Prognostic information for known genetic carriers of *RB1* pathogenic variants (germline and mosaic)

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Abstract

Objective: To compare the number of tumors per eye for mosaic carriers of *RB1* pathogenic variants with full germline variants and the conversion from unilateral to bilateral disease.

Design: Retrospective cohort study comparing patients with retinoblastoma and different genetic subtypes (HP: high penetrant, LP: low penetrant & mosaicism).

Subjects: Data were analysed between 1992 and 2018 at the Retinoblastoma Unit, Royal London Hospital, London UK. All familial patients had a parent with a known pathogenic variant even if the parent did not manifest the disease.

Main outcome measures: Number of tumors per eye in children who developed retinoblastoma in that eye. Other outcomes included total number of tumors per patient, age at diagnosis, laterality at presentation and later, sex and stage according to International Intraocular Retinoblastoma Classification

Results: 111 patients were included: 64 full germline, familial patients (53 HP and 11 LP) & 47 were mosaic patients. 12 (23%) of HP patients were unilateral and 8 of 12 (67%) developed tumors in their previously unaffected eye. 34 (72%) of mosaic patients were unilateral and only 2 (6%) developed tumors in their unaffected eye. Age at diagnosis was higher in mosaic patients (median 22 months) than HP patients (median 7) ($p<0.00002$). Number of tumors per eye was fewer in patients with mosaic alleles (median 1.0 range 1-6) compared to patients with HP alleles (median 3.0 range 1-8) ($p<0.0003$). All three children (4 eyes) with mosaicism and more than 2 tumors per eye had high levels of mosaicism.

Conclusions: Children with mosaic alleles have fewer tumors per eye compared to those with known high penetrant pathogenic variants and are more likely to remain unilateral. The level of mosaicism has an impact on laterality and number of tumors.

INTRODUCTION

Retinoblastoma is the most common paediatric primary ocular cancer and can be heritable. The majority is caused by pathogenic variants (previously known as disease causing mutations) in the *RB1* tumor suppressor gene which is located at 13q14. Potentially heritable disease can be divided into 2 groups. One group consists of heterozygous, germline pathogenic variant carriers with the first *RB1* allele altered in all cells due to an event during gametogenesis or zygote formation. The second group consists of mosaic *RB1* pathogenic variant carriers with 2 or more different genotypes present due to post-zygotic alterations[1, 2]. Variant alleles can be further subdivided into alleles associated with high penetrance (HP) and low penetrance (LP). Traditionally the definition and classification of pathogenic variant alleles have been based upon disease eye ratio (DER) for patients with retinoblastoma i.e. the proportion of eyes affected with retinoblastoma[3]. With the development of a clinical classification system[4] and screening of at-risk patients from birth, it is feasible to quantify the impact of a genetic category according to number of tumors[5] and also the risk of conversion from unilateral to bilateral disease.

In this study, we compared the number of tumors per eye and conversion from unilateral to bilateral disease in mosaic *RB1* pathogenic variant carriers with that of full germline carriers. To add certainty regarding familial patients, patients whose parent carried a pathogenic variant were considered familial full germline carriers: 'de novo' pathogenic variants were not included.

MATERIALS AND METHODS

This retrospective study was approved by the Barts Health Clinical Effectiveness Unit (#7343) and followed the tenets of the Declaration of Helsinki. A retrospective analysis of mosaic and full germline heterozygous *RB1* pathogenic variant carriers from 1992 to 2018 was conducted in the Retinoblastoma Genetic Screening Unit (RGSU) at the Royal London Hospital, Barts Health NHS Trust.

Genetic Testing

Peripheral blood and tumor samples were collected from patients referred to the RGSU for genetic analysis. Consent was obtained from parents/guardians. Techniques used to identify pathogenic variants included conformation analysis, Sanger sequencing, MLPA, QF-PCR and hypermethylation testing as previously described [6,7]. Levels of mosaicism were based upon areas under the peak for sequencing/sizing analysis and titration for standardisation (mixing normal and variant DNA at certain ratios). Levels of mosaicism were defined as high (31-40%), medium (21-30%) and low (less than 20%).

The pathogenic variant type was categorised into High Penetrant (HP) or Low Penetrant (LP), and either type could be mosaic. LP variants included promoter, missense and splicing variants (Supplementary tables). Clinical data were collected from notes if available.

Group Definitions

Three groups of patients were included: full germline children with HP alleles, full germline children with LP alleles and children who carried mosaic *RB1* pathogenic

variants. To add certainty regarding familial patients, patients whose parent carried a pathogenic variant were considered familial even if they did not manifest the disease: 'de novo' pathogenic variants were not included.

In order to ensure all heritable cases were full germline, and not children with high level mosaics, only familial cases were included. It was essential that one of the parents carried a pathogenic variant even if the parent did not manifest the disease. Heterozygous familial cases were screened soon after birth (H1[8]), but some patients presented with inherited pathogenic variants sporadically at a later age. All children with mosaicism presented sporadically. If unilateral, they were staged as Hx according to the AJCC TNM 8th edition[8] and converted to H1 once the molecular testing results were available. Data included age at diagnosis, tumor group according to IIRC[4], treatment with systemic chemotherapy (for primary treatment or post-enucleation adjuvant chemotherapy: carboplatin, etoposide and vincristine) or external beam radiation (whole eye and lens sparing), number of tumors or foci (including retinomas) per patient and per eye.

Number of Tumors

The number of tumors was assessed in patients classified with O, A, B and C tumors using the IIRC system: '**gaugeable'** eyes. We were keen to assess the number of tumors accurately and not confuse new tumors with subretinal seeds or implants from vitreous seeds. We calculated the total number of tumors in patients with two gaugeable eyes in the 3 groups.

As patients often presented with advanced disease in one eye (Groups D or E) and O/A/B/C (gaugeable) in the other, the number of tumors per eye was recorded in the

gaugeable eye. These eyes were included as tumors per eye. In view of the possibility that there may be a large number of eyes that would never develope a tumor (ie patients staying unilateral), tumor numbers per eye were analysed in 2 different ways. (1) in A,B,C eyes that developed tumors excluding eyes that did not manifest disease and (2) in all O,A,B,C eyes including eyes that did not develop tumors.

Age

Only patients with D or E group eyes were assessed based upon age at diagnosis as they presented sporadically and not following routine screening of the eyes from birth under general anaesthetic. Laterality of disease was also examined and age of conversion from unilateral to bilateral disease. Patients missing large amounts of data were excluded.

Statistics

A one-tailed Mann-Whitney U test ($p<0.05$ was deemed statistically significant) was used to determine if there was a significant difference between the three categories: full germline groups (one category of HP variants and the other LP variants) and mosaic patients.

The Shapiro-Wilk test was used to mathematically determine whether the data followed a normal distribution. A one-tailed Mann-Whitney *U* Test ($p<0.05$ was deemed as statistically significant) was used for the comparison of full germline and mosaic patients. Statistical analysis was performed using the Real Statistics Resource Pack[9], a statistical package add-on for Microsoft Excel (Utah, USA).

RESULTS

We identified 137 patients with full germline and mosaic pathogenic variants. After excluding 26 patients (15 full germline and 11 mosaic patients) with insufficient clinical information, data were analysed for 111 patients: 64 were full germline, familial patients (53 HP and 11 LP) as shown in Table 1. 47 were mosaic patients. Figure 1 shows a flow chart demonstrating the selection of the groups.

Patient demographics

All 64 full germline patients were familial; 59/64 (92%) had a family history at presentation with an affected parent. Five probands were included who had unaffected carrier parents with a pathogenic variant but were still deemed familial. No children with mosaic disease had a family history and were deemed non-heritable. Proportionally, the gender of patients was similar with 41% and 40% of male patients in the full germline and mosaic groups respectively. The remaining 59% and 60% of both groups were female patients.

Classification and number of tumors

All eyes were classified according to the International Intra-ocular Retinoblastoma Classification and were recorded in Table 1. 85 (80%) of HP eyes, 16 (72%) of LP and 54 (57%) of mosaic eyes had O/A/B/C tumors and were deemed gaugeable such that the number of tumors could be assessed.

Total number of tumors in patients with two gaugeable eyes

The total number of tumors was calculated in the 3 groups for patients who had 2 eyes staged as O,A,B or C. For 33 patients in the HP group, the median number was 6.0 (mean 6.42 range 1-14) for 2 eyes. For 5 patients in the LP group, the median number was 2.0 (mean 2.8 range 1-5). For 9 patients in the mosaic group, the median number per patient was 1.0 (mean 2.0 range 1-11).

Number of tumors per eye for gaugeable eyes

1) We assessed the number of tumors per eye in gaugeable eyes that developed new tumors and **excluded** eyes that **never developed tumors**, as this reflected clinical experience when parents were keen to know how many **more** tumors would develop in affected eyes (Table 1). Retinomas were included in this group of tumor foci. 81 (94%) of gaugeable HP eyes had eyes that developed tumors compared to 8 (50%) of LP eyes and 22 (41%) of mosaics. The number of tumors per eye was fewer in patients with mosaicism (median 1.0 mean 1.9 range 1-6) compared to full germline patients with highly penetrant alleles (median 3.0 mean 3.3 range 1-8) ($p<0.0003$ 95% CI 0.5, 2.0). Patients in the LP group had a median of 2.0 tumors (mean 2.4 range 1-4) but only 8 eyes were affected.
2) In addition, we evaluated **all** eyes (including those that never developed a tumor) which were gaugeable. In patients with mosaicism, the median number was 0.0 tumors per eye (mean 0.7 range 0-6) whereas in patients in the HP group, the median number was 3.0 tumors per eye (mean 3.1 range 0-8). Patients with LP pathogenic alleles had a median of 1.0 tumor per eye (mean 1.1 range 0-4) but only 16 eyes were included.

As the practicality of discussing a median of 0 tumors per eye to parents was questionable, we used different methods to assess tumor numbers.

Age at presentation

Age at diagnosis was calculated for patients with Group D or E eyes who presented sporadically. 38 of 47 patients (81%) with mosaicism presented at median age 22 months (range 2-117) compared to 19 of 53 (33%) of HP patients who presented sporadically at median 7 months (range 0.75-33) ($p<0.00002$ 95% CI 8,21). Only 6 of 11 patients with LP had Groups D or E and they presented at median age 27 months (range 12 to 36 months).

Screening under anaesthetic from birth

Despite 59 of 64 full germline cases having a family history at presentation, conventional examination under anaesthesia strategies from birth had been in place for only 29/53 (55%) of the HP group, and only 5/11 (45%) of the LP group were screened. This reflects an earlier era when the screening strategy was being developed. As expected, no child with mosaicism was screened from birth.

Laterality and age for bilaterality

Presentation with bilateral retinoblastoma was seen in the majority 41/53 (77%) of HP cases in contrast to LP cases with 3/11 (27 %) and 13/47 (28%) of mosaic patients. Conversion from unilateral disease to bilateral disease occurred in 8/12 (67%) of unilateral HP group cases (median age 5.5 months, mean 6.2, range 3-12) with 49/53 (92%) of all cases eventually being bilateral. All eventual bilateral cases

were screened from birth. None of the eight LP group patients with unilateral disease converted to bilateral disease. Only 2 of 34 unilateral patients with mosaicism converted to bilateral disease (mean age 8.5 months, median 8.5, range 8-9). HP patients with unilateral disease were at 11 times increased risk of developing bilateral disease when compared to mosaic patients with unilateral disease (RR 11.3, 95% CI 2.8, 46.1).

Level of leukocyte DNA mosaicism and correlation with laterality and number of tumors

All patients with mosaicism (32% bilateral; 68% unilateral) had pathogenic variants that were deemed HP. Levels of leukocyte mosaicism were classified as low if the variant was less than 20%, medium if 21-30% and high if 31-40%. Nine of 15 (60%) children who had high levels of mosaicism presented as, or became, bilateral..This compares with only 3 of 22 (14%) patients with low level mosaicism who were bilateral. 7/10 (70%) of patients with medium level and 19/22 (86%) of patients with low level mosaicism were associated with unilateral disease. The number of tumors in affected eyes with mosaicism ranged from 1 to 6 and all 3 children who had more than 2 tumors in one or either eye (unilateral or bilateral) had high levels of mosaicism.

Genotype and number of tumors

We attempted to assess the number of tumors for the same genotype in either HP or LP groups and compare with the mosaic group. In this cohort of patients, we did not see LP pathogenic variants in any mosaic carriers. Only 3 genotypes (all HP)

overlapped as shown in Table 2. (1) c. 958C>T (exon 10): 11 tumors between 2 eyes in the HP group, but 1 tumor between 2 eyes in the low level mosaic group. (2) c. 1654C>T (exon 17): 7 tumors between 2 eyes in the HP group, but 1 tumor between 2 eyes in the medium level mosaic group. (3) c. 2501C>G (exon 24): 5 tumors between 2 eyes in the HP group, but 1 tumor between 2 eyes in the high level mosaic group.

Treatments

Patients within this cohort were categorised into no systemic treatment (use of laser/cryotherapy/radioactive plaque/enucleation), intravenous chemotherapy (both primary and post-enucleation adjuvant chemotherapy) and/or external beam radiation therapy (EBRT: both lens-sparing and whole eye). In the HP group, treatment information was available for 46/53 (87%) patients. 19 of those patients (41%) had an enucleation and 2 received adjuvant chemotherapy. Altogether 25/46 (54%) had systemic chemotherapy, 14/46 (30%) had EBRT and only 7/46 (6%) had neither. 2 patients had both systemic chemotherapy and EBRT. In the LP group, of 11 patients, six had systemic chemotherapy (55%), 2 had external beam radiotherapy (18%) and 3 (27%) had local treatment throughout. In the mosaic group, 25/47 (53%) had systemic chemotherapy, 3/47 (6%) had EBRT and 19/47 (40%) had neither with 18 (38%) having enucleations.

DISCUSSION

Parents who have suffered from retinoblastoma themselves are keen to know the number of new retinoblastoma tumors that might develop in their children's eyes as soon as they are diagnosed. Parents of children with one eye affected also want to know the chance of bilaterality. This study attempts to address these questions. Hence, we provide prognostic information from the identification of different genetic categories of potentially heritable retinoblastoma.

Giving parents information of the number of tumors per eye is practical and useful. This is because parents are often distressed when a tumor develops in a previously unaffected eye and they would like to know how many more might develop. We analysed the data in two ways. When all O,A,B,C eyes in patients with mosaic *RB1* alleles were considered, the median number of tumors per eye was 0.0 tumors and we felt this was not meaningful. When only eyes that were affected were included (excluding eyes that did not express disease), the median number of tumors per eye was 1.0 for the mosaic group. We felt this was more useful for parents who had a child with one tumor in one eye and were concerned if more tumors would develop. The median number per eye was 3.0 for HP cases using both analyses.

We found that in mosaic carriers, 15/47 (35%) were or became bilateral. Interestingly, of the 34 mosaic patients with unilateral disease, 32 (32/34; 94%) remained unilateral, which is important information to provide to parents. The two patients who converted from unilateral disease to bilateral disease presented very early (under 3 months of age) with a group E eye and converted 6 months later. No patient with a mosaic pathogenic variant converted to bilateral disease after 9 months of age.

Genotype-phenotype correlations with respect to the genetic subcategories of HP and LP have been based upon DER as defined by ratio of affected eyes to patients

carrying pathogenic variants. Historically, a disease eye ratio of greater than 1.5 denoted high penetrance disease and less than 1.0 low penetrance[3, 10]. With the advent of increased genetic knowledge, the definition of HP and LP are based upon genetic databases[1, 3] rather than DER.

Although one would expect mosaic carriers[11, 12] to be unilateral rather than bilateral and to have an older age at presentation compared to high penetrant disease, this has not been borne out in some previous studies. Rushlow *et al*[13] analysed 45 patients with mosaicism and demonstrated that 23 (51%) were bilateral and only 22 (49%) were unilateral compared to 28% and 72% respectively in this study. Kivela[14] assessed 13q14 deletions and demonstrated no difference in age and laterality between the 29 mosaics and 107 non-mosaics. However, large deletions including the *MED4* gene have a milder non-ocular phenotypic expression[15] and may behave as LP variants with respect to retinoblastoma. In such cases, the differences will not be clear cut in contrast to HP disease and mosaicism. Neither study assessed the number of tumors. Nor did Rodriguez-Martin *et al*[16] whose study showed 14% of 100 bilateral, and 31% of 45 unilateral patients displayed mosaicism. However, they reported that mosaicism was associated with late onset retinoblastoma particularly in unilateral patients which we have also found. In addition, our criteria for high level mosaicism (31%-40%) is below their conservative upper threshold for high level mosaicism (43%)[16] which is reassuring.

We found a correlation between variant percentage in leukocyte DNA and laterality with 60% (9 of 15) of mosaic patients with bilateral retinoblastoma (at final follow-up) having a high level of mosaic pathogenic variant compared to only 20% (3 of 15) with low level mosaicism. In addition, all 3 patients (4 eyes) with more than 2 tumors per eye had high level mosaicism. The percentage of white blood cells affected

355 correlates with the eye involvement and also the number of tumors per eye. Of
356 interest, all pathogenic variants in mosaic carriers were considered as high penetrant
357 pathogenic variants and there was a stark contrast regarding number of tumors
358 between full germline and mosaic carriers (Table 2). There are no reported LP
359 mosaic carriers to our knowledge. It is possible that very low level LP mosaic carriers
360 may not develop the disease due to maintaining sufficient levels of active
361 retinoblastoma protein.

362 In the literature, the number of tumors in affected eyes with familial retinoblastoma
363 has been reported in germline (HP together with LP), with means of 2.19[17] and
364 3.15[5] recorded. Using calculations from original data (Lohmann[18] *et al*), we found a
365 mean of 2.8 tumors per eye in HP (nonsense variants) and 2.5 in LP variants (splice
366 site and frameshift). In this study, we found a mean of 3.3 tumors per eye (median
367 3.0) in HP patients and a mean of 2.4 tumors (median 2.0) in 8 LP eyes and present
368 data for patients carrying mosaic pathogenic variants for the first time.

369 The treatments given may affect the number of tumors formed. New tumor formation
370 has been assessed with systemic chemotherapy for Reese-Ellsworth Groups I to III
371 (equivalent to A, B and C in IIRC or cT1 and cT2a in the AJCC)[19]:for seven patients,
372 36 new tumors developed in 11 eyes (mean 3.2).This is comparable to the mean of
373 3.3 tumors per eye in HP patients noted in this study with different treatment
374 modalities. It has been suggested that systemic chemotherapy may delay the onset
375 of new tumors, but ethically it is difficult to conduct a comparative trial to prove this.
376 We had similar proportions of HP, LP and mosaic patients who had systemic
377 chemotherapy (53-55%). Similarly for EBRT, new tumor development can be
378 retarded but comparison with purely local treatment groups has proven difficult[20, 21].

Giving figures related to conversion of unilateral disease to bilateral disease is useful for families. Only a small proportion (6%) of mosaic patients converted from unilateral to bilateral disease and this may be related to the older age of non-screening (sporadic) presentation compared to the majority of germline patients who were screened from birth. But only 32% (15/47) of mosaics were eventually bilateral compared to 92% (49/53) of HP patients. Although we did not find any child who converted from unilateral to bilateral disease after the age of 12 months, Temming *et al*[22] noted 3 patients who converted to bilateral disease after this age in their 1961-2006 cohort.

Next generation sequencing is better able to detect low level mosaics[16] and future studies may be able to delineate these findings more accurately.

Limitations

We assessed LP patients for the number of tumors but we had data for only 8 eyes with gaugeable eyes and tumor development.It is difficult to make conclusions based upon this limited sample size. We had insufficient clinical information for 26 patients which may have affected results.

Treatment may have had an impact on the number of tumors per eye recorded. We limited our assessment of number of tumors to only eyes without substantial subretinal and vitreous seeding which reduced the number of eyes being assessed. Systemic chemotherapy and temporal approach EBRT (including lens sparing) may have treated the eye with the more aggressive disease, but also the fellow eye without disease. Similar proportions of HP and mosaic patients had systemic chemotherapy. We only had 7 patients in the HP group who had neither treatment.

No patients had first line intra-arterial chemotherapy, which can be systemically absorbed and may have an impact on both eyes despite being given to one eye.

We may have been unable to detect low level mosaics with unilateral disease and instead labelled them as having non-heritable somatic pathogenic variants (and excluded them from this study) due to the limitations of technology used. We are reassured as we screened the offspring of the patients via examinations under anaesthesia and did not find any affected. However, this is not completely confirmatory.

Conclusions

In summary, this is the first study to demonstrate increased unilateral disease (rather than bilateral) and fewer tumors per eye for mosaicism compared to high penetrant disease in retinoblastoma. The expected number of tumor foci in patients with somatic mosaicism is lower compared to full germline patients heterozygous for the same variant *RB1* allele. Details regarding number of tumors can be provided to parents/guardians for prognostic information for different categories of potentially heritable retinoblastoma

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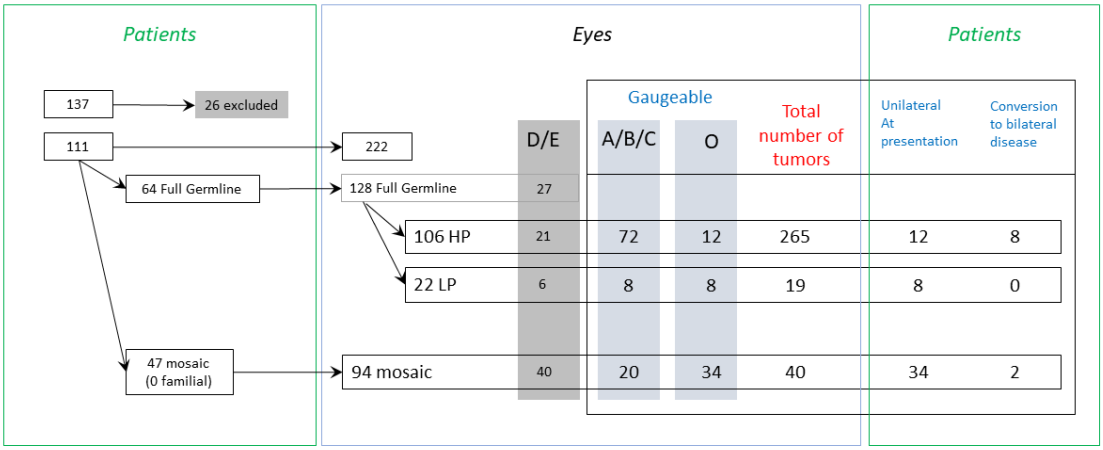
Figure 1. A flow chart demonstrating the selection of the groups



491 Table 1. Characteristics for genetic subtypes of *RB1* pathogenic variants

	HP	LP	Mosaic
Patients (eyes)	53 (106)	11 (22)	47 (94)
Group O	12 (12%)	8 (36%)	34 (36%)
Group A-C	72 (68%)	8 (36%)	20 (21%)*
Group D-E	21 (20%)	6 (28%)	40 (43%)
Gaugeable eyes that developed tumors	81	8	22
No of Tumors per eye (affected eyes only) Median (mean,range)	3.0 (3.9,1-7)	2.0 (2.4,1-4)	1.0 (1.9, 1-6)
Age (months) at diagnosis for sporadic cases Median (mean, range)	7.00 (8.42,0.75-33)	27.00 (25.00,12-36)	21.00 (25.11, 2-117)
Management			
Screened under anaesthetic	29 (55%)	5 (45%)	0
Systemic Chemotherapy	21	4	22
Radiotherapy	12	2	3
Both	6	1	1
None	5	3	20
Incomplete Information	9	1	1
Enucleation	19 (36%)	6	37 (79%)
Unilateral at presentation	12	8	34
Stayed unilateral	4 (33%)	8	32 (94%)
Became bilateral	8	0	2

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493 *including 2 eyes with retinomas

Table 2. *RB1* genotypes present in both the full germline and mosaic groups

RB1 Nonsense Variant	Full germline HP group (both eyes)	Mosaic group (both eyes)
c. 958C>T exon 10	11 tumours	1 tumour (low level)
c. 1654C>T exon 17	7 tumours	1 tumour (medium level)
c. 2501C>G exon 24	5 tumours	1 tumour (high level)

Supplementary Tables

Presentation	Age at diagnosis (m)	*RB1* g. no. L11910.1	*RB1* c. no. LRG_517t1 (*RB1*)	*RB1* ex/int/pro	Putative consequence	Final laterality (U/B)	Tumour no/eye
A/A	0.50		c.(?_-166)_(264+1_265-1)del	pro-int2 del	expression	B	3
A/A	0.50	g.162298C>A	c.2420C>A	ex23	p.(S807*)	B	2.5
A/A	1.50	del(13)(q14.1q21)		del*RB1*x1	no pRb	B	2
A/A	3.00		c.(1215+1_1216-1)_(1332+1_1333-1)del	ex13 del	p.(406_444)del	B	2.5
A/A	3.00	g.73801dupA	c.1264dupA	ex13	p.(I422Nfs*6)	B	3.5
A/B	0.25		c.(?_-166)_(264+1_265-1)del	pro-int2 del	expression	B	7
A/B	0.25	g.64348C>T	c.958C>T	ex10	p.(R320*)	B	5.5
A/B	1.00		c.(1695+1_1696-1)_(*1815_?)del	ex18-beyond 3'del	no pRb	B	2.5
A/B	1.00	g.170383C>G	c.2501C>G	ex24	p.(S834*)	B	2.5
A/B	1.25	g.59759_59778del20	c.827_846del20	ex8	p.(L277*)	B	3
A/B	3.00		c.(1695+1_1696-1)_(*1815_?)del	ex18-beyond 3'del	no pRb	B	1.5
A/B	4.00	g.56,963-56,964insAT	c.718_719insAT	ex7	p.(K240Nfs*25)/splice	B	1.5
A/D	7.00	g.59683C>T	c.751C>T	ex8	p.(R251*)	B	3
A/O	0.50	g.45867G>T	c.607+1G>T	int6	sd/ex 6 skip/ p.(I181Gfs*8)	B	1.5
A/O	0.50	g.45867G>C	c.607+1G>C	int6	sd/ex 6 skip/ p.(I181Gfs*8)	B	3
A/A	1.00	g.162112T>G	c.2325+2T>G	int22	sd	B	5.5
B/A	0.25	g.150062_150071del10	c.1760_1769del10	ex18	p.(E587Vfs*21)	B	1
B/A	1.00	g.56862T>A	c.617T>A	ex7	p.(L206*)	B	2.5
B/A	10.00	g.2104_2135del32	c.45-76del32	ex1	p.(A17Pfs*3)	B	1
B/B	0.25	g.39478G>A	c.297G>A	ex3	p.(W99*)	B	2
B/B	0.75	g.41954G>T	c.409G>T	ex4	p.(E137*)	B	4.5
B/B	1.50		c.(1695+1_1696-1)_(*1815_?)del	ex18-beyond 3'del	no pRb	B	5.5
B/B	1.00	g.77080A>T	c.1498+3A>T	int16	sd	B	1.5
B/B	6.00	g.70240A>G	c.1128-2A>G	int11	sa	B	3.5
B/B	6.00	g.39562G>T	c.380+1G>T	int3	sd/ex 3 skip/ p.(G89Cfs*3)	B	3.5
D/O	2.00		c.(?_-166)_(*1815_?)del	del*RB1*x1	no pRb	B	2
B/D	0.75	g.42018T>A	c.473T>A	ex4	p.(L158*)	B	4

B/D	9.00	g.45867G>C	c.607+1G>C	int6	sd/ex 6 skip/ p.(I181Gfs*8)	B	1
B/E	2.00	g.76460C>T	c.1363C>T	ex14	p.(R455*)	B	2
B/E	24.00	g.162364C>A	c.2486C>A	ex23	p.(S829*)	B	7
B/O	0.50	g.45844G>A	c.585G>A	ex6	p.(W195*)	B	3.5
B/O	1.00	g.64348C>T	c.958C>T	ex10	p.(R320*)	B	2.5
B/O	7.00		c.(1695+1_1696-1)_(*1815_?)del	ex18-beyond 3'del	no pRb	U	1.5
C/B	10.00	g.76894delA	c.1395delA	ex15	p.(E466Nfs*12)	B	5.5
B/B	2.00	g.78238C>T	c.1654C>T	ex17	g.(R552*)	B	3.5
C/C	4.00	g.61733A>T	c.865A>T	ex9	p.(K289*)	B	5
D/D	5.00	g.70330G>A	c.1215+1G>A	int12	sd/ex 12 skip/ p.(V378Afs*3)	B	NA
C/D	9.00	g.64348C>T	c.958C>T	ex10	p.(R320*)	B	5
C/E	1.00	g.39445G>A	c.265-1G>A	int2	sa	B	6
C/O	1.25	g.70330G>A	c.1215+1G>A	int12	sd/ex 12 skip/ p.(V378Afs*3)	B	5
C/O	36.00	g.150050delC	c.1748delC	ex18	p.(T583Mfs*28)	U	0.5
D/B	1.25	g.170405_170408delGAGT	c.2520+3_2520+6delGAGT	int24	sd/ex 24 skip/ (p.I831Lfs*8)	B	4
D/B	9.00	g.77078G>T	c.1498+1G>T	int16	sd	B	1
D/C	4.00	g.153354_153359delGTTAGTins22	c.1960+1_1960+6delGTTAGTins22	int19	sd/ex 19 skip/ p.(M605Ifs*14)	B	5
D/C	33.00	g.2079delG	c.20delG	ex1	p.(R7Qfs*58)	B	1
E/B	2.00	g.59646_59649delTACAins18	c.719-5_719-2delTACAins18	int7	sa	B	6
E/B	7.00	g.73809_73818dup10	c.1272_1281dup10	ex13	p.(E428Hfs*3)	B	4
D/D	11.00	g.162237C>T	c.2359C>T	ex23	p.(R787*)	B	NA
O/D	13.00	g.162237C>T	c.2359C>T	ex23	p.(R787*)	U	NA
E/O	16.00	g.45867G>T	c.607+1G>T	int6	sd/ex 6 skip/ p.(I181Gfs*8)	U	NA
E/C	4.00	g.76460C>T	c.1363C>T	ex14	p.(R455*)	B	2
O/A	2.00	g.149997G>A	c.1696-1G>A	int17	sa	B	5.5

O/C	0.50	g.59683C>T	c.751C>T	ex8	p.(R251*)	B	1.5

Table 2a. Pathogenic variant data for High Penetrant *RB1* variant patients.

Genomic (g.) nucleotide numbering is according to GenBank sequence accession number L11910.1. In cDNA (c.) nucleotide numbering c.1 is the A of the ATG translation initiation codon based on the Locus Reference Genomic Sequence LRG_517t1 (*RB1*). Variant nomenclature is according to Human Genome Variation Society guidelines (www.hgvs.org). ex – exon, int- intron, pro- promoter, sd- splice donor, sa- splice acceptor, g- germline, U- unilateral, B- bilateral. NA- not applicable

Presentation	**Age at diagnosis (m)**	***RB1* g. no. L11910.1**	***RB1* c. no. LRG_517t1 (*RB1*)**	***RB1* ex/int/pro**	**Putative consequence**	**Final laterality(U/B)**	**Tumour no/eye**
B/B	0.25	g.160834G>C	c.2211G>C	ex21	p.(E737D)/sd	B	2.5
O/B	4	g.149996A>G	c.1696-2A>G	int17	sa	U	1
A/D	12	g.45867G>T	c.607+1G>T	int6	sd/ex 6 skip/p.(I181Gfs*8)	B	2
B/O	14	g.2104_2135del32	c.45_76del32	ex1	p.(A17Pfs*3)	U	2
B/O	14	g.1867T>A	c.-193T>A	pro	expression	U	0.5
O/D	14	g.1862G>A	c.-198G>A	pro	expression	U	0.5

E/O	20	g.156713C>T	c.1981C>T	ex20	p.(R661W)	U	NA
B/O	32	g.65378_65379delGA	c.1064_1065delGA	ex11	p.(R355Nfs*6)	U	1
E/B	34	g.156713C>T	c.1981C>T	ex20	p.(R661W)	B	3
E/O	34	g.59793G>A	c.861G>A	ex8	p.(E287=)/sd	U	NA
O/E	36	g.156713C>T	c.1981C>T	ex20	p.(R661W)	U	NA

Table 2b. Pathogenic variant data for Low Penetrant *RB1* variant patients.
Genomic (g.) nucleotide numbering is according to GenBank sequence accession number L11910.1. In cDNA (c.) nucleotide numbering c.1 is the A of the ATG translation initiation codon based on the Locus Reference Genomic Sequence LRG_517t1 (*RB1*). Varaint nomenclature is according to Human Genome Variation Society guidelines (www.hgvs.org). ex – exon, int- intron, pro- promoter, sd- splice donor, sa- splice acceptor, g- germline, U- unilateral, B- bilateral. NA- not applicable

Presentation	Age at diagnosis (m)	*RB1* g. no. L11910.1	*RB1* c. no. LRG_517t1(*RB1*)	Exon/intron	Putative consequence	Final Laterality U/B	Tumour no/eye	Mosaic level
			27					
E/O	2	g.59794G>A	c.861+1G>A	int8	sd	B	1	Medium
O/E	3	g.150117G>C	c.1814+1G>C	int18	sd	B	4	High
B/C	7	g.162317T>G	c.2439T>G	ex23	p.(Y813*)	B	5.5	High
A/C	8	g.73843C>T	c.1306C>T	ex13	p.(Q436*)	B	1.5	Low
D/C	9	g.150037C>T	c.1735C>T	ex18	p.(R579*)	B	2	Medium
O/D	9	g.2121delC	c.62delC	ex1	p.(P21Rfs*44)	U	NA	Low
O/D	20	g.65363G>A	c.1050-1G>A	int10	sa	U	NA	Low
E/O	9	g.76898C>T	c.1399C>T	ex15	p.(R467*)	U	NA	Medium
A/E	10	g.78217G>T	c.1633G>T	ex17	p.(E545*)	B	1	Medium
D/O	10	g.76430C>T	c.1333C>T	ex14	p.(R445*)	U	NA	Low
E/O	10	g.156774G>A	c.2042G>A	ex20	p.(W681*)	U	NA	Low
D/C	11	g.70298_71084delinsTG	c.1184_1215+755delinsTG	ex12	p.(Q395_N405 delins43)	B	2	High
O/C	11	g.78,152_78,155dupTAAA	c.1568_1571dupTAAA	ex17	p.(K524Nfs*5)	U	0.5	Medium
O/D	11		c.(?_-166)_(*1815_?)del	del*RB1*x1	no pRb	U	NA	High
D/O	11		c.(?_-166)_(*1815_?)del	del*RB1*x1	no pRb	U	NA	Low
C/O	11	g.78238C>T	c.1654C>T	ex17	p.(R552*)	U	0.5	Medium
O/D	12	g.153352dupA	c.1959dupA	ex19	p.(V654Sfs*14)	U	NA	Medium
D/E	12	g.76921G>C	c.1421+1G>C	int15	sd	B	NA	High
E/B	13	g.70004_70672del	c.1128-238_1215+343del	ex 12 skip	p.(V378Afs*3)	B	2	High
O/E	13	g.56,903-56,909del7	c.658_664del7	ex7	p.(L220Sfs*42)	U	NA	High
O/D	14	g.76910C>T	c.1411C>T	ex15	p.(Q471*)	U	NA	Low
O/D	18	g.64348C>T	c.958C>T	ex10	p.(R320*)	U	NA	Low
O/D	20	g.150037C>T	c.1735C>T	ex18	p.(R579*)	U	NA	Low
E/B	22	g.156785C>T	c.2053C>T	ex20	p.(Q685*)	B	1	Low
D/B	24	g.70280T>A	c.1166T>A	ex12	p.(L389*)	B	5	High
O/D	24	g.76975_77081del107	c.1422-26_1498+4del107	int15_int16	p.(S474Rfs*)	U	NA	Low
O/D	25	g.59695C>T	c.763C>T	ex8	p.(R255*)	U	NA	Medium
O/C	25	g.64348C>T	c.958C>T	ex10	p.(R320*)	U	0.5	Low
C/O	27		c.(1695+1_1696-1)_(*1815_?)del	ex18-beyond 3'del	no pRb	U	0.5	High
A/E	28	g.153352delA	c.1959delA	ex19	p.(V654Cfs*4)	B	1	High
D/O	28	g.76460C>T	c.1363C>T	ex14	p.(R455*)	U	NA	Medium
E/O	30	g.78250C>T	c.1666C>T	ex17	p.(R556*)	U	NA	Low
O/B	30	g.170383C>G	c.2501C>G	ex24	p.(S834*)	U	0.5	High

O/E	54	g.156785C>T	c.2053C>T	ex20	p.(Q685*)	U	NA	Low
O/D	43	g.76932_76952del21	c.1421+12_1421+32del21	int15	sd	U	NA	Low
D/O	43	delint23_int26	c.2489+1_2490-1)_(2713+1_2714-1)del	ex24-26	p.(R830Sfs*14)	U	NA	Low
D/O	44	g.78238C>T	c.1654C>T	ex17	p.(R552*)	U	NA	Low
O/E	45	g.76898C>T	c.1399C>T	ex15	p.(R467*)	U	NA	Low
O/D	57	g.153352delA	c.1959delA	ex19	p.(V654Cfs*4)	U	NA	High
O/C	59	g.65386C>T	c.1072C>T	ex11	p.(R358*)	U	0.5	Low
O/D	60	g.59695C>T	c.763C>T	ex8	p.(R255*)	U	NA	Low
Retinoma/O	100		gainex3_ex23	tandem repeat of ex3_23 at the transcript level	p.(I831Efs*22)	U	0.5	High
D/ Retinoma	117	g.162093C>T	c.2308C>T	ex22	p.(Q770*)	B	2	High
E/D	24	g.162069C>T	c.2284C>T	ex22	p.(Q762*)	B	NA	High
B/D	24	g.160785_160791dupTCAAAAT	c.2152_2168dupTCAAAAT	ex21	p.(I724Qfs*29)	B	1	Low
O/D	45	g.150037C>T	c.1735C>T	ex18	p.(R579*)	U	NA	Medium
O/D	29	g.162237C>T	c.2359C>T	ex23	p.(R787*)	U	NA	Low

Table 2c. Genetic mutation data for mosaic carriers of the *RB1* mutation. Genomic (g.) nucleotide numbering is according to GenBank sequence accession number L11910.1. In cDNA (c.) nucleotide numbering c.1 is the A of the ATG translation initiation codon based on the Locus Reference Genomic Sequence LRG_517t1 (*RB1*). Mutation nomenclature is according to Human Genome Variation Society guidelines (www.hgvs.org). ex – exon, int- intron, pro- promoter, sd- splice donor, sa- splice acceptor, g- germline, m- mosaic, U- unilateral, B- bilateral. NA- not applicable