Experimental Eye Research 251 (2025) 110233

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Research article

Mosaicism and intronic variants in *RB1* gene revealed by next generation sequencing in a cohort of Spanish retinoblastoma patients

Gema Gomez-Mariano [a,*], Esther Hernandez-SanMiguel [a], Marta Fernandez-Prieto [a], Sheila Ramos del Saz [a], Beatriz Baladrón [a], Lidia Mirela Mielu [a], Daniel Rivera [a], Victoria Moneo [a], Lidia Lopez [a], Carlos Rodriguez-Martin [b], Ana Fernandez-Teijeiro Álvarez [c], Constantino Sabado [d], Eva Bermejo [a], Francisco Javier Alonso [e,f], Beatriz Martinez-Delgado [a,f]

[a] Genetic Diagnosis Unit, Institute for Rare Diseases Research (IIER), Institute of Health Carlos III (ISCIII), Madrid, Spain
[b] Unit of Human Disease Mechanisms in Drosophila, Institute for Rare Diseases Research (IIER), Institute of Health Carlos III (ISCIII), Madrid, Spain
[c] Virgen Macarena University Hospital, Sevilla, Spain
[d] Vall D'Hebron Hospital, Barcelona, Spain
[e] Childhood Solid Tumors Unit, Institute for Rare Diseases Research (IIER), Institute of Health Carlos III (ISCIII), Madrid, Spain
[f] CIBER of Rare Diseases (CIBERER), U758. Institute of Health Carlos III (ISCIII), Madrid, Spain

ARTICLE INFO

Keywords:
Retinoblastoma
RB1 gene
Mosaicism
Next generation sequencing
MLPA

ABSTRACT

Constitutional variants in the *RB1* gene predispose individuals to the development of Retinoblastoma (RB) and the occurrence of second tumors in adulthood. Detection of causal *RB1* gene variants is essential to establish the genetic diagnosis and to performing familial studies and counseling. In our cohort of 579 Spanish RB patients, 15% of cases suspected to have a genetic origin remained negative after traditional Sanger sequencing and Multiplex Ligation-dependent Probe Amplification (MLPA) of *RB1* gene, likely due to the possibility of mosaicism or non-coding variants. A specific next-generation sequencing (NGS) gene panel was designed to analyze the complete sequence of the *RB1* gene. While many familial RB cases showed variants through Sanger and MLPA, the analysis of 65 available sporadic RB patients using the NGS gene panel identified a causative variant in an additional 6 of 26 (23%) bilateral cases and 6 of 39 (15.4%) unilateral cases. Seven of these cases exhibited different degrees of mosaicism (26%, 20%, 15.8%, 8%, 6%, 5.9% and 3%) while 5 cases had heterozygous deep intronic variants, all of them previously described in RB patients. Additional cases with suspected variants, not detected in blood but present in tumor tissue, were also analyzed using NGS PCR amplicons, and mosaicism was confirmed in other 10 sporadic cases. Altogether, the use of NGS increased the diagnostic yield, particularly for patients with sporadic RB in 10 bilateral cases and in 12 unilateral cases.

1. Introduction

Retinoblastoma (RB; OMIM #180200) is an embryonic tumor of the retina that occurs in children before the age of five and presents in two forms of presentation, unilateral with involvement of one eye, or bilateral with both eyes affected. The onset of the disease is due to the biallelic inactivation of the *RB1* gene (OMIM #614041), the first tumor suppressor gene identified, which is located on chromosome 13q14.2 and consists of 27 exons distributed across 183 kb of genomic sequence. Retinoblastoma is the cause of 5% of childhood blindness and contributes to childhood cancer deaths through second tumors in germline carriers (Kanski, 2010; Valverde et al., 2005). It has an incidence of 1 in 18,000 live births and can occur as either a hereditary (40%) or sporadic (60%) disease (Alonso et al., 2006; Donaldson and Smith, 1989; Imperatore et al., 2018). Patients with familial, bilateral or multifocal forms are indicative of hereditary disease with risk of transmission to offspring, while patients with unilateral tumors are more likely to have the non-hereditary form of RB (Vogel, 1979a). Furthermore, patients with hereditary RB are at risk of developing second tumors in adulthood, mainly osteosarcomas, which are more aggressive and have a worse prognosis than retinoblastoma. Therefore, the detection of germline variants in the *RB1* gene is crucial for these patients (Fujiwara et al.,

* Corresponding author. Genetic Diagnosis Unit, Institute for Rare Diseases Research (IIER), Institute of Health Carlos III (ISCIII), Ctra. Majadahonda-Pozuelo Km 2, Madrid, 28220, Spain.
E-mail address: ggomezm@isciii.es (G. Gomez-Mariano).

https://doi.org/10.1016/j.exer.2025.110233
Received 16 October 2024; Received in revised form 31 December 2024; Accepted 5 January 2025
Available online 6 January 2025

2015; Marees et al., 2008).

Patients with RB can acquire variants in the *RB1* gene during embryogenesis, leading to mosaicism. The existence of mosaicism in patients with RB was first described in 1979 by some authors (Carlson and Desnick, 1979; Vogel, 1979b), and later in 1998 Sippel et al. published that mosaicism occurs in 10% of families with RB, either in the proband or one of the proband's parents (generally involving the paternal germ cell) (Biesecker and Spinner, 2013; Sippel et al., 1998). The detection of somatic mosaicism in families with RB has been facilitated by the advent of massive sequencing techniques (NGS). Its application has led to an increase in the detection of mosaicism cases in RB patients (Amitrano et al., 2015; Rodríguez-Martín et al., 2020). Previously used techniques, such as Sanger sequencing, had low sensitivity and often failed to detect mosaicism, especially at low levels. NGS techniques have a high sensitivity and specificity, and offer an excellent opportunity to detect mosaicism (Chen et al., 2014; Grotta et al., 2015).

The incorporation of NGS into the study of RB is improving the identification of variants, especially in sporadic cases where the detection success has traditionally been lower. In addition to mosaicism, this technique has enabled the detection of variants in regulatory or intronic regions in cases where no variant had been previously identified (Li et al., 2016; Zou et al., 2021).

In the present study we have performed an analysis of the distribution and frequency of constitutional variants in a cohort of 579 Spanish RB patients RB (353 unilateral cases and 226 bilateral cases) initially studied using Sanger sequencing and MLPA techniques. Furthermore, we have applied an NGS gene panel including the entire sequence of *RB1* gene to detect mosaicism and variants in regulatory and intronic regions, thereby increasing diagnostic rates.

2. Materials and methods

2.1. Patients and clinical diagnosis

A cohort of 579 Spanish patients with RB was sent for genetic study from different hospitals in Spain, mainly Hospital La Paz (Madrid), Hospital Universitario Virgen Macarena (Sevilla) and Hospital Vall d'Hebron (Barcelona). The patients included 353 unilateral (339 sporadic and 14 familial) and 226 bilateral (194 sporadic and 32 familial) (Fig. 1). Informed consent was obtained from the patients or their legal guardians. For the genetic diagnosis of the patients, DNA was extracted from blood samples using the QIAamp DNA Blood mini kit (QIAGEN) and the DNA concentration was determined using the NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.2. Sanger sequencing and MLPA copy number analysis

The 27 exons and adjacent intronic regions of *RB1* gene were

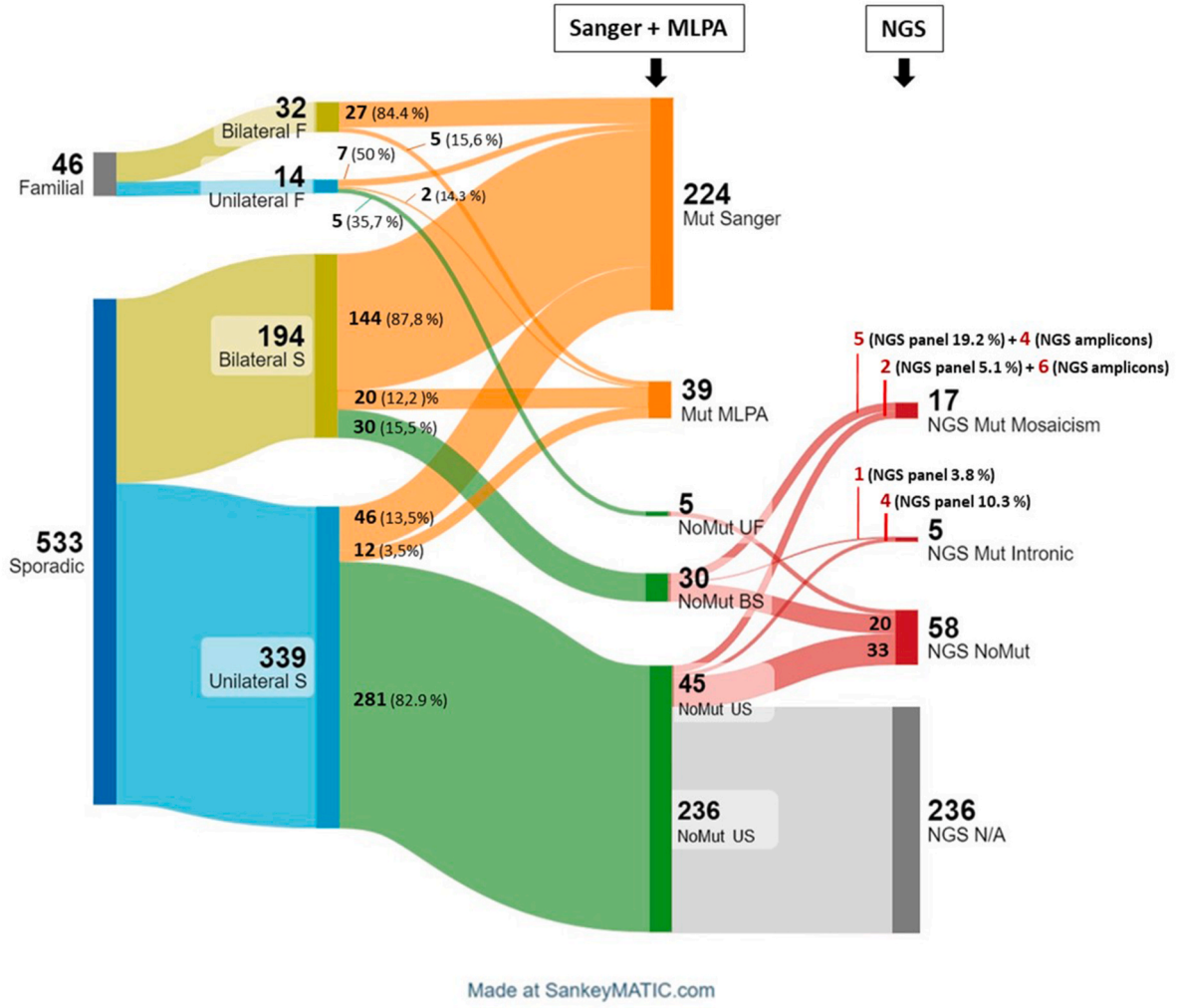

Fig. 1. Diagram showing the cohort of 579 patients, the techniques applied for their study and some of the results based on the phenotype and the type of variant identified. Unilateral Familial (UF); Bilateral Sporadic (BS); Unilateral Sporadic (US); Mutation (Mut); Not analyzed (N/A); Multiplex Ligation-dependent Probe Amplification (MLPA).

analyzed by Sanger sequencing to identify single nucleotide variants (SNV), and by multiplex ligation-dependent probe amplification (MLPA) to analyze large deletions and duplications in the gene. PCR amplification of exons from 1 to 27 and their adjacent intronic regions using specific oligonucleotides (Table 1) and sequenced on an automatic sequencer (ABI PRISM 377 Applied Bio-Systems) by Sanger sequencing. SALSA MLPA KIT P047 *RB1* (MRC-Holland, Amsterdam, The Netherlands) was used to identify deletions or duplications. The MLPA assay was performed according to the manufacturer's protocol and the data were analyzed using the Coffalyser.Net™ software developed and supported by MRC-Holland.

2.3. Next generation sequencing (NGS-RB1 panel and amplicons)

Among the 579 cases included in this study, 80 cases were analyzed by NGS techniques (70 with NGS-*RB1* panel and 10 with deep sequencing PCR amplicons). Two customized NGS-*RB1* panels (Roche-NimbleGen and IDT) were used for the study of 70 patients with RB who did not have any variant detected by conventional techniques. The Roche-NimbleGen panel was used for the analysis of 38 patients (26 unilateral and 12 bilateral) and the IDT panel for the study of 32 patients (16 unilateral and 16 bilateral). SeqCap EZ HyperCap (KAPA Hyper Prep Kit) was used to generate genomic DNA (gDNA) libraries for the Roche panel and xGen hybridization capture of DNA libraries (xGen DNA Library Prep EZ Kit") for the IDT panel. Libraries were sequenced directly with the MiSeq Illumina sequencing instrument. A high-performance visualization tool, Integrative Genomics Viewer (IGV), was used to review the results.

Additionally, we performed deep sequencing of PCR amplicons of specific regions, in 10 patients with RB (6 unilateral and 4 bilateral) suspected of having mosaicism after performing the Sanger sequencing study on DNA extracted from blood or tumor samples. PCR amplicons were subsequently sequenced using an Illumina MiSeq sequencer.

Table 1
Oligonucleotides used for the amplification and sequencing of the 27 exons of the *RB1* gene and adjacent intronic regions are shown. All oligonucleotides are designed based on the specific sequence of the *RB1* gene *(Homo sapiens (human) genome assembly GRCh38 (hg38) from Genome Reference Consortium)*.

Exon *RB1* Gene	Sequences Oligonucleotides 5'→ 3'	
	Forward	Reverse
1	TTGTAACGGGAGTCGGGA	CAACCCAGAATCCTGTCA
2	TTCACAGTAGTGTTATGTGC	CAATTTCCTCTGGGTAATGG
3	CACTTTTAACATAGTATCCA	CACAAAAGTCTATTGAGAG
4	AGTAGTGATTTGATGTAGAGC	CTAACATTAAAAGGGAC
5	GAGAAAACTACTATGACTTCTAA	CAAGATGTTTGAGATTATTCC
6	TGGAAAACTTTCTTTCAG	GAATTTAGTCCAAAGGAA
7	ATACTCTACCCTGCGATT	CTTAGAACCATGTTTGGTA
8	GTTATCCTTCTAATGAAA	CATGCTCATAACAAAAG
9	TTGACACCTCTAACTTACC	TAGACAATTATCCTCCCTC
10	GTGCTGAGAGATGTAATGA	ACCTATATCAGTATCAACCT
11	TGGGTCAATCTATTTTCTA	CTGAAACACTATAAAGCCA
12	GAGGCAGTGTATTTGAAGAT	TTCAAGTTTCTTTGCCAAGATA
13	TTCAGTAGTTGTGGTTACCTA	ATACGAACTGGAAAGATGC
14	TTCTAAAATAGCAGGCTCT	CTTGATGCCTTGACCTC
15	CAATGCTGACACAAATAA	TACTTACTTCTATAAAAAGAA
16	CTTTTTATAGAAGTAAGTA	CTTCTCCTTAACCTCACAC
17	CTATTTCCTATGAGTCCG	TGTTAAGAAACACCTCTC
18	TGACTTTTAAATTGCCACTGT	GAATGTTACATTGCACTTATG
19	GTACAACCTTGAAGTGTA	CATGATTTGAACCCAGT
20	GGAAAGAAAAGAGTGGTAG	CAGTTAACAAGTAAGTAGG
21	TGAGCTCAGTATGGAAA	CTATGTTATGTTATGGATA
22	TATGTGCTTCTTACCAGTCA	CGAGGAATGTGAGGTATT
23	TCTAATGTAATGGGTCCAC	CCCTCTCATTCTTTACTACT
24	GCTCATCTCTGCAAAATTGT	GAGGTGTTTGAATAACTGCA
25	CCTTTGCCTGATTTTTGACA	TGAGCCATTCTCACAACTTC
26	AGTAAGTCATCGAAAGCATC	CGAAAAGACTTCTTGCAG
27	TCAGTTTGACATGAGCAT	TCTGTGAGAGACAATGA

2.4. Bioinformatics analysis

Quality control of fastq files was performed with fastQC v0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and any adapter sequences were removed using Trimmomatic v0.33 (Bolger et al., 2014), as well as low quality 3′ ends, reads shorter than 50 nucleotides and regions with quality lower than 20Q Phred33. The high-quality reads were then mapped against UCSC's hg38 human genome (https://hgdownload.soe.ucsc.edu/goldenPath/hg38/bigZips/analysisSet/) and UCSC's hg19 human genome (https://hgdownload.soe.ucsc.edu/goldenPath/hg19/bigZips/analysisSet/) using the BWA-MEM aligner v0.7.12 (Li and Durbin, 2009) and Samtools v1.2 (Li et al., 2009) with default parameters. Mapping quality control was performed using Picard's v1.140 (http://broadinstitute.github.io/picard/) CalculateHsMetrics module. Aligned reads were processed with Samtools mpileup v1.2 and variants with an allele frequency higher than 5% were extracted using VarScan's v2.3.9 (Koboldt et al., 2012) mpileup2cns module. Variants were annotated using KGGSeq v1.2 (Li et al., 2013). Finally, MultiQC v1.9 (Ewels et al., 2016) was used to generate summary statistics reports.

2.5. In silico analysis of intronic variants

Pathogenicity and the possible effect on splicing of intronic variants were evaluated *in silico* with SpliceAI to predict potential splicing impacts (SpliceAI Lookup (broadinstitute.org)) (Jaganathan et al., 2019).

2.6. Statistical analysis

The Chi-square test was used to estimate differences in the distribution of variant types among unilateral and bilateral cases. Statistical calculations were performed using GraphPad Prism v9.0 software (GraphPad Software, San Diego, CA, USA) and the level of significance was set at $p < 0.05$.

3. Results

3.1. Mutational spectrum of RB1 gene

Constitutional variants in the *RB1* gene were initially studied using conventional Sanger sequencing and MLPA to detect the variants responsible for the disease in a cohort of 579 patients with RB. Applying both techniques, a pathogenic variant leading to a diagnosis was found in 263 patients (45.4%). Conversely, 316 patients (54.6%) did not show any variant using these conventional techniques, most of them being sporadic RB patients (Fig. 1).

Among cases with variants, 224 patients had SNVs (85.2%) and 39 patients (14.8%) had large deletions of the *RB1* gene, 25 of which were complete gene deletions (64%) and 14 were partial deletions affecting one or more exons (36%). The distribution of these SNV and gene deletions along the gene is represented in Fig. 2. Some recurrent nonsense variants were found p.Arg320* in exon 10 was found in eleven unrelated cases; p.Arg445*, in exon 14, was identified in twelve cases; p.Arg255*, in exon 8 was identified in seven cases and p.Arg358* in exon 11 was identified in six cases. Table 2 describes 78 SNVs that have not been described previously in other studies by our group (Alonso et al., 2001, 2005; Rodríguez-Martín et al., 2016, 2020). This table includes 8 variants classified as variants of unknown significance (VUS) since there is not enough information to confirm their pathogenicity, although they had a low allelic frequency in GnomAD (MAF:<0.0001). Most of these VUS were non-coding or intronic variants (7 cases) and only one was a missense variant.

The distribution of variants based on tumor phenotype (bilateral or unilateral) revealed that bilateral RB exhibited a higher percentage of SNV than unilateral ones (Fig. 3A). In contrast, unilateral RB, either familial or sporadic, presented a higher percentage of large deletions of

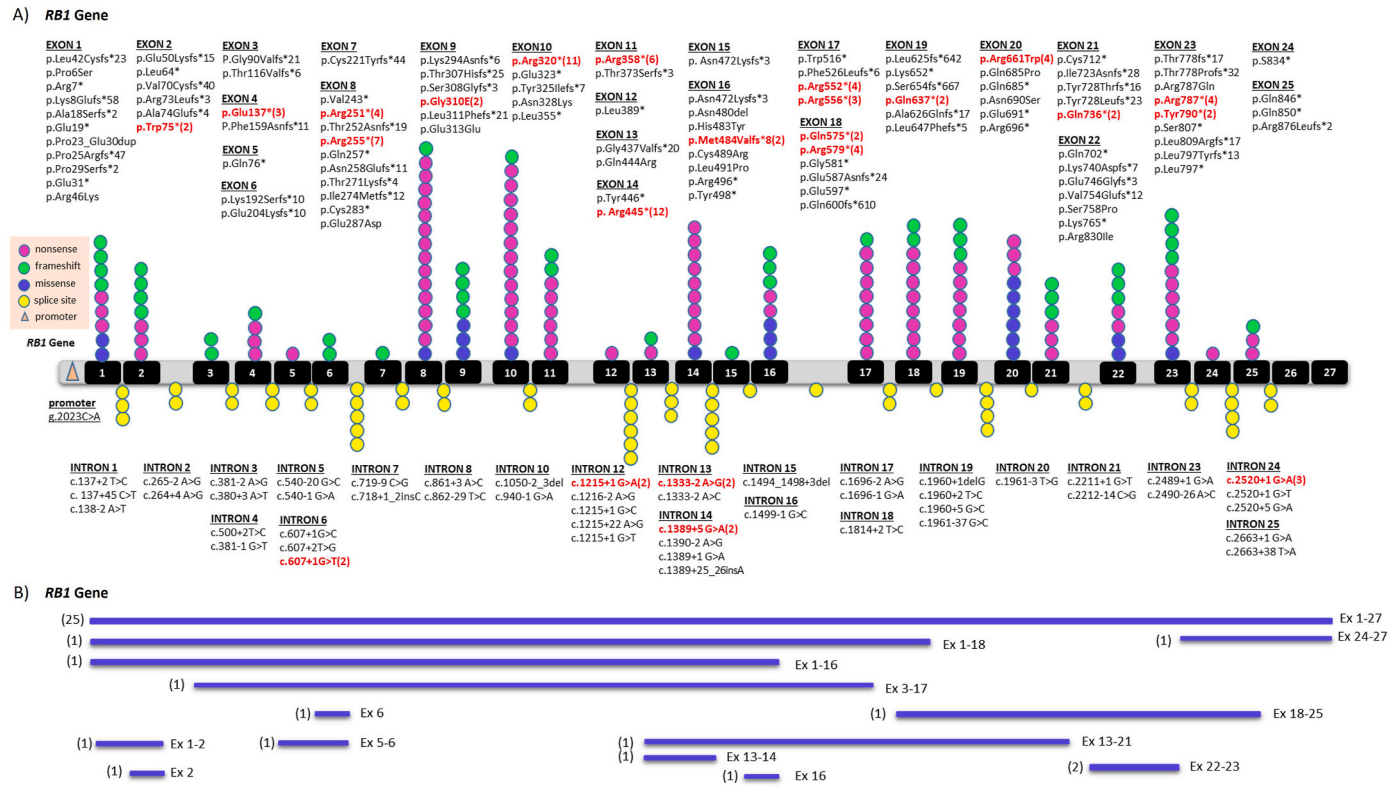

Fig. 2. Mutational spectrum along the *RB1* gene (27 exons and 26 introns) in 263 Spanish patients. A) In the central part of the figure, the 27 exons of the *RB1* gene are shown in black boxes and the promoter with an orange triangle. The upper part shows the nonsense (pink circle), frameshift (green circle) and missense (blue circle) variants, the lower part those of splice (yellow circle) and promoter (triangle). SNV in each of the exons and introns (*RB1* protein) are shown. Recurrent variants are marked in red, indicating the number of patients in parentheses. B) Large deletions in the *RB1* gene. The number of patients with the deletion is indicated in parentheses on the left and the exons included in the deletion on the right. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the gene compared to bilateral patients.

Regarding the type of variant, the most frequent were loss of function variants, distributed as follows: 43% nonsense, 26% splice-site, 23% frameshift, and only 9% were missense variants. Among bilateral cases nonsense variants were more prevalent (46%), while for the unilateral patients, splice-site variants were predominant (36 %) (Fig. 3B).

The comparison between the distribution along the gene of SNVs detected in our study (224 cases) and those registered in Lohmann *RB1* database (3290 cases) showed a similar profile with a good correlation ($r = 0.7348$, $p < 0.0001$) (Fig. 3C). Exons with a high concentration of variants in the Lohmann *RB1* database also showed a high frequency of variants in our study. However, a slightly higher mutational frequency was observed in exons 1, 2, 8, 9, 10, 11, 16 and 22, and a lower frequency was found in exons 17 and 18 in our series than in Lohmann *RB1* database.

3.2. *Identification of variants by NGS-RB1 panel in patients with sporadic RB*

A group of 65 patients with sporadic RB, comprising 39 unilateral cases and 26 bilateral cases, who had no variant detected by conventional methods and had available blood DNA was further analyzed using the NGS-*RB1* gene panel to search for mosaic-level variants, and variants in intronic or regulatory regions.

As a result, in seven sporadic cases (two unilateral and five bilateral) we found variants with different degrees of mosaicism (Table 3). The variants c.2107-1G > T (Fig. 4) and c.1666C > T were detected with 6% and 8%, respectively, in unilateral patients. In bilateral patients, the variant c.763C > T; p.Arg255* was detected at 3%, the variant c.1389+2T > C was detected at 15.8%, the variant c.2092-2093del; p. Arg698Alafs*22 was detected at 5.9%, and the variant c.1363C > T; p. Arg455* was detected at 20% (Fig. 4), and the variant c.1333C > T; p. Arg445* was detected at 26%. As shown in Table 3, five of these variants were described in the Lohmann-LOVD-rb1-lsdb-RB (*RB1*) database several times, in patients with RB.

On the other hand, 5 rare deep intronic variants were detected in 4 unilateral and 1 bilateral sporadic patient (Fig. 1). All of them are reported in the Lohmann-LOVD-rb1-lsdb-RB (*RB1*) in RB patients. Moreover, one of these variants in intron 23 of the *RB1* gene has been described as pathogenic in ClinVar database (ID number: 13096; *RB1*: NM_000321.3: c.2490-1398A > G) and was identified in a sporadic bilateral patient. Variants located in intron 6 (*RB1*: NM_000321.3: c.608-91T > C) and intron 18 (*RB1*: NM_000321.3: c.1815-104A > G) of the *RB1* gene were found in genomic databases (GnomAD) with a MAF frequency ≤0.005. The other 3 variants located in intron 18 (*RB1*: NM_000321.3: c.1814 + 72T > G), intron 23 (*RB1*: NM_000321.3: c.2490-1398A > G) and intron 24 (*RB1*: NM_000321.3: c.2520 + 1393T > C) have no population frequency data (Table 4). *In silico* analysis of the effect of intronic variants using SpliceAI showed no clear effect. All intronic variants were present at nearly 50% of heterozygosity.

3.3. *Deep sequencing of PCR amplicons confirmed mosaicism in suspected cases with sporadic RB*

Deep sequencing of PCR amplicons was performed on 10 sporadic cases: 6 unilateral cases and 4 bilateral cases, with signs of possible mosaicism. Analysis using deep sequencing of the blood DNA of the variant identified in the tumor detected different degrees of mosaicism (Table 5).

In a sporadic bilateral patient, a pathogenic nonsense variant was

Table 2

The following table shows 78 variants of the 263 detected by conventional techniques in the cohort of 579 Spanish patients. Sporadic Bilateral (BS), Sporadic Unilateral (US), Familial Bilateral (BF), Familial Unilateral (UF). Not applicable (#), Not described (−). GnomAD Frequency (*f*). Classification in ClinVar Variation: Conflicting classifications of pathogenicity (C), Pathogenic (P), Likely Pathogenic (LP), Likely Benign (LB), and Variant of Uncertain Significance (VUS). SpliceAI scores: delta scores range from 0 to 1 and can be interpreted as the probability that the variant affects splicing at any position within a window around it (± 500bp by default), Acceptor (A), Donor (D), Loss (L), Gain (G) and Splice (S). In the SpliceAI paper (Jaganathan et al., 2019), a detailed characterization is provided for 0.2 (high recall), 0.5 (recommended), and 0.8 (high precision) cutoffs. *(Homo sapiens (human) genome assembly GRCh38 (hg38) from Genome Reference Consortium)*.

ID	Phenotype	Variant description Genome position (hg38)	Variant description (NM_000321.3)	Exon/ Intron	Protein Effect	Variant Type	Lohmann-LOVD-rb1 Nº cases	ClinVar Variation	Author's classification	Splice AI Scores	GnomAD (*f*)
1	BS	13:48303928 C > T	c.16 C > T	Ex 1	p.Pro6Ser	missense	–	C	VUS	#	0.00004604
2	BS	13:48303932–48303933 dup	c.20-21 dup	Ex 1	p.Lys8Glufs*58	frameshift	–	P	P	#	–
3	US	13:48303931 C > T	c.19 C > T	Ex 1	p.Arg7*	nonsense	1	P	P	#	0.000000736
4	BS	13:48303957–48303982 del	c.45-70 del	Ex 1	p.Glu19Profs*3	frameshift	1	–	P	#	–
5	BS	13:48303958–48303977 dup	c.46-65 dup	Ex 1	p.Pro25Argfs*47	frameshift	–	–	P	#	–
6	US	13:48303964–48303995 del	c.52-83 del	Ex 1	p.Ala18Serfs*2	frameshift	–	–	P	#	–
7	US	13:48303967 G > T	c.55G > T	Ex 1	p.Glu19*	nonsense	–	P	P	#	–
8	UF	13:48303978–48304001 dup	c.66-89 dup	Ex 1	p. Pro23_Glu30dup	frameshift	–	–	P	#	–
9	BS	13:48303995 dup	c.83 dup	Ex 1	p.Pro29Serfs*2	frameshift	1	–	P	#	–
10	BS	13:48304003 G > T	c.91 G > T	Ex 1	p.Glu31*	nonsense	1	–	P	#	–
11	BS	13:48304036 del	c.124 del	Ex 1	p.Leu42Cysfs*23	frameshift	1	–	P	#	–
12	BS	13:48304049 G > A	c.137 G > A	Ex 1	p.Arg46Lys	missense	7	P	P	#	–
13	BS	13:48307359–48307362del	c.217-220 del	Ex 2	p.Arg73Leufs*3	frameshift	1	P	P	#	–
14	BS	13:48307367 G > A	c.225 G > A	Ex 2	p.Trp75*	nonsense	2	–	P	#	–
15	BS	13:48342617 A > T	c.283 A > T	Ex 3	p.Lys95*	nonsense	1	P	P	#	–
16	BS	13:48362868–48362872del	c.769-773 del	Ex 8	p. Asn258Glufs*11	frameshift	–	P	P	#	–
17	BS	13:48362957 G > C	c.861 G > C	Ex 8	p.Glu287Asp	missense	3	P	P	#	–
18	BF	13:48364914–48364917del	c.882-885 del	Ex 9	p.Lys294Asnfs*6	frameshift	–	–	P	#	–
19	BS	13:48364951 del	c.919 del	Ex 9	p. Thr307Hisfs*25	frameshift	–	–	P	#	–
20	BS	13:48367537 dup	c.983 dup	Ex 10	p.Asn328Lysfs*2	nonsense	1	P	P	#	–
21	BS	13:48367558 T > A	c.1004 T > A	Ex 10	p.Leu355*	nonsense	1	–	P	#	–
22	BF	13:48373443 T > G	c.1166 T > G	Ex 12	p.Leu389*	nonsense	–	–	P	#	–
23	UF	13:48377033 A > G	c.1331 A > G	Ex 13	p.Gln444Arg	missense	3	–	P	#	–
24	BF	13:48379594 C > T	c.1333 C > T	Ex 14	p.Arg445*	nonsense	79	P	P	#	–
25	BS	13:48379599 C > A	c.1338 C > A	Ex 14	p.Tyr446*	nonsense	1	P	P	#	–
26	BS	13:48380078 dup	c.1415 dup	Ex 15	p.Asn472Lysfs*3	frameshift	–	–	P	#	–
27	BS	13:48380215 T > C	c.1472 T > C	Ex 16	p.Leu491Pro	missense	–	–	LP	#	–
28	US	13:48380237 T > G	c.1494 T > G	Ex 16	p.Tyr498*	nonsense	4	P	P	#	–
29	BS	13:48380239–48381251 del	c.1496-1503 del	Ex 16	p.Ser499Asnfs*6	frameshift	–	–	P	#	–
30	US	13:48381419 C > T	c.1671 C > T	Ex 17	p.Arg552*	nonsense	–	–	P	#	–
31	BS	13:48453055 del	c.1758 del	Ex 18	p. Glu587Asnfs*24	frameshift	–	–	P	#	–
32	BS	13:48459781–48459782 del	c.2054–2055 del	Ex 20	p.Gln685Profs*6	frameshift	–	–	P	#	–
33	BS	13:48459797 dup	c.2070 dup	Ex 20	p.Glu691*	nonsense	–	–	P	#	–
34	BS	13:48459823 A > T	c.2096 A > T	Ex 20	p.Arg696*	nonsense	–	–	P	#	–
35	BS	13:48463791 dup	c.2167 dup	Ex 21	p.Ile723Asnfs*28	frameshift	1	–	P	#	–
36	BS	13:48463805del	c.2181 del	Ex 21	p. Tyr728Thrfs*16	frameshift	–	–	P	#	–
37	BF	13:48463806–48463807 dup	c.2182-2183 dup	Ex 21	p. Tyr728Leufs*23	frameshift	–	–	P	#	–
38	US	13:48465003–48465013 del	c.2215-2225 del	Ex 22	p.Lys740Aspfs*7	frameshift	–	–	P	#	–
39	BS	13:48465045–48465046 dup	c.2259-2260 dup	Ex 22	p. Val754Glufs*12	frameshift	–	–	P	#	–
40	BS	13:48465209 dup	c.2330 dup	Ex 23	p.Thr778fs*17	frameshift	1	–	P	#	–
41	BS	13:48465210 del	c.2331 del	Ex 23	p. Thr778Profs*32	frameshift	1	–	P	#	–

(*continued on next page*)

Table 2 (*continued*)

ID	Phenotype	Variant description Genome position (hg38)	Variant description (NM_000321.3)	Exon/ Intron	Protein Effect	Variant Type	Lohmann-LOVD-rb1 Nº cases	ClinVar Variation	Author's classification	Splice AI Scores	GnomAD (*f*)
42	US	13:48465368 G > T	c.2489 G > T	Ex 23	p.Arg830Ile	missense	1	–	P	#	–
43	BS	13:48476716 C > T	c.2536 C > T	Ex 25	p.Gln846*	nonsense	2	P	P	#	0.00000658
44	BS	13:48304051 T > C	c.137 + 2 T > C	In 1	#	splice	1	–	P	DL 0.32	–
45	US	13:48304094 C > T	c.137 + 45 C > T	In 1	#	splice	–	–	VUS	DL 0.01	0.00006542
46	BS	13:48307410 A > G	c.264 + 4 A > G	In 2	#	splice	1	–	P	SL 0.12	–
47	BF	13:48342717 A > T	c.380 + 3 A > T	In 3	#	splice	2	P	P	AL 0.39	–
48	BS	13:48345079 G > T	c.381-1 G > T	In 4	#	splice	1	–	P	AL 1.00	–
49	BS	13:48348936 G > C	c.540-20 G > C	In 5	#	splice	–	LB	VUS	0	–
50	BS	13:48362958 G > A	c.861 + 1 G > A	In 8	#	splice	1	P	P	DL 1.00	–
51	BS	13:48364865 T > C	c.862-29 T > C	In 8	#	splice	–	–	VUS	0	0.0001690
52	BF	13:48367493 G > A	c.940-1 G > A	In 10	#	splice	1	P	P	AL 0.98	–
53	BS	13:48368525–48368529 del	c.1050-2_1052del	In 10	#	splice	–	–	P	AL 0.97	–
54	UF	13:48373493 G > T	c.1215 + 1 G > T	In 12	#	splice	1	P/LP	P	DL 0.98	–
55	US	13:48373514 A > G	c.1215 + 22 A > G	In 12	#	splice	–	–	VUS	0	0.00000657
56	BS	13:48376916 A > G	c.1216-2 A > G	In 12	#	splice	–	P	P	AL 1.00	–
57	US	13:48377035 G > T	c.1332 + 1 G > T	In 13	#	splice	1	P	P	AL 0.97	–
58	BF	13:48379592 A > C	c.1333-2 A > C	In 13	#	splice	3	–	P	AL 0.96	–
59	BS	13:48379592 A > G	c.1333-2 A > G	In 13	#	splice	–	P	P	AL 0.96	–
60	BS	13:48373493 G > A	c.1215 + 1 G > A	In 14	#	splice	64	P	P	DL 0.98	–
61	BS	13:48456354 G > C	c.1960 + 5 G > C	In 19	#	splice	1	P	P	DL 0.40	–
62	US	13:48459651 G > C	c.1961-37 G > C	In 19	#	splice	–	–	VUS	0	0.000008074
63	BS	13:48459685 C > G	c.1961-3 C > G	In 20	#	splice	1	–	P	AL 0.97	–
64	US	13:48476881 T > A	c.2663 + 38 T > A	In 25	#	splice	–	–	VUS	0	0.00006910
65	US	13:48303876 C > A	c.-37C > A	Prom.	#	promoter	–	#	VUS	#	0.0000617
66	BF	#	#	Ex13-Ex14	#	del	–	#	#	#	#
67	UF	#	#	Ex13-Ex21	#	del	–	#	#	#	#
68	US	#	#	Ex16	#	del	–	#	#	#	#
69	BS	#	#	Ex18-Ex25	#	del	–	#	#	#	#
70	US	#	#	Ex01-Ex16	#	del	–	#	#	#	#
71	BS	#	#	Ex01-Ex18	#	del	–	#	#	#	#
72	BS	#	#	Ex01-Ex02	#	del	–	#	#	#	#
73	BS	#	#	Ex02	#	del	–	#	#	#	#
74	BF	#	#	Ex22-Ex23	#	del	–	#	#	#	#
75	BF	#	#	Ex24-Ex27	#	del	–	#	#	#	#
76	BS	#	#	Ex03-Ex17	#	del	–	#	#	#	#
77	BS	#	#	Ex05-Ex06	#	del	–	#	#	#	#
78	BS	#	#	Ex06	#	del	–	#	#	#	#

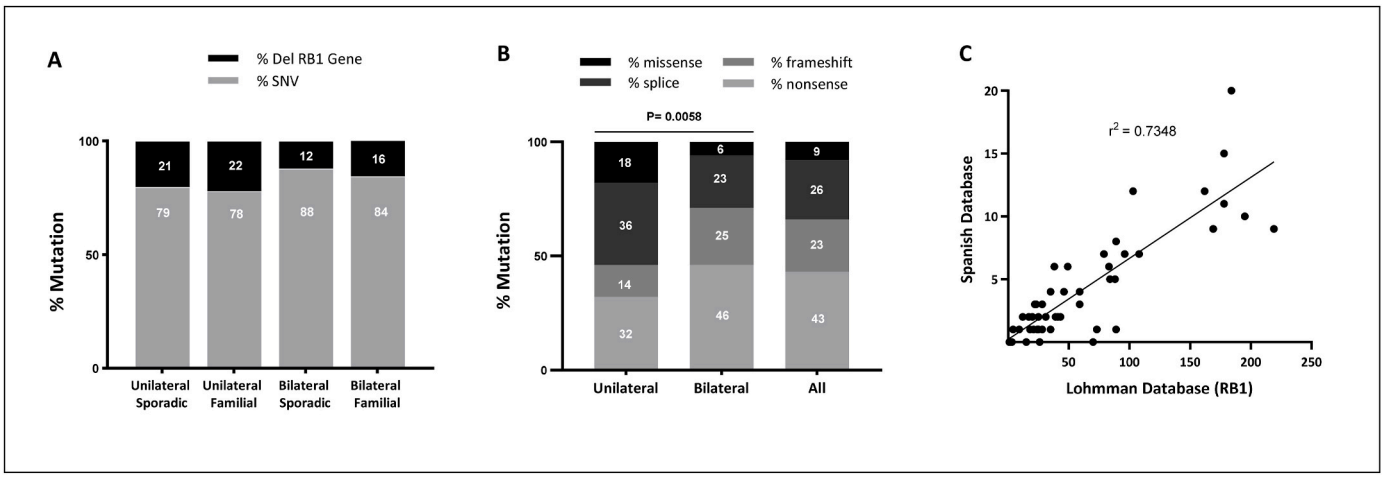

Fig. 3. Distribution of 263 variants according to phenotype in Spanish patients. A) Frequency of SNV and deletions of the *RB1* gene. B) Frequency of distribution by type of variant in all patients and by phenotype (unilateral and bilateral). Statistically significant differences were observed between sporadic bilateral and sporadic unilateral P = 0.0069 (Chi-square test). C) Comparison between the SNV distribution of patients in the Spanish database (224 cases) and those in the Lohmann database (*RB1*) (3290 cases) shows a similar profile with a good correlation ($r^2 = 0.7348$, $p < 0.0001$).

Table 3
The different degrees of mosaicism of 2 sporadic unilateral and 5 sporadic bilateral patients studied with a panel of NGS-*RB1* are shown. Ŧ: The variant c.2107G > A is described 3 times (pathogenic in ClinVar) and c.2107G > C (likely pathogenic in ClinVar) once in LOVD-Lohmann. US: Sporadic unilateral, BS: sporadic bilateral. (–) Not described. (*Homo sapiens (human) genome assembly GRCh38 (hg38) from Genome Reference Consortium)*.

Clinical Diagnosis (IDLab)	Variant NGS-*RB1* Panel *RB1*: NM_000321.3 Genome position (hg38)	Protein RB	Variant Exon/ Intron *RB1*Type	% Mosaicism NGS-*RB1* Panel	Reads NGS-*RB1* Panel REF/ALT	ClinVar Variation (ID)	Lohmann-LOVD-rb1 Nº of cases
BS (4723)	c.763C > T (13:48362859)	p.Arg255*	Ex 8 (nonsense)	3	11231/33	Pathogenic (126820)	46
BS (2993)	c.1363C > T (13:48379624)	p.Arg455*	Ex 14 (nonsense)	20	1052/211	Pathogenic (126837)	62
BS (3063)	c.1389+2T > C (13:48379652)	–	In 14 (splice)	15.8	1032/164	–	2
BS (3713)	c.2092_2093del (13:48459818)	p. Arg698Alafs*22	Ex 20 (frameshift)	5.9	3841/229	–	–
BS (0033)	c.1333C > T (13:48379594)	p.Arg445*	Ex 14 (nonsense)	26	380/100	Pathogenic (126837)	79
US (3723)	c.2107-1G > T (13:48463730)	–	In 20 (splice)	6	41164/2729	Likely pathogenic (428698)	Ŧ
US (5863)	c.1666C > T (13:48381414)	p.Arg556*	Ex 17 (nonsense)	8	43807/3585	Pathogenic (13090)	49

suspected by Sanger sequencing in exon 3 (NM_000321.3.3: c.277C > T; p.Gln93*) (Fig. 5A). The analysis of DNA isolated from tissues of the three embryonic layers of the patient, blood (mesoderm), saliva (mesoderm), urine (endoderm/mesoderm) and hair bulbs (ectoderm) showed mosaicism in the patient. The variant was found at different rates of mutant/reference alleles, being in 30% in blood (41.901/96.766 reads), 9% in saliva (8.524/28.676 reads), 23% in urine (9.815/136.862 reads) and 7% in hair bulbs (12.957/131.011 reads), confirming the mosaicism. This variant is described once in the Lohmann *RB1* database.

Two bilateral sporadic patients showed blood mosaicism of the variants detected in the tumor. The percentages of mosaicism were 8% in a case with a frameshift variant (p.Pro374Argfs*2), and 35% in a case with a nonsense variant (p.Arg320*), both described in other patients with RB (Lohmann *RB1* database).

Another patient with sporadic bilateral RB showed in the Sanger sequencing a possible deletion in low proportion in exon 16 (NM_000321.3.3: c.1450-1451del; Met484Valfs*8) (Fig. 5B). Deep sequencing revealed a proportion of 12% of the mutant allele in peripheral blood DNA (23.682/198.016 reads). This variant is also described once in the Lohmann *RB1* database.

In another patient with sporadic unilateral RB, the two variants responsible for the RB were identified in the patient's tumor sample. One variant was a 5 nucleotides deletion in exon 22 (NM_000321.3.3: c.2235-2239del; p. Glu746Glyfs*3) (Fig. 5C), and the second variant was a deletion in the 5′ proximal region of the *RB1* gene detected by MLPA. None of these alterations were detected in the peripheral blood DNA by Sanger sequencing. Exon 22 was analyzed in blood DNA by deep sequencing, obtaining a 1% mosaicism in blood (22/2.000 reads). In addition, another unilateral sporadic patient was detected with a 4% mosaicism, presenting a frameshift variant (p.Met825Lysfs*10) in exon 23 of the *RB1* gene has not been previously described. The remaining sporadic unilateral patients studied with deep sequencing amplicons showed different degrees of mosaicism in blood (12–33%) and variants were also previously described in the Lohmann *RB1* database and in ClinVar as pathogenic.

4. Discussion

Thanks to the increased sensitivity of new sequencing technologies, mosaicism can now be easily detected, even when it occurs in very low levels (Grotta et al., 2015; Gudiseva et al., 2019). The identification of mosaicism in patients with RB is of highly significant, as it can improve genetic counseling for families (Amitrano et al., 2015). Moreover, the use of an RB1 panel that includes the complete sequence of the gene

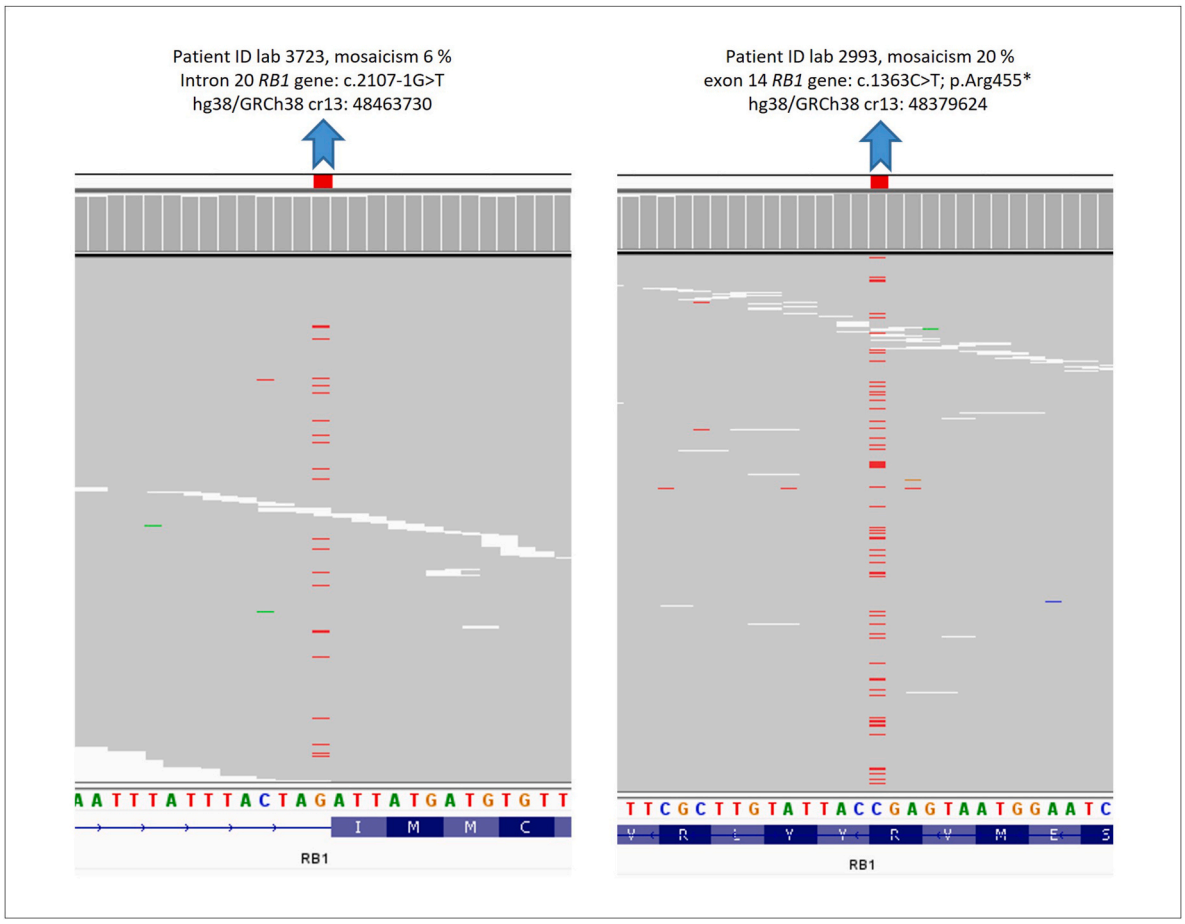

Fig. 4. Zoom images of variant mapped reads from two sporadic patients with mosaicism are shown. A) Patient Lab ID 3723 (unilateral), hg38/GRCh38 cr13:48463730 (intron 20 *RB1* gene): c.2107-1G > T, 6% mosaicism. B) Patient Lab ID 2993 (bilateral), hg38/GRCh38 cr13:48379624 (exon 14 *RB1* gene): c.1363C > T; p. Arg455*, 20% mosaicism.

Table 4
Deep intronic variants detected by the NGS-*RB1* panel in sporadic RB patients. US Sporadic unilateral, BS: sporadic bilateral. (–) Not described. *(Homo sapiens (human) genome assembly GRCh38 (hg38) from Genome Reference Consortium).*

Clinical Diagnosis (IDLab)	Variant NGS-*RB1* Panel *RB1*: NM_000321.3 (Genome Position hg38)	Intron RB	% Heterozygosity	Reads NGS-*RB1* Panel REF/ALT	Allelic Frequency GnomAD (MAF)	Spanish Variant Server Frec. (MAF)	1000G ALL/ EUR (MAF)	Lohmann-LOVD-rb1 Nº of cases
US (4673)	c.608-91T > C (13:48359926)	In6	46,6	538/470	0,001	0,005	0,001/ 0,002	1
US (5523)	c.1815-104A > G (13:48456100)	In18	51,3	650/684	0,0031	0,005	0,054/ 0,008	1
US (3623)	c.1814 + 72T > G (13:48453183)	In18	46,6	789/688	0,00016	–	–	1
US (5413)	c.2520 + 1393T > C (13:48474783)	In24	48,8	717/682	–	–	–	1
BS (3533)	c.2490-1398A > G (13:48471962)	In23	49,2	1380/1335	–	–	–	2

(including exons, regulatory regions and introns) has allowed the identification of variants that remained undetected in some patients. The possibility of finding new causal variants in deep intronic regions is challenging, but it would significantly influence the diagnostic rates of RB patients. However, establishing the functional relevance of rare intronic and regulatory variants is challenging and must be addressed by specific experimental validation.

Analysis of our cohort of 579 Spanish RB patients using conventional Sanger sequencing and MLPA techniques allowed us to identify the causative variant in 45.4% of patients. This success rate is consistent with those obtained in other studies of RB patients RB (Mendonça et al., 2022; Valverde et al., 2005). In our study, using Sanger/MLPA techniques, pathogenic variants were identified in 196/226 (86.7%) of bilateral cases and in 68/353 (19%) of unilateral cases. These results are similar to other studies, with variants detected in 80–85% of bilateral patients and 10–15% in unilateral patients (Kugalingam et al., 2023). Analysis of the distribution by type of variant also revealed agreement with other studies (Dommering et al., 2014; Harbour, 1998), where nonsense variants were the most frequent variants (43%). Although variants are distributed throughout the *RB1* gene, this study and previous research by our group identified recurrent variants in exons 1, 8, 10, 11, 14, 17, 18, 19, 20 and 23 that could be considered hotspots in our

Table 5
The different degrees of mosaicism in blood of 6 sporadic unilateral and 4 sporadic bilateral patients studied with deep sequencing are shown. US: sporadic unilateral, BS: sporadic bilateral. (–) Not described. *(Homo sapiens (human) genome assembly GRCh38 (hg38) from Genome Reference Consortium).*

Clinical Diagnosis (IDLab)	Variant NGS-*RB1* Panel *RB1*: NM_000321.3 Genome position (hg38)	Protein RB	Variant Exon/ Intron *RB1* (Type)	% Mosicism NGS-*RB1*	Reads NGS-*RB1* REF/ALT	ClinVar (Variation ID)	LOVD Lohmann Nºcases
BS (413)	c.1450_1451del (13:48380172)	p.Met484Valfs*8	Ex 16 (frameshift)	12	198016/23682	Pathogenic (855427)	7
US (733)	c.2235_39del (13:48465000)	p.Glu746Glyfs*3	Ex 22 (frameshift)	1	2000/22	–	–
US (1163)	c.1215+1G > A (13:48373472)	–	In 12 (splice)	33	1730/859	Pathogenic (126832)	64
BS (1593)	c.1121_1122del (13:48368577)	p.Pro374Argfs*2	Ex 11 (frameshit)	8	2332/200	–	3
BS (1603)	c.958C > T (13:48367491)	p.Arg320*	Ex 10 (nonsense)	35	1344/728	Pathogenic (126824)	113
US (2223)	c.2359C > T (13:48465217)	p.Arg787*	Ex 23 (nonsense)	16	914/173	Pathogenic (13073)	68
US (2593)	c.2474_2481del (13:48465332)	p. Met825Lysfs*10	Ex 23 (frameshift)	4	2236/93	–	–
US (3543)	c.1981C > T (13:48459687)	p.Arg661Trp	Ex 20 (missense)	17	1887/374	Pathogenic (13087)	35
US (3843)	c.1363C > T (13:48379603)	p.Arg455*	Ex 14 (nonsense)	12	502/69	Pathogenic (126837)	62
BS (4803)	c.277C > T (13:48342590)	p.Gln93*	Ex 3 (nonsense)	30	96766/41901	Pathogenic (428746)	1

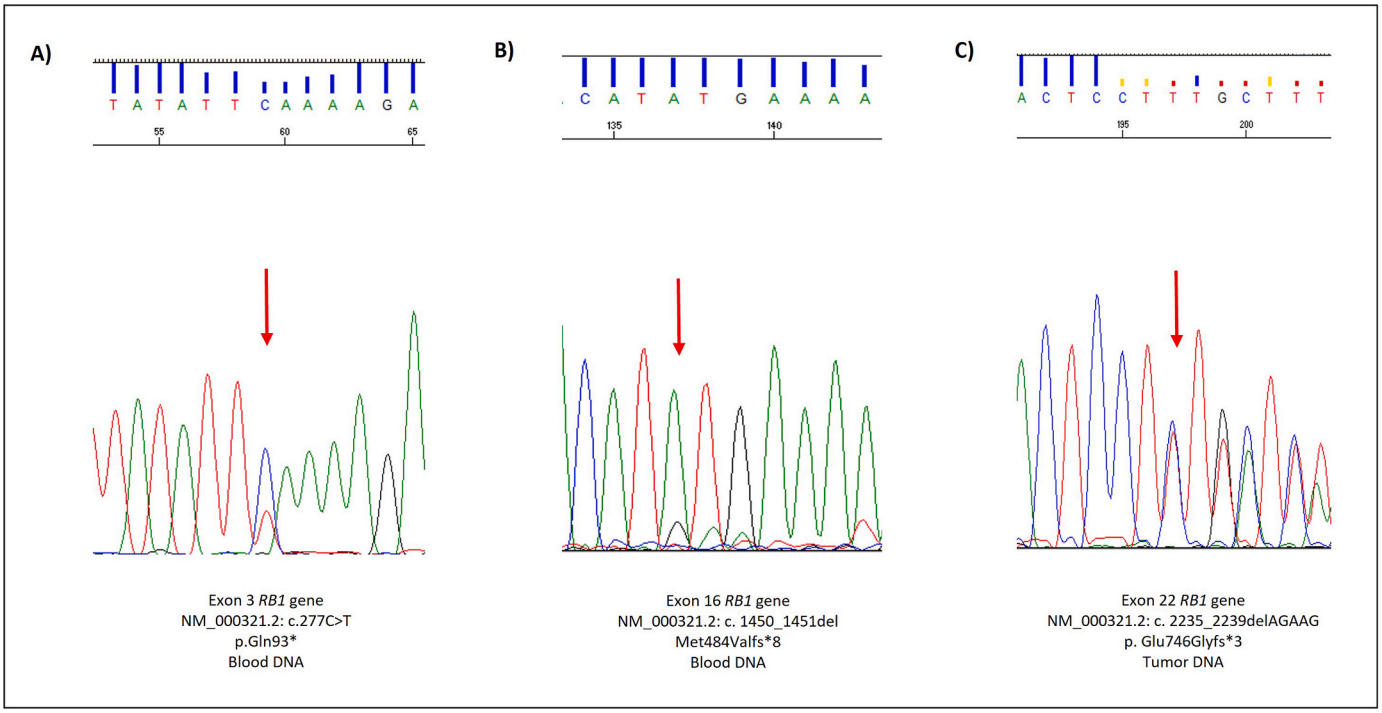

Fig. 5. Electropherograms of Sanger sequencing: A) Bilateral sporadic patient (blood DNA): exon 3 of *RB1* gene, NM_000321.3: c.277C > T, p.Gln93*. B) Bilateral sporadic patient (blood DNA): exon 16 of *RB1* gene, NM_000321.3: c.1450_1451del, Met484Valfs*8. C) Unilateral sporadic patient (tumor DNA): exon 22 of *RB1* gene, NM_000321.3: c.2235_2239delAGAAG; p. Glu746Glyfs*3.

population. Moreover, a concentration of recurrent splice variants was identified in introns 6, 12, 13, 14, and 24, which constituted 19% of all identified intron variants. Our results are consistent with those reported by other authors (Valverde et al., 2005). It is important to be aware of these population specific hot spots that may facilitate interpretation and counseling.

According to the phenotype, unilateral RB patients have a higher frequency of complete or partial deletions of the *RB1* gene compared to bilateral patients, who contain a greater number of SNV (Alonso et al., 2006). The combination of the different genetic techniques was important to improve diagnostic success. The causal variant was detected in 100% of bilateral familial cases and in 84.5% of bilateral sporadic cases, while in unilateral RB, the percentage was lower, especially in sporadic cases, with 17.1%, since they are mostly cases in which the variant occurred somatically in the retina.

However, in bilateral cases in which a variant has not been detected, mosaicism or non-coding variants might be suspected. It has also been described that causal variants occur because of biallelic inactivation of *RB1* by epigenetic mechanisms (Greger et al., 1989; Singh et al., 2016).

The use of NGS gene panel for RB genetic diagnosis allowed us to detect variants with mosaicism in 10% of analyzed cases, and deep intronic variants in 7.1% of cases, which is in agreement with other

studies (Devarajan et al., 2015; Le Gall et al., 2021). Regarding the RB subtypes, bilateral sporadic RB patients showed the rate of mosaicism of 19.2% of the cases. The *RB1*-panel also proved useful for detecting one intronic pathogenic variant. In our study, this approach resolved 23% of bilateral cases with mosaic or intronic variants. This indicates that all bilateral RB patients without detected variants should undergo further analysis using the NGS panel. In the case of sporadic unilateral cases, an improvement in the diagnosis was also observed, mainly due to the detection of 5.1% of cases with mosaicism and additional 10.3% of cases with intronic variants RB. This finding indicates that intronic variants could also be causing the disease, and they should be functionally characterized. Furthermore, the detection of mosaicism in this group reinforces the importance of using the *RB1*-panel also in unilateral cases. On the other hand, application of deep sequencing of PCR amplicons was very useful to confirm suspected mosaicism, or when a variant identified in the tumor is not detected by Sanger sequencing in blood samples.

In conclusion, our work describes the variant landscape in Spanish RB patients and highlights hotspots in specific exons and introns of the *RB1* gene. The use of the NGS *RB1*-panel in sporadic RB cases without previously identified variant allowed to find new variants in 23% of bilateral cases and 15.4% of unilateral cases. It is important to extend the mutational analysis outside the exons since some causal intronic variants were demonstrated. Detecting mosaicism through NGS techniques has direct implications for family genetic counseling, and improved patient management.

The authors of this article have no conflicts of interest.

CRediT authorship contribution statement

Gema Gomez-Mariano: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Esther Hernandez-SanMiguel:** Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis. **Marta Fernandez-Prieto:** Validation, Supervision, Methodology, Investigation, Formal analysis. **Sheila Ramos del Saz:** Validation, Supervision, Methodology, Formal analysis. **Beatriz Baladrón:** Supervision, Methodology, Formal analysis. **Lidia Mirela Mielu:** Visualization, Methodology, Formal analysis. **Daniel Rivera:** Methodology. **Victoria Moneo:** Methodology, Investigation, Formal analysis. **Lidia Lopez:** Formal analysis. **Carlos Rodriguez-Martin:** Validation, Methodology, Formal analysis. **Ana Fernandez-Teijeiro Álvarez:** Supervision, Resources. **Constantino Sabado:** Supervision, Resources. **Eva Bermejo:** Validation, Supervision, Project administration. **Francisco Javier Alonso:** Visualization, Validation, Supervision, Investigation, Formal analysis, Conceptualization. **Beatriz Martinez-Delgado:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Funding

Sources of support: institutional funding, Institute of Health Carlos III (ISCIII)

Acknowledgments

The Instituto de Salud Carlos III of Madrid has supported this work (PT23CIII/00003). We acknowledge the genomics and bioinformatics services of the Instituto de Salud Carlos III for their participation in this study. We would like to thank Belén Marugán Gómez for the illustration of the figure presented in Highlights.

Data availability

The data that has been used is confidential.

References

Alonso, J., Frayle, H., Menéndez, I., López, A., García-Miguel, P., Abelairas, J., Sarret, E., Vendrell, M.T., Navajas, A., Artigas, M., Indiano, J.M., Carbone, A., Torrenteras, C., Palacios, I., Pestaña, A., 2005. Identification of 26 new constitutional RB1 gene mutations in Spanish, Colombian, and Cuban retinoblastoma patients. Hum. Mutat. 25, 99. https://doi.org/10.1002/humu.9299.

Alonso, J., García-Miguel, P., Abelairas, J., Mendiola, M., Sarret, E., Vendrell, M.T., Navajas, A., Pestaña, A., 2001. Spectrum of germline RB1 gene mutations in Spanish retinoblastoma patients: phenotypic and molecular epidemiological implications. Hum. Mutat. 17, 412–422. https://doi.org/10.1002/humu.1117.

Alonso, J., Palacios, I., Gámez, A., Camino, I., Frayle, H., Menéndez, I., Kontic, M., García-Miguel, P., Sastre, A., Abelairas, J., Sarret, E., Sabado, C., Navajas, A., Artigas, M., Indiano, J.M., Carbone, A., Rosell, J., Pestaña, A., 2006. [Molecular diagnosis of retinoblastoma: molecular epidemiology and genetic counseling]. Med. Clin. 126, 401–405. https://doi.org/10.1157/13086125.

Amitrano, S., Marozza, A., Somma, S., Imperatore, V., Hadjistilianou, T., De Francesco, S., Toti, P., Galimberti, D., Meloni, I., Cetta, F., Piu, P., Di Marco, C., Dosa, L., Lo Rizzo, C., Carignani, G., Mencarelli, M.A., Mari, F., Renieri, A., Ariani, F., 2015. Next generation sequencing in sporadic retinoblastoma patients reveals somatic mosaicism. Eur. J. Hum. Genet. EJHG 23, 1523–1530. https://doi.org/10.1038/ejhg.2015.6.

Biesecker, L.G., Spinner, N.B., 2013. A genomic view of mosaicism and human disease. Nat. Rev. Genet. 14, 307–320. https://doi.org/10.1038/nrg3424.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinforma. Oxf. Engl. 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Carlson, E.A., Desnick, R.J., 1979. Mutational mosaicism and genetic counseling in retinoblastoma. Am. J. Med. Genet. 4, 365–381. https://doi.org/10.1002/ajmg.1320040408.

Chen, Z., Moran, K., Richards-Yutz, J., Toorens, E., Gerhart, D., Ganguly, T., Shields, C.L., Ganguly, A., 2014. Enhanced sensitivity for detection of low-level germline mosaic RB1 mutations in sporadic retinoblastoma cases using deep semiconductor sequencing. Hum. Mutat. 35, 384–391. https://doi.org/10.1002/humu.22488.

Devarajan, B., Prakash, L., Kannan, T.R., Abraham, A.A., Kim, U., Muthukkaruppan, V., Vanniarajan, A., 2015. Targeted next generation sequencing of RB1 gene for the molecular diagnosis of Retinoblastoma. BMC Cancer 15, 320. https://doi.org/10.1186/s12885-015-1340-8.

Dommering, C.J., Mol, B.M., Moll, A.C., Burton, M., Cloos, J., Dorsman, J.C., Meijers-Heijboer, H., van der Hout, A.H., 2014. RB1 mutation spectrum in a comprehensive nationwide cohort of retinoblastoma patients. J. Med. Genet. 51, 366–374. https://doi.org/10.1136/jmedgenet-2014-102264.

Donaldson, S.S., Smith, L.M., 1989. Retinoblastoma: biology, presentation, and current management. Oncol. Williston Park N 3, 45–51. ; discussion 51-52.

Ewels, P., Magnusson, M., Lundin, S., Käller, M., 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinforma. Oxf. Engl. 32, 3047–3048. https://doi.org/10.1093/bioinformatics/btw354.

Fujiwara, T., Fujiwara, M., Numoto, K., Ogura, K., Yoshida, A., Yonemoto, T., Suzuki, S., Kawai, A., 2015. Second primary osteosarcomas in patients with retinoblastoma. Jpn. J. Clin. Oncol. 45, 1139–1145. https://doi.org/10.1093/jjco/hyv140.

Greger, V., Passarge, E., Höpping, W., Messmer, E., Horsthemke, B., 1989. Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. Hum. Genet. 83, 155–158. https://doi.org/10.1007/BF00286709.

Grotta, S., D'Elia, G., Scavelli, R., Genovese, S., Surace, C., Sirleto, P., Cozza, R., Romanzo, A., De Ioris, M.A., Valente, P., Tomaiuolo, A.C., Lepri, F.R., Franchin, T., Ciocca, L., Russo, S., Locatelli, F., Angioni, A., 2015. Advantages of a next generation sequencing targeted approach for the molecular diagnosis of retinoblastoma. BMC Cancer 15, 841. https://doi.org/10.1186/s12885-015-1854-0.

Gudiseva, H.V., Berry, J.L., Polski, A., Tummina, S.J., O'Brien, J.M., 2019. Next-generation technologies and strategies for the management of retinoblastoma. Genes 10, E1032. https://doi.org/10.3390/genes10121032.

Harbour, J.W., 1998. Overview of RB gene mutations in patients with retinoblastoma. Implications for clinical genetic screening. Ophthalmology 105, 1442–1447. https://doi.org/10.1016/S0161-6420(98)98025-3.

Imperatore, V., Pinto, A.M., Gelli, E., Trevisson, E., Morbidoni, V., Frullanti, E., Hadjistilianou, T., De Francesco, S., Toti, P., Gusson, E., Roversi, G., Accogli, A., Capra, V., Mencarelli, M.A., Renieri, A., Ariani, F., 2018. Parent-of-origin effect of hypomorphic pathogenic variants and somatic mosaicism impact on phenotypic expression of retinoblastoma. Eur. J. Hum. Genet. EJHG 26, 1026–1037. https://doi.org/10.1038/s41431-017-0054-6.

Jaganathan, K., Kyriazopoulou Panagiotopoulou, S., McRae, J.F., Darbandi, S.F., Knowles, D., Li, Y.I., Kosmicki, J.A., Arbelaez, J., Cui, W., Schwartz, G.B., Chow, E. D., Kanterakis, E., Gao, H., Kia, A., Batzoglou, S., Sanders, S.J., Farh, K.K.-H., 2019. Predicting splicing from primary sequence with deep learning. Cell 176, 535–548. e24. https://doi.org/10.1016/j.cell.2018.12.015.

Kanski, J.J., 2010. Chapter 9 - pupils. In: Kanski, J.J. (Ed.), Signs in Ophthalmology. Mosby, London, pp. 193–209. https://doi.org/10.1016/B978-0-7234-3548-8.50012-7.

Koboldt, D.C., Zhang, Q., Larson, D.E., Shen, D., McLellan, M.D., Lin, L., Miller, C.A., Mardis, E.R., Ding, L., Wilson, R.K., 2012. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res. 22, 568–576. https://doi.org/10.1101/gr.129684.111.

Kugalingam, N., De Silva, D., Abeysekera, H., Nanayakkara, S., Tirimanne, S., Ranaweera, D., Suravajhala, P., Chandrasekharan, V., 2023. RB1 screening of retinoblastoma patients in Sri Lanka using targeted next generation sequencing

(NGS) and gene ratio analysis copy enumeration PCR (GRACE-PCR). BMC Med. Genom. 16, 279. https://doi.org/10.1186/s12920-023-01721-6.

Le Gall, J., Dehainault, C., Benoist, C., Matet, A., Lumbroso-Le Rouic, L., Aerts, I., Jiménez, I., Schleiermacher, G., Houdayer, C., Radvanyi, F., Frouin, E., Renault, V., Doz, F., Stoppa-Lyonnet, D., Gauthier-Villars, M., Cassoux, N., Golmard, L., 2021. Highly sensitive detection method of retinoblastoma genetic predisposition and biomarkers. J. Mol. Diagn. JMD S1525–1578 (21), 323–328. https://doi.org/10.1016/j.jmoldx.2021.08.014.

Li, H., Durbin, R., 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinforma. Oxf. Engl. 25, 1754–1760. https://doi.org/10.1093/bioinformatics/btp324.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., 2009 Aug 15. 1000 Genome Project Data Processing Subgroup. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25 (16), 2078–2079. Epub 2009 Jun 8. https://doi.org/10.1093/bioinformatics/btp352.

Li, M.-X., Kwan, J.S.H., Bao, S.-Y., Yang, W., Ho, S.-L., Song, Y.-Q., Sham, P.C., 2013. Predicting mendelian disease-causing non-synonymous single nucleotide variants in exome sequencing studies. PLoS Genet. 9, e1003143. https://doi.org/10.1371/journal.pgen.1003143.

Li, W.L., Buckley, J., Sanchez-Lara, P.A., Maglinte, D.T., Viduetsky, L., Tatarinova, T.V., Aparicio, J.G., Kim, J.W., Au, M., Ostrow, D., Lee, T.C., O'Gorman, M., Judkins, A., Cobrinik, D., Triche, T.J., 2016. A rapid and sensitive next-generation sequencing method to detect RB1 mutations improves care for retinoblastoma patients and their families. J. Mol. Diagn. JMD 18, 480–493. https://doi.org/10.1016/j.jmoldx.2016.02.006.

Marees, T., Moll, A.C., Imhof, S.M., de Boer, M.R., Ringens, P.J., van Leeuwen, F.E., 2008. Risk of second malignancies in survivors of retinoblastoma: more than 40 years of follow-up. J. Natl. Cancer Inst. 100, 1771–1779. https://doi.org/10.1093/jnci/djn394.

Mendonça, V., Pereira Sena, P., Evangelista Dos Santos, A.C., Rodrigues Bonvicino, C., Ashton-Prolla, P., Epelman, S., Ferman, S.E., Lapunzina, P., Nevado, J., Grigorovski, N., Mattosinho, C., Seuànez, H., Regla Vargas, F., 2022. Diverse mutational spectrum in the 13q14 chromosomal region in a Brazilian cohort of retinoblastoma. Exp. Eye Res. 224, 109211. https://doi.org/10.1016/j.exer.2022.109211.

Rodríguez-Martín, C., Cidre, F., Fernández-Teijeiro, A., Gómez-Mariano, G., de la Vega, L., Ramos, P., Zaballos, Á., Monzón, S., Alonso, J., 2016. Familial retinoblastoma due to intronic LINE-1 insertion causes aberrant and noncanonical mRNA splicing of the RB1 gene. J. Hum. Genet. 61, 463–466. https://doi.org/10.1038/jhg.2015.173.

Rodríguez-Martín, C., Robledo, C., Gómez-Mariano, G., Monzón, S., Sastre, A., Abelairas, J., Sábado, C., Martín-Begué, N., Ferreres, J.C., Fernández-Teijeiro, A., González-Campora, R., Rios-Moreno, M.J., Zaballos, Á., Cuesta, I., Martínez-Delgado, B., Posada, M., Alonso, J., 2020. Frequency of low-level and high-level mosaicism in sporadic retinoblastoma: genotype-phenotype relationships. J. Hum. Genet. 65, 165–174. https://doi.org/10.1038/s10038-019-0696-z.

Singh, U., Malik, M.A., Goswami, S., Shukla, S., Kaur, J., 2016. Epigenetic regulation of human retinoblastoma. Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med. 37, 14427–14441. https://doi.org/10.1007/s13277-016-5308-3.

Sippel, K.C., Fraioli, R.E., Smith, G.D., Schalkoff, M.E., Sutherland, J., Gallie, B.L., Dryja, T.P., 1998. Frequency of somatic and germ-line mosaicism in retinoblastoma: implications for genetic counseling. Am. J. Hum. Genet. 62, 610–619. https://doi.org/10.1086/301766.

Valverde, J.R., Alonso, J., Palacios, I., Pestaña, A., 2005. RB1 gene mutation up-date, a meta-analysis based on 932 reported mutations available in a searchable database. BMC Genet. 6, 53. https://doi.org/10.1186/1471-2156-6-53.

Vogel, F., 1979a. Genetics of retinoblastoma. Hum. Genet. 52, 1–54. https://doi.org/10.1007/BF00284597.

Vogel, F., 1979b. Genetics of retinoblastoma. Hum. Genet. 52, 1–54. https://doi.org/10.1007/BF00284597.

Zou, Y., Li, J., Hua, P., Liang, T., Ji, X., Zhao, P., 2021. Spectrum of germline mutations in RB1 in Chinese patients with retinoblastoma: application of targeted next-generation sequencing. Mol. Vis. 27, 1–16.