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RESEARCH REPORT

Spectrum of *RB1* Mutations in Argentine Patients: 20-years Experience in the Molecular Diagnosis of Retinoblastoma[1]

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ABSTRACT

Background: Retinoblastoma is a hereditary cancer of childhood caused by mutations in the *RB1* tumor suppressor gene. An early diagnosis is critical for survival and eye preservation, thus identification of *RB1* mutations is important for unequivocal diagnosis of hereditary retinoblastoma and risk assessment in relatives.

Methods: We studied 144 families for 20 years, performing methodological changes to improve detection of mutation. Segregation analysis of polymorphisms, MLPA, FISH and cytogenetic assays were used for detection of "at risk haplotypes" and large deletions. Small mutations were identified by heteroduplex/DNA sequencing.

Results: At risk haplotypes were identified in 11 familial and 26 sporadic cases, being useful for detection of asymptomatic carriers, risk exclusion from relatives and uncovering *RB1* recombinations. Ten large deletions (eight whole gene deletions) were identified in six bilateral/familial and four unilateral retinoblastoma cases. Small mutations were identified in 29 cases (four unilateral retinoblastoma patients), being the majority nonsense/frameshift mutations. Genotype-phenotype correlations confirm that the retinoblastoma presentation is related to the type of mutation, but some exceptions may occur and it is crucial to be considered for genetic counseling. Three families included second cousins with retinoblastoma carrying different haplotypes, which suggest independent mutation events.

Conclusion: This study enabled us to obtain information about molecular and genetic features of patients with retinoblastoma in Argentina and correlate them to their phenotype.

Keywords: at-risk haplotype; genotype-phenotype correlation; penetrance; *RB1* mutations; *RB1* tumor suppressor gene

INTRODUCTION

Retinoblastoma (RB) is a rare disease of childhood. As it is a hereditary cancer in 40% of cases, molecular analysis is important for an unequivocal diagnosis of a hereditary RB, since there is a risk for secondary tumors and for RB predisposition in relatives.[1] The most common presenting signs are leukocoria (54%) and strabismus (19%) which correlate with a high survival rate of the patient in developed countries (>86%) and a poor ocular survival rate (9–17%).[2]

Retinoblastoma is caused by mutations in the *RB1* tumor suppressor gene (13q14). Genetic studies revealed that loss of both alleles is required but it is not sufficient for RB development since chromosomal gains and losses and also epigenetic deregulation were described.[3,4] There is an intermediate stage, a

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benign tumor retinoma, which contains the same *RB1* mutations.[5]

The incidence of retinoblastoma varies in different geographical locations (15.3–42.5 per million children aged 0–4) and appears to be higher in less affluent regions.[6] There are suggestions that variation in incidence correlates with the degree of health care systems and also with environmental exposure.[7]

An early diagnosis is critical for survival and eye preservation because retinoblastoma is a potentially curable tumor when it is intraocular and becomes fatal when it disseminates.[8] In order to prevent late diagnosis and to develop new strategies of treatment there are several organizations for collaborative work between institutions, such as the Fund for Ophthalmic Knowledge (New York, USA) and the Hospital Garrahan (Buenos Aires, Argentina).

The diagnosis based on DNA analyses of affected families provides useful information for genetic counseling to reduce the risk of tumor development in relatives. Identification of the *RB1* mutation allows early detection of tumor in children who carry the mutation, and avoids repeated invasive surveillance procedures under anesthesia in young relatives (offspring, siblings and first cousins) who do not carry the mutation. In addition this analysis allows the detection of asymptomatic mutation carriers.[17,22]

Although the genetic testing of RB is relevant for health care, it has not been widely implemented in developing countries because of the heterogeneity of inactivating mutations, their distribution along the promoter region and the 27 exons of the *RB1* gene and the cost related to it. We have carried out molecular studies on RB patients for 20 years and performed methodological changes to improve mutation detection. Our study aimed to identify *RB1* mutations in as many RB patients as possible and to correlate them to the patient phenotype, which allows better genetic counseling and clinical management of affected families.

MATERIALS AND METHODS

Patients

A total of 144 retinoblastoma families, referred from Hospital Garrahan (Buenos Aires, Argentina) and other pediatric hospitals and health care centers in Argentina, were analyzed: 15 familial cases, 59 sporadic bilateral, 64 sporadic unilateral and three cases including two second cousins with RB. Retinoblastoma diagnosis was established by current ophthalmologic/histological criteria. Cytogenetic analysis of peripheral blood lymphocytes was performed in patients showing developmental problems indicative of a 13q deletion syndrome. After approval of the study protocol by the Bioethical Committee of Hospital de Clinicas Jose de San Martin (Buenos Aires, Argentina), informed consent for genetic analyses was signed by parents of affected children.

DNA Isolation and Genotyping of Polymorphic Loci

The deoxyribonucleic acid (DNA) was obtained from peripheral blood leukocytes using the cetyltrimethylammonium bromide (CTAB) method, and from paraffin-embedded tumors by treatment with proteinase K.[9] Segregation analysis of six polymorphic loci within *RB1* gene included: (i) three intragenic restriction fragment length polymorphisms (RFLPs): BamHI, XbaI and Tth111I (introns 1, 17 and 24), and (ii) three microsatellites: RBi2, RBi4 and RB1.20 (introns 2, 4 and 20).[10,11] The RFLPs amplified fragments were digested with the appropriate restriction enzyme and visualized in a 2% agarose gel by etidium bromide staining. RBi4 was initially assayed with a radioactively-labeled (γ^{32}P-dATP) forward primer and since the year 2008 the RBi4 and also RBi2 fragments were visualized by silver staining on a 6% polyacrylamide sequencing gel. The same methodology was used for RB1.20 on a 10% polyacrylamide gel. Southern analysis was performed by DNA digestion with the appropriate restriction endonuclease, separation by electrophoresis, transfer onto the membrane and hybridization with a probe as described.[12]

Mutation Screening

Fluorescent in situ hybridization analysis (FISH) on metaphase spreads was performed using 13q14 (*RB1*) specific green probe (Qbiogene Molecular Cytogenetics, Irvine, CA, USA) according to the manufacturer's instructions. This probe spans most of the *RB1* gene, from upstream of the promoter to near the 3′ region. Multiplex ligation-dependent probe amplification assay (MLPA) was performed using the Salsa MLPA kit PO47-B1 RB1 (MRC-Holland).

The heteroduplex/DNA sequence assay was performed as described.[13] Initially, electrophoresis of the radioactively-labeled fragments and autoradiography were used for sequence analysis. Thereafter, since 2006, the altered amplicons were analyzed by fluorescent automated sequencing on an ABI 3130XL genetic analyzer (Applied Biosystems, Seoul Korea). Finally, since 2009, the screening for small mutations has been performed by direct sequencing of the 27 exons and promoter region of the *RB1* gene. Mutations were described according to the nomenclature[14] using the *RB1* sequence from the GenBank, accession No L11910. The identified mutations were submitted to the Retinoblastoma Database.[15]

RESULTS

Clinical and Genetic Features of RB Patients

The results are summarized in Table 1. The average age at diagnosis was similar to that previously reported[16] except for one 15-year-old unilateral patient, who developed RB in adult life or had had an undetected retinoma in childhood which was activated later. Five patients developed a second non ocular tumor in adult life: (i) osteosarcoma (patients #126, #142, #211) two of whom died; (ii) melanoma and sarcomas of soft tissue (#112), and (iii) epithelioma (#501). The daughter (#400) of the unilateral patient #112 developed a rare trilateral tumor: bilateral retinoblastoma and pinealoblastoma. In total, six patients died as a result of second tumors, trilateral RB or other causes.

The haplotype analysis was performed in all patients and the at-risk haplotype was identified in 11 familial and 26 sporadic cases showing loss of heterozygosity (LOH) (Figure 1A). The families including second cousins with RB (#438/443, #420/424 and #321/570) showed different haplotypes (Figure 2A–C). This analysis also revealed a recombination in the *RB1* gene in two of these families (Figure 2A & C).

Mutational Analysis

The methodology was changed over this period of 20 years in order to improve the detection of mutations, as is depicted in Figure 1B. Heteroduplex prescreening/sequence analysis showed a low detection rate, which was increased by performing the sequencing of all the *RB1* exons and MLPA analysis. In the course of our study we found a total of 35 mutations in 39 not related patients, 15 of which were novel as they were not reported in the Retinoblastoma Mutation Database[15] (Table 2, Figure 3). Some of these results were already published.[9,10,12,13]

Mutations were widely distributed throughout the *RB1* gene and no hot spots were found. Most of the mutations had occurred de novo and differ from one patient to another. The type of mutation varied between familial, sporadic bilateral and sporadic unilateral cases. Frameshift and nonsense mutations occurred at a higher rate than other small mutations in familial cases and sporadic bilateral patients (20 out of 25 patients), whereas mutations presumably resulting in residual protein, such as missense, promoter and some splice mutations prevailed in unilateral hereditary cases (3 out of 4 patients).

Nonsense and Frameshift Mutations (18/35)
These were mostly base pair substitutions (11/18) (predominantly C → T or G → A transitions), 1–2 bp deletions (6/18) and one insertion; which were identified in six familial, 14 sporadic bilateral and one sporadic unilateral case. Among the familial cases two of them included patients with bilateral RB showing complete penetrance (#1 and #163); two others included one unilateral progenitor (#316 and #112) and offspring with bilateral (#317) and trilateral RB (#400); and still two other families had one unaffected mutation carrier progenitor and bilaterally affected offspring (#51 and #132) exhibiting incomplete penetrance.

Splice Site Mutations (4/35)
Two out of four mutations disrupted the highly conserved AG/GT sites and two others occurred at less conserved positions: IVS21+5 and IVS23-3 splice site. The former (intron 21) was already reported for the same intron as well as for introns 17, 22 and 24 of the *RB1* gene.[15,17,18] Mutations at +5 splice site in the *RB1* gene and in other genes involved in genetic diseases support their pathogenic nature.[19] Mutations at −3 splice site were reported in other RB patients.[15] These results support the fact that the evolutionary conserved bases at the splice junctions are six at the 5′ and three at the 3′ ends of all introns.[19]

Promoter Mutations (2/35)
Two G → A transitions were detected in the proximal promoter region: (i) c.-197, in a bilateral sporadic patient (#449) and ii) c.-192, in two cases: one unilateral familial case (#160) where the mutation cosegregated with the RB predisposition, and one unilateral sporadic patient (#501) who had inherited the mutation from his asymptomatic father. The unilateral sporadic patient had developed a second non ocular tumor above the enucleated eye (epithelioma), and the identified mutation is being used for a preimplantation genetic test.

Missense Mutation (1/35)
This mutation was identified in a unilateral patient (#176) in exon 13 (encoding for the pocket domain A) by heteroduplex prescreening and sequence analysis.

Deletions (10/35)
Five large deletions, spanning the polymorphic sites of introns 1–20, were identified and then confirmed as whole *RB1* gene deletions by FISH and MLPA analyses. One of these deletions represents a rare case of familial mutation (#336), usually expected to be a small mutation. The other four deletions were identified in sporadic cases (#10, #331, #360, #432). Another whole *RB1* gene deletion was identified by MLPA in one of two second cousins with RB, being absent in the other, consistent with the different haplotypes (#420: II-2 Figure 2B). One deletion was detected by Southern blot and later confirmed by MLPA as a whole gene deletion (#19). Five of the

TABLE 1. Disease phenotype, at risk haplotype and information for relatives.

No. of patients/ families analyzed	Familial/ sporadic	Average age at diagnosis (months)	Enucleated patients	2nd tumors/ patients died	At risk hap/ recom	LOH	AsCar/ RelExc	Prenatal/ neonatal diagnosis
15 (11%)	Familial	1st patient: 24 2nd patient: 3 DER: 0.5–2.0	One eye: 22 Both eyes: 5 NE: 3	1: #112 1: #400 T	11	Yes: 2 ND: 13	As Car: 7 Rel Exc:12	Prenatal Family#1: Mutant *RB1*
59 (43%)	Sporadic bilateral	10	One eye: 43 Both eyes: 9 NE: 7	3: #126,142,211 3: #15,126,142	11	Yes: 9 No: 4 ND: 46	Rel Exc:30	Neonatal 5 Rel Exc
64 (46%)	Sporadic unilateral	17 (15 years, one patient)	One eye: 58 NE: 6	1: #501 2: #79,148	15	Yes: 14 No: 12 ND: 38	As Car: 1 Rel Exc: 4	Prenatal and Neonatal Family#227:Wd type *RB1*
Three Families: second cousins with RB	U/U U/B U/B	20/60 3/7 27/6	One eye: 2 One eye: 2 One eye: 1 NE: 1	–	Dif Hap/R Dif Hap Dif Hap/R	ND	Rel Exc: 1	ND
Total 144 (100%)			One eye: 128 Both eyes: 14 NE:17	5 patients with 2nd tumors 6 patients died	37/2	Yes: 25 No: 16 ND: 97	As Car: 8 Rel Exc:47	Prenatal: 2 Neonatal: 6

DER, diseased eye ratio (ratio of eyes with tumor to mutation carriers); NE, not enucleated; Dif Hap, different haplotypes; R, recombination; As Car, asymptomatic carrier; Rel Exc, relatives excluded from risk; ND, not determined; Wd type, wild type; U, unilateral RB; B, bilateral RB; T, trilateral RB.

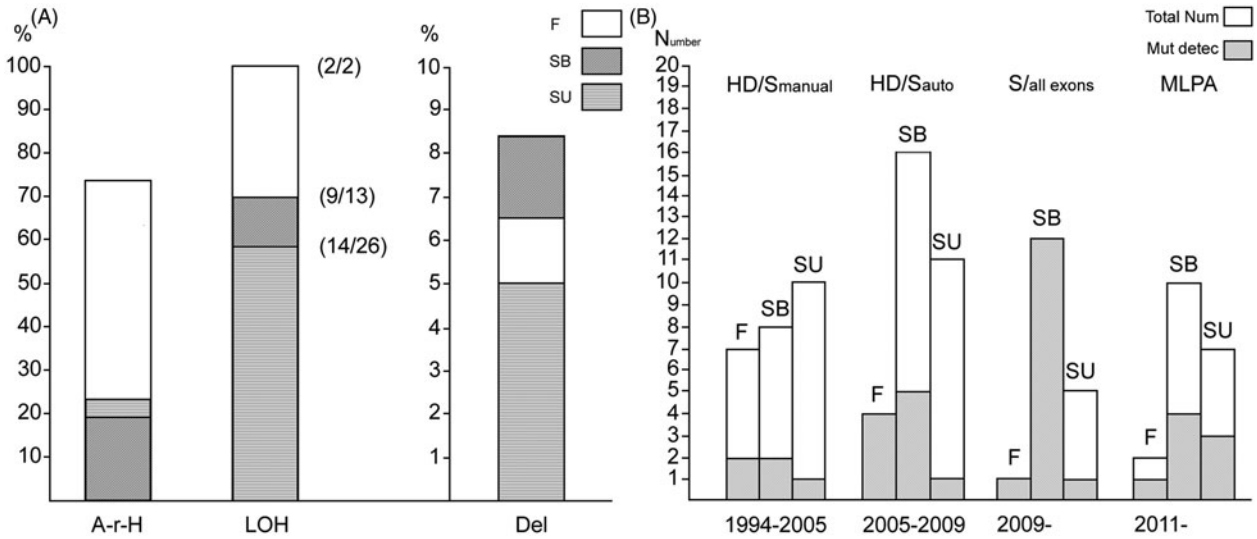

FIGURE 1. Summary of the methodology used for *RB1* mutation detection throughout the 20-year period study. The three groups of patients analyzed were: familial RB (F), sporadic bilateral (SB) and sporadic unilateral (SU). (A) Haplotype analysis. The bar graph shows the percentage (%) of cases with at-risk haplotype (A-r-H), loss of heterocigosity (LOH) and large deletion (Del), identified in the three groups of patients during the whole study period (1990–2012). LOH was tested in a subset of patients with an available tumor sample. The number of tumors with LOH relative to the total number assayed is shown in brackets. (B) Different groups of patients with RB analyzed by: Heteroduplex assay and Manual Sequencing (HD/Smanual), Heteroduplex assay and Automated Sequencing (HD/Sauto), Sequencing of the 27 Exons and the Promoter region of the *RB1* Gene (S/all exons) and MLPA analysis (MLPA). The number of patients analyzed of each group is indicated by white bars and the number of identified mutations is denoted by the gray bars.

patients carrying whole gene deletions were unilateral and four were bilateral (including all familial RB individuals). Additionally, a chromosomal 13q13-21 deletion was uncovered by cytogenetic assay in one unilateral patient with dismorphic signs and mental deficiency (#236). Deletions spanning several exons were found in two bilateral patients: #498 (exons 1–17) detected by segregation of polymorphisms and confirmed by MLPA; and #72 (exons 18–27) identified in tumor DNA with a LOH.

Penetrance

The Disease Eye Ratio (DER) is defined as the ratio of the sum of the eyes affected by tumors to the number of mutation carriers in a family. Families with a DER of $\geq$1.5 are considered as displaying high penetrance, whereas families with a DER of $\leq$1 are designated as low penetrance cases.[20]

The average DER in 13 families was 1.78, corresponding to complete penetrance. However, two of these families (#51 and #132) carrying frameshift/nonsense mutations, showed lower penetrance than expected for these types of mutations (DER = 1.33). They included asymptomatic carrier progenitors and siblings with bilateral RB (#51/51′ and #132/133). The lowest penetrance was found in two families: (i) #336 with a whole *RB1* deletion (DER = 0.8) including two asymptomatic carriers, two unilateral patients and one bilateral, and (ii) #160 with a promoter mutation (DER = 0.5) including two asymptomatic carriers and two unilateral patients. In addition, the missense mutation (#176) and the whole gene deletions identified in three sporadic unilateral patients (#236, #420, #432) are correlated to a low penetrance phenotype, in agreement with the previous reports.[20,21]

Recombinations in the *RB1* Gene

Two recombinations were found in two families including second cousins with RB: (i) in the sister of the patient #438 between maternal chromosomes (Figure 1A, II-2), and (ii) in the sister of the patient #321 between paternal chromosomes (Figure 1C, II-1).

DISCUSSION

The incidence of retinoblastoma is higher in regions with poor living conditions and delayed diagnosis is a major problem in developing countries.[6–8] The Hospital Garrahan (Buenos Aires, Argentina), a referral center for RB in Argentina, is contributing to the study and management of retinoblastoma in developing countries, implementing efficient diagnostic methods and advanced strategies for treatment. The hospital mentioned above has received an increasing number of patients over the last years, up to 40 patients per year, most of them with poor living conditions. Only 38% of them have been tested for

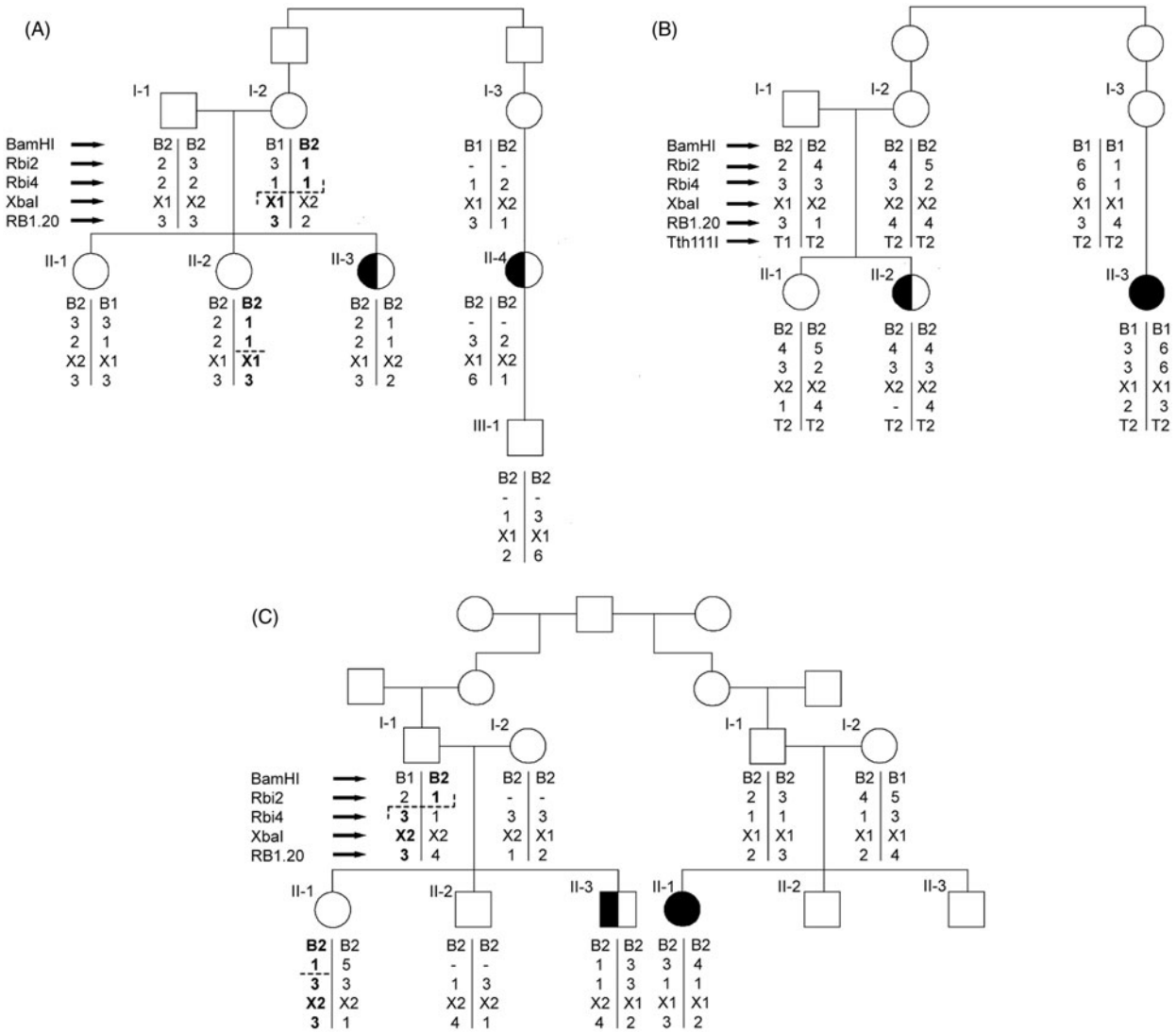

FIGURE 2. Pedigree and haplotype analysis of three families including second cousins with RB. The families analyzed were #438 (A-II3), #420 (B-II2) and #321 (C-II3). The polymorphisms are indicated at the left of the pedigree. Both second cousins in each family show different haplotypes. A recombination in the *RB1* gene in families A and C is represented as a horizontal line in the chromosomes of the mother (I2) and father (I1) respectively, and in the recombinant chromosome of the sisters of both patients. The break-point location is either between RBi4 and XbaI or RBi2 and RBi4 polymorphisms in A because the mother is homozygous for RBi4, and between RBi2 and RBi4 in C.
Blackened symbols: bilateral RB. Half blackened symbols: unilateral RB.

RB1 mutations due to the insufficient economical support. The aim of our group is to ascertain by molecular study most of the RB patients in Argentina and also in the neighboring countries, in order to prevent the late diagnosis.

We performed molecular characterization and correlation of genetic and clinical features in retinoblastoma patients, and our results represent the first comprehensive study of retinoblastoma, as far as we know, in a developing country. It is worth mentioning the problems and difficulties encountered in the performance of these analyses, such as limited grants and a poor communication between distant collaborative centers.

The methodology has been changed from laborious and low efficient techniques, such as Southern blot and sequencing by electrophoresis/autoradiography to MLPA and fluorescent automated sequencing, increasing the detection rate from 44% to 94% (17 out of 18 hereditary cases, taking into account four cases not included in the manuscript).

The analysis of haplotypes used at the beginning for risk assessment allowed the identification of children at risk or those not at risk, and the detection of asymptomatic carriers who could transmit the *RB1* mutation (Table 1). However, this analysis used mostly in familial cases for detection of at-risk haplotype (70%) and in all cases for uncovering large deletions (8%) (Figure 1A), has limited usefulness since most patients with RB are sporadic and carry small mutations. Tumor DNA, obtained previously from paraffin-embedded samples, is now

TABLE 2. Distribution of *RB1* mutations in Argentine patients.

Patient/family # ID	Description	Expected consequence	Recurrence number	Disease phenotype: Type	Age at diagnosis (months)	Relatives	References
449	g.1863G>A Prom	c.-197 Sp1	1	SB	6	ND	Unpublished
160/172	g.1868G>A Prom	c.-192 ATF	NR	FU	18/40	1 SibEx-2AC ND	Unpublished
501				SU	30		
523	g.5510G>A Exon 2	p.W75X	4	SB	0.5	ND	Unpublished
297	g.41966delA Exon 4	p.S141fsX152	NR	SB	8	ND	9
316/317	g.42001delG Exon 4	p.L152fsX174	NR	FU	36/2	ND	9
15	g.56944insGTTG Exon 7	p.L235fsX263	NR	SB	24	ND	11
527	g.56946T>A Exon 7	p.L234X	NR	SB	18	1 SibEx	Unpublished
538	g.59755G>T Exon 8	p.E275X	NR	SB	2	ND	Unpublished
163/165	g.65386C>T Exon 11	p.R358X	64	FB	24/4	1 SibEx	12
534	g.70261C>T Exon 12	p.Q383X	6	SB	1.5	ND	Unpublished
176	g.73835C>T Exon 13	p.A431V	NR	SU	33	ND	12
507	g.76460C>T Exon 14	p.R455X	57	SB	11	1 SibEx	Unpublished
459	g.77000 IVS16-1G>T	Exon 17 skipped	NR	SB	13	ND	Unpublished
112/400	g.78221delT Exon 17	p.M546fsX547	NR	FU	11/4	2 RelEx	Unpublished
1/1′	g.150037C>T Exon 18	p.R579X	89	FB	3	1 AC	11
132/133	g.150037C>T Exon 18	p.R579X	FB	22/3	1 AC	12	
227	g.150037C>T Exon 18	p.R579X		SU	36	2 OffEx	12
375	g.150037C>T Exon 18	p.R579X		SB	9	2 SibEx	Unpublished
51	g.153331delCTExon19	p.S646fsX651	NR	FB	11/1	1 AC	11
483	g.153347A>T Exon19	p.K652X	1	SB	3	ND	Unpublished
515	g.156803G>T Exon 20	p.E691X	NR	SB	18	1 SibEx	Unpublished
60	g.156820delA Exon 20	p.R696fsX704	NR	SB	12	2 SibEx	11
194	g.160829C>T Exon 21	p.Q736X	1	SB	2	1 SibEx	Unpublished
435	g.160833delA Exon 21	p.E737fsX747	NR	SB	3	2 SibEx	Unpublished
491	g.160839 IVS21+5G>A	Exon 21 skipped	1	SB	8	ND	Unpublished
237	g.162237C>T Exon 23	p.R787X	66	SB	4	ND	12
541	g.162368 IVS23+1G>A	Exon 23 skipped	NR	SB	6	3 SibEx	Unpublished
274	g.170369 IVS23-3C>G	Exon 24 skipped	NR	SU	15	ND	Unpublished
498	Del Exons 1–17	Del exons 1–17	ND	SB	12	1 SibEx	Unpublished
10	Del (polymorphisms)	Del exons 2–20	ND	SB	12	ND	11
336/337/380	Del ITM2B-RCBTB2	Del *RB1* + other genes	ND	FU/FB	31/2/11	6 RelEx-2AC	9
331	Del ITM2B-RCBTB2	Del *RB1* + other genes	ND	SB	18	6 SibEx	9
360	Del ITM2B-DLFU1	Del *RB1* + other genes	ND	SU	30	4 SibEx	9
19	Del ITM2B-RCBTB2	Del *RB1* + other genes	ND	SB	11	3 SibEx	11
72	Del Exons 18–27	Del exons 18–27	ND	SB	18	1 SibEx	8
420	Del ITM2B- *RB1*	Del *RB1* + other genes	ND	SU	3	1 SibEx	Unpublished
432	Del ITM2B-DLFU1	Del *RB1* + other genes	ND	SU	48	ND	Unpublished
236	Del 13q14	Chromosomic Del	ND	SU	2	ND	8

Mutation description according to den Dunen and Antonarakís nomenclature using the genomic sequence of GenBank (L11910.1)
SB, sporadic bilateral RB; SU, sporadic unilateral RB; FB, familial bilateral RB; FU, familial unilateral RB; Prom, promoter; Sp1 – ATF, transcription factors recognition sequences in the RB1 promoter; Del, large deletion; ITM2B, centromeric gene; RCBTB2, proximal telomeric gene; DLFU1, distal telomeric gene, NR, not reported; ND, not determined; AC, asymptomatic carrier; Ex, excluded from RB risk; Sib, sibling; Rel, relatives; Off, offspring.

available from frozen tumor tissues (Tumor Bank, Hospital Garrahan). Therefore, the availability of more efficient methodology/equipment, cost reduction and the awareness of the relevance of mutation analysis have improved the molecular diagnosis of patients with RB in Argentina.

Mutation Analysis

The pattern of identified mutations is similar to that which has been previously reported[22–24] being small mutations the most frequent (71%) and large deletions less common (29%).

Nonsense/Frameshift Mutations

These were the most frequent among the small mutations (51%). All but one of them correlated with bilateral/familial RB. The C→T transition in exon 18 was the most recurrent, occurring in four cases of our study: two familial patients (DER: 2 and 1.33), one sporadic bilateral patient, and one sporadic unilateral patient. This latter was a rare case of a unilateral patient with a nonsense

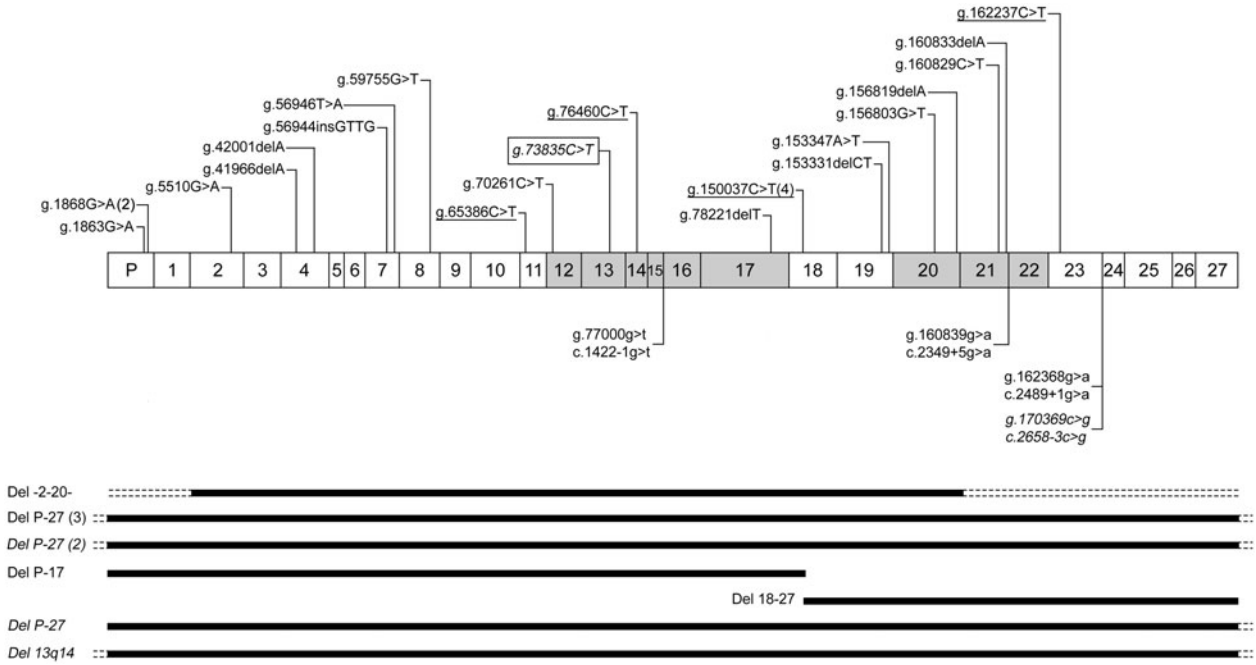

FIGURE 3. Distribution of *RB1* mutations identified throughout the study period. The boxes with numbers represent *RB1* exons, those shown in grey are part of the pocket domains A and B. Nonsense and frameshift mutations are above the coding sequence, one missense mutation is indicated in a square. The underlined mutations are recurrent and the recurrence number in our study is indicated in brackets. Splice site mutations are below the coding sequence. Large deletions are represented as black lines, with an extension related to the deleted exons. Dashed lines indicate uncertainty in the endpoints of the deletions. Unilateral cases are in italics.

mutation, whose two offspring do not carry the mutation.

Splice Site Mutations

These were less frequent mutations (11%). Three of them were out of frame and one was in frame, affecting the pocket domain of the RB protein (pRB). The expected phenotype should have been bilateral in the four cases, however one patient with an out-of-frame mutation was unilateral (#274). The lower disease severity in this case may be explained by the fact that the mutation occurred at a less conserved splice site (−3).

Promoter Mutations

Mutations in the proximal promoter region (6%) occurred within the recognition sites for transcription factors ATF and Sp1. Changes in these sequences were shown to impair the binding of these transcription factors, affecting the expression of *RB1*.[25] Interestingly, two unilateral unrelated cases carried the same mutation (c.-192G > A) not reported before: one familial case, and one sporadic patient with his father being an asymptomatic carrier. Both cases show low penetrance (DER = 0.5) since this mutation may not fully inhibit the expression of the *RB1* gene.[20]

Large Deletions

Deletions spanning the whole *RB1* gene (23%) were found in different cases: (i) familial RB, with intrafamilial variation of tumor presentation; (ii) sporadic bilateral RB, and (iii) sporadic unilateral RB, one of them carrying a chromosomal 13q14-21 deletion. The identification of large deletions in unilateral patients emphasizes the statement that whole-gene deletions originate fewer tumors, frequently unilateral.[21] Deletions spanning part of the *RB1* gene (6%) were found in two bilateral patients, consistent with previous reports stating that deletions with breakpoints located within the *RB1* gene exhibited high penetrance.[26]

Penetrance

The nonsense and frameshift mutations are highly penetrant in the majority of cases; however, variations may occur due to the influence of different factors. Two families in our study included one asymptomatic carrier despite carrying nonsense and frameshift mutations respectively. These mutations commonly lead to tumor development, but sometimes the tumor regresses or remains in a retinoma state.[5] Two other families (mentioned before) exhibited low penetrance and contrasting mutations: a point mutation in the *RB1* promoter and a whole-gene deletion. The identification of asymptomatic mutation carriers in these families was useful for genetic counseling.

Second Cousins with Retinoblastoma and *RB1* Recombinations

The occurrence of second cousins with RB carrying different mutations is an uncommon event, considering the incidence of RB is very low. Interestingly, recombinations in the *RB1* gene were found in two of these families and represent an estimated rate of 0.512%, calculated on 391meiosis; it is higher than expected, based on the size of the *RB1* gene (0.2%). Of note, both families were from the same geographic region, suggesting that epidemiologic factors may influence genetic changes that predispose to recurrent mutations and recombinations.

CONCLUSIONS

This study enabled us to obtain information about molecular and genetic characteristics of Argentine RB patients and correlate them to their phenotype. Changes in methodology throughout the study period allowed a gradual increase in the rate of mutation detection.

Our findings are consistent with the fact that penetrance varies with the type of mutation. Thus, nonsense/frameshift/splice mutations and large exonic deletions correlate with high penetrance and were found mainly in bilateral patients. On the other hand, whole gene deletions, missense and promoter mutations display incomplete penetrance. However, uncommon mutation consequences were observed, such as a correlation of nonsense/frameshift mutations with a not complete penetrance in several families. These findings are crucial to be considered for genetic counseling.

Several rare events in the *RB1* gene, such as recombinations and second cousins with RB carrying different mutations have been uncovered.

These data allow an approach to a better outcome for patients with retinoblastoma in our country.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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