Splicing aberrations caused by constitutional *RB1* gene mutations in retinoblastoma

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Analysis of RB1 mRNA from blood leukocytes of patients with retinoblastoma identified the effects of mutations involving consensus splice site, exonic substitution and whole-exon deletions identified in genomic DNA of these patients. In addition, this study identified mutations in cases in which no mutations were detectable in the genomic DNA. One proband had mutation at the canonical splice site at +5 position of IVS22, and analysis of the transcripts in this family revealed skipping of exon 22 in three members of this family. In one proband, a missense substitution of c.652T>G (g.56897T>G; Leu218Val) in exon 7 led to splicing aberrations involving deletions of exons 7 and 8, suggesting the formation of a cryptic splice site. In two probands with no detectable changes in the genomic DNA upon screening of RB1 exons and flanking intronic sequences, transcripts were found to have deletions of exon 6 in one, and exons 21 and 22 in another family. In two probands, RNA analysis confirmed genomic deletions involving one or more exons. This study reveals novel effects of RB1 mutations on splicing and suggests the utility of RNA analysis as an adjunct to mutational screening of genomic DNA in retinoblastoma.

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1. Introduction

Retinoblastoma (Rb) is the most common pediatric intraocular malignancy and occurs at ages below 5 years. The incidence is 1 in 20000 in India, and is similar in other parts of the world. It is brought about by the biallelic inactivation of the human retinoblastoma susceptibility gene *RB1* (GenBank accession number: L11910) on chromosome 13q14. Rb can manifest either as hereditary or non-hereditary disease, and in the case of hereditary disease, one allele is mutated in the germline and the other at the cellular level. In the non-hereditary disease, both the alleles are mutated at the cellular level (Knudson Jr 1971). The hereditary disease generally manifests bilaterally and is inherited as an autosomal dominant disorder with high penetrance (90% or more), characterized by early onset (within the first year of life) and presence of multiple tumors (multifocal). The non-hereditary form of the disease typically involves one eye (unilateral) and occurs as a single tumor (unifocal) with late onset i.e. after the first year of life.

A high degree of mutational heterogeneity has been reported in the *RB1* gene with over 900 mutations reported till date (Valverde *et al.* 2005). With the use of multiple approaches and highly sensitive techniques, detection rates have mostly been between 89% and 92% (Richter *et al.* 2003; Houdayer *et al.* 2004; Parsam *et al.* 2009). The main barriers to achieving efficient detection of *RB1* mutations is the large size of the gene, the presence of mosaicism and the possibility of mutations within non-coding regions that are not routinely screened.

Transcript analysis of the *RB1* mRNA is potentially valuable in revealing pathogenic mutations that affect splicing such as those involving consensus splice sites, deep intronic mutations as well as silent changes in the exons. Apart from these, mRNA analysis can reveal somewhat unexpected effects of other types of mutations such as nonsense and

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missense changes on splicing, and thereby provide insight into additional sequence elements regulating mRNA splicing. Analysis of *RB1* mRNA has been employed to detect mutations (Dunn *et al.* 1989; Dehainault *et al.* 2007; Zhang *et al.* 2008). Effect of mutations on splicing have been studied in the aetiology of several genetic diseases (Cartegni *et al.* 2002). For example, several germline mutations – missense, nonsense, frameshift types of mutations in the neurofibromatosis type 1 (*NF1*) gene and ataxia telangiectasia (*ATM*) gene – cause aberrant splicing either by exon skipping or by activating cryptic splice sites (Teraoka *et al.* 1999; Ars *et al.* 2000).

A premature termination codon (PTC) in exon 51 of fibrillin 1 (*FBN1*) gene that causes Marfan syndrome (Dietz *et al.* 1993) and in exon 18 (E1694X) of the *BRCA1* gene (Mazoyer *et al.* 1998) are associated with exon skipping. Skipping of an exon might confer an advantage as this might lead to a protein with residual function rather than an inactive protein. This situation is depicted in Duchenne muscular dystrophy (DMD) and its milder variant, Becker muscular dystrophy (BMD), caused by mutations in the dystrophin gene (*DMD*) (Ahn and Kunkel 1993). It has been reported that skipping of PTC-containing internal exons preserves the *DMD* open reading frame, leading to BMD (Shiga *et al.* 1997). Exon skipping has also been reported with missense and translationally silent changes (Cartegni *et al.* 2002).

We have in a previous study employed a screening strategy using multiple approaches for *RB1* mutation screening using genomic DNA and obtained a detection rate of >80% for bilateral Rb using peripheral blood DNA (Parsam *et al.* 2009). In this study we employed RNA analysis to explore the possible effects of *RB1* mutations on mRNA splicing to confirm whole-exon deletions detected by quantitative PCR and to detect additional mutations not evident in genomic DNA through their effects on splicing.

2. Materials and methods

This study included 5 families with more than 1 affected member (including a total of 11 affected and 4 unaffected) and 1 proband with sporadic bilateral Rb. Genomic DNA analysis from peripheral blood was as described by Parsam *et al.* (2009).

2.1 *RNA isolation*

Fresh blood (2–3 mL) was collected from the probands and other family members for RNA isolation. Equal volume of 1× PBS was used to wash and obtain the leukocyte pellet by centrifuging at 4000 rpm at 4°C for 5 min. Total RNA was isolated from the leukocyte pellet using Trizol™ (Invitrogen, Carlsbad, CA) reagent according to the manufacturer's protocol.

2.2 *RT-PCR analysis*

Approximately 3–4 μg of RNA was used for the first-strand synthesis using Superscript III with oligo dT primer (Invitrogen, Carlsbad, CA). The quality of cDNA was verified by amplification of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA. Five sets of overlapping primers for amplifying the entire length of the *RB1* mRNA transcript of 2787 bp were designed (table 1). All the *RB1* cDNA fragments were amplified using a touchdown PCR protocol. Amplification of exons 7–27 were carried out using initial denaturation at 95°C for 2 min,

Table 1. Primers used for RT-PCR of RB1 mRNA

No.	Primer name	Primer sequence	Size of amplicon
1	GAPDH-FP GAPDH-RP	GCCAAGGTCATCCATGACAAC GTCCACCACCCTGTTGCTGTA	498
2	RB-E-22-FP RB-E-22-RP	CGCCTTCTGTCTGAGCACCC GTCGCTGTTACATACCATCTGATTTA	567
3	RB-E-1-6-FP RB-E-1-6-RP	GAGGGCGCGTCCGGTTTTTC CGTGCACTCCTGTTCTGACCTCGC	932
4	RB-E-7-12-FP RB-E-7-12-RP	CAGCAAATTGGAAAGGACATGTGA CGGTAATACAAGCGAACTCCAAG	879
5	RB-E-13-18-FP RB-E-13-18-RP	AATTCCTCCACACACTCCAGTTAG CTGGGTCTGGAAGGCTGAGGT	814
6	RB-E-19-22-FP RB-E-19-22-RP	AAAGGACCGAGAAGGACCAACTG GACCTTCTGAAATTTTATATGGACTCTT	728
7	RB-E-23-27-FP RB-E-23-27-RP	ATGCAAAGTGAAGAATATAGACCTT ATGGCAGGATTTACACAAGATTTTCA	883

15 cycles of 95°C for 30 s, 60.8°C for 30 s (touchdown protocol with decrease of 0.5°C per cycle), 72°C for 90 s, 20 cycles of 95°C for 30 s, 53.8°C for 30 s and 72°C for 90 s with a final elongation of 72°C for 5 min. The cDNA fragment for exons 1–6 was amplified using the same protocol with the exception that it was amplified at a higher annealing of 69.4°C with touchdown protocol for 15 cycles and then for 20 cycles at a annealing of 62.4°C with 5% DMSO (dimethyl sulfoxide) as the region is GC-rich. PCR amplification was carried out in a reaction mix containing 2 µl of cDNA, 10 pmols of each of forward primer and reverse primer, 1× PCR reaction buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, in 25 µl reaction volume. PCR products were analysed on 2% agarose gel and scanned using an ultraviolet (UV) gel doc (UVtec Ltd, Cambridge, UK). PCR products were purified after elution from agarose gels, and subjected to bidirectional sequencing by standard methods on the ABI 3130XL genetic analyser (Applied Biosystems, Foster City, USA). Mutations identified were on the genomic *RB1* sequence (GenBank accession number: L11910) and cDNA sequence (NM_000321.2).

3. Results

3.1 *Effects of mutations at the conserved 5′ donor splice site*

RNA analysis of a family RB-40 (shown in figure 1a) having a mutation at IVS22+5G>C, (g.162115G>C) revealed two *RB1* transcripts. These are depicted by the RT-PCR products shown in figure 1b. One product corresponded to the wild-type transcript (size 567 bp) and the other smaller product (size 453 bp) was an abnormal transcript. Elution and sequencing showed deletion of exon 22 (114 bp) due to the mutation (r.2106.2211del; figure 1c). When we analysed the amounts of the two cDNAs by quantitative PCR (qPCR), we found the the ratio of normal to mutant transcript to be 2.5 in the mother and 0.4 and 0.6 in the proband (unilateral Rb) and sibling (bilateral Rb), respectively (data not shown).

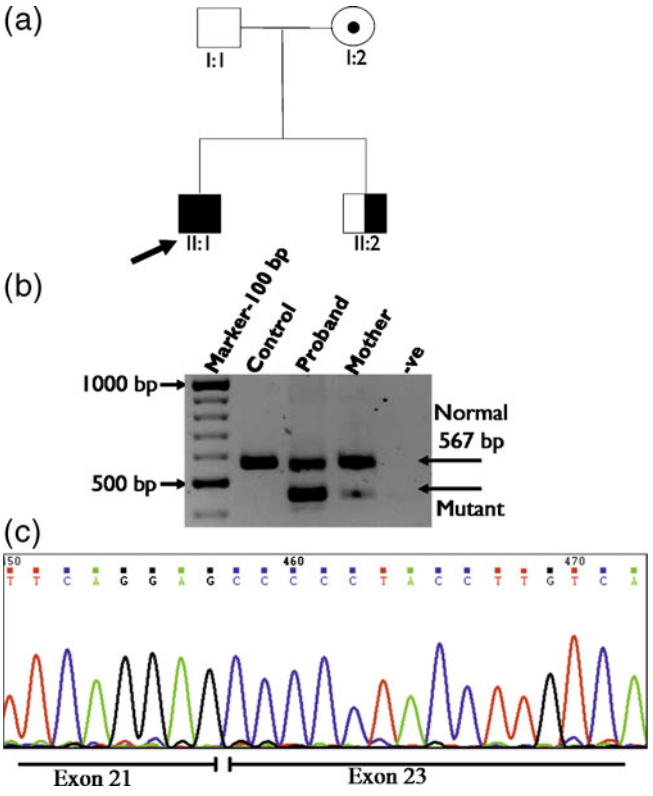

Figure 1. RT-PCR analysis of family RB-40 (IVS22+5G>C). (**a**) Pedigree of family RB-40. Open symbols represent unaffected individuals, fully shaded and half-shaded symbols represent individuals with bilateral and unilateral retinoblastoma, respectively. The arrow indicates the proband. A dot within the symbol (I:2) represents an unaffected mutation carrier. (**b**) Agarose gel electrophoresis of cDNA fragments obtained from RT-PCR analysis with primer set RB-E-22-FP/RP containing exon 22 for family RB-40. (**c**) Sequence electropherogram of the mutant cDNA showing deletion of exon 22.

3.2 *Effects of substitution mutations in the exonic region*

In a case of sporadic bilateral Rb (RB51), a T>G change in exon 7 (g.56897T>G; c.652T>G) corresponding to a codon change of Leu218Val was identified by genomic DNA analysis. Analysis of the *RB1* mRNA from this patient showed three transcripts (figure 2a). Gel elution and sequencing of each product revealed that one transcript was a wild-type transcript and the other two were mutant transcripts (M1 and M2). M1 had a part of exon 7 deleted – this deletion included sequences from c.652, the site of the change T>G, to position c.718 at the end of exon 7. A second mutant transcript (M2) had the above sequences (c.652.718del) of exon 7 and all of exon 8 skipped, i.e. r.652.861del (figure 2b).

3.3 *Identification of mutations by RNA analysis in patients in whom DNA analysis could not identify any mutation*

In two families, no mutations (no large deletions identified by QM-PCR or no point mutation identified in the coding regions by sequencing) were detected by genomic DNA screening of coding regions as described by Parsam *et al.* (2009). In these two cases, the entire length of the *RB1* mRNA was screened for mutations by RT-PCR and sequence analysis. In family

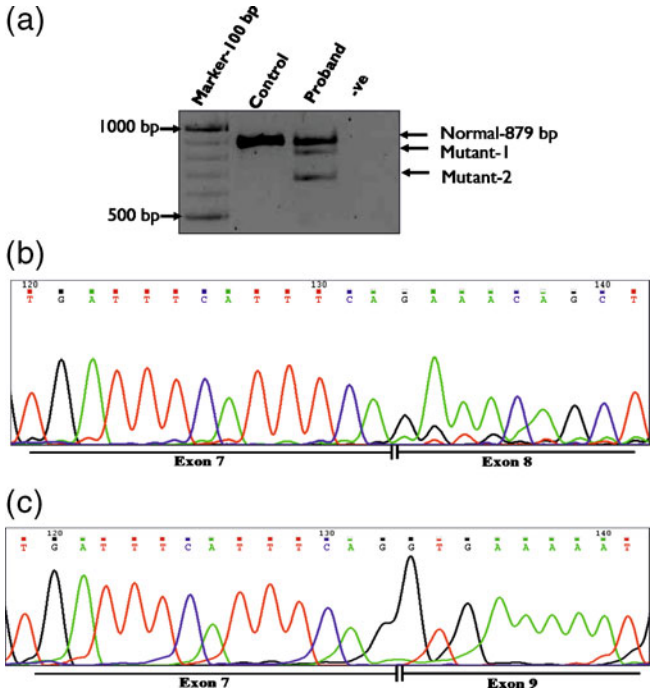

Figure 2. RT-PCR analysis of RB-51 (c.652T>G; Leu218Val). (**a**) RT-PCR and agarose gel analysis with primer set RB-E-7-12 FP/RP. Sequence electropherograms of mutant transcripts (**b**) M1 (deletion of part of exon 7) and (**c**) M2 (deletion of part of exon 7 and exon 8).

RB-14, the proband had bilateral Rb, and the father had a history of enucleation in both eyes, the cause of which could not be ascertained. RNA analysis showed one aberrant transcript along with the wild-type transcript (879 bp) in the proband and not in the father (figure 3a). The aberrant transcript had a deletion of exon 6 (r.540.607del; figure 3b).

In another family (RB-70), the proband had bilateral Rb and his mother had unilateral Rb (pedigree in figure 4a). RNA analysis for exons 19–22 showed two products in the proband and three products in the mother (figure 4b). Sequence analysis of all these fragments revealed that one corresponded to the wild-type transcript (band at 728 bp). A mutant (M1), present in both proband and mother, corresponded to a transcript with exon 22 (114 bp) deleted (r.2106.2211del; figure 4c). The smaller transcript M2, observed in the mother, was found to have exons 21 and 22 deleted (r.2106.2211del; total of 219 bp).

3.4 *Detection of whole-exon deletions*

A large deletion of exons 24, 25 and 26 was identified by DNA analysis using quantitative PCR in three affected members of a family (RB-74; Parsam *et al.* 2009) (pedigree in figure 5a).

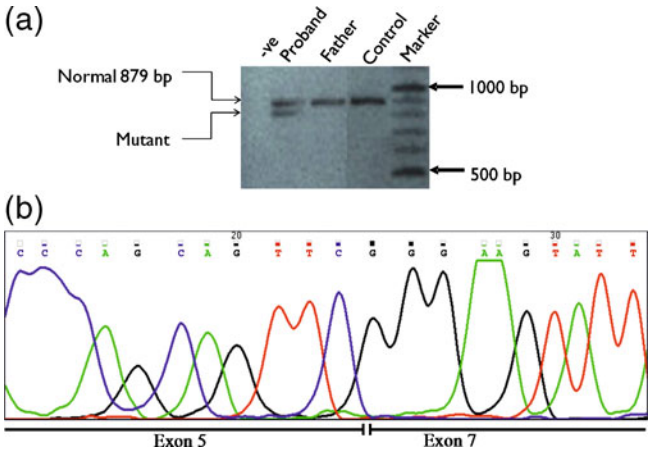

Figure 3. RT-PCR analysis in RB14. (**a**) RT-PCR and agarose gel analysis of cDNA fragments with primer set RB-E-7-12-FP/RP spanning exon 6. Sequence electropherogram of mutant transcript showing deletion of exon 6 (**b**).

The proband had unilateral Rb, the sibling had bilateral Rb and the father had pthisis bulbi (a shrunken non-functional eye) in one eye. RT-PCR and sequencing analysis of *RB1* mRNA for all three affected members confirmed the same deletion (figure 5b and c). A deletion of exon 14 was revealed by DNA analysis in a proband (RB-76 with bilateral Rb), and the same deletion was observed in the father (with unilateral Rb). RNA analysis by RT-PCR showed two transcripts detectable in the proband and in the father (figure 6a) – one

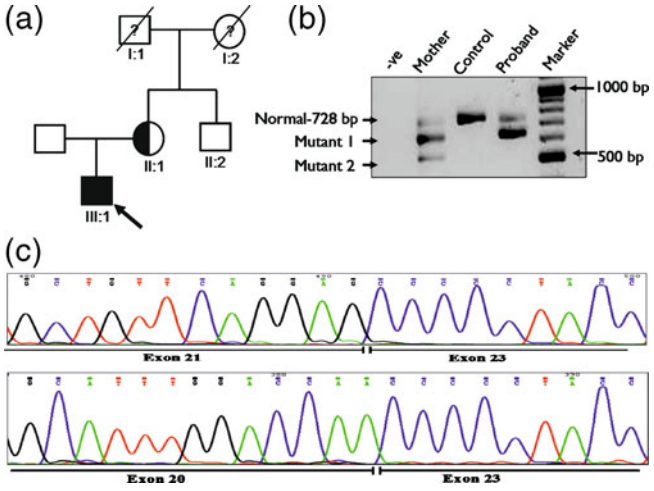

Figure 4. RT-PCR analysis of family RB-70. (**a**) Pedigree of RB70. Symbols are as denoted in figure 1a. (**b**) RT-PCR and agarose gel analysis of cDNA fragments obtained with primer set RB-E-19-22-FP/RP. (**c, d**) Electropherogram of the mutant transcripts, M1 and M2, showing deletion of exon 22 and exon 21 and 22, respectively, in family RB70.

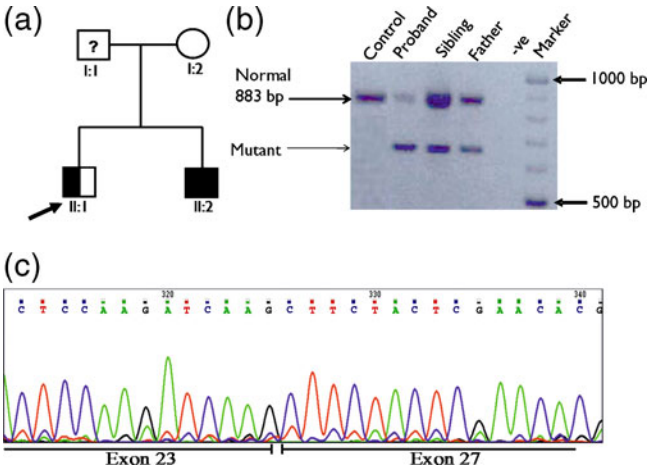

Figure 5. RT-PCR analysis of family RB-74 (del24_26). (**a**) Pedigree of family RB-74. Explanation of symbols is as given in figure 1a. The question mark denotes uncertain affection status. (**b**) RT-PCR analysis of cDNA fragments with primer set RB-E-23-27-FP/RP in RB-74. (**c**) Sequence electropherogram of mutant transcript showing deletion of exons 24, 25 and 26.

was the wild-type transcript (814 bp) and the smaller (758 bp) was a transcript with deletion of exon 14 (figure 6b).

4. Discussion

Transcript analysis of *RB1* mRNA in retinoblastoma in this study revealed different patterns of missplicing due to oncogenic *RB1* mutations (table 2). We found one splice site mutation (IVS22+5G>C), identified in family RB-40, to be associated with variable expressivity and penetrance. The ratios of expression of normal and mutant transcripts varied between the three members of the family. The mother, who was an unaffected carrier, showed a relative abundance of the normal transcript, whereas the two affected children showed an abundance of the mutant transcript. We cannot rule out the possibility at present that the mother is a mosaic for this mutation and therefore does not manifest the disease, rather than being a non-penetrant carrier. The ratios of 0.4 and 0.6 in the two affected siblings are fairly similar and may reflect a lower stability of the mutant mRNA relative to the wild type. It is also possible that these quantitative differences may be the basis for variable expressivity and penetrance in this family. Differences in the proportions of normal and mutant mRNAs in affected vs unaffected carriers have been reported although the patterns are not always the same in different studies (Klutz *et al.* 2002; Taylor *et al.* 2007). In addition, the *RB1* gene is imprinted with favoured expression of the maternal allele (Kanber *et al.* 2009). Imprinting may explain the relative abundance of the two different alleles, but the relation between abundance of the mutant allele and phenotype is not explainable by imprinting alone. Variable levels of aberrantly spliced RNA have been linked to parent of origin as well as penetrance by Klutz *et al.* (2002). They found that reduced relative abundance of the mutant transcript produced as a result of mutation IVS6+1G>T was associated with paternal transmission and high penetrance, whereas similar levels of normal and mutant transcripts were associated with maternal transmission and reduced penetrance. In contrast, Taylor *et al.* (2007) observed a higher expression of the wild-type allele in unaffected carriers. *In silico* analysis of the mutant cDNA sequence showed an in-frame deletion leading to protein short by 34 amino acids at the C-terminal end with the formation of a mutant protein of predicted size 890 aa). It is possible that this in-frame deletion of 34 amino acids (due to exon 22 skipping) has a mild effect on the cellular function of the RB protein. This phenotype resembles that from a low-penetrance mutation that causes skipping of exon 21 from the *RB1* transcript due to G>A transition in the last base of exon 21 (Schubert *et al.* 1997). A different substitution at the same position in IVS22, i.e. G>A at +5 position in IVS22, was reported earlier in a family with low penetrance (Weir-Thompson *et al.* 1991), suggesting that mutation at this position has partial effects on *RB1* function.

In RB-51 a novel mutation of c.652T>G, predicting a missense change of Leu218Val located in exon 7, led to spicing defects. Two mutant transcripts were formed in addition to the normal transcript. Transcript M1 had deletion of exon 7 sequences downstream from the site of mutation; transcript M2 had deletion of exon 7 sequences as in M1 with additional deletion of exon 8. We presume that the mutation is creating a new splice donor site and this in combination with the acceptor site at intron 7–exon 8 junction created the splice variant, M1, and in combination with acceptor site at intron 8–exon 9 junction, led to formation of mutant M2. An earlier study has shown that in normal human breast and prostrate tissues, three splice variants of *RB1* were observed, one

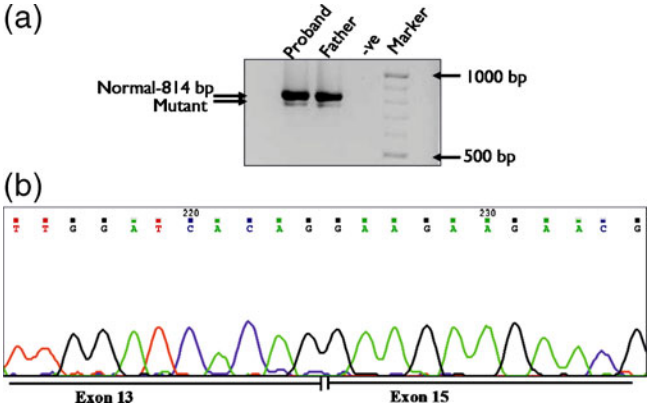

Figure 6. RT-PCR analysis of family RB-76 (del14). (**a**) RT-PCR analysis of cDNA fragments with primer set RB-E-13-18-FP/RP in RB-76. (**b**) Sequence electropherogram of the mutant transcript showing deletion of exon 14.

Table 2. Summary of RB1 mutants detected by RNA analysis

Family ID	Type of mutation	Mutation in genomic DNA	No of mutant transcripts	Splicing alterations	Mutation at RNA level	Consequences of the mutation
RB-40 (familial Rb)	Splice	g.162115G>C, (IVS22+5)	1	Skipping of exon 22 (in-frame)	r.2106.2211del	In-frame deletion
RB-51 (sporadic bilateral Rb)	Substitution with predicted missense (Leu218Val)	g.56897T>G , in exon 7 (c.652T>G)	2	M1-Skipping of part of exon 7 (out-of-frame) M2-Skipping of part of exon 7 and exon 8 (in-frame)	Mutant 1-r.652.718del Mutant 2- r.652.861del	M1-Results in termination codon M2-In-frame deletion
RB-14 (sporadic bilateral Rb)	Splice (presumed)	No mutation identified at DNA level	1	Skipping of exon 6 (out-of-frame)	r.540.607del	Results in termination codon
RB-70 (familial Rb)	Splice (presumed)	No mutation identified at DNA level	Proband-1	M1-skipping of exon 22, (in-frame)	r.2106.2211del	In-frame deletion
			Mother-2	M1-skipping of exon 22; M2-skipping of exons 21 and 22 (in-frame)	Mutant 1- r.2106.2211del Mutant 2- r.2106.2325del	Both M1 and M2 are predicted to cause in-frame deletion
RB-74 (familial Rb)	Deletion of exons 24, 25 and 26	c.2490-?.2713+?del	1	Skipping of exons 24, 25 and 26 (out-of-frame)	r.2490.2713del	In-frame deletion
RB-76 (familial Rb)	Deletion of exon 14	c.1333-?.1389+?del	1	Skipping of exon 14 (in-frame)	r.1333.1389del	In-frame deletion

Details are shown of families screened by RNA analysis, mutations if any identified in genomic DNA and alterations in *RB1* mRNA. Mutations in columns 3 and 6 indicate DNA and RNA mutations as per the nomenclature guidelines of Human Genome Variation Society (*http://www.hgvs.org/mutnomen/recs.html#general*).

missing either exon 2 or exon 8 and another splice variant missing both exons 2 and 8 (Latil *et al.* 1999). The M2 transcript might have formed simply because of the transcript that is found normally missing exon 8, and so it may not be specifically related to the disease. The deletion of exon 7 sequences (present in M1) predicts a frameshift with a premature stop codon after 23 amino acids, whereas the deletion of exons 7 and 8 (in M2) is a in-frame deletion of 70 amino acids. The mutant sequence when analysed by Human Splice Finder software version 2.4 (*http://www.umd.be/HSF/*) (Desmet *et al.* 2009) also predicted the same. Previous studies on two missense changes – Gly449Arg (Taylor *et al.* 2007) and Leu220Val (Zhang *et al.* 2008) in *RB1* – have reported exon skipping. In the case of Leu220Val (g.56903C>G), two splice variants were observed, presumably through usage of two different acceptor sites (Zhang *et al.* 2008).

Genomic DNA analysis carried out in our previous study (Parsam *et al.* 2009) included coding sequences and 20 bp or more of flanking intronic sequence at the exon–intron junctions. We show that in two cases, (RB-14 and RB-70), with no detectable mutations by genomic DNA analysis, *RB1* mRNA produced in both these patients was structurally defective, indicating aberrant splicing due to undetected sequence alterations. In RB-14 family, with deletion of exon 6, we did not attempt to look at any intronic changes since the intron 6 is quite large (~10 kb). In family RB-70, different patterns of missplicing were observed in the proband (bilateral Rb) and in the mother (unilateral Rb). Curiously, both mutant transcripts, i.e. M1 with deletion of exon 22 and M2 with deletion of exons 21 and 22, are predicted to cause in-frame deletions. The presence of an additional mutant M2 in the mother cannot be correlated with variable expressivity in this family. Sequence analysis of entire introns 20, 21 and 22 did not reveal any sequence variations except for the reported SNP (rs198589) in the genomic DNA. Previous screening of the exons and flanking sequences as well as quantitative PCR to detect large exonic deletions/duplications did not reveal any alterations in these two families. The causative genomic changes resulting in the skipping of exons is not known at present. One cannot rule out the possibility of an artifact (such as allelic dropout) that prevented us from picking up the sequence change responsible for splicing aberration. It is also possible that there is a large genomic rearrangement in this region that cannot be detected by the methods employed here including quantitative PCR and PCR-based sequencing. An intronic change involving a change IVS23-1398A>G affecting splicing in a case of familial Rb (Dehainault *et al.* 2007) was found to create a new 3′ splice acceptor site that resulted in the exonisation of 103 bp of intron 23 between exon 23 and 24. This mutation was associated with incomplete penetrance (Dehainault *et al.* 2007).

The deletions identified by quantitative PCR were confirmed by mRNA analysis in two families, RB-74 and RB-76. Our

study reveals novel effects of *RB1* mutations on splicing. The foregoing data suggest that *RB1* mutations should be investigated at the cDNA level and that different forms of aberrantly spliced mRNAs as well as the relative amounts of mutant and wild-type mRNAs may affect penetrance and expressivity of retinoblastoma. Our study also provides support for the idea that cDNA analysis is valuable as an adjunct for screening of Rb patients when a mutation is not identified by routine DNA analysis and as a confirmation of deletions detected by quantitative PCR. The overall detection rate was improved from 83% to 86% in the bilateral Rb patients by combining both RNA analysis along with the DNA analysis (Parsam *et al.* 2009).

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