RESEARCH ARTICLE

A comprehensive, sensitive and economical approach for the detection of mutations in the *RB1* gene in retinoblastoma

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Abstract

Retinoblastoma (Rb) is the most common primary intraocular malignancy in children. It is brought about by the mutational inactivation of both alleles of *RB1* gene in the developing retina. To identify the *RB1* mutations, we analysed 74 retinoblastoma patients by screening the exons and the promoter region of *RB1*. The strategy used was to detect large deletions/duplications by fluorescent quantitative multiplex PCR; small deletions/insertions by fluorescent genotyping of *RB1* alleles, and point mutations by PCR-RFLP and sequencing. Genomic DNA from the peripheral blood leucocytes of 74 Rb patients (53 with bilateral Rb, 21 with unilateral Rb; 4 familial cases) was screened for mutations. Recurrent mutations were identified in five patients with bilateral Rb, large deletions in 11 patients (nine with bilateral Rb and two with unilateral Rb), small deletions/insertions were found in 12 patients all with bilateral Rb, and point mutations in 26 patients (14 nonsense, six splice site, five substitution and one silent change). Three mutations were associated with variable expressivity of the disease in different family members. Using this method, the detection rates achieved in patients with bilateral Rb were 44/53 (83%) and with unilateral Rb, 5/21 (23.8%). This approach may be feasible for clinical genetic testing and counselling of patients.

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Introduction

Retinoblastoma (Rb) is a pediatric tumour of the retina and the most common primary intraocular malignancy of childhood. It has an incidence of approximately 1 in 20,000 in USA (Young *et al*. 1999) and is considered to have a similar incidence rate in various parts of the world. It is the most common type of malignant tumour in children below the age of 5 years in India (Pratap *et al*. 1973; Das *et al*. 1994). Both males and females are equally affected, and the median age at diagnosis is 12 months in those with bilateral tumour, and 24 months in those with unilateral disease (Young *et al*. 1999). Leukocoria (white pupillary reflex) is the most common presenting sign.

Rb is brought about by the biallelic inactivation of the human retinoblastoma susceptibility gene, *RB1* (GenBank accession number L11910) on chromosome 13q14, that codes for the RB protein. Cytogenetic deletions examined in retinoblastoma have assigned the genetic locus of the disease to q14 of chromosome 13 linked with the polymorphic marker gene enzyme esterase D (Friend *et al*. 1986). Rb arises due to two genetic events involving both the alleles of *RB1* and occurs in two forms, hereditary and nonhereditary. Mutation of both the alleles of *RB1* is required for tumour initiation. In case of hereditary transmission of the disease, one allele is mutated in the germline and the other at the cellular level. In non-hereditary disease, both the alleles are mutated at the cellular level (Knudson 1971). Other genetic alterations in addition to *RB1* inactivation are commonly found in Rb, and these events possibly contribute to tumour progression (Mairal *et al*. 2000; Corson and Gallie 2007).

The heritable form of the disease is mostly bilateral and can be transmitted to successive generations in an autosomal dominant fashion. It is characterized by high penetrance (90% or more), early onset (within the first year of life),

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and presence of multiple tumours (multifocal) in both the eyes. Majority of patients with bilateral Rb have sporadic (isolate) disease with no familial transmission that evidently arises through *de novo* mutation of *RB1* either in the germ line or embryo. About 5%–10% of patients have familial Rb with more than one affected member in the family. The non-hereditary form of the disease typically involves one eye (unilateral) and occurs as a single tumour (unifocal) with late onset i.e., after the first year of life.

Precise identification of the *RB1* gene mutation could help in enhancing the clinical management of the relatives at risk (Gallie *et al*. 1995). If the mutation is known in the proband, molecular testing of the family members at risk allows for prompt management of the disease and better visual outcome in affected children (Gallie *et al*. 1999). Identification of the *RB1* mutation in the family followed by *in vitro* fertilization (IVF) and pre-implantation genetic diagnosis (PGD) have been employed to successfully select against mutation-carriers (Xu *et al*. 2004).

The *RB1* gene shows a high degree of mutational heterogeneity in Rb with over 900 mutations reported till date (Valverde *et al*. 2005). Approaches using multiple techniques including quantitative multiplex PCR and sequencing (Richter *et al*. 2003), or DHPLC (denaturing high-performance liquid chromatography) along with quantitative multiplex PCR for short fluorescent fragments (QMPSF) (Houdayer *et al*. 2004) could achieve detection rates of 80%–89%. Techniques that detect small mutations such as single-strand conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE) or heteroduplex analysis followed by sequencing allow the identification of mutations in a subset of patients (Shimizu *et al*. 1994; Blanquet *et al*. 1995; Liu *et al*. 1995; Alonso *et al*. 2001; Ata-ur-Rasheed *et al*. 2002; Kiran *et al*. 2003). In the present study, we developed a combinatorial approach for detection of recurrent mutations, large deletions/insertions and point mutations for screening 74 patients with Rb seen at our institution, a tertiary care referral centre.

Materials and methods

Patients

Seventy-four Rb patients who were clinically evaluated in our institute with age at diagnosis ranging from 3–48 months were included in the study. Of these, 53 had bilateral Rb and 21 had unilateral Rb. Four patients belonged to families who had one or more affected individuals in addition to the proband. After the prior approval of the study protocol by the Institutional Review Board of L. V. Prasad Eye Institute, Hyderabad, India, informed consent for genetic analyses was taken from parents/guardians of children affected with Rb. Peripheral blood samples (2–4 mL) were collected from the probands in heparinized vaccutainers during examination under anesthesia. In familial cases, blood from parents and other affected members (seven members from four families) was collected and included in our study. Blood samples were stored in −20°C until DNA was extracted.

DNA isolation

Genomic DNA was isolated from the peripheral blood leukocytes using phenol–chloroform method. DNA was dissolved in TE (Tris-EDTA) buffer, quantitated by spectrophotometry and diluted to a concentration of 10 ng/μL.

PCR-RFLP (restriction fragment length polymorphism) analysis

PCR-RFLP was designed for five recurrent mutations. The details of the five mutations tested, and enzymes used for PCR-RFLP are given in table 1. PCR for five exons (8, 12, 14, 20 and 23) was done using the genomic DNA of 74 Rb patients using exon-specific primers (table 2). PCR products were digested with 2 to 4 units of the restriction enzyme (MBI Fermentas, Life Sciences, Maryland, USA) in a final volume of 20 μL using the recommended restriction enzyme buffer. All reactions were incubated overnight at appropriate temperatures. Following digestion, fragments were separated on 2% agarose gels, stained with ethidium bromide (0.5 μg/mL in 1x TAE) and DNA was visualized on a UV transilluminator (UVITec, Cambridge, England).

Table 1. Recurrent mutations detected by PCR-RFLP.

	Mutation	Nucleotide change	Exon	RE
1	p.Arg251X	g.59683C>T	E08	*Taq*I (-)
2	IVS12+1G>C	g.70330G>C	IVS12	*Tai*I (-)
3	p.Arg787X	g.162238C>T	E23	*Taq*I (-)
4	p.Arg661Trp	g.156713C>T	E20	*Msp*I (-)
5	p.Arg455X	g.76460C>T	E14	*BseL*I (-)

IVS, intervening sequence; RE, restriction enzyme. (-) denotes loss of restriction enzyme site.

Table 2. Multiplex PCR reactions for *RB1*.

Multiplex	Exons
Group 1	12, 19, 17, IC
Group 2	14, 13, 21, IC
Group 3	20, 18, 25, IC
Group 4	10, 7, 5, IC
Group 5	6, 9, 8, IC
Group 6	3, 11, 4, IC
Group 7	24, 23, 2, IC
Group 8	22, 26, 27, IC
Group 9	15-16, IC
Group 10	1, IC

IC, internal control.

Universal primer quantitative fluorescent multiplex PCR (UPQFM-PCR)

The strategy followed here was as described by Heath *et al*. (2000) and is based on quantitative fluorescent multiplex PCR (Heath *et al*. 2000). All the primers were designed to have a universal tag at the 5′ end. The strategy

involves two PCR reactions: the first (P1) reaction amplifies the selected *RB1* exons, using unique gene-specific primer sequences tagged with a common 5′ tag sequence of 22 nucleotides for forward and reverse primers; and the second (P2) PCR reaction amplifies the P1 amplicons using 'universal' primers that are complementary to the 5′ tag sequences. The tag sequences were same as those used by Heath *et al*. (2000). The sequence 5′ TCC GTC TTA GCT GAG TGG CGTA 3′ was the tag for the forward primer and 5′ AGG CAG AAT CGA CTC ACC GCTA 3′ was the tag for the reverse primers. One of the universal primers was labelled with 6-Fam, and labelled PCR products were resolved on the ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, USA).

An internal control (IC) consisting of exon 6 of the *βig-H3* gene on chromosome 5 was also amplified in each multiplex PCR. The ratio of the area of the product peak obtained from amplification of each *RB1* exon to that of the internal control was determined. The sequences of primers used are provided in table 1 of appendix.

In each experiment, multiplex PCR was performed on DNA from Rb patients as well as three normal controls. Multiplex PCR reactions were set up in duplicate or triplicate for each template. P1 reaction volume was 20 μL with 20 ng of template, 5 pmol of each primer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1x PCR buffer and 1.5 units of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India). Two μL from the P1 product was used as template for P2. The P2 reaction was set up with the same components except for universal primers instead of specific primers. Two μL of the P2 products were mixed with 10 μL of Hi-Di formamide (Applied Biosystems, Foster City, USA) and denatured at 94°C for 5 min and quick chilled before loading the samples on the ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, USA) along with the size standard LIZ (Applied Biosystems, Foster City, USA). The P1 reactions were carried out by initial denaturation at 94°C for 3 min, 10–12 cycles of 94°C for 30 s, 58°C–60°C for 35 s and 72°C for 50 s with a final elongation at 72°C for 10 min. The P2 reactions were carried out by initial denaturation at 94°C for 3 min, 20 cycles of 94°C for 30 s, 57°C for 35 s and 72°C for 50 s with a final elongation at 72°C for 10 min.

Data and statistical analysis

The peak area and the size of all the amplicons of RB1 in the multiplex reaction along with the internal control were determined for patients and normal controls using GeneMapper Software v 4.0 (Applied Biosystems, Foster City, USA). The ratios of the area of each exon with that of the internal control was calculated for each patient in duplicate and for the normal control in triplicate. Mean ratios and standard deviation (s.d.) were calculated for each exon in three different normal controls, and the means across normal controls (M_n) were taken for evaluation of patients. $M_n \pm 2$ s.d. for each exon in three controls were calculated as the range (R) and this was used to evaluate the deletions/insertions in the patients. Mean peak area ratios for exons amplified in duplicate from patients' DNA that were within the range (obtained from the controls) were interpreted as having no copy number changes. If less than the range by 20% or more, deletion of that exon was inferred and if the mean ratio for an exon was more than range by 20% or more, a duplication of that exon was inferred. All 27 exons were grouped into 10 multiplex reactions are given in table 2. Small deletions/insertions were detected by size change of the fluorescent labelled PCR products of each exon on the ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, USA).

Sequencing

Exons from 2–27 were amplified with 1.5 mM $MgCl_2$ at 52°C. Primer sequences of the *RB1* promoter and exon 1 were GC-rich requiring annealing temperatures of 60°C and 62°C respectively, with 5% dimethyl sulfoxide (DMSO). Amplification was done using *Taq* polymerase (1–1.4 U) (Bangalore Genei, Bangalore, India), with 0.2 mM dNTPs, 1x PCR reaction buffer, 1.5 mM $MgCl_2$, 5 pmol of each forward and reverse primers and 20 ng of genomic DNA template in a 25 or 50 μL reaction. The PCR reactions were carried out by initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 s, 52°C–62°C for 30–35 s and 72°C for 35–45 s with a final elongation at 72°C for 10 min.

Sequencing was done using the dideoxy method by fluorescent automated sequencing on the ABI 3130XL genetic analyzer (Applied Biosystems, Foster City, USA). The sequence data were analysed using SeqScape software v2.6 (ABI, Foster City, USA). Mutations are designated on the genomic *RB1* sequence with reference to GenBank accession number L11910.

Results

Recurrent mutations detected by PCR-RFLP

The strategy used for screening all the 74 patients is depicted in figure 1. Accordingly, they were first screened for the five recurrent mutations (listed in table 1). Mutations were identified in five patients (shown in table 3). Two nonsense mutations, Arg455X and Arg787X were found in patients RB-45 and RB-23 respectively, with bilateral disease. A missense mutation Arg661Trp was identified in two patients, RB-60 and RB-73, both with bilateral disease. A G>C change at splice junction in intron 12 at +1 position was found in the proband RB-65 with bilateral disease and also in the affected father.

Large deletions/duplications

The remaining 69 patients were screened by UPQFM-PCR. Deletions found in 11 patients (nine bilateral Rb, two unilateral Rb) are listed in table 4. Whole gene deletions were found in two patients with bilateral Rb (RB-28 and RB-62).

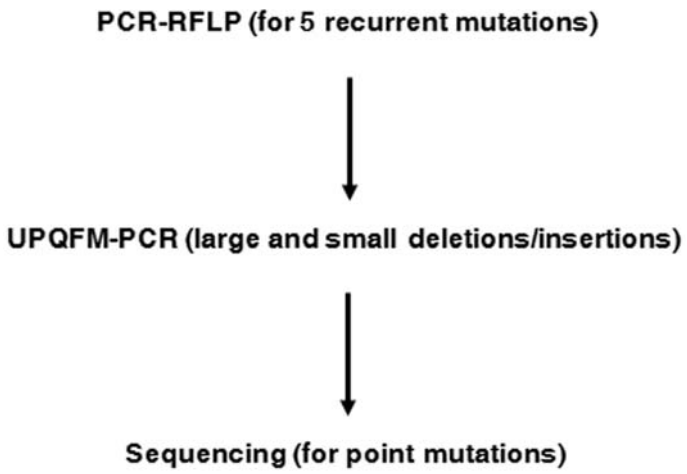

Figure 1. Strategy employed for screening retinoblastoma patients.

Table 3. Mutations identified with PCR-RFLP.

Patient	Laterality	Exon	Mutation	Consequence
RB-45	Bilateral	14	p.Arg455X	Nonsense
RB-56	Bilateral	23	p.Arg787X	Nonsense
RB-60	Bilateral	20	p.Arg661Trp	Missense
RB-65*	Bilateral	IVS12	IVS12+1G>C	Splice
RB-73	Bilateral	20	p.Arg661Trp	Missense

*Patient with familial Rb.

Table 4. Large deletions identified in *RB1* gene by UPQFM-PCR.

Patient	Laterality	Deletion
RB-28	Bilateral	exons 1-27
RB-62	Bilateral	exons 1-27
RB-43	Bilateral	**exons 4-27**
RB-8	Bilateral	**exons 18-24**
RB-74*	Bilateral	**exons 24-26**
RB-46	Bilateral	**exons 3-4**
RB-18	Bilateral	exon 20
RB-32	Bilateral	**exon 4**
RB-33	Bilateral	exon 3
RB-7	Unilateral	exons 15-16
RB-47	Unilateral	exon 20

*Patient with familial Rb. Mutations shown in bold represent novel mutations

A large deletion of exons 4–27 was found in one patient RB-43, with bilateral disease. One patient RB-8 (bilateral Rb) had a deletion of seven exons (exons 18–24). A deletion of two exons, 3 and 4 was found in one patient RB-46 (bilateral Rb). Single exon deletions of exons 3, 4 and 20 were found in RB-33, RB-32 and RB-18, respectively, all with bilateral disease. In family RB-74, deletion of exons 24–26 was identified in the proband, sibling and father, all affected. Multiplex peak data for this patient for three multiplex reactions are shown in figure 2 and table 5. The same deletion resulted in different phenotypes in this family. The proband had bilateral Rb, the father had phthisis bulbi in one eye (phthisis bulbi is a shrunken non-functional eye suspected to have arisen because of spontaneous regression of the tumour due to necrosis) and the affected sibling had unilateral Rb. Exonic deletions were also identified in two patients with unilateral disease RB-7 and RB-47, with deletions of exons 15–16 and 20 respectively. Of all the large deletions identified five are novel.

Small deletions/insertions:

Small deletions/insertions were found in 12 patients, all with bilateral Rb (table 6). Three patients had changes within introns, including a 4-bp deletion in intron 24 in two patients RB-24 and RB-72, beginning at +1 and +3 positions of the intron respectively. One patient RB-70 had a 1-bp insertion at −22 of intron 5. The significance of this change is not known and any possible effect on mRNA splicing would have to be determined by RNA analysis. Deletions of 1 bp occurred in six patients, deletion of 3 bp in exon 17 in one patient, a 14-bp deletion in exon 14 in one patient), and an insertion of 5 bp in exon 8 in one patient. The genomic positions of the deletion/insertion were determined by bidirectional sequencing of the PCR products.

Point mutations:

The remaining 46 patients were screened by bidirectional sequencing of all the 27 exons and the promoter region to detect point mutations. Nonsense mutations were identified in 12 patients (10 bilateral and two unilateral Rb; listed in table 7a). Splice site mutations were identified in five patients all with bilateral disease. They were found at IVS2-2, IVS3+1, IVS11-1, IVS22+5, and IVS24+5 in RB-21, RB-15, RB-59, RB-40 and RB-63 respectively (table 7b). In RB-40 and RB-63, which were familial cases, the same mutation was found in the affected members of the families.

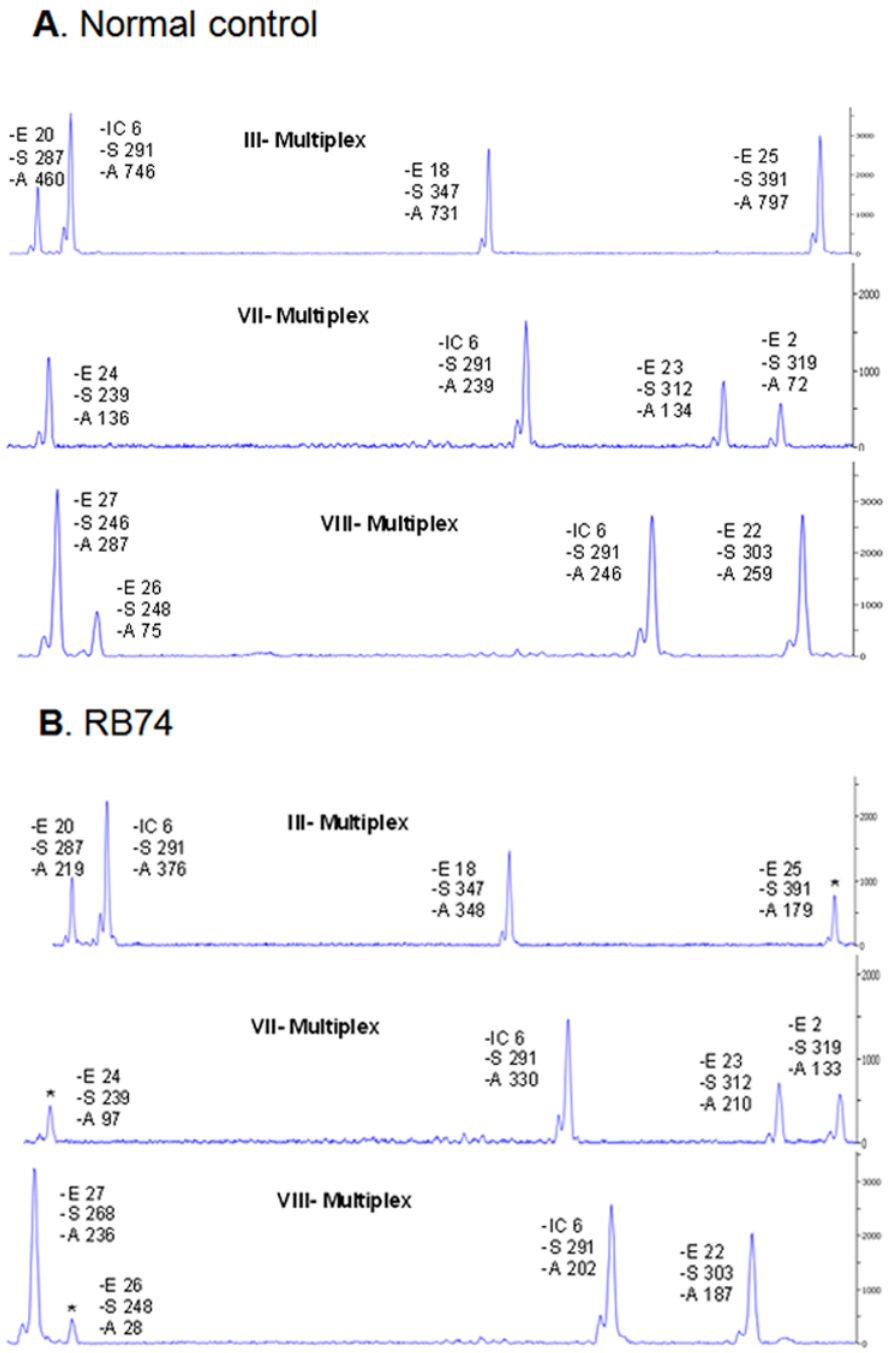

Figure 2. Multiplex PCR reactions III, VII and VIII for control and patient RB-74. Multiplex PCR peak data for patient RB74 and normal control are shown above to illustrate copy number changes in exons 24, 25 and 26. The peaks are labelled for exon, size and area. E, exon; IC, internal control; S, fragment size; A, peak area. Peaks for exons showing copy number change in RB74 are marked by asterisks.

Base substitutions resulting in missense changes were found in three patients, RB-29 (unilateral Rb), RB-51 and RB-52 (bilateral disease), listed in table 7c. In RB-67, change of g.56857A>G was identified, leading to a synonymous change (Glu203Glu).

Discussion

By combining three different approaches, mutations were identified in 49 out of 74 patients. Two changes, a 1-bp insertion at 22 of intron 5 (RB-70, table 6) and a silent change

Table 5. Mean peak areas of exons 24, 25 and 26 in multiplex PCR reactions VII, III and VIII for RB-74 and comparison with the normal range obtained from three normal controls.

Exon		Size	Peak area	Ratio	Mean ratio	Normal range
Multiplex VII						
74A	**Exon 24**	**239**	**28**	**0.29**	**0.29 (42% less)**	**0.50 to 0.72**
74A	E-6	291	95	1.00	1.00	
74A	Exon 23	312	52	0.55	0.52	0.47 to 0.58
74A	Exon 2	318	40	0.42	0.41	0.40 to 0.45
74B	**Exon 24**	**239**	**41**	**0.29**		
74B	E-6	291	139	1.00		
74B	Exon 23	312	69	0.50		
74B	Exon 2	318	56	0.40		
Multiplex III						
74A	Exon 20	287	184	0.63	0.61	0.52 to 0.92
74A	E-6	291	292	1.00	1.00	
74A	Exon 18	347	286	0.98	0.95	0.92 to 1.33
74A	**Exon 25**	**391**	**147**	**0.50**	**0.49 (51% less)**	1.00 to 1.39
74B	Exon 20	287	219	0.58		
74B	E-6	291	376	1.00		
74B	Exon 18	347	348	0.93		
74B	**Exon 25**	**391**	**179**	**0.48**		
Multiplex VIII						
74A	Exon 27	246	236	1.17	1.20	1.12 to 1.30
74A	**Exon 26**	**248**	**28**	**0.14**	**0.14 (53% less)**	**0.30 to 0.33**
74A	E-6	291	202	1.00	1.00	
74A	Exon 22	303	187	0.93	0.87	0.81 to 1.16
74B	Exon 27	246	245	1.24		
74B	**Exon 26**	**248**	**27**	**0.14**		
74B	E-6 2	91	198	1.00		
74B	Exon 22	303	162	0.82		

'A' and 'B' denote duplicate PCRs of the same exon. Peak areas were obtained from GeneMapper Software v 4.0 (ABI, Foster City, USA). Data in bold represents the exons the showed copy number changes.

Table 6. Small insertions/deletions in the RB1 gene.

Patient	Laterality	Exon	del/ins	Genomic position
RB-6	Bilateral	**8**	**5-bp ins**	**g.59730_59731insAACAA**
RB-11	Bilateral	**1**	**1-bp del**	**g.2189delC**
RB-17	Bilateral	**8**	**1-bp del**	**g.59792delA**
RB-20	Bilateral	**17**	**3-bp del**	**g.78155_78157delAAG**
RB-24	Bilateral	**IVS24**	**4-bp del**	**g.170403_170406delGTGA**
RB-50	Bilateral	**6**	**1-bp del**	**g.45863delA**
RB-64	Bilateral	**13**	**1-bp del**	**g.73804delG**
RB-66	Bilateral	**14**	**14-bp del**	**g.76464_76477del14**
RB-68	Bilateral	**1**	**1-bp del**	**g.2124delC**
RB-70	Bilateral	**IVS5**	**1-bp ins**	**g.45776insT**
RB-71	Bilateral	3	2-bp del	g.39552_39553delTA
RB-72	Bilateral	IVS24	4-bp del	g.170405_170408delGAGT

Novel mutations are indicated in bold.

Glu204Glu (RB-67, table 7c) are of uncertain pathogenic impact. When the mutant sequence of Rb-70 was analysed by Human Splice Finder (http://www.umd.be/HSF/), the enhancer motif for SR protein (Serine-Arginine proteins) was disrupted completely thereby implicating that it might have an impact on splicing (Desmet *et al*. 2009). The presence

Table 7a. Nonsense mutations.

Patient	Laterality	Exon	Genomic change	Mutation in amino acid
RB-1	Bilateral	17	g.78250C>T	p.Arg556X
RB-2	Bilateral	10	g.64348C>T	p.Arg320X
RB-12	Bilateral	8	g.59728C>T	p.Gln266X
RB-37	Unilateral	**3**	**g.39458C>T**	**p.Gln93X**
RB-39	Bilateral	**21**	**g.160741T>A**	**p.Cys706X**
RB-41	Unilateral	10	g.64348C>T	p.Arg320X
RB-48	Bilateral	10	g.64348C>T	p.Arg320X
RB-53	Bilateral	17	g.78238C>T	p.Arg552X
RB-54	Bilateral	14	g.76430C>T	p.Arg445X
RB-57	Bilateral	**23**	**g.162348A>T**	**p.Lys824X**
RB-58	Bilateral	13	g.73774G>T	p.Glu413X
RB-75	Bilateral	18	g.150037C>T	p.Arg579X

Table 7b. Splice site mutations.

Patient	Laterality	Position	Mutation
RB-15	Bilateral	IVS 3+1	g.39562G>T
RB-21	Bilateral	**IVS 2-2**	**g.39444A>G**
RB-59	Bilateral	**IVS 11-1**	**g.70241G>A**
RB-63*	Bilateral	IVS 24+5	g.170407G>C
RB-40*	Bilateral	**IVS 22+5**	**g.162115G>C**

*Patients with familial Rb.

Table 7c. Missense and silent changes.

Patient	Laterality	Exon	Mutation	Amino acid change
RB-29	Unilateral	9	g.61788C>T	p.Thr307Ile
RB-51	Bilateral	**7**	**g.56897T>G**	**p.Leu218Val**
RB-52	Bilateral	1	g.2196G>A	p.Arg46Lys
RB-67	Bilateral	**7**	**g.56857A>G**	**p.Glu204Glu**

Novel mutations are indicated in bold font.

of the Glu204Glu change in normal controls was tested in 80 unrelated controls and it was found to be absent. Further studies at the mRNA level would be required to know whether the two changes affect splicing.

Mutations were identified in 44 patients with bilateral Rb at about 83% (44/53) detection rate and in five patients with unilateral Rb with a detection rate of 21% (5/21). In four cases with familial Rb, the causative mutation was identified in the proband and also in the affected relatives. Variable expressivity was found in three families with splice mutations and in one family with deletion of the C-terminal region of RB1 (exons 24–26). In family RB-40, a G>C change in IVS22 at +5 position was identified in the proband (unilateral Rb), sibling (bilateral Rb) and in the mother who was not affected as determined by clinical examination. The same genotype resulted in variable expressivity and incomplete penetrance. In RB-63, a G>C change in IVS24+5 was found in the proband (bilateral Rb), and probands mother (unilateral Rb). Analysis of the two splice site mutations by Human Splicing Finder (http://www.umd.be/HSF/) showed that the consensus values of the wild-type splice donor sites were lowered by 13%–14%, thereby predicting disruption of normal splicing (Desmet *et al*. 2009). A third mutation with variable expressivity was deletion of three exons (24–26) identified in the proband RB-74 (bilateral Rb), sibling (unilateral Rb) and probands father (pthisis bulbi with a spontaneously regressed tumour in one eye).

Of the total mutations identified, 22.4% (11/49) were large deletions, 25% (12/49) were small deletions/insertion, 28.5% (14/49) were nonsense mutations, 12.3% (6/49) were splice mutations, and 10.2% (5/49) were missense mutations. The consequence of all the small deletions/insertions except the 3-bp deletion in exon 17 in RB-20 (table 6) are predicted to cause frame-shift and premature termination upon analysis by ExPASy proteomic tools (http://www.expasy.ch/tools/#translate) (Gasteiger *et al*. 2003).

There are 46 arginine codons in *RB1* gene, of which 14 are encoded by CGA/CGG, and 13 are targets for recurrent mutations (Richter *et al*. 2003). Of the C>T changes observed in 12 patients in our study, majority i.e., 11 patients had a change of CGA (arginine) codon to TGA (stop codon). Similar findings involving mutations at CpG dinucleotides have been described earlier (Cowell *et al*. 1994). Only one C>T change in RB-29 resulted in change of threonine to isoleucine in the protein.

Of the three missense substitutions observed, one mutation in RB-51, a T>G change at g.56897 (Leu218Val) is a novel mutation. This change was absent in 80 unrelated normal control individuals. When the Rb protein from human was aligned with other species by ClustalW alignment (http://www.ebi.ac.uk/Tools/clustalw2/) the leucine residue is fairly well conserved amongst closely related species (figure 3) suggesting that such mutation might be deleterious to the function of the protein.

Of the 49 mutations identified, 23 mutations were not reported earlier to our knowledge and these are novel mutations identified in our study. They are distributed as five large deletions, 10 small deletions, three nonsense mutations, three splice site mutations, one missense and one silent change.

A detection rate of 83% was achieved with this approach in bilateral Rb. Earlier studies have reported that detection rates of 80% to 90% were achievable with a comprehensive approach using a combination of two or three different techniques - cytogenetics/fluorescent *in situ* hybridization (FISH) / Southern blot / quantitative multiplex PCR for large gene rearrangements and PCR-sequencing or denaturing high performance liquid chromatography (DHPLC) for point mutations (Lohmann *et al*. 1996; Richter *et al*. 2003; Houdayer *et al*. 2004; Bamne *et al*. 2005; Nichols *et al*. 2005; Brichard *et al*. 2006; Fernandez *et al*. 2007). An approach of MLPA (multiplex ligation-dependent probe amplification) in combination with DHPLC also identified large mutations that comprised of 22% of the total mutations (Sellner *et al*. 2006). Since only one primer needs to be fluorescently labelled in the method used by us, it is more economical that other methods requiring multiple fluorescent probes. Multiplexing is more robust and less time-consuming. Mutations were not identified in 10 patients with bilateral Rb, and might be located deeper within the introns or were not detected in blood due to mosaicism. Overall, this approach is sensitive and cost effective. It may be suitable for use in molecular testing of the families who seek genetic counselling.

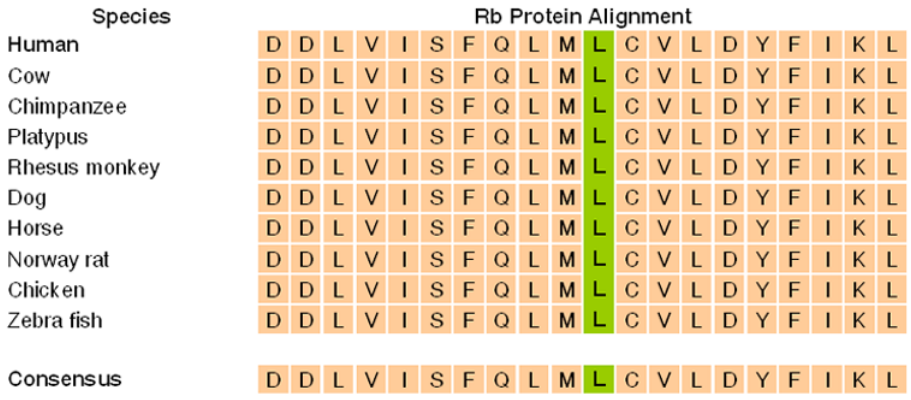

Figure 3. Clustal W alignment of Rb proteins of humans versus other species.

Appendix

Table 1. Primers used for PCR of *RB1* exons.

Exon	FP/RP	Primer sequence	Amplicon size (bp)
Promoter	FP	GCGAATTCCCAAAAGGCCAGC AAGTGTCTAAC	709
Exon 1-Seq	1-F	TCCGTCTTAGCTGAGTGGCGTA TTTTGTAACGGGAGTCGGGAGAGG AGG	356
	1-R	AGGCAGAATCGACTCACCGCT ACCTTGCGCCCGCCCTACG	
Exon-1-qPCR	1-qFP	TCCGTCTTAGCTGAGTGGCGT ATCCTCCACAGCTCGCTGGCT	255
	1-qRP	AGGCAGAATCGACTCACCGCT	

Table 1 (*contd*)

Exon	FP/RP	Primer sequence	Amplicon size (bp)
		ACTTCCCGCGTGAGGCGAC	
Exon 2	2-F	TCCGTCTTAGCTGAGTGGCGT	318
		ATTTTCATTTGGTAGGCTTGAGTTT	
	2-R	AGGCAGAATCGACTCACCGCT	
		ACCTCTGGGTAATGGAATTATTATT	
Exon 3	3-F	TCCGTCTTAGCTGAGTGGCGT	234
		AATTTGATCTTTATTTTTTGTTCCC	
	3-R	AGGCAGAATCGACTCACCGCTA	
		TTCATTTCCTTTTATGGCAGAG	
Exon 4	4-F	TCCGTCTTAGCTGAGTGGCGTATG	340
		TAGAGCTGATAATCTTTTGAATTG	
	4-R	AGGCAGAATCGACTCACCGCTAAT	
		TCCCAGAATCTAATTGTGAACA	
Exon 5	5-F	TCCGTCTTAGCTGAGTGGCGTATT	340
		GGGAAAATCTACTTGAACTTTG	
	5-R	AGGCAGAATCGACTCACCGCT	
		AGCTATAATCGATCAAACTAACCCT	
Exon 6	6-F	TCCGTCTTAGCTGAGTGGCGT	222
		ATTTCCTGTTTTTTTTCTGCTTTCT	
	6-R	AGGCAGAATCGACTCACCGCT	
		AATTTAGTCCAAAGGAATGCCAA	
Exon 7	7-F	TCCGTCTTAGCTGAGTGGCGT	284
		ACCTGCGATTTTCTCTCATACAA	
	7-R	AGGCAGAATCGACTCACCGCTAAG	
		ACATTCAATAAGCAACTGCTGA	
Exon 8	8-F	TCCGTCTTAGCTGAGTGGCGTATG	300
		ATGGATGTACAATTGTTCTTAT	
	8-R	AGGCAGAATCGACTCACCGCTAAT	
		ATTGTTAGGGAGAACTTACATC	
Exon 9	9-F	TCCGTCTTAGCTGAGTGGCGTACC	254
		TGCATTGTTCAAGAGTCAAGAG	
	9-R	AGGCAGAATCGACTCACCGCT	
		ACCTCCCTCCACAGTCTCAAAAC	
Exon 10	10-F	TCCGTCTTAGCTGAGTGGCGTACT	262
		GTGTGCTGAGAGATGTAATGAC	
	10-R	AGGCAGAATCGACTCACCGCTACA	
		ACCTATATCTAAAGCAAATCAATC	
Econ 11	11-F	TCCGTCTTAGCTGAGTGGCGT	262
		ATGATGCATAAAGCACAAATTGT	
	11-R	AGGCAGAATCGACTCACCGCTAAA	
		CGTGAACAAATCTGAAACACTA	
Exon 12	12-F	TCCGTCTTAGCTGAGTGGCGT	236
		ATTCTCCCTTCATTGCTTAACAC	
	12-R	AGGCAGAATCGACTCACCGCTAAA	
		TTTCAAGTTTCTTTGCCAAGAT	
Exon 13	13-F	TCCGTCTTAGCTGAGTGGCGTATC	283
		TGATTACACAGTATCCTCGACA	
	13-R	AGGCAGAATCGACTCACCGCT	
		ATACGAACTGGAAAGATGCTGCT	
Exon 14	14-F	TCCGTCTTAGCTGAGTGGCGTATT	255
		GTGATTTTCTAAAATAGCAGGC	
	14-R	AGGCAGAATCGACTCACCGCT	
		ACTTGACCTCCTGATCTGCCC	
Exon 15-16	15-F	TCCGTCTTAGCTGAGTGGCGTAAA	420
		TGCTGACACAAATAAGGTTTC	
	15-R	AGGCAGAATCGACTCACCGCTAGA	
		TCTAAAATAAGCATTCCTTCTCC	

Table 1 (*contd*)

Exon	FP/RP	Primer sequence	Amplicon size (bp)
Exon 17	17-F	TCCGTCTTAGCTGAGTGGCGTACT	345
		AGCTCAAGGGTTAATATTTCAT	
	17-R	AGGCAGAATCGACTCACCGCTATT	
		GTTAGCCATATGCACATGAATG	
Exon 18	18-F	TCCGTCTTAGCTGAGTGGCGTATT	346
		CTGACTTTTAAATTGCCACTGT	
	18-R	AGGCAGAATCGACTCACCGCTAAA	
		TCTATTTGCAGTTTGAATGGTC	
Exon 19	19-F	TCCGTCTTAGCTGAGTGGCGTACT	305
		ACTGTGATTCTTAGCCAACTTGAAA	
	19-R	AGGCAGAATCGACTCACCGCT	
		AATGATTTGAACCCAGTCAGCCTA	
Exon 20	20-F	TCCGTCTTAGCTGAGTGGCGTATT	286
		GTAATTCAAAATGAACAGTAAA	
	20-R	AGGCAGAATCGACTCACCGCTACA	
		AGTAAGTAGGGAGGAGAGAAGG	
Exon 21	21-F	TCCGTCTTAGCTGAGTGGCGTAGA	314
		GGCTAAAAGAAAGAAAATGGTATTT	
	21-R	AGGCAGAATCGACTCACCGCTAAA	
		ATGAGATCAAATGAATTACCTATG	
Exon 22	22-F	TCCGTCTTAGCTGAGTGGCGTATT	303
		CCTCAGACATTCAAACGTG	
	22-R	AGGCAGAATCGACTCACCGCTATA	
		AGGGCTTCGAGGAATGTG	
Exon 23	23-F	TCCGTCTTAGCTGAGTGGCGTAAA	312
		TGTAATGGGTCCACCAAAAC	
	23-R	AGGCAGAATCGACTCACCGCTATC	
		CCCCTCTCATTCTTTACTACTTC	
Exon 24	24-F	TCCGTCTTAGCTGAGTGGCGTACT	238
		CATCTCTGCAAAATTGTATATGG	
	24-R	AGGCAGAATCGACTCACCGCTATT	
		TGAATAACTGCATTTGGAAATT	
Exon 25	25-F	TCCGTCTTAGCTGAGTGGCGTATT	392
		TGCCTGATTTTTGACACACC	
	25-R	AGGCAGAATCGACTCACCGCTAAA	
		CTTGGCATGAAAGAAATTGG	
Exon 26	26-F	TCCGTCTTAGCTGAGTGGCGTAAT	247
		CGAAAGCATCATAGTTACTGGA	
	26-R	AGGCAGAATCGACTCACCGCTATA	
		ACGAAAAGACTTCTTGCAGTGT	
Exon 27	27-F	TCCGTCTTAGCTGAGTGGCGTAGC	242
		ATAATATATATGGCAGCCACTT	
	27-R	AGGCAGAATCGACTCACCGCTAAA	
		TGAATCCAGAGGTGTACACAGT	
Exon 6 of BIGH3	IC-6F	TCCGTCTTAGCTGAGTGGCGTACT	291
		TTGGGACTATGCCTCTGTTG	
	IC-6R	AGGCAGAATCGACTCACCGCTAGC	
		AATGTGTCCCAAGCCTGG	
Universal Primer	Univ-F	TCCGTCTTAGCTGAGTGGCGTA	
	Univ-R	6 Fam-AGGCAGAATCGACTCACCGCTA	

Sequences of primers used for amplifying the *RB1* gene exons as well as those for the *BIGH3* gene used as an internal control are given in the table. FP/RP stand for forward and reverse primers respectively. Details are in text.

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