 24 and 25 [11], alternative splicing mutation in exon 21 [12], p. L662P [13], promoter mutations [14], in-frame deletions affecting the N-terminal region of pRB [15], or alternative translation initiation associated with nonsense mutations in exon 1 [16] among other molecular changes. Genetic modifying factors or residual protein function due to either missense mutation or alternative translation initiation may influence phenotype expression, particularly in low-penetrant RB.

Studies of patients from different parts of the world can help explain the spectrum of *RB1* mutations and thus improve detection rate. Risk prediction is mandatory for current RB management [5,17] and justifies the continuous search for *RB1* novel mutations and for phenotypic correlations. To characterize the spectrum of *RB1* mutations and to analyze genotype-phenotype correlation, we performed a phenotype and mutation analysis of 65 patients with isolated or familial RB who underwent treatment in our institution. Ten patients with sporadic and familial bilateral RB in whom novel mutations were detected during a non-systematic mutation screening program occurring between 1995 and 1998 are reported separately.

METHODS

Between March 2004 and January 2006, we performed a mutational screening of the *RB1* gene in 65 consecutive

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proband with RB. Ten additional patients in whom mutations were detected before 2004 were included in the study.

Patients were referred to us either from university eye clinics and private Swiss practitioners or from neighboring European countries. All patients were examined and treated at the Retinoblastoma Clinic of the Jules-Gonin Eye Hospital, Lausanne, Switzerland. All patients underwent physical examination at our institution with special attention for dysmorphic features. Age ranged from 2 months to 12 years. Informed consent was obtained from all parents to draw blood and perform genetic analysis. Control DNA were obtained from 96 ethnically matched anonymous blood donors after informed consent. They were all above 18 years of age, had a history of good health but were not investigated by us. The study was conducted in accordance with the tenets of the Declaration of Helsinki. Specific clinical features such as tumor laterality, type (endophytic or exophytic), number of tumor foci, and systemic dysmorphic features were obtained from charts and photographs of the patients. Genetic analysis was performed

at the Institute for Research in Ophthalmology (IRO) at Sion, Switzerland, between March 2004 and January 2006 for the 65 probands, and at the Service of Medical Genetics, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, between 1995 and 1998 for the ten added patients. DNA was extracted from blood leukocytes and used for PCR amplifica-

TABLE 2. WAVE CONDITIONS FOR GENOMIC SCREENING

Exon	Temp (°C)	%B start	Time shift
Promoter	64.3	57.8	
	67.8	54.3	0.7
1	67.6	57.9	
	68.6	56.9	0.2
2	52	57.6	
	56.3	53.3	0.86
3	54.4	56.1	
	55.8	54.7	0.28
	57	53.5	0.52
4	53.5	56.9	
	56	54.4	0.5
5	53.7	52.4	
6	52.6	53.8	
	54.7	51.7	0.42
7	55	55.3	
8	53.1	58.7	
	62	49.7	1.8
9	53.9	53.8	
10	54.1	56.5	
	55.1	55.5	0.2
11	52.8	55.6	-0.16
	53.6	54.8	
	55.3	53.1	0.34
12	53.2	57	
	55	55.2	0.36
13	54	57.8	
	55.8	56	0.36
14	56.3	53.4	
	64.6	45.1	1.66
17	54.4	57.8	
	55.7	56.5	0.26
	56.4	55.8	0.4
18	53.3	57.8	
	57.5	53.6	0.84
19	55.5	55.9	
	56.3	55.1	0.16
	59.8	51.6	0.86
20	54.3	57.7	
	59	53	0.94
21	52.9	57.5	
	55.9	55.2	0.46
22	51.8	57.1	
	55.7	53.2	0.78
23	55.8	56.3	
	57.6	54.5	0.36
	59.2	52.9	0.68
24	53.1	56	
	54.7	56	
25	55.4	56.6	
	59.2	52.8	0.76
26	55.3	53.2	
27	58.7	54.5	

TABLE 1. SEQUENCE OF PRIMERS AND POLYMERASE CHAIN REACTION CONDITIONS

Region	Primer	Sequence (5'-3')	Amplicon (bp)	Annealing (°C)
Promoter	PromF	CTGGACCCACGCGGTTTC	340	61
	PromR	GTTTGGGCGCATGACGCCCTT		
RB exon 1	1F	CCGTTTTTCTCAGGGACGTTG	340	56.4
	1R	TTGGCCCCGCCCTACGCACAC		
RB exon 2	2F	CTATTGAAACAAGTATGTACTG	331	54.3
	2R	GGTAAATGGAATTATTATTAGC		
RB exon 3	3F	CAGTTTTAACATAGTATCCAG	281	52.8
	3R	AGCAITTTCTCACTAATTCAC		
RB exon 4	4F	GTAGTGTATTGATGTAGAGC	305	55
	4R	CCCAGAATCTAATTGTGAAC		
RB exon 5	5F	GCATGAGAAAACACTATGAC	194	54.3
	5R	CTAACCCCTAACTATCAAGATG		
RB exon 6	6F	CACCCAAAAGATATATCTGG	222	54.3
	6R	ATTTAGTCCAAAGGAATGCC		
RB exon 7	7F	CCTGCGATTTTCTCTCATAC	256	55
	7R	ATGTTTGGTACCCTAGAC		
RB exon 8	8F	AGTAGTAGAATGTTACCAAG	380	50.8
	8R	TACTGCAAAAAGAGTTAGCAC		
RB exon 9	9F	TGCATTTTCAAGAGTCAAG	222	56
	9R	AGTTAGACAATTTATCCCTCC		
RB exon 10	10F	TCTTTAATGAAATCTGTGCC	291	56
	10R	GATATCTAAAGGTCACTAAG		
RB exon 11	11F	GAGACAACAGAAGCATTATAC	245	54.2
	11R	CGTGAAACAAATCTGAACAC		
RB exon 12	12F	GGCAGTGTATTTGAAGATAC	310	52.5
	12R	AACACATGTTAGATAGGAG		
RB exon 13	13F	CITATGTTCACTAGTTGTGG	342	54.3
	13R	TATACGAACTGAAAGATGC		
RB exon 14	14F	GTGATTTTCTAAAATAGCAGG	212	58.9
	14R	TGCCTTGACCTCCTGATCTG		
RB exon 15+16	15/16F	CAATGCTGACACAAATAAGG	366	55
	15/16R	AGCAITCTTCTCCTTAACC		
RB exon 17	17F	AAAAATACCTAGCTCAAGGG	339	56
	17R	TGTTAAGAAACACCTCTCAC		
RB exon 18	18F	TGTACCTGGGAAAATTTATGC	340	56.4
	18R	CTTTATTTGGGTCAATGTACC		
RB exon 19	19F	ATAATCTGTGATTTCTAGCC	273	56
	19R	AAGAAACATGATTTGAACCC		
RB exon 20	20F	AAAGAGTGTAGAAAAGAGG	335	56.4
	20R	CAGTTAACAGTAAGTAGGG		
RB exon 21	21F	AAACCTTTCTTTTTTGAGGC	328	54
	21R	TACATAATAAGGTGACAGAG		
RB exon 22	22F	TAATATGTGTTCTTACCAGTC	313	56
	22R	TTTAATGTTTGGTGGACCC		
RB exon 23	23F	ATCTAATGTAATGGGTCCAC	287	54.2
	23R	CTTGGATCAAAAATAATCCCC		
RB exon 24	24F	GAATATAGTTTGTGAGTGGTTC	273	52 53
	24R	GTGTTTGAATACTGCATTTGG		
RB exon 25	25F	GGTTGCTAACTATGAAACAC	297	54.2 55
	25R	AGAAATGGTATAAGCCAGG		
RB exon 26	26F	AGTAAGTCATCGAAAGCATC	209	52.8
	26R	AACGAAAAGACTTCTTGACG		
RB exon 27	27F	CGCCATCAGTTTGACATGAG	237	54.2

Forward (F) and reverse (R) primers used to amplify the promoter region and the various RB1 exons. Expected size and polymerase chain reaction annealing temperatures are provided.

Analysis of each fragment was performed on a WAVE system using the following conditions, including temperature, percentage of buffer B at the beginning of the run and applied time shifts.

tion. Denaturing high-performance liquid chromatography (DHPLC) and sequencing were used. Primers, and PCR reactions were described by Houdayer et al. (2004). In short, amplification was performed in a thermal cycler (GeneAmp 9700,

Applied Biosystems, Foster City, CA), in a total volume of 30 µl. Each polymerase chain reaction (PCR) contained 100 ng genomic DNA, 0.9 nanomoles of each primer, and 15 µl master mix 2X (Qiagen, Hombrechtikon, Switzerland), with or

TABLE 3. PATIENTS AND RESULTS

2004-2006	Familial			Sporadic			Total	Novel mutations
	Unilateral	Bilateral	Total	Unilateral	Bilateral	Total		
	3	4	7	28 (48%)	30 (52%)	58 (88%)	65	
Detected mutations	1	3	4 (57%)	3 (10.7%)	22 (73%)	25 (43%)	29 (44%)	6/29 (21%)

1995-1998	Familial			Sporadic			Total	Novel mutations
	Unilateral	Bilateral	Total	Unilateral	Bilateral	Total		
	0	3	3	0	7	7	10	
Detected mutations	0	3	3	0	7	7	10	4/10 (40%)

Table presenting the number of detected mutations for each category of RB patient with the total number of novel mutations per category.

TABLE 4. RETINOBLASTOMA I DETECTED MUTATIONS

Patient number	RB	Disease	Age at diagnosis (months)	Location	DNA	Protein	RNA	Tumor foci	
								RE	LE
1	F	B	3	Exon 1*	g.2157dupC	p.P34RfsX15		4	1
2	S	B	2	Intron 2*	g.39445G>A		splicing	1	4
3	S	B	13	Exon 4*	g.41983delT	p.N146KfsX7		5	1
4	S	B	9	Exon 5*	g.44699_44706delAGCAGTTC	p.S178DfsX4		1	3
5	S	U	10	Intron 6*	g.56851A>G		splicing		1
6	S	B	8	Exon 8	g.59683C>T	p.R251X		NA	3
7	F	B	21	Exon 8	g.59683C>T	p.R251X		3	3
8	S	B	2	Exon 10	g.64348C>T	p.R320X		5	2
9	S	B	12	Exon 10	g.64348C>T	p.R320X		2	5
10	S	B	14	Intron 12	g.70330G>A		splicing	2	1
11	S	B	8	Intron 12	g.70330G>A		splicing	5	NA
12	S	B	3	Exon 14	g.76430C>T	p.R445X		NA	1
13	S	B	3	Exon 14	g.76460C>T	p.R455X		1	1
14	S	B	2	Exon 15	g.76898C>T	p.R467X		NA	NA
15	S	B	4	Exon 17	g.78238C>T	p.R552X		2	2
16	F	B	7	Exon 17	g.78244G>T	p.E554X		3	1
17	S	B	18	Exon 17*	g.78253delA	p.I560SfsX54		NA	NA
18	S	B	13	Exon 18	g.150037C>T	p.R579X		1	2
19	S	U	17	Exon 18	g.150037C>T	p.R579X		1	
20	S	U	33	Exon 18	g.150037C>T	p.R579X		3	
21	S	B	20	Intron 19	g.153354G>A		splicing	3	4
22	F	U	5	Exon 20	g.156713C>T	p.R661W			1
23	S	B	5	Exon 21	g.160799delA	p.T726QfsX18		1	5
24	S	B	29	Exon 23	g.162237C>T	p.R787X		4	2
25	S	B	13	Exon 23	g.162237C>T	p.R787X		5	1
26	S	B	2	Exon 23	g.162237C>T	p.R787X		2	1
27	S	B	6	Intron 23	g.162368G>C		splicing	5	1
28	S	B	2		g.(?_47'727'284)_(49'209'610_?)del NCBI build 36.1			1	2
29	S	B	5		46,XY,del(13; q13.2q14.3)			3	1
30	F	B	6	Exon 1*	g.2179_2183dupGGACC	p.L42RfsX25		6	NA
31	S	B	2	Intron 3*	g.41924A>G		splicing	NA	2
32	S	B	13	Exon 8	g.59704_59708delAACAG	p.N258EfsX11		3	NA
33	S	B	17	Exon 8	g.59695C>T	R255X		NA	1
34	S	B	3	Exon 8	g.59695C>T	R255X		9	5
35	F	B	21	Exon 11	g.65386C>T	R358X		2	2
36	S	B	3	Exon 13*	g.73788_73789delAA	p.R418SfsX9		3	1
37	S	B	4	Exon 17	g.78238C>T	R552X		2	2
38	F	B	13	Exon 20*	g.156795T>G	p.L688R		2	4
39	S	B	3		46,XX,inv(13; q14.2q21.3)			NA	1

The following abbreviations were used: retinoblastoma (RB), familial (F), sporadic (S), bilateral (B), unilateral (U), novel mutation (*), right eye (RE), left eye (LE), and not available (NA).

without betaine. Reactions were subjected to 35 cycles of 94 °C for 1 m, annealing at the specific temperature for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 10 min (Table 1).

After PCR amplification, products were screened for mutations using DHPLC on a WAVE system (TEAA, Transgenomics, Crewe, Cheshire, UK). Buffer A contained 0.1 M triethylammonium acetate (TEAA, Transgenomics). Buffer B contained 0.1 M TEAA (Transgenomic) and 25%

acetonitrile HPLC grade (Sigma-Aldrich, Suffolk, UK). The flow rate was set at 1.5 ml/min and the Buffer B gradient increased by 5% per minute for 2 min. The optimum temperature was determined by the Wavemaker software (Transgenomic) for each DNA fragment, and a time shift was applied as needed (Table 2). When multiple melting domains were established, each domain was analyzed at the appropriate temperature. Initial Buffer B concentrations and temperatures for each fragment are listed in Table 2.

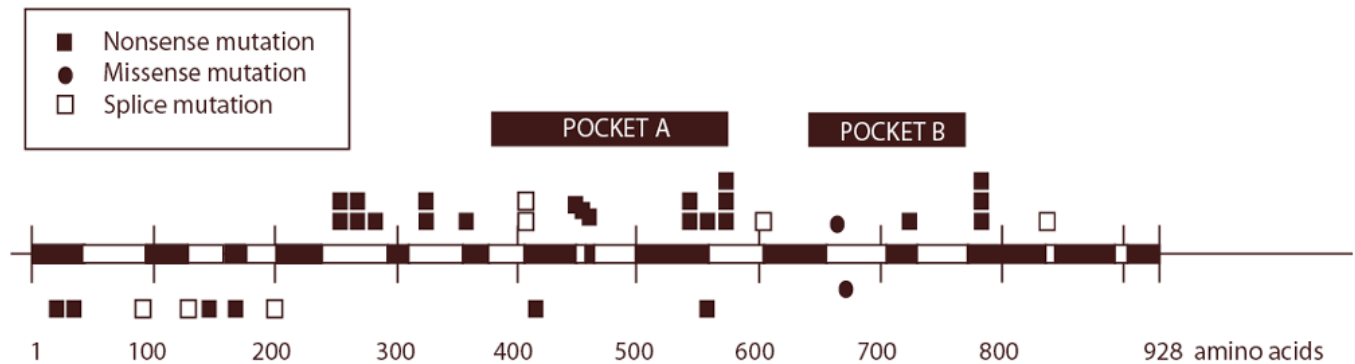


Figure 1. Distribution of point mutations in *retinoblastoma 1*. A schematic drawing of the cDNA, with amino acid in axis, exons alternatively in black and white and pocket A and B. Novel mutations are shown under the bar, mutations already reported in the literature are depicted on top.

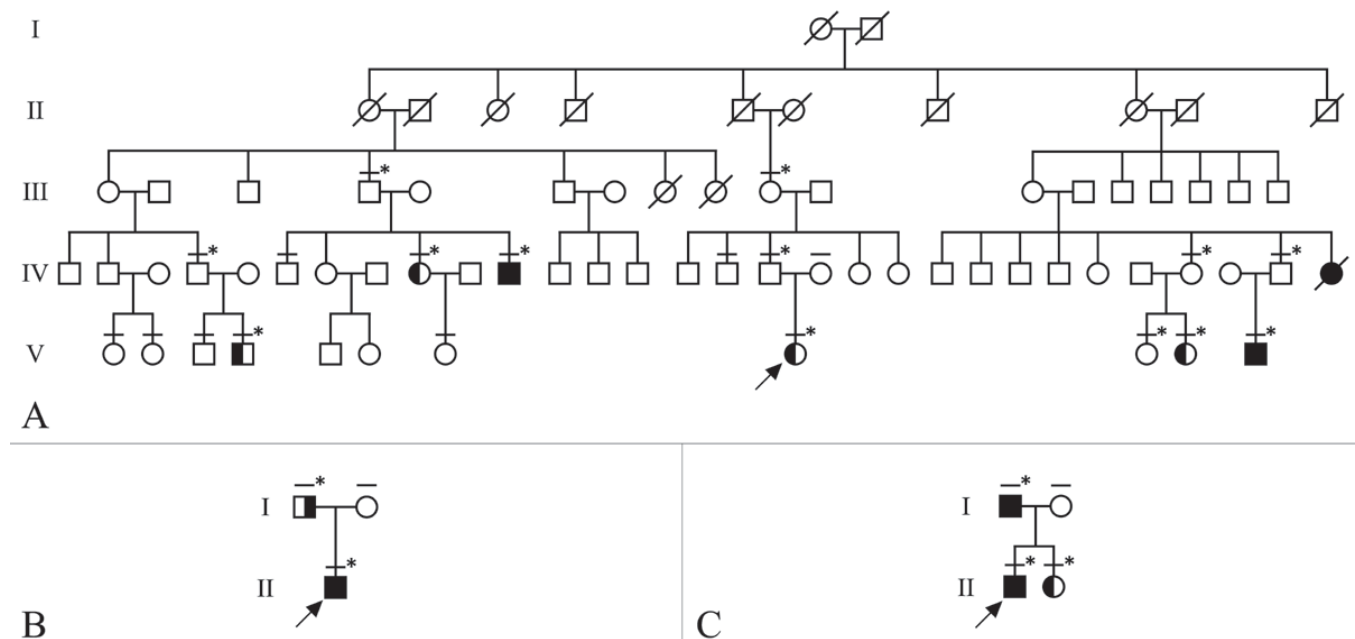


Figure 2. Three families with low-penetrance retinoblastoma. **A:** Family segregating the previously described p.R661W mutation. Mutation carriers of this family presented uni- or bilateral retinoblastoma (RB) or were unaffected. **B:** Family segregating a novel nonsense mutation g.2157dupC in exon 1 that was carried by both the proband, who had bilateral RB and his father who had unilateral RB. Mutations in the first exon could be associated with low-penetrance by alternative in-frame translation. **C:** Family segregating a novel duplication mutation in exon 1, g.2179_2183dupGGACC that was carried by the proband, who had bilateral retinoblastoma, his father who had unilateral RB and unilateral retinocytoma in separate eyes and his only sister who had unilateral RB. Bar on top of symbol represent tested individuals, asterisk represents mutation carriers, shaded symbols represent bilateral RB; half-shaded symbols represent unilateral RB, dashed symbols represents deceased individuals, and arrow represents proband.

PCR fragments displaying DHPLC abnormal retention times were further sequenced on both strands using ABI Dye Terminator, version 1 or 3, in a final reaction volume of 10 µl and electrophoresed on a 3130XL ABI genetic analyzer (Applied Biosystems). Sequences were aligned using the Chromas version 2.23 (Technelysium, Tewantin, Australia).

Denaturing gradient gel electrophoresis was used in the ten added patients studied at the Service of Medical Genetics, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland, between 1995 and 1998.

Screening for large deletions was performed by haplotype analysis using *RBI* flanking microsatellites D13S161, D13S164, D13S153, D13S1307, and D13S273. One primer was fluorescently labeled, and the product was separated on an automated sequencer (ABI XL3100; Applied Biosystems).

RESULTS

Out of the 65 RB patients participating in this study, we found seven familial cases, 30 sporadic bilateral cases, and 28 sporadic unilateral cases (Table 3). Screening of the *RBI* gene resulted in the identification of four mutations in the familial cases (57%), 22 in the sporadic bilateral cases (73%) and three in the sporadic unilateral cases (10.7%), as detailed in Table 3. We discovered 22 mutations which were the single-base substitution type and four (14%) showed single-base deletion (3) or duplication (1; Table 4).

Figure 1 shows the distribution of the mutations. In addition, one small deletion (8 bp) was detected as well as two large deletions associated with dysmorphic features of 13q-deletion syndrome [18-20].

Of the 22 single-base substitutions, 15 (68%) were nonsense mutations, six (27%) were missense mutations affecting splice sites and one (5%) was a missense mutation in the coding region of the gene. In the 65 index patients, four recurrent mutations were observed once; they were all of the nonsense type (g.59683C>T, g.78238C>T, g.150037C>T, and g.162237C>T). This recurrence is attributed to the fact that the C>T transition that changes arginine codons (CGA) to stop codons is the most common point mutation in the *RBI* gene due to spontaneous deamination of methylcytosine to thymine in CpG dinucleotides [15,21].

In the ten patients screened during a non-systematic approach and added to our series, four of the ten detected mutations were nonsense mutations (one redundant), two missense, two small deletions, one small duplication, and one paracentric inversion (Table 4). Four out of these 10 mutations were not previously reported. We suggest that the two missense mutations (g.156795 and g.41924A>G) are pathogenic. Indeed, each new mutation has only been observed in single families and has not been found in any other patients.

The g.156795 mutation was located at amino acid L688, in a region that is conserved in many species from *Canis familiaris* to *Mus musculus*, *Cavia porcellus*, *Rattus norvegicus*, *Gallus gallus*, and even *Takifugu rubripes* and *Oryzias latipes*. The g.41924A>G mutation is part of the two conserved nucleotides of the 3' cononical splice site.

In total, ten novel mutations were identified that were not detected in 96 ethnically matched healthy individuals. They included a majority of single base mutations with four missense mutations, two single base deletion, two small deletions, one single base insertion, and one small duplication (Table 4). The previously reported g.156713C>T (R661W) mutation known to induce low-penetrant RB was also identified in our cohort (Patient 22, Table 4), and again showed reduced penetrance as mutations carriers of the family harbored only unilateral RB or were unaffected carriers (Figure 2) [10]. The nonsense mutation g.2157dupC in exon 1 detected in Patient 1 (Table 4) was also carried by his father, who presented unilateral RB but not by his mother (Figure 2). Patient 30 (Table 4) harboured a duplication mutation in exon 1, g.2179_2183dupGGACC, which was also carried by his father who had retinoblastoma in one eye and retinocytoma in the other and by his only sister, who had a unilateral RB (Figure 2). It has been reported that nonsense mutations in the first exon could be associated with low-penetrance by alternative in-frame translation involving methionines at position 113 or 233 [16,22]. Modulation of disease penetrance in these two families could be due to the described phenomenon, although we did not perform expression analysis to confirm this hypothesis.

No correlation between the type or localization of mutation and the number of tumor foci per eye, the laterality or the type of RB (endophytic or exophytic) could be established neither in the 65 patients nor in the 10 added patients.

DISCUSSION

The detection rates of 73% in the sporadic bilateral cases of 10.7% in the sporadic unilateral cases in our series are similar to previous reports [2,3,6]. Our pattern of mutations confirms the predominantly gene-inactivating mutations, i.e. single-base non-sense mutations and splice site mutations [3,6].

The reasons why not all mutations were detected remains controversial. Aside from technical limitations of mutation detection, epigenetic changes could contribute to this phenomenon to an underestimated degree [23]. Meanwhile, other described causes such as mosaicism or non-coding sequence variants may play a role in lowering mutation detection rate as well [24].

Clinical care of RB families includes disease prediction, which carries a significant socio-economic impact. Mutation detection in the family members helps differentiate those who will need specific care and follow-up from those who can avoid invasive and costly procedures. Carriers of germline mutation can benefit from early management, especially with prenatal diagnosis. This is of particular importance when considering tumor growth. For sporadic cases, it is mandatory to perform mutational analysis to know if the patient has a constitutive mutation that can potentially be transmitted to offspring. In sporadic cases, disease expression may be modified by the secondary somatic mutation. Therefore it is important to perform molecular studies on tumor material whenever available.

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