RESEARCH ARTICLE

Mutation spectrum of RB1 mutations in retinoblastoma cases from Singapore with implications for genetic management and counselling

Swati Tomar1, Raman Sethi1, Gangadhara Sundar2, Thuan Chong Quah1, Boon Long Quah3, Poh San Lai1*

1 Department of Paediatrics, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, Singapore, 2 Department of Ophthalmology, National University Hospital, Singapore, Singapore, 3 Singapore National Eye Centre, Singapore, Singapore

* paelaps@nus.edu.sg

Abstract

Retinoblastoma (RB) is a rare childhood malignant disorder caused by the biallelic inactivation of RB1 gene. Early diagnosis and identification of carriers of heritable RB1 mutations can improve disease outcome and management. In this study, mutational analysis was conducted on fifty-nine matched tumor and peripheral blood samples from 18 bilateral and 41 unilateral unrelated RB cases by a combinatorial approach of Multiplex Ligation-dependent Probe Amplification (MLPA) assay, deletion screening, direct sequencing, copy number gene dosage analysis and methylation assays. Screening of both blood and tumor samples yielded a mutation detection rate of 94.9% (56/59) while only 42.4% (25/59) of mutations were detected if blood samples alone were analyzed. Biallelic mutations were observed in 43/59 (72.9%) of tumors screened. There were 3 cases (5.1%) in which no mutations could be detected and germline mutations were detected in 19.5% (8/41) of unilateral cases. A total of 61 point mutations were identified, of which 10 were novel. There was a high incidence of previously reported recurrent mutations, occurring at 38.98% (23/59) of all cases. Of interest were three cases of mosaic RB1 mutations detected in the blood from patients with unilateral retinoblastoma. Additionally, two germline mutations previously reported to be associated with low-penetrance phenotypes: missense-c.1981C>T and splice variant-c.607+1G>T, were observed in a bilateral and a unilateral proband, respectively. These findings have implications for genetic counselling and risk prediction for the affected families. This is the first published report on the spectrum of mutations in RB patients from Singapore and shows that further improved mutation screening strategies are required in order to provide a definitive molecular diagnosis for every case of RB. Our findings also underscore the importance of genetic testing in supporting individualized disease management plans for patients and asymptomatic family members carrying low-penetrance, germline mosaicism or heritable unilateral mutational phenotypes.
Introduction

Retinoblastoma (RB) is a retinal cancer associated with biallelic loss of \textit{RB1} gene. The global incidence of this disease is 1 case in 15,000 to 20,000 live births [1] with the average annual incidence in Singapore reported as 2.4 to 11.1 cases per million children [2,3] occurring equally among males and females [4]. On a global scale, an estimated 3001 to 3376 children die due to retinoblastoma annually [1]. The mortality rate in Asia (39%) is much higher than that of Europe, Canada, and the USA (3–5%) [1] due to the gap in healthcare access which primarily refers to the fact that majority of RB patients are diagnosed in low- and middle-income countries, whereas the bulk of retinoblastoma-specific health care facilities are available in high-income countries [5]. In more developed countries in Asia such as in Singapore, the overall 5-year survival rate can be much higher ranging between 88.1% to 91% [3,4].

Most RB cases are diagnosed by 5 years of age and occur in either heritable or non-heritable forms. Non-heritable RB arises from somatic mutations occurring on both alleles of \textit{RB1} gene in the developing retina, whereas heritable RB arises from the inheritance of at least one germline mutation along with an acquired \textit{RB1} somatic mutation [6]. All bilateral retinoblastomas are heritable, of which about 10% are inherited [6,7]. Fifteen percent of unilateral retinoblastoma occur due to \textit{de novo} germline \textit{RB1} mutations which is transmissible in subsequent generations [8]. In heritable RB, offspring have a 50% chance of inheriting the mutant \textit{RB1} allele from an affected parent. Such an inheritance of the mutant \textit{RB1} allele results in a 97% risk of developing the disease and a high lifelong risk of secondary cancers [8].

\textit{RB1} inactivation has been implicated in more than 97% of all RB cases with mutations in this gene being undetectable in the remaining cases [5]. Recent reports suggest that other genes may play a role in either driving tumor initiation or progression [9,10]. It has been postulated that probable candidate genes may be located in chromosomal regions with recurrent gains [11–15] and losses [16–18] observed in RB tumors. Rushlow et al provided evidence that retinoblastoma could also be caused by \textit{MYCN} oncogene amplification and predicted that 18% of cases who are diagnosed with non-familial unilateral RB before the age of 6 months would harbour only \textit{MYCN} amplification and no \textit{RB1} mutations [9]. They also quoted another 1.5% of unilateral non-familial RB whose pathogenesis could not be explained as they harboured normal \textit{RB1} and \textit{MYCN} genes.

Genetic testing in RB is essential to not only identify the spectrum of underlying mutations but also to delineate heritable RB for non-heritable ones for efficient genetic counselling [5]. Hence, this study aims to characterize the spectrum of \textit{RB1} mutations in RB cases seen among patients in Singapore in order to aid disease management.

Materials and methods

Patients

This study was conducted on DNA samples from a cohort of 59 retinoblastoma cases (18 bilateral and 41 unilateral), collected over a period of 15 years. Diagnosis of retinoblastoma was established by standard ophthalmologic and histological criteria. Thirty-four cases were female and twenty-five were male. When an \textit{RB1} mutation was found in the peripheral blood of the proband, DNA samples from the parents were tested for presence of the identified mutation. If parents tested positive for the proband’s mutation, siblings’ blood were collected and analysed similarly. In addition, parental DNA was sought in cases where a gross deletion in \textit{RB1} gene was identified, to determine the parental origin of the loss of \textit{RB1} allele. Samples from all patients and family members were collected with written informed consent and in accordance
with the principles of the Declaration of Helsinki. The study was approved by the institutional review board of the National University of Singapore.

DNA isolation

DNA samples used were extracted from matched peripheral blood (10ml in EDTA tubes) and fresh tumor samples (100–200 mg), collected after enucleation. DNA isolation protocol was adapted from the high salt extraction method of Miller et al [19].

**RB1** gene sequencing

The DNA obtained from all 59 tumors and corresponding blood samples was sequenced for 27 exons and promoter region of **RB1** gene after PCR amplification using 27 sets of primers as described previously [20]. Some cases were sent out to an international laboratory (Impact Genetics Inc., Canada) for **RB1** gene sequence analysis and Allele-specific PCR (AS-PCR) for eleven recurrent **RB1** mutations. Additional information about **RB1** gene mutations were confirmed from gene locus specific mutation database (rb1-lsdb) and The Human Gene Mutation Database (HGMD). Predictive analysis tools were used to determine the pathogenicity status of novel variants. Missense mutations were analyzed by SIFT (http://sift.jcvi.org/www/SIFT_BLink_submi.html), CADD (http://cadd.gs.washington.edu/score) and Mutation taster (http://www.mutationtaster.org/), while all frameshift variants were predicted by PROVEAN (http://provean.jcvi.org) and Mutation Taster, respectively.

Gross **RB1** deletions analysis

**Multiplex ligation-dependent probe amplification (MLPA) analysis.** To screen for deletions or duplications in the **RB1** gene, MLPA analysis was done using the SALSA MLPA kit P047-B1 **RB1** (MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer’s protocol with 100 ng of genomic DNA from matched tumor and blood. The PCR amplicons were separated on Genetic Analyzer 3130 (Applied Biosystems, Foster City, CA), and the results were analyzed using Coffalyser Software available at http://www.mlpa.com/coffalyser/. Based on the normalized signal value ratio of 1:1; threshold ratios of 0.75 (deletion) and 1.30 (duplication) were used to indicate loss or gain of probe copy numbers respectively.

**Microsatellite analysis and SNP genotyping.** The extent of loss of heterozygosity (LOH) was assayed in matched tumor and blood DNA using 20 flanking extragenic microsatellite markers (S1 Table). Allelic imbalance affecting **RB1** gene locus at 13q14 was examined using three intragenic microsatellites: D13S153—located within intron 2 of **RB1**, dinucleotide repeats (TG)$_{22}$—located within intron 4 and tetra nucleotide repeats (TTCT)$_{16}$—located within intron 20 of **RB1** along with four previously reported SNP markers [21–24] (S2 Table). The SNP markers and Microsatellite markers were typed using standard PCR-based methods as described previously [17] and samples were scored as informative if the lymphocyte DNA showed heterozygosity of alleles for each marker, or non-informative for homozygosity or positive for LOH when the tumour showed complete loss of one allele [25]. LOH was ascertained when loss of one of the alleles in the tumour samples was observed whereas the matched lymphocyte sample showed heterozygous alleles.

Methylation specific PCR (MSP)

Methylation analysis at the CpG islands of **RB1** Promoter in tumor and blood was analyzed using CpGenome™ DNA Modification kit (Intergen) and methylation specific PCR using specific primers as previously described [26–28]. For **MGMT** promoter hypermethylation
analysis, primers were synthesized using Primo MSP 3.4 software (http://www.changbioscience.com/primo/primom.html) based on MGMT promoter sequence (GenBank Acc. No. X61657). MSP was performed in two separate reactions to identify unmethylated and methylated DNA as described previously [28].

Quantitative multiplex PCR (QM-PCR)
QM-PCR studies were performed on tumor samples to determine copy number of TNF (6p21.3) using methods previously described [29–31]. A positive control, DNA from the WERI-RB1 retinoblastoma cell line which has isochromosome 6p (i6p) and therefore carrying 4 copies of the chromosomal region 6p [32], and a normal DNA as external control was amplified together with tumour samples in each PCR reaction. The PCR products were prepared as described previously for genotyping on the ABI PRISM 3130xl Sequencer [17]. The results were analyzed using the GeneMapper® Software v4.0. The copy number in tumour sample was compared with the normal and positive control sample in each case. The range for diploid or normal two-copy number was calculated using a series of normal DNA samples. The values obtained from QM-PCR of the WERI-RB1 cell line were used to indicate minimum value above which more than two copies of the gene are expected in the test sample.

Statistics
To determine differences in the frequencies of observed types of RB1 point mutations in our cohort and those from worldwide mutation frequencies from a reported meta-analysis by Valverde et al 2005 [33], the χ² goodness-of-fit test was performed. Fisher’s exact t test was performed to test the significance of all contingency tables in the study. Welch’s t test was done to test the significance of age distribution by different categories. P value of <0.05 was considered significant.

Results
Age at diagnosis
The mean age at presentation of the disease in our data set of 59 cases (22.1± 16.5 months) was slightly lower than what was reported in a previous clinical study on 51 Singaporean patients (25.7± 19.9 months) [4]. However, the overall frequency of bilateral cases in our study (30.5%, 18/59) was similar to the above study (31.4%, 16/51) [4]. A summary of cases by their respective clinicopathological characteristics is given in Table 1. When the age of patients with somatic point mutations (25 unilateral cases) was compared to those with germline point mutations (18 bilateral + 7 unilateral cases), the distribution was found to be statistically significant by Welch’s t test of unpaired groups (Somatic group: mean age at diagnosis = 28.71 months, standard deviation = 18.51 months; Germline group: mean age at diagnosis = 14.71, standard deviation = 11.46 months; p = 0.001824, 95% CI [-22.5448, -5.469]). The overall trend of age at diagnosis for the three categories of patients, as given in Fig 1, shows that the patients with heritable mutational events (Familial and Non-Familial) were diagnosed earlier than those with non-heritable mutations (Welch’s t test, p = 0.001351). We did not have age at diagnosis available for three cases and hence those cases were not considered for this comparison. Additionally, patients with germline nonsense mutations presented at a younger age as compared to those with somatic nonsense mutations (p = 0.07244). Lastly, there was a significant correlation between earlier age of diagnosis for bilateral cases with point mutations to
that of unilateral cases with point mutations \((p = 0.02649; 95\% \text{ CI } [-18.301, -1.1901])\). This correlation did not hold significant when compared by gender of the cases \((p = 0.7733; 95\% \text{ CI } [-7.8444, 10.4827])\).

### Mutations identified in RB1

Among the 118 RB1 alleles examined from the 59 RB cases, 98 mutant alleles were identified \((83.05\%)\) (Table 2). The percentage of mutated alleles identified within the unilateral and bilateral cases are shown in Table 2. The types of RB1 mutations carried by these alleles were point mutations, gross deletions and promoter methylation.

|                      | Heritable | Non-Heritable | Uncategorized* | Total |
|----------------------|-----------|---------------|----------------|-------|
| All patients         | 25        | 31            | 3              | 59    |
| Laterality           |           |               |                |       |
| Bilateral            | 17        | 1             | 0              | 18    |
| Unilateral           | 8         | 30            | 3              | 41    |
| Age at Diagnosis (months)* |          |               |                |       |
| ≤12                  | 12        | 6             | 2              | 20    |
| ≤24                  | 8         | 9             | 0              | 17    |
| <36                  | 2         | 4             | 0              | 6     |
| ≥36                  | 1         | 11            | 1              | 13    |
| Family History       |           |               |                |       |
| Familial             | 2         | 0             | 0              | 2     |
| Non-Familial         | 23        | 31            | 3              | 57    |

* Three cases had unknown age at diagnosis and hence not reflected.

\(^*\) No RB1 mutations could be detected in these cases.

https://doi.org/10.1371/journal.pone.0178776.t001

![Fig 1. Age distribution of heritable and non-heritable RB cases.](https://doi.org/10.1371/journal.pone.0178776.g001)
Spectrum of **RB1** point mutations. A total of 61 point mutations were identified in 84.7% (50/59) of all our retinoblastoma cases. This comprised 100% (18/18) of bilateral cases and 78% (32/41) of unilateral cases (Fig 2). The spectrum of different mutation types among the 61 point mutations were; nonsense mutations occurring at 55.7% (34/61), followed by 24.6% (15/61) frameshift, 9.8% (6/61) splicing, 8.2% (5/61) missense and 1.64% (1/61) promoter alterations (Fig 3). Exons coding for pocket domains of pRB, involved in regulation of transcription, (exons 12–18, domain A and 19–23, domain B) harbored 57.4% (35/61) of all point mutations. In our cohort, no mutations were observed for exons 1, 4, 5, 6, 9, 13, 22 and 25–27 (Fig 4). The graphical representation of all identified mutations spanning the entire **RB1** gene is shown in Fig 4. Ten novel **RB1** variants, representing 16.4% (10/61) of all the mutations identified were found in 3 bilateral (germline) and 7 unilateral (somatic) tumors (Table 3). All novel mutations except the promoter variant were predicted to be deleterious by four commonly used *in silico* analyses tools namely: PROVEAN, Mutation Taster, SIFT and CADD. The chi square test for independence for novel variants v/s known variants; germline v/s somatic variants and variants present in bilateral v/s unilateral cases did not show any statistical significance. In addition, 9 point mutations occurred more than once in 23 unrelated RB cases (16 unilateral and 7 bilateral) as shown in Table 4. These recurrent mutations comprised 44.3% (27/61) of all the identified point mutations. The most frequent recurring mutation was—p.Arg320 (Exon 10), which was found in five different unrelated cases. It was followed by p.Arg358 (Exon 11) and p.Arg455 (Exon 14) variants which occurred four times, respectively (Table 4). Another two mutations occurred three times, namely p.Arg445 (Exon 14) and p.Tyr498 (Exon 16). Lastly, four mutations: p.Arg579Glnfs29 (Exon 18), p.Arg787 (Exon 23),
p.Arg255* (Exon 8) and p.Arg552* (Exon 17); affected two cases each. The complete list of 
$RB1$ mutations is given in S3 Table.

**Germline alterations (blood).** Overall, 42.4% (25/59) cases tested positive for 
$RB1$ mutations in the peripheral blood, which included 24 cases with point mutations and one case of 
gross deletion of germline origin (Table 5). All but one bilateral tumor presented with germ-
lime $RB1$ mutations (17/18; 94.4%) (Fig 2). The remaining one bilateral case had a single 
somatic point mutation not present in blood, which suggests the presence of another germline

![Diagram](https://doi.org/10.1371/journal.pone.0178776.g003)

**Fig 3. Distribution of total $RB1$ point mutations by Type.** The frequency of nonsense, frameshift, splice site, missense and promoter mutations among all the point mutations identified in our cohort.

![Diagram](https://doi.org/10.1371/journal.pone.0178776.g004)

**Fig 4. Schematic representation of sequence mutations across $RB1$ gene.** (GenBank Accession Number: L11910.1). The pocket domains are highlighted in orange (Pocket A) and purple (Pocket B) and exons are numbered respectively. Exons known to be mutational hotspots are highlighted with yellow boxes (Valverde et al 2005). Novel mutations are shown in callout boxes. Gross $RB1$ deletions are shown in blue for paternal loss of allele and pink for maternal loss of allele. Grey indicates unknown inheritance. The respective frequencies of gross $RB1$ deletions are given in brackets.

https://doi.org/10.1371/journal.pone.0178776.g004
### Table 3. Novel RB1 point mutations.

| Case | Codon Change | Protein Change | Type | Location | Prediction# |
|------|--------------|----------------|------|----------|-------------|
| 367T | c.175delG    | p.Ala59His*5   | Frameshift deletion | Exon 2 | Deleterious (Mutation Taster, PROVEAN) |
| 381T | c.2494_2495delTT | p.Leu832Serfs*5 (C-terminus) | Frameshift deletion | Exon 24 | Deleterious (Mutation Taster, PROVEAN) |
| 208T | c.1831A>T    | p.Arg611*      | Nonsense | Exon 19 | Deleterious (Mutation Taster, CADD) |
| 182T | c.301delA    | p.Ile101Serfs*9 | Frameshift deletion | Exon 3 | Deleterious (Mutation Taster, PROVEAN) |
| 210T | c.948_951delTCTTT | p.Ser318Asnsfs*13 | Frameshift deletion | Exon 10 | Deleterious (Mutation Taster, PROVEAN) |
| 122T | c.1604_1605delTT | p.Phe535Tyrfs*19 | Frameshift deletion | Exon 17 | Deleterious (Mutation Taster, PROVEAN) |
| 224T | c.2174_2175insGT | -               | Frameshift insertion | Exon 21 | Deleterious (Mutation Taster, PROVEAN) |
| 410T | c.958C>T     | p.Arg320*      | Nonsense | Exon 20 | Deleterious (Mutation Taster, CADD) |

*Pathogenicity prediction was done using Mutation Taster (http://www.mutationtaster.org/) and PROVEAN (http://provean.jcvi.org)*

https://doi.org/10.1371/journal.pone.0178776.t003

### Table 4. Recurrent RB1 point mutations.

| Case | cDNA Change | Putative Consequence | Mutation Type | Exon | Previously Reported LOVD ID |
|------|-------------|----------------------|---------------|------|----------------------------|
| 280T | c.958C>T    | p.Arg320*            | Nonsense      | 10   | RB1_000072                 |
| 332T | c.958C>T    | p.Arg320*            | Nonsense      | 10   | RB1_000072                 |
| 423T | c.1363C>T   | p.Arg455*            | Nonsense      | 14   | RB1_000096                 |
| 572T | c.1363C>T   | p.Arg455*            | Nonsense      | 14   | RB1_000096                 |
| 545T | c.1494T>G   | p.Tyr498*            | Nonsense      | 16   | RB1_00314                  |
| 583T | c.1494T>G   | p.Tyr498*            | Nonsense      | 16   | RB1_00314                  |
| 212T | c.2359C>T   | p.Arg787*            | Nonsense      | 23   | RB1_00005                  |
| 212T | c.1072C>T   | p.Arg358*            | Nonsense      | 11   | RB1_00008                  |
| 583T | c.1736_1745del10 | p.Arg579Glnfs*29  | Frameshift deletion | 18   | RB1_000014                 |
| 545T | c.1736_1745del11 | p.Arg579Glnfs*29  | Frameshift deletion | 18   | RB1_000014                 |
| 182T | c.1654C>T   | p.Arg552*            | Nonsense      | 17   | RB1_00121                  |
| 227T | c.763C>T    | p.Arg255*            | Nonsense      | 8    | RB1_000063                 |
| 578T | c.958C>T    | p.Arg320*            | Nonsense      | 10   | RB1_000072                 |
| 232T | c.1072C>T   | p.Arg358*            | Nonsense      | 11   | RB1_00008                  |
| 244T | c.1072C>T   | p.Arg358*            | Nonsense      | 11   | RB1_00008                  |
| 345T | c.1072C>T   | p.Arg358*            | Nonsense      | 11   | RB1_00008                  |
| 244T | c.1333C>T   | p.Arg445*            | Nonsense      | 14   | RB1_00003                  |
| 320T | c.1333C>T   | p.Arg445*            | Nonsense      | 14   | RB1_00003                  |
| 394T | c.1333C>T   | p.Arg445*            | Nonsense      | 14   | RB1_00003                  |
| 150T | c.1363C>T   | p.Arg455*            | Nonsense      | 14   | RB1_000096                 |
| 435T | c.1363C>T   | p.Arg455*            | Nonsense      | 14   | RB1_000096                 |
| 329T | c.1494T>G   | p.Tyr498*            | Nonsense      | 16   | RB1_00314                  |
| 440T | c.1654C>T   | p.Arg552*            | Nonsense      | 17   | RB1_00121                  |
| 304T | c.2359C>T   | p.Arg787*            | Nonsense      | 23   | RB1_00005                  |
| 575T | c.763C>T    | p.Arg255*            | Nonsense      | 8    | RB1_000063                 |
| 122T | c.958C>T    | p.Arg320*            | Nonsense      | 10   | RB1_000072                 |
| 537T | c.958C>T    | p.Arg320*            | Nonsense      | 10   | RB1_000072                 |

LOVD- Leiden Open (source) Variation Database

https://doi.org/10.1371/journal.pone.0178776.t004
RB1 mutation which was not detected by the methods used in this study. Among the unilateral cases, 19.5% (8/41) were reported to harbour RB1 mutations in blood. The frequencies of the different types of RB1 germline point mutations (nonsense, frameshift, splicing, missense) are shown in Table 6. Further analysis of parental transmission of mutant alleles in all the 25 pairs of unaffected parents of cases with germline mutations revealed only two cases with positive transmission of the variant alleles (c.607+1G>T and c.1981C>T). Both mutations occurred in the two familial cases among our cohort. The origin of variant allele was paternal in both

| Case | Laterality | Mutation | Putative Consequence | Mutation Type | Location | Reported (LOVD) |
|------|------------|----------|----------------------|---------------|----------|-----------------|
| 189T | Bilateral  | c.1568T>G (Homo) | p.Leu523* | Nonsense | Exon 17 | RB1_01352 |
| 208T | Bilateral  | c.1735_1736insGA (Homo) | Gly581Lysfs*31 | Frameshift insertion | Exon 18 | Novel |
| 280T | Bilateral  | c.958C>T (Homo) | p.Trp320* | Nonsense | Exon 10 | RB1_00072 |
| 308T | Bilateral  | c.225G>A (Homo) | p.Trp75* | Nonsense | Exon 2 | RB1_01495 |
| 367T | Bilateral  | c.175delG (Homo) | p.Ala59Hisfs*5 | Frameshift deletion | Exon 2 | Novel |
| 381T | Bilateral  | c.175delG (Homo) | p.Leu832Serfs*5 (C-terminus) | Frameshift deletion | Exon 24 | Novel |
| 432T | Bilateral  | c.224G>A (Homo) | p.Trp75* (N-terminus) | Nonsense | Exon 2 | RB1_00494 |
| 436T | Bilateral  | c.265-1G>T (Homo) | Removal of acceptor site | Splicing | Intron 2 | RB1_01476 |
| 527T | Bilateral  | c.1363C>T (Homo) | p.Arg455* | Nonsense | Exon 14 | RB1_00096 |
| 212T | Bilateral  | c.2359C>T (Het) | p.Arg787* | Nonsense | Exon 23 | RB1_00005 |
| 336T | Bilateral  | c.265-2A>G (Het) | Removal of acceptor site | Splicing | Intron 2, In14 | RB1_00322 |
| 423T | Bilateral  | c.1363C>T (Het) | p.Arg455* | Nonsense | Exon 14 | RB1_00096 |
| 462T | Bilateral  | c.1981C>T (Het) | p.Arg661Trp | Missense | Exon 20 | RB1_00019 |
| 545T | Bilateral  | c.1494T>G (Het) | p.Tyr498* | Nonsense | Exon 16 | RB1_00314 |
| 583T | Bilateral  | c.1494T>G (Het) | p.Tyr498* | Nonsense | Exon 16 | RB1_00314 |
| 592T | Bilateral  | c.658C>G (Het) | Leu220Val | Missense | Exon 7 | RB1_00251 |
| 604T | Bilateral  | c.1510C>T (Het) | Gln504X | Nonsense | Exon 17 | RB1_00668 |
| 111T | Unilateral  | c.2455C>G (Homo) | p.Leu819Val | Missense | Exon 23 | RB1_02045 |
| 227T | Unilateral  | c.763C>T (Homo) | p.Arg255* | Nonsense | Exon 8 | RB1_00063 |
| 519T | Unilateral  | c.940-1G>C (Homo) | p.Met484Valfs*8; Altered splicing | Splicing (mosaic) | Intron 9 | RB1_00195 |
| 578T | Unilateral  | c.958C>T (Homo) | p.Arg320* | Nonsense (mosaic) | Exon 10 | RB1_00072 |
| 182T | Unilateral  | c.1654C>T (Het) | p.Arg552* | Nonsense | Exon 17 | RB1_00121 |
| 477T | Unilateral  | c.607+1G>T (Het) | Altered splicing | Splicing | Intron 6 | RB1_00191 |
| 569T | Unilateral  | c.1450_1451delAT (Het) | p.Val484Valfs*8; Altered splicing | Frameshift deletion | Exon 16 | RB1_00105 |
| 558T | Unilateral  | - | - | Whole Gene deletion (mosaic) | - |

Total 24 RB1 point mutations and 1 whole gene deletion were identified in 25 cases. Homozygous mutation is shown in bold.

https://doi.org/10.1371/journal.pone.0178776.t005

| Mutation Type | Unilateral | Bilateral | Total* |
|---------------|------------|-----------|--------|
| Nonsense      | 3          | 10        | 13/24 (54.17%) |
| Frameshift    | 1          | 3         | 4/24 (16.67%) |
| Splicing      | 2          | 2         | 4/24 (16.67%) |
| Missense      | 1          | 2         | 3/24 (12.5%) |

Distribution of germline mutation by laterality (%)

7/24 (29.17%) | 17/24 (70.83%) | 24/24 (100%)

* A total of 24 germline point mutations were detected in blood from both unilateral and bilateral cases.

https://doi.org/10.1371/journal.pone.0178776.t006

RB1 mutation which was not detected by the methods used in this study. Among the unilateral cases, 19.5% (8/41) were reported to harbour RB1 mutations in blood. The frequencies of the different types of RB1 germline point mutations (nonsense, frameshift, splicing, missense) are shown in Table 6. Further analysis of parental transmission of mutant alleles in all the 25 pairs of unaffected parents of cases with germline mutations revealed only two cases with positive transmission of the variant alleles (c.607+1G>T and c.1981C>T). Both mutations occurred in the two familial cases among our cohort. The origin of variant allele was paternal in both
families, whereby one was a unilateral (family F1, Fig 5) case and the another, a bilateral (family F2, Fig 5) case. These mutations have previously been reported to cause low penetrance phenotype of RB and a summary of all reported cases harbouring similar (c.607+1G>T and c.1981C>T) low penetrance mutations is shown in Table 7. Within the bilateral cases, 5.6% (1/18) harboured a low penetrance phenotype.

Of the unilateral probands, who tested positive for \(RB1\) mutations in blood, 37.5% (3/8) were found to be mosaics. The three probands showed mosaicism for a splice site mutation - c.940-1G>C, 4% mosaic in blood (family F3, Fig 5); a nonsense mutation - p.Arg320*, 2%

Table 7. Germline mutations identified in this study that were previously reported as low-penetrance mutations.

| Mutation (Location) | Number of times reported as \(RB1\) mutation in LOVD | Cases previously reported as carriers of Low Penetrance mutation | Reference |
|---------------------|--------------------------------------------------------|----------------------------------------------------------------|-----------|
| c.1981C>T; p.Arg661Trp (Exon 20) | 33 | 35 | [34–37] |
| c.607+1G>T; Splicing (Intron 6) | 21 | 19 | [38–40] |

LOVD- Leiden Open Variation Database

https://doi.org/10.1371/journal.pone.0178776.t007
mosaic in blood (family F4, Fig 5) and a deletion of one RB1 allele, 60% mosaic in blood (family F5, Fig 5), respectively. Parents of all 3 probands tested negative for RB1 mutations (families F3-F5, Fig 5). A summary of the three mosaic mutations identified in this study, one of which has been previously described (c.958C>T), is given in Table 8.

### Somatic alterations (tumor).

Among the different types of mutations arising only in the somatic cells, there were 37 which were point mutations (Table 9). These mutations were detected in 26 non-heritable cases which did not carry any germline mutation and in 6 heritable cases which carried one germline mutation in the tumor cell. The frequency of different types of mutations as observed within the somatic point mutations is shown in Table 10.

The remaining somatic alterations observed were RB1 gross deletion and promoter methylation. There were 36 gross RB1 deletions found in 47.45% (34/59) tumors, of which 9 were bilateral and 25 were unilateral. Of the cases with gross RB1 deletions, only 26 cases could be further analysed for parental origin of the lost RB1 allele due to limited availability of parental DNA. We found 61.5% (16/26) cases showing preferential loss of maternal allele, while 38.5% (10/16) cases showed preferential loss of paternal RB1 allele (Table 11). The graphical representation of gross RB1 deletions with respective origin of allelic loss is given in Fig 4. RB1 promoter was found to be hypermethylated in only one unilateral case (410T), who also harboured a gross deletion within RB1 gene (spanning Exons 17–24).

### Discussion

The spectrum of RB1 mutations in cases diagnosed with RB in Singapore show small sequence mutations and gross RB1 deletions as the major mechanisms of RB1 inactivation. The majority of RB1 point mutations are known to be distributed throughout the gene, with specific patterns of recurrent mutations and mutational hotspots encompassing retinoblastoma pocket domain coding regions: exons 12–23 [14,33,38,42,44–48]. We observed a high mutation rate in the sequence coding for these pocket domains within our cohort (58.1%) which was comparable to the previously reported rates of 58.6% [49] and 40% [42]. In addition, 44.3% (27/61) of the point mutations are known recurrent mutations and located in the CpG rich RB1 mutational hotspots spanning exons 8, 10, 11, 14, 15, 17, 18 and 23. Point mutations in these 8 exons have been reported previously at variable frequencies of 50% (15/30) [50] and 35.7% (5/14) [51] in Chinese population. Of these identified recurrent mutations, seven belonged to a group of recurrent RB1 CGA (Arg)>TGA(STOP) nonsense mutations (Arg255*, Arg320*, Arg358*, Arg445*, Arg455*, Arg552* and Arg787*) [41,52]. A meta-analysis on the RB1 mutation spectrum across published databases previously revealed a 40% recurrent mutation frequency across 16 mutational hotspots in the CpG islands of RB1 gene, out of which, 79% variants were associated with the recurrent C to T change in 11 CGA codons [33]. Within our cohort of 41
### Table 9. List of somatic RB1 point mutation identified only in tumor cells.

| Case | Laterality | Mutation                  | Putative Consequence | Mutation Type | Location | Reported (LOVD) |
|------|------------|---------------------------|----------------------|---------------|----------|-----------------|
| 143T | Unilateral | c.1666C>T (Homo)          | p.Arg556*            | Nonsense      | Exon 17  | RB1_00124       |
| 224T | Unilateral | c.2174_2175insGT (Homo)   | -                    | Frameshift ins| Exon 21  | Novel           |
| 232T | Unilateral | c.1072C>T (Homo)          | p.Arg358*            | Nonsense      | Exon 11  | RB1_00008       |
| 304T | Unilateral | c.2369C>T (Homo)          | p.Arg787*            | Nonsense      | Exon 23  | RB1_00005       |
| 320T | Unilateral | c.1333C>T (Homo)          | p.Arg445*            | Nonsense      | Exon 14  | RB1_00003       |
| 341T | Unilateral | c.1399C>T (Homo)          | p.Arg467*            | Nonsense      | Exon 15  | RB1_00089       |
| 345T | Unilateral | c.1072C>T (Homo)          | p.Arg358*            | Nonsense      | Exon 11  | RB1_00008       |
| 349T | Unilateral | c.1959_1960insA (Homo)    | Val654Serfs*14       | Frameshift ins| Exon 19  | RB1_01687       |
| 378T | Unilateral | c.1439_1441del (Homo)     | p.Asn480del          | Frameshift del| Exon 16  | RB1_00102       |
| 394T | Unilateral | c.1333C>T (Homo)          | p.Arg445*            | Nonsense      | Exon 14  | RB1_00003       |
| 410T | Unilateral | c.490A>T (Homo)           | -                    | Promoter      | Upstream| Novel           |
| 414T | Unilateral | c.2067G>C (Homo)          | p.Gln689His          | Missense      | Exon 20  | Novel           |
| 420T | Unilateral | c.1831A>T (Homo)          | p.Arg611*            | Nonsense      | Exon 19  | Novel           |
| 435T | Unilateral | c.1363C>T (Homo)          | p.Arg455*            | Nonsense      | Exon 14  | RB1_00096       |
| 440T | Unilateral | c.1654C>T (Homo)          | p.Arg552*            | Nonsense      | Exon 17  | RB1_00121       |
| 550T | Unilateral | c.1450_1451insAT (Homo)   | p.Met684Asnfs*12    | Frameshift ins| Exon 16  | RB1_01736       |
| 575T | Unilateral | c.763C>T (Homo)           | p.Arg255*            | Nonsense      | Exon 8   | RB1_00063       |
| 122T | Unilateral | c.1604_1605delTT (Het)    | p.Phe535Tyrfs*1      | Frameshift del| Exon 17  | Novel           |
| 122T | Unilateral | c.958C>T (Het)            | p.Arg320*            | Nonsense      | Exon 10  | RB1_00072       |
| 150T | Unilateral | c.1363C>T (Het)           | p.Arg455*            | Nonsense      | Exon 14  | RB1_00096       |
| 182T#| Unilateral | c.301delA; (Het)          | p.Ile101Serfs*9;     | Frameshift del| Exon 3   | Novel           |
| 210T | Unilateral | c.948_951delTCTT (Het)    | p.Ser318Asnfs*13    | Frameshift ins| Exon 10  | Novel           |
| 244T | Unilateral | c.1333C>T (Het)           | p.Arg445*            | Nonsense      | Exon 14  | RB1_00003       |
| 244T | Unilateral | c.1072C>T (Het)           | p.Arg358*            | Nonsense      | Exon 11  | RB1_00008       |
| 329T | Unilateral | c.1494T>G (Het)           | p.Tyr498*            | Nonsense      | Exon 16  | RB1_00314       |
| 456T | Unilateral | c.1735delC (Het)          | p.Arg579Glufs*32    | Frameshift del| Exon 18  | RB1_00451       |
| 456T | Unilateral | c.1653_1654insCG (Het)    | p.Cys553Aspfs*59    | Frameshift ins| Exon 17  | RB1_01738       |
| 533T | Unilateral | c.1466G>A (Het)           | p.Cys489Tyr         | Missense      | Exon 16  | RB1_00081       |
| 533T | Unilateral | c.1150C>T (Het)           | p.Gln384*           | Nonsense      | Exon 12  | RB1_01684       |
| 537T | Unilateral | c.1735C>T (Het)           | p.Arg579*            | Nonsense      | Exon 18  | RB1_00129       |
| 537T | Unilateral | c.958C>T (Het)            | p.Arg320*            | Nonsense      | Exon 10  | RB1_00072       |
| 569T#| Unilateral | c.2106+2T>G (Het)         | Altered splicing    | Splicing      | In 20    | RB1_01791       |
| 212T#| Bilateral  | c.1072C>T (Het)           | p.Arg358*            | Nonsense      | Exon 11  | RB1_00008       |
| 332T | Bilateral  | c.958C>T (Het)            | p.Arg320*            | Nonsense      | Exon 10  | RB1_00072       |
| 336T#| Bilateral  | c.1390-14A>G (Het)        | Removal of acceptor site | Splicing | RB1_00919 |
| 545T#| Bilateral  | c.1736_1745del10 (Het)    | p.Arg579Glnfs*29    | Frameshift del| Exon 18  | RB1_00014       |
| 583T#| Bilateral  | c.1736_1745del10 (Het)    | p.Arg579Glnfs*29    | Frameshift del| Exon 18  | RB1_00014       |

*Heritable cases with a germline point mutation detected in tumor cell. Homozygous mutation is shown in bold.

https://doi.org/10.1371/journal.pone.0178776.t009

unilateral cases, 40 were sporadically affected and one was a familial case. We observed 17.5% (7/40) sporadic unilateral cases carrying germline RB1 mutations which is comparable to previous reports indicating an incidence of between 10% - 18% [41,53–58]. The frequency of heritable and non-heritable RB1 mutational events in this Singaporean cohort of 59 RB cases primarily comprised of familial or de novo sequence point mutations (50 cases), acquired intragenic and extragenic deletions (35 cases) and epigenetic changes (1 case). Although RB1 promoter hypermethylation has been observed to play an important role as one of the two hits
in RB with varying frequencies ranging from 0–27% [49,51,55,59,60]; we found only one instance of this, concurring with the previous observation by Choy et al 2002, that it is not a major inactivating mechanism in our population which had a predominance of Chinese patients. The unilateral patient with promoter hypermethylation in our study also harboured a novel somatic upstream RB1 variant: c.-490A>T (Table 3 and S3 Table). This represents an incidence of 1.7% (1/59 cases) in the Singaporean population. Methylation of the RB1 promoter is known to be the causative ‘first hit’ in about 8% of unilateral non-heritable tumours and about 88% of those were reported to have RB1 gross deletions or loss of heterozygosity as ‘second hit’ [53]. The cumulative impact of the identified somatic variations occurring in the regulatory region of RB1 in our proband is unknown as we were unable to perform any functional studies due to the samples from this case being depleted. However, based on previous RB1 promoter methylation and sequence alteration studies [61,62], reduced gene expression of RB1 can be postulated in such patients.

Occurrence of novel mutations can be as high as 20% to 53.3% of all mutations in patient populations of Chinese ethnicity reported from different countries [51,60,63,64]. In this study, we report 10 novel likely pathogenic variants which add to the genetic spectrum of RB disease. Reporting of novel variants is important as line of evidence for attributing pathogenicity when the same variants are subsequently found in other unrelated patients. This studied population with predominant Chinese ethnicity represents the first report from Singapore and contributes towards the reported variants in the literature.

**Tumors with one or no mutations identified**

Among the 59 tumors, sixteen tumors presented with either one (13/59, 22.03%) or no RB1 mutations (3/59, 5.1%) as shown in S4 Table. RB1 independent means of tumorigenesis have been reported in a fraction of cases with somatic MYCN gene amplification [9,65]. These patients with unilateral RB1+/MYCN+ retinoblastomas are usually diagnosed at a younger age (mean age = 4.5 months) with distinct histological features and harbour fewer genomic copy-number changes characteristic of retinoblastoma [9]. Aberrant methylation of MGMT has

| Mutation Type | Unilateral | Bilateral | Total* |
|---------------|------------|-----------|--------|
| Nonsense      | 19         | 2         | 21/37 (56.75%) |
| Frameshift    | 9          | 2         | 11/37 (29.73%) |
| Splicing      | 1          | 1         | 2/37 (5.4%) |
| Missense      | 2          | 0         | 2/37 (5.4%) |
| Promoter      | 1          | 0         | 1/37 (2.7%) |

**Distribution of somatic mutation by laterality (%)**

| Distribution of somatic mutation by laterality (%) | Unilateral | Bilateral | Total |
|-----------------------------------------------------|------------|-----------|-------|
| 32/37 (86.48%)                                      | 4/37 (10.8%) | 37/37 (100%) |

* A total of 37 somatic point mutations were detected in tumor only. Germline mutations found in both tumor and blood were excluded.

https://doi.org/10.1371/journal.pone.0178776.t010

Table 11. Parental origin of allelic loss.

| Cases with Maternal allelic loss | Cases with Paternal allelic Loss | Total allelic loss |
|----------------------------------|----------------------------------|-------------------|
| Heritable                        | 7                                | 10/26 (38.5%) |
| Non-Heritable                    | 9                                | 16/26 (61.5%) |
| Total                            | 16/26 (61.5%)                    | 26/26 (100%) |

Data from 26 cases in which parental samples were available for this analysis.

https://doi.org/10.1371/journal.pone.0178776.t011
been suggested as an additional epigenetic dysregulation mechanism underlying retinoblastoma [66]. Tumor Necrosis Factor (TNF) gene is located on chromosome 6p, a region of recurrent chromosomal gain often observed in RB [14,65,67]. In RB, TNFs were found to be overexpressed in the membrane compartments and cytoplasm of WERI-Rb1 (with i(6p)) and Y79 (without i(6p)) retinoblastoma cell lines [67]. Of these 18 cases, we further analysed 10 samples which had sufficient remaining genomic DNA from tumor, for alterations in other candidate genes previously suspected to be associated with RB, viz. MYCN and TNF genes amplification and MGMT promoter methylation, using the screening methods described in this study. However, no MYCN amplification could be detected in any of the 10 analyzed RB cases. Hypermethylation of MGMT promoter was observed in 20% (2/10) cases, which included one case carrying both TNF gene amplification as well as MGMT promoter hypermethylation events (S5 Table). For TNF gene amplification analysis, 30% (3/10) retinoblastoma tumour samples harboured more than two copies of TNF gene (S5 Table).

In the remaining 5% (3/59) of cases, no RB1 mutations were found. This could be due to the fact that conventional methods of RB1 screening have limited scope as they might miss out on variants in deep intronic and untranslated regions (UTRs) of the gene, which could further be analyzed by incorporating other screening methods such as next generation sequencing technology [68]. A recent case of familial RB caused by retrotransposition of a Long interspersed element-1 (LINE-1) into the RB1 gene also suggests that such events might be missed by most commonly used mutation detection platforms which are based on amplification of small fragments [69]. Thus, a combinatorial approach of RNA-based techniques and massive parallel sequencing is recommended for cases where no RB1 alteration can be identified. Nevertheless, our mutation detection rate of 92.5% for unilateral tumors and 100% for bilateral tumors, are comparable to other studies that have employed direct sequencing and MLPA as a combinatorial approach towards determining the RB1 mutational status with detection figures ranging between 92–100% for Bilateral and 10–61% in unilateral cases [45,50,55,70,71]. The high detection rates of RB1 gene mutations reported in our study shows that conventional techniques are still effective as clinical screening methods for most RB cases even in the era of next-generation sequencing due to the type of mutations that occur frequently in this disease.

Low-penetrance mutation

Low-penetrance phenotype in RB explains the phenomena of familial cases inheriting potentially deleterious mutated variants from an unaffected parent who lacks any disease related phenotype. Such asymptomatic carriers may have the proband’s mutation as a single recessive mutant allele or in some rare cases, even a dominant variant. However, they may present with a clinical phenotype which is either within the range of normal healthy variations, or is too mild to get noticed or would become apparent in later decades of life [72]. In addition, it was recently shown that both RB1 gene and the upstream inhibitor of pRB: CDKN1C (cyclin-dependent kinase) gene are evolutionarily selected for maternal inhibition of cell proliferation [73]. Hence, this imprinting of RB1 gene due to a differentially methylated CpG island in intron 2 results in parent-of-origin-specific DNA methylation and gene expression patterns [74]. We observed that both the identified familial cases in our cohort presented with low-penetrance retinoblastoma phenotype. The first case (Family F1; Fig 5) was a 2-year-old boy with unilateral RB who had inherited the low-penetrance splice site mutation (c.607+1G>T) from his asymptomatic father in a heterozygous state. Family history revealed that the proband had a deceased elder sibling (at 2-year-10-months of age) who was known to have metastasized RB tumor of unknown laterality and was not tested for the given mutation. The
proband also had a younger brother (14-months) who presented with unilateral RB and harboured the same mutation. Typically, low-penetrance mutations are known to have a disease-eye ratio \((\text{der}) \leq 1\), which is the ratio of the total number of affected eyes to the number of carriers in the family, while high penetrance mutations have \(\text{der} \geq 1.5\) ratio [75,76]. In family F1, \(\text{der}\) was found to be 0.66 (2/3) which concurs with the low-penetrance phenotype. To the best of our knowledge, our study reports the first Asian family (Family F1; Fig 5) harbouring c.607+1G\(\triangleright\)T low-penetrance mutation, which is known to present a variable expressivity phenotype in RB [38,39]. This mutation has been listed 21 times in the RB database (rb1-lsdb, Version RB1 150518) with a total of 19 individuals reported as carriers of this mutation [38–40]. Variable expressivity of this mutation is linked with the sex of the transmitting parent and was shown to cause RB in the progeny only when it is inherited from the father; as reported previously in Spanish [38] and German [39] patients. The second low-penetrance phenotype was due to the missense mutation: p.Arg661Trp, in family F2 (Family F2; Fig 5). The proband was diagnosed with bilateral RB at 10 months of age and was found to inherit the given germline mutation (heterozygous) from his normal father. His unaffected younger sibling’s DNA was analyzed and he was found to carry the same familial mutation in heterozygous state, thus leading to a \(\text{der} = 0.66\) (2/3) in the family, which is according to the expected low-penetrance values. This low-penetrance mutation- p.Arg661Trp, has been reported 33 times in RB database (rb1-lsdb, Version RB1 150518) being present in 35 individuals [34–37] and its resultant protein is shown to have a temperature-sensitive pocket activity whose reversible fluctuations may result in low-penetrance phenotype [37]. A recent publication has linked this missense mutation to variable expressivity phenotype, with a parent-of-origin gender effect determining the probability of developing the disease [35]. The study reported that the probability of not developing RB was 90.3% when the mutation was inherited from the mother and 32.5% when inherited from father (\(p\)-value = \(7.10^{-7}\)) [35]. Thus, in families which carry the low-penetrance mutations, a reduced number of carriers develop retinoblastoma rather than the expected rate of \(>99\%\) [43,52] which is commonly expected for most RB1 mutations. Apart from the dominant inheritance pattern of RB, a few families have been reported to display a phenomena characterized by reduced penetrance and may not develop RB or may result in the development of unilateral RB or retinomas instead of bilateral RB (reduced expressivity) [76]. It has been hypothesised that reduction in the quantity or quality of cellular pRB activity is central to these low-penetrance mutations. In addition, pRB may be partially inactivated by subtle mutations that globally reduce the stability and binding affinity of the protein or that locally perturb semi essential functions [76]. Among the reasons postulated for this male-specific transmission of disease are differential regulation of genes in males and females [77] and RB1 genomic imprinting [73]. In both our families carrying the reduced penetrance mutation, although the offsprings had retinoblastoma through paternal inheritance of the mutant alleles, there was reduced penetrance associated with the mutant alleles as fathers in the two families did not have the disease. Additionally, there also appears to be reduced expressivity in both siblings carrying the c.607+1G\(\triangleright\)T mutation as they presented with unilateral retinoblastoma and no further tumors were detected in follow-ups. Less than 10% of familial RB are known to show low penetrance phenotype. A meta-analysis from Valverde et al showed 20% (27/133) of all familial cases and 3.5% (27/753) of all germline RB1 mutations to be linked to low penetrance phenotype in patients, whereby this distribution in familial cases was suggested to be an over-estimation of the true incidence due to research bias for low penetrance phenotypes. In our smaller study, we encountered two such cases out of 25 cases bearing germline mutations suggesting that such mutations may not be that uncommon because they are recurrent mutations previously associated with low penetrant phenotype. For further identification of novel
splice site and missense mutations that could be associated with low penetrance, genotype-phenotype correlations in larger families are necessary.

Mosaic mutation

The phenomena of mosaicism in RB occurs when a mutation in the RB1 gene arises at some time during embryogenesis - 'post zygotic' [43,78]. Therefore, a mosaic may have the initial mutation in some but not all cells of the body. Overall RB1 mosaicism has been reported between 10–20% of all RB cases, previously [43]. Low-level germline mosaicism in sporadic bilateral RB has been reported in about 5% [41] cases, while a higher detection rate was achieved by using Deep Semiconductor Sequencing, 30% [58]. Around 12–14% of sporadic unilateral cases are known to carry de novo, germline mutations, of which 1.2% may be mosaics, and could be transmitted to the offspring [41,53]. The incidence of germline mosaicism in sporadic unilateral RB cases has been reported at varying frequencies of 3.8% [41], 6% [58], 8.7% [49], 16.6% [79], 22.2% [59] and 33% [42]. These varying frequencies observed are linked to the type of detection platform employed and the sample size of the study. A proband with sporadic RB possesses a 10% risk of bearing an undetectable low level RB1 mosaicism when conventional genetic testing techniques fail to identify any RB1 mutations [80]. In this study, we detected germline RB1 mosaicism in three RB cases, which are all unilateral. The first patient (578T), who was diagnosed with unilateral RB at 26 months of age, carried 2% mosaicism for the stop-gain mutation in exon 10 (p.Arg320). Mosaicism for this recurrent mutation- p.Arg320 has been reported previously in 5 cases (4 bilateral and 1 unilateral) at frequencies of <5, 10 and <50% (Table 8), whereby the unilateral case with unknown age carried a mosaicism at 10% frequency [41] (Table 8). The overall incidence of mosaicism among all tumors in our study was 5.1% (3/59), and 12% (3/25) among cases where mutation was found in peripheral blood. However, this may not reflect the true incidence of mosaicism in our patient population as mosaic mutations are not routinely screened in our set-up. While Sanger sequencing can detect some cases of mosaicism through recognition of unequally reduced heterozygous peaks on sequence traces, accurate detection of low level mosaicism requires sensitive technique such as allele specific amplification or next generation sequencing [41,58,78].

Genetic counselling and disease management

The ultimate goal of retinoblastoma therapy is to ensure high survival rate while minimizing collateral damage to surrounding tissues and low recurrence of the disease through efficient genetic counselling. On one hand, owing to increased awareness and advancements in RB therapeutics, the overall survival rate in high-income countries has improved from <5% to 99%; significantly poor survival rates in low-income countries still remains a cause of concern [5,81]. Based on the genetic make-up of the individual, there are three major scenarios where an RB Patient would require lifelong follow-up for future family planning or to avoid any risk of secondary cancers: 1) germline RB1 mutations, 2) germline RB1 Mosaicism, or 3) low penetrance RB1 Mutations. Identification of germline mosaicism is important as it could lead to an increased recurrence risk to future siblings. While the rate of second malignancies in retinoblastoma survivors with low-penetrance or mosaic RB1 mutations is still unknown, it is presumed to be lower than those with germline null RB1 alleles [8]. A recent study reported cumulative incidence of developing a second malignancy by the age of 10 in patients with heterozygous germline RB1 alterations was 5.2% (95%CI 1.7; 8.7%) [82]. One of the existing challenges of genetic counselling in retinoblastoma is the applicability of the identified heterogeneous spectrum of mutations in the patient which may in turn lead to variable disease
phenotypes viz., low penetrance and mosaicism. Thus, efficient genetic testing would add to the growing knowledge and enable accurate genetic counselling with customized follow-up schedules for cases where the underlying mutation heterogeneity could be easily missed either due to a low-penetrance phenotype or because only a fraction of cells harbour the causative mutations (mosaicism). Hence, it is imperative to provide life-long follow-up to both the patients and their families with heritable mutations as the former carry a higher risk for developing secondary cancers and a probability of passing risk mutations to their future offspring, while the latter may require advice on family planning due to their asymptomatic carrier status.

With respect to therapeutics, disease outcomes have improved due to intra-arterial and intravitreal chemotherapy which are focused on salvaging the eyes, which otherwise would have been lost in conventional treatment [5]. Since the first successful report of preimplantation genetic testing [83], this preventive intervention appears promising for future disease management in families at risk for having children with inherited retinoblastoma. With respect to RB1 mutational signature, since nonsense mutations comprise majority of the reported point mutations in RB, as also shown in our study, nonsense suppression therapy which allows readthrough of premature termination codon (PTCs), restoring the protein function [84]; offers possible future targeted therapeutics of such cases. Generation 4 polyamidoamine (G4PAMAM) dendrimers, which act as delivery system of vascular endothelial growth factor antisense oligodeoxynucleotides were recently reported to have antitumor properties, both in vitro and in vivo [85]. In addition to gene therapy, few sustained drug release platforms are also being developed for targeted intraocular drug delivery in RB [6]. Thus, information of the mutational signatures in RB patients would further aid in targeted therapeutics besides ensuring effective disease management and life-long follow up, where indicated.

Conclusions

Our report expands the spectrum of RB1 mutations and further emphasizes on the need to not only identify the causative mutations but also to detect special disease phenotypes viz., low-penetrance mutations and germline mosaicism. Thus, our study on identifying the genetic signatures from Singaporean patients with RB will further aid in developing appropriate screening programmes and devising efficient disease management measures for such patients and their families.

Supporting information

S1 Table. Sequences of primers for extragenic microsatellite markers used in this study. (DOCX)

S2 Table. Sequences of primers for intragenic RB1 markers used in this study. (DOCX)

S3 Table. Overview of RB1 mutations identified in total 59 Retinoblastoma cases. (DOCX)

S4 Table. List of RB cases with number of RB1 mutations. (DOCX)

S5 Table. List of 10 cases analyzed for TNF and MGMT mutations, where only one or no RB1 mutations could be identified. (DOCX)
Acknowledgments

We acknowledge grant funding support from National Medical Research Council of Singapore. RB1 mutation screening for selected samples were performed at RB Solutions/Impact Genetics as indicated in the S3 Table. We thank Rong Rong and Priya Kadam for technical assistance with some of the experiments.

Author Contributions

Conceptualization: GS TCQ BLQ PSL.
Funding acquisition: PSL.
Investigation: ST RS GS BLQ TCQ PSL.
Methodology: GS TCQ BLQ PSL.
Resources: BLQ GS TCQ PSL.
Supervision: PSL.
Visualization: ST RS.
Writing – original draft: ST PSL.
Writing – review & editing: ST RS GS BLQ TCQ PSL.

References

1. Kivela T. The epidemiologic challenge of the most frequent eye cancer: retinoblastoma, an issue of birth and death. Br J Ophthalmol. 2009; 93: 1129–1131. https://doi.org/10.1136/bjo.2008.150292 PMID: 19704035

2. Saw SM, Tan N, Lee SB, Au Eong KG, Chia KS. Incidence and survival characteristics of retinoblastoma in Singapore from 1968–1995. J Pediatr Ophthalmol Strabismus. 2000; 37: 87–93. Available: http://www.ncbi.nlm.nih.gov/pubmed/10779266 PMID: 10779266

3. Aung L, Chan YH, Yeoh EJ, Tan PL, Quah TC. Retinoblastoma: a recent experience at the National University Hospital, Singapore. Ann Acad Med Singapore. 2009; 38: 693–8. Available: http://www.ncbi.nlm.nih.gov/pubmed/19736573 PMID: 19736573

4. Lim FPM, Soh SY, Iyer JV, Tan AM, Swati H, Quah BL. Clinical Profile, Management, and Outcome of Retinoblastoma in Singapore. J Pediatr Ophthalmol Strabismus. 2013; 50: 106–112. https://doi.org/10.3928/01913913-20121211-01 PMID: 23244241

5. Dimaras H, Corson TW, Cobrinik D, White A, Zhao J, Munier FL, et al. Retinoblastoma. Nat Rev Dis Prim. 2015; 15062. https://doi.org/10.1038/rndp.2015.62

6. Mendoza PR, Grossniklaus HE. The Biology of Retinoblastoma. Prog Mol Biol Transl Sci. Department of Ophthalmology, Emory University, School of Medicine, Atlanta, GA, United States; 2015; 134: 503–516. https://doi.org/10.1016/bs.pmbts.2015.06.012 PMID: 26310174

7. Lohmann DR, Gallie BL. Retinoblastoma: Revisiting the model prototype of inherited cancer [Internet]. American Journal of Medical Genetics. 2004. pp. 23–28. https://doi.org/10.1002/ajmg.c.30024 PMID: 15264269

8. Dimaras H, Kimani K, Dimba EAO, Grondsahl P, White A, Chan HSL, et al. Retinoblastoma. Lancet. 2012; 379: 1436–1440. https://doi.org/10.1016/S0140-6736(11)6137-9 PMID: 22414599

9. Rushlow DE, Mol BM, Kennett JY, Yee S, Pajovic S, Thériault BL, et al. Characterisation of retinoblastomas without RB1 mutations: Genomic, gene expression, and clinical studies. Lancet Oncol. Impact Genetics and the Toronto Western Hospital Research Institute, University Health Network, Toronto, ON, Canada; 2013; 14: 327–334. https://doi.org/10.1016/S1470-2045(13)70045-7

10. Kooi IE, Mol BM, Massink MPG, De Jong MC, De Graaf P, Van Der Valk P, et al. A meta-analysis of retinoblastoma copy numbers refines the list of possible driver genes involved in tumor progression. PLoS One. Department of Clinical Genetics, VU University Medical Center, Amsterdam, Netherlands; 2016; 11: e0153323. https://doi.org/10.1371/journal.pone.0153323 PMID: 27115612
11. Bowles E, Corson TW, Bayani J, Squire JA, Wong N, Lai PBS, et al. Profiling genomic copy number changes in retinoblastoma beyond loss of RB1. Genes Chromosom Cancer. 2007; 46: 118–129. https://doi.org/10.1002/gcc.20383 PMID: 17099872

12. Laurie NA, Donovan SL, Shih C-S, Zhang J, Mills N, Fuller C, et al. Inactivation of the p53 pathway in retinoblastoma. Nature. Nature Publishing Group; 2006; 444: 61–66. https://doi.org/10.1038/nature05194 PMID: 17080083

13. Grasemann C, Gratias S, Stephan H, Schüler A, Schramm A, Klein-Hitpass L, et al. Gains and overexpression identify DEK and E2F3 as targets of chromosome 6p gains in retinoblastoma. Oncogene. University Children’s Hospital of Essen, Universitätssklinikum Essen, D-45122 Essen, Germany, England; 2005; 24: 6441–6449. https://doi.org/10.1038/sj.onc.1208792 PMID: 16007192

14. Mol BM, Massink MPG, van der Hout AH, Dommering CJ, Zaman JMA, Bosscha MI, et al. High resolution SNP array profiling identifies variability in retinoblastoma genome stability. Genes Chromosom Cancer. 2011; 50: 1–14. https://doi.org/10.1002/gcc.21111 PMID: 24249257

15. Benavent CA, Dyer MA. Genetics and Epigenetics of Human Retinoblastoma. Annu Rev Pathol Mech Dis. 2015; 10: 547–562. https://doi.org/10.1146/annurev-pathol-012414-040259 PMID: 25621664

16. Marchong MN, Chen D, Corson TW, Lee C, Harmandayan M, Bowles E, et al. Minimal 16q genomic loss implicates cadherin-11 in retinoblastoma. Mol Cancer Res. 2004; 2: 495–503. 2/9/495 [pii] PMID: 15383628

17. Priya K, Jada SR, Boon LQ, Thuan CQ, Poh SL. High incidence of allelic loss at 16q12.2 region spanning RBL2/p130 gene in retinoblastoma. Cancer Biol Ther. Division of Human Genetics, Department of Paediatrics, National University of Singapore, Singapore, Singapore: Taylor & Francis; 2009; 8: 714–717. https://doi.org/10.4161/cbt.8.8.7921

18. McEvoy J, Flores-Otero J, Zhang J, Nemeth K, Brennan R, Bradley C, et al. Coexpression of Normally Incompatible Developmental Pathways in Retinoblastoma Genes. Cancer Cell. United States: NIH Public Access; 2011; 20: 260–275. https://doi.org/10.1016/j.ccr.2011.07.005 PMID: 21840489

19. Miller SA, Dykes DD, Polesky HF. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1988; 16: 1215. https://doi.org/10.1093/nar/16.3.1215 PMID: 3344211

20. Hogg A, Onadim Z, Baird PN, Cowell JK. Detection of heterozygous mutations in the RB1 gene in retinoblastoma patients using single-strand conformation polymorphism analysis and polymerase chain reaction sequencing. Oncogene. 1992; 7: 1445–1451. Available: http://www.ncbi.nlm.nih.gov/pubmed/1352398 PMID: 1352398

21. Bookstein R, Lai CC, Hoang T, Lee WH. PCR-based detection of a polymorphic BamHI site in intron 1 of the human retinoblastoma (RB) gene. Nucleic Acids Res. 1990; 18: 1666. https://doi.org/10.1093/nar/18.6.1666 PMID: 2326211

22. Mcgee TL, Cowley GS, Yandell DW, Dryja TP. Detection of the XbaI RFLP within the retinoblastoma locus by PCR. Nucleic Acids Res. 1990; 18: 207. https://doi.org/10.1093/nar/18.1.207-a

23. Vaughn GL, Toguchida J, Mcgee T, Dryja TP. Pcr detection of the Tth 111 I RFLP at the RB locus. Nucleic Acids Res. Oxford University Press; 1990; 18: 4965. https://doi.org/10.1093/nar/18.16.4965

24. Toguchida J, Ishizaki K, Sasaki MS. Mutation Spectrum of the Retinoblastoma Gene in Osteosarcoma. Cancer Res. 1994; 54: 3032–3048. Available: http://www.ncbi.nlm.nih.gov/pubmed/8187094 PMID: 8187094

25. Canzian F, Salovaara R, Hemminki A, Kristo P, Chadwick RB, Aaltonen LA, et al. Semiautomated assessment of loss of heterozygosity and replication error in tumors. Cancer Res. 1996; 56: 3331–3337. Available: http://www.ncbi.nlm.nih.gov/pubmed/8764130 PMID: 8764130

26. Stirzaker C, Millar DS, Paul CL, Warnecke PM, Harrison J, Vincent PC, et al. Extensive DNA methylation spanning the Rb promoter in retinoblastoma tumors. Cancer Res. 1997; 57: 2229–2237. Available: http://www.ncbi.nlm.nih.gov/pubmed/9187126 PMID: 9187126

27. Zeschnigk M, Lohmann D, Horshemke B. A PCR test for the detection of hypermethylated alleles at the retinoblastoma locus. J Med Genet. 1999; 36: 793–4. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=13744233&tool=pmcentrez&rendertype=abstract PMID: 10528863

28. Choy KW, Pang CP, To KF, Yu CBO, Ng JSK, Lam DSC. Impaired expression and promoter hypermethylation of O6-methylguanine-DNA methyltransferase in retinoblastoma tissues. Invest Ophthalmol Vis Sci. 2002; 43: 1344–1349. Available: http://www.ncbi.nlm.nih.gov/pubmed/11980845

29. Chen D, Galle L, Squire JA. Minimal regions of chromosomal imbalance in retinoblastoma detected by comparative genomic hybridization. Cancer Genet Cytogenet. 2001; 129: 57–63. https://doi.org/10.1016/S0165-4608(01)00427-7 PMID: 11520568

30. Rowland JS, Barton DE, Taylor GR. A comparison of methods for gene dosage analysis in HMSN type 1. J Med Genet. 2001; 38: 90–9. https://doi.org/10.1136/jmg.38.2.90 PMID: 11158172
31. Yau SC, Bobrow M, Mathew CG, Abb Süb SJ. Accurate diagnosis of carriers of deletions and duplications in Duchenne/Becker muscular dystrophy by fluorescent dosage analysis. J Med Genet. 1996; 33: 550–8. https://doi.org/10.1136/jmg.33.7.550 PMID: 8818939

32. Horsthemke B, Gregor V, Becher R, Passarge E. Mechanism of i(6p) formation in retinoblastoma tumor cells. Cancer Genet Cytogenet. 1989; 37: 95–102. https://doi.org/10.1016/0165-4608(89)90079-4 PMID: 2917337

33. Valverde JR, Alonso J, Palacios I, Pestaña A, Pestaña A. RB1 gene mutation up-date, a meta-analysis based on 932 reported mutations available in a searchable database. BMC Genet. 2005; 6: 53. https://doi.org/10.1186/1471-2156-6-53 PMID: 16269091

34. Lohmann DR, Brandt B, Höpping W, Passarge E, Horsthemke B. Spectrum of small length germline mutations in the RB1 gene. Hum Mol Genet. 1994; 3: 2187–2193. https://doi.org/10.1093/hmg/3.12. 2187 PMID: 7881418

35. Eloy P, Dehainault C, Sefia M, Aerts I, Doz F, Cassoux N, et al. A Parent-of-Origin Effect Impacts the Phenotype in Low Penetrance Retinoblastoma Families Segregating the c.1981C>T/p.Arg661Trp Mutation of RB1. PLoS Genet. Service de Génétique, Institut Curie, Paris, France; 2016; 12: e1005888. https://doi.org/10.1371/journal.pgen.1005888 PMID: 26925970

36. Onadim Z, Hogg A, Baird PN, Cowell JK. Oncogenic point mutations in exon 20 of the RB1 gene in families showing incomplete penetrance and mild expression of the retinoblastoma phenotype. Proc Natl Acad Sci U S A. 1992; 89: 6177–81. https://doi.org/10.1073/pnas.89.13.6177 PMID: 1352863

37. Otterson GA, Modi S, Nguyen K, Coxon AB, Kaye FJ. Temperature-sensitive RB mutations linked to incomplete penetrance of familial retinoblastoma in 12 families. Am J Hum Genet. 1999; 65: 1040–6. https://doi.org/10.1086/302581 PMID: 10483322

38. Alonso J, García-Miguel P, Abella J, Mendiola M, Sarret E, Teresa Vendrell M, et al. Spectrum of germline RB1 gene mutations in Spanish retinoblastoma patients: Phenotypic and molecular epidemiological implications. Hum Mutat. 2001; 17: 412–422. https://doi.org/10.1002/humu.1117 PMID: 11317357

39. Klutz M, Brockmann D, Lohmann DR. A parent-of-origin effect in two families with retinoblastoma is associated with a distinct splice mutation in the RB1 gene. Am J Hum Genet. 2002; 71: 174–9. https://doi.org/10.1086/341284 PMID: 12016598

40. Taylor M, Dehainault C, Desjardins L, Doz F, Levy C, Sastre X, et al. Genotype-phenotype correlations in hereditary familial retinoblastoma. Hum Mutat. 2007; 28: 284–293. https://doi.org/10.1002/humu.20443 PMID: 17096365

41. Rushlow D, Piovesan B, Zhang K, Prigoda-Lee NL, Marchong MN, Clark RD, et al. Detection of mosaic RB1 mutations in families with retinoblastoma. Hum Mutat. Retinoblastoma Solutions, Toronto Western Hospital, Toronto, ON, Canada; 2015; 14: 2044–2187. PMID: 2917337

42. Sagi M, Frenkel A, Eilat A, Weinberg N, Frenkel S, Pe’er J, et al. Genetic screening in patients with Retinoblastoma in Israel. Fam Cancer. American Academy of Human Genetics and Metabolic Diseases, Hadassah-Hebrew University Medical Center, Karem Hadassah, POB 12000, Jerusalem, Israel; 2015; 14: 471–480. https://doi.org/10.1007/s10689-015-9794-z PMID: 25754945

43. Sippel KC, Fraioli RE, Smith GD, Schalkoff ME, Sutherland J, Gallie BL, et al. Detection of mosaic RB1 mutations in families with retinoblastoma. Hum Mutat. Retinoblastoma Solutions, Toronto Western Hospital, Toronto, ON, Canada; 2009; 30: 842–851. https://doi.org/10.1002/humu.20940 PMID: 19280657

44. Price E a, Price K, Kolukkaewicz K, Hack S, Reddy MA, Hungerford JL, et al. Spectrum of RB1 mutations identified in 403 retinoblastoma patients. J Med Genet. Retinoblastoma Genetic Screening Unit, Barts Health NHS Trust, London, United Kingdom; 2014; 51: 208–14. https://doi.org/10.1136/jmedgenet-2013-101821 PMID: 24225018

45. Price E A, Price K, Kolukkaewicz K, Hack S, Reddy MA, Hungerford JL, et al. Spectrum of RB1 mutations identified in 403 retinoblastoma patients. J Med Genet. Retinoblastoma Genetic Screening Unit, Barts Health NHS Trust, London, United Kingdom; 2014; 51: 208–14. https://doi.org/10.1136/jmedgenet-2013-101821 PMID: 24225018

46. Ayari Jeridi H, Bouguila H, Anspenger-Rescher B, Baroudi O, Mdimegh I, Omran I, et al. Genetic testing in Tunisian families with heritable retinoblastoma using a low cost approach permits accurate risk prediction in relatives and reveals incomplete penetrance in adults. Exp Eye Res. Elsevier Ltd; 2014; 124: 48–55. https://doi.org/10.1016/j.exer.2014.04.013 PMID: 24810223

47. Bamne MN, Ghule PN, Jose J, Banavali SD, Kurkure P a, Amare Kadam PS. Constitutional and somatic RB1 mutation spectrum in nonfamilial unilateral and bilateral retinoblastoma in India. Genet Test. 2005; 9: 200–11. https://doi.org/10.1089/gte.2005.9.200 PMID: 16225399

48. Nichols KE, Houseknecht MD, Godmilow L, Bunin G, Shields C, Meadows A, et al. Sensitive multistep clinical molecular screening of 180 unrelated individuals with retinoblastoma detects 36 novel mutations in the RB1 gene. Hum Mutat. Division of Pediatric Oncology, Children’s Hospital of Philadelphia, Philadelphia, PA, United States; 2005; 25: 566–574. https://doi.org/10.1002/humu.20184 PMID: 15884040
48. Shi W, Kato H, Perez-Ordoranza B, Pintiliie M, Huang S, Hui A, et al. Comparative prognostic value of HPV16 E6 mRNA compared with in situ hybridization for human oropharyngeal squamous carcinoma. J Clin Oncol. 2009; 27: 6213–6221. https://doi.org/10.1200/JCO.2009.23.1670 PMID: 19884544

49. Price E A, Price K, Kolkiewicz K, Hack S, Reddy MA, Hungerford JL, et al. Spectrum of RB1 mutations identified in 403 retinoblastoma patients. J Med Genet. 2014; 51: 208–14. https://doi.org/10.1136/jmedgenet-2013-101821 PMID: 24225018

50. He M, An Y, Gao Y, Qian X, Li G, Qian J. Screening of RB1 gene mutations in Chinese patients with retinoblastoma and preliminary exploration of genotype-phenotype correlations. Mol Vis. Department of Hematology and Oncology, Children’s Hospital of Fudan University, Shanghai, China; 2014; 20: 545–52. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4007138&tool=pmcentrez&rendertype=abstract

51. Choy KW, Pang CP, Yu CBO, Wong HL, Ng JSK, Fan DSP, et al. Loss of heterozygosity and mutations are the major mechanisms of RB1 gene inactivation in Chinese sporadic retinoblastoma. Hum Mutat. 2002; 20: 408. https://doi.org/10.1002/humu.9077 PMID: 12401220

52. Lohmann DR, Brandt B, Hopping W, Passarge E, Horsthemke B. The Spectrum of RB1 Germ-Line Mutations in Hereditary Retinoblastoma. Am J Hum Genet. 1996; 58: 940–949. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1914612&tool=pmcentrez&rendertype=abstract PMID: 8851278

53. Richter S, Vandezaende K, Chen N, Zhang K, Sutherland J, Anderson J, et al. Sensitive and efficient detection of RB1 gene mutations enhances care for families with retinoblastoma. Am J Hum Genet. Elsevier; 2003; 72: 253–69. https://doi.org/10.1096/ajhg.03-203 PMID: 12641200

54. Dimaras H, Khent B, Halliday W, Orlov M, Prigoda NL, Piovesan B, et al. Loss of RB1 induces non-proliferative retinoma: Increasing genomic instability correlates with progression to retinoblastoma. Hum Mol Genet. Ontario Cancer Institute, Princess Margaret Hospital, University Health Network, 610 University Avenue, Toronto, ON M5G 2M9, Canada, England; 2008; 17: 1363–1372. https://doi.org/10.1093/hmg/ddn024 PMID: 18211953

55. Dommering CJ, Mol BM, Moll AC, Burton M, Cools J, Dorsman JC, et al. RB1 mutation spectrum in a comprehensive nationwide cohort of retinoblastoma patients. J Med Genet. Department of Clinical Genetics, VU University Medical Center, PO Box 7057, Amsterdam 1007 MB, Netherlands; 2014; 51: 366–374. https://doi.org/10.1136/jmedgenet-2014-102264 PMID: 24688104

56. Schüller A, Weber S, Neuhausser M, Jurklies C, Lehnert T, Heimann H, et al. Age at diagnosis of isolated unilateral retinoblastoma does not distinguish patients with and without a constitutional RB1 gene mutation but is influenced by a parent-of-origin effect. Eur J Cancer. 2005; 41: 735–740. https://doi.org/10.1016/j.ejca.2004.12.022 PMID: 15763650

57. Vogel F. Genetics of retinoblastoma. Hum Genet. Department of Pediatric Hematology and Oncology, Cliniques Universitaires Saint Luc, Université Catholique de Louvain, Avenue Hippocrate 10, B-1200 Brussels, Belgium; 1979; 52: 1–54. https://doi.org/10.1007/BF00284597

58. Chen Z, Moran K, Richards Yutz J, Tooren P, Gerard D, Ganguly T, et al. Enhanced sensitivity for detection of low-level germline mosaic RB1 mutations in sporadic retinoblastoma cases using deep semiconductor sequencing. Hum Mutat. Genetic Diagnostic Laboratory, Department of Genetics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, United States; 2014; 35: 384–391. https://doi.org/10.1002/humu.22488 PMID: 24282159

59. Ayari-Jeridi H, Moran K, Chebbi A, Bouguila H, Abbes I, Charraki K, et al. Mutation spectrum of RB1 gene in unilateral retinoblastoma cases from Tunisia and correlations with clinical features. PLoS One. Laboratoire de Génétique, Immunologie et Pathologies Humaines, Faculté des Sciences de Tunis, Université de Tunis EL MANAR, Tunis, Tunisia: Public Library of Science; 2015; 10: e0116615. https://doi.org/10.1371/journal.pone.0116615 PMID: 25602518

60. Cheng, G, Wang, Y, Li, B, Shi, J, Zhao, J, Jonas JB. Genetic and Epigenetic Profile of Retinoblastoma in a Chinese Population. Asia-Pacific J Ophthalmol. 2013; 2: 414–417. https://doi.org/10.1097/APO.0000000000000016 PMID: 26107153

61. Ohtani-Fuji ta N, Fujita T, Aoike A, Osifchin NE, Robbins PD, Sakai T. CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. Oncogene. 1993; 8: 1063–7. Available: http://www.ncbi.nlm.nih.gov/pubmed/8459933 PMID: 8459933

62. Sakai T, Othani N, McGee T, Robbins P, Dryja T. Oncogenic germ-line mutations in SP1 and ATF sites in the human retinoblastoma gene. Nature. 1991; 353: 83–86. https://doi.org/10.1038/353083a0 PMID: 1881452

63. Zhang L, Jia R, Zhao J, Fan J, Zhou YX, Han B, et al. Novel mutations in the RB1 gene from Chinese families with a history of retinoblastoma. Tumor Biol. Department of Ophthalmology, Shanghai Ninth People’s Hospital, Shanghai Jiao Tong University School of Medicine, No. 639 Zhi Zao Ju Road, Shanghai, China; 2015; 36: 2409–2420. https://doi.org/10.1007/s13277-014-2851-7 PMID: 25424699
64. He MY, An Y, Li G, Qian J, Gao YJ. Characteristics of RB1 gene mutations in Chinese patients with retinoblastoma. Chinese J Med Genet. Department of Hematology and Oncology, Children's Hospital of Fudan University, Shanghai 201102, China; 2013; 30: 509–512. https://doi.org/10.3760/cma.j.issn.1003-9406.2013.05.001 PMID: 24078560

65. McEvoy J, Nagahawatte P, Finkelstein D, Richards-Yutz J, Valentine M, Ma J, et al. RB1 gene inactivation by chromothripsis in human retinoblastoma. Oncotarget. Department of Developmental Neurobiology, St. Jude Children’s Research Hospital, Memphis, TN, United States; 2014; 5: 438–450. https://doi.org/10.18632/oncotarget.1686 PMID: 24509483

66. Choy KW, Lee TC, Cheung KF, Fan DSP, Lo KW, Beaverson KL, et al. Clinical Implications of Promoter Hypermethylation in RASSF1A and MGMT in Retinoblastoma, Neoplasia. Dept. of Ophthalmol. and Vis. Sci., Chinese University of Hong Kong, Hong Kong, Hong Kong; 2005; 7: 200–206. https://doi.org/10.1593/neo.044556 PMID: 15799820

67. Conson TW, Gallie BL. One hit, two hits, three hits, more? Genomic changes in the development of retinoblastoma. Genes Chromosom Cancer. 2007; 46: 617–634. https://doi.org/10.1002/gcc.20457 PMID: 17437278

68. Devarajan B, Prakash L, Kannan TR, Abraham AA, Kim U, Muthukkaruppan V, et al. Targeted next generation sequencing of RB1 gene for the molecular diagnosis of Retinoblastoma. BMC Cancer. Department of Bioinformatics, Aravind Medical Research Foundation, Madurai, India; 2015; 15: 320. https://doi.org/10.1186/s12888-015-1340-8 PMID: 25928201

69. Rodriguez-Martín C, Cedre F, Fernández-Tejeiro A, Gómez-Mariano G, de la Vega L, Ramos P, et al. Familial retinoblastoma due to intrinsic LINE-1 insertion causes aberrant and noncanonical mRNA splicing of the RB1 gene. J Hum Genet. 2016; 61: 463–466. https://doi.org/10.1038/jhg.2015.173 PMID: 26763876

70. Sellner LN, Edkins E, Smith N. Screening for RB1 mutations in tumor tissue using denaturing high performance liquid chromatography, multiplex ligation-dependent probe amplification, and loss of heterozygosity analysis. Pediatr Dev Pathol. 2006; 9: 31–37. PMID: 16808635

71. Ottaviani D, Parma D, Giliberto F, Ferrer M, Fandino A, Davila MT, et al. Spectrum of RB1 mutations in Argentine patients: 20-years experience in the molecular diagnosis of retinoblastoma. Ophthalmic Genet. Genetica y Biologia Molecular, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina; 2013; 34: 189–98. https://doi.org/10.3109/13816810.2012.755553 PMID: 23301675

72. Cooper DN, Krawczak M, Polychronakos C, Tyler-Smith C, Kehrer-Sawatzki H. Where genotype is not predictive of phenotype: Towards an understanding of the molecular basis of reduced penetrance in human inherited disease. Hum Genet. Springer; 2013; 132: 1077–1130. https://doi.org/10.1007/s00439-013-1331-2 PMID: 23820649

73. Kanber D, Berulava T, Ammerpohl O, Richter J, Siebert R, et al. The human retinoblastoma gene is imprinted. PLoS Genet. Institut für Humangenetik, Universitätsklinikum Essen, Essen, Germany; 2009; 5: e1000790. https://doi.org/10.1371/journal.pgen.1000790 PMID: 20041224

74. Kanber D, Buiting K, Roos C, Gromoll J, Kaya S, Horsthemke B, et al. The origin of the RB1 imprint. PLoS One. Public Library of Science; 2013; 8: e81502. https://doi.org/10.1371/journal.pone.0081502 PMID: 24282601

75. Lohmann DR, Brandt B, Höpping W, Passarge E, Horsthemke B. Distinct RB1 gene mutations with low penetrance in hereditary retinoblastoma. Hum Genet. 1994; 94: 349–354. https://doi.org/10.1007/BF00205191 PMID: 7927327

76. Harbour JW. Molecular Basis of Low-Penetrate Retinoblastoma. Arch Ophthalmol. Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St Louis, MO, United States: American Medical Association; 2001; 119: 1699. https://doi.org/10.1001/archophht.119.11.1699

77. Dimas AS, Nica AC, Montgomery SB, Stranger BE, Raj T, Bui L, et al. Sex-biased genetic effects on gene regulation in humans. Genome Res. 2012; 22: 2368–2375. https://doi.org/10.1101/gr.134981.111 PMID: 22960374

78. Amitrano S, Marozza A, Somma S, Imperatore V, Hadjistilianou T, De Francesco S, et al. Next generation sequencing in sporadic retinoblastoma patients reveals somatic mosaicism. Eur J Hum Genet. Department of Medical Biotechnologies, University of Siena, Policlinico “Santa Maria alle Scotte”, Viale Bracci 2, Siena, Italy; 2015; 23: 1523–1530. https://doi.org/10.1038/ejhg.2015.6 PMID: 25712084

79. Lohmann DR, Gerick M, Brandt B, Oelschläger U, Lorenz B, Passarge E, et al. Constitutional RB1-gene mutations in patients with isolated unilateral retinoblastoma. Am J Hum Genet. 1997; 61: 282–94. https://doi.org/10.1086/514845 PMID: 9311732

80. Gallie B. Canadian guidelines for retinoblastoma care. Can J Ophthalmol / J Can d’Ophtalmologie. 2009; 44: 639–642. https://doi.org/10.3129/09-0229
81. Singh G, Daniels AB. Disparities in Retinoblastoma Presentation, Treatment, and Outcomes in Developed and Less-Developed Countries. Semin Ophthalmol. 2016; 538: 1–7. https://doi.org/10.3109/08820538.2016.1154177 PMID: 27127937

82. Temming P, Viehmann A, Arendt M, Eisele L, Spix C, Bornfeld N, et al. Pediatric second primary malignancies after retinoblastoma treatment. Pediatr Blood Cancer. Department of Pediatric Hematology and Oncology, University Hospital Essen, Essen, Germany; 2015; 62: 1799–1804. https://doi.org/10.1002/pbc.25576 PMID: 25970657

83. Xu K, Rosenwaks Z, Beaverson K, Cholst I, Veeck L, Abramson DH. Preimplantation genetic diagnosis for retinoblastoma: The first reported liveborn. Am J Ophthalmol. 2004; 137: 18–23. https://doi.org/10.1016/S0002-9394(03)00872-9 PMID: 14700639

84. Wang X, Gregory-Evans CY. Nonsense suppression therapies in ocular genetic diseases. Cell Mol Life Sci. 2015; 72: 1931–1938. https://doi.org/10.1007/s00018-015-1843-0 PMID: 25651836

85. Xin G-H, Zhao X-H, Liu D, Gong Q, Hou L, Li J-Y, et al. Effect of VEGF-targeted antisense gene therapy on retinoblastoma cell line SO-RB50 in vitro and in vivo. Int J Ophthalmol. 2012; 5: 440–7. https://doi.org/10.3980/j.issn.2222-3959.2012.04.07 PMID: 22937502