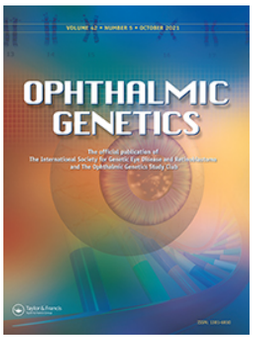

Ophthalmic Genetics

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iopg20

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To cite this article: Xiaolian Fang, Jun Chen, Yizhuo Wang, Minchao Zhao, Xin Zhang, Lei Yang, Xin Ni, Junyang Zhao & Brenda L. Gallie (2021) *RB1* germline mutation spectrum and clinical features in patients with unilateral retinoblastomas, Ophthalmic Genetics, 42:5, 593-599, DOI: 10.1080/13816810.2021.1946703

To link to this article: https://doi.org/10.1080/13816810.2021.1946703

Published online: 30 Jun 2021.

OPHTHALMIC GENETICS
2021, VOL. 42, NO. 5, 593–599
https://doi.org/10.1080/13816810.2021.1946703

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RESEARCH REPORT

RB1 germline mutation spectrum and clinical features in patients with unilateral retinoblastomas

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ABSTRACT

Background: Retinoblastoma is the most common intraocular cancer in children in which above 90% of bilateral cases and 10–25% of unilateral cases have germline *RB1* mutations. We summarized the spectrum of *RB1* germline mutations and the clinical manifestations of unilateral retinoblastomas to guide clinical treatments.

Methods: Two hundred and sixty-three unrelated patients with unilateral retinoblastoma and their parents were included between February 2014 and August 2020. Next-generation sequencing and Sanger sequencing analysis of the core promoter region and exons 1–27 including flanking intronic regions of the *RB1* gene were performed. If a germline mutation was identified in a retinoblastoma patient, the parental blood sample was requested to test for the identified mutation.

Results: *RB1* germline mutations were identified in 39/263 (14.8%) unilateral retinoblastoma patients and 11 (28.2%) had a missense mutation, 10 (25.6%) had nonsense mutations, 2 (5.1%) had frameshifts, 1 (2.6%) had synonymous mutation, and 7 (17.9%) had a large deletion, 2 (5.1%) had splice site mutations, 6 (15.4%) had variant of uncertain significance. Moreover, 27 (69.2%) of 39 patients identified *RB1* mutations were predicted to have pathogenic mutation. The median age at diagnosis of patients with identified *RB1* pathogenic mutations was 16.9 months and the patients with the wild-type allele was 21.1 months ($P = .323$).

Conclusion: The rate of germline *RB1* mutations is 14.8% in our cohort of unilateral retinoblastomas. The high incidence of germline mutations indicates that genetic testing and counseling for families of unilateral retinoblastoma patients would be beneficial.

ARTICLE HISTORY
Received February 08, 2021
Revised May 22, 2021
Accepted June 10, 2021

KEYWORDS
Children; genetic testing; germline mutation; retinoblastoma; next-generation sequencing

Retinoblastoma is the most common intraocular cancer in children. It is caused by the biallelic inactivation of the human retinoblastoma transcriptional corepressor 1 (*RB1*) gene on chromosome 13q14, which encodes the retinoblastoma protein (1–4). Generally, above 90% of bilateral cases and 10–25% of unilateral cases have germline *RB1* mutations (5–8). *RB1*, the first tumor suppressor gene discovered, is 183kb long (7,9). Mutations in the *RB1* gene are highly heterogeneous and scattered along the promoter and 27 exons (10,11). The mutation spectrum of the *RB1* gene ranges from large deletions to single nucleotide and can affect the structure and impair function of pRb, eventually leading to the formation of retinoblastoma (1,12,13).

With genetic testing, retinoblastoma can be diagnosed earlier, increasing the potential to cure this disease (6,14). Moreover, the detection of disease-causing mutations contributes to appropriate treatment, long-term follow-up, prenatal testing, and assessment of the development of *RB1* related tumors (15,16). In addition, identification of asymptomatic carriers of *RB1* mutations can facilitate genetic counseling for family planning (17). However, little attention has been paid to the genetic testing of unilateral retinoblastomas in China, and few studies have been conducted. Therefore, we summarized the spectrum of *RB1* mutations in unilateral retinoblastomas in a long-term follow-up of a large cohort to determine the frequency and type of mutations. In addition, we explored the correlation between genotype and age at diagnosis, clinical features, and prognosis, to guide clinical treatments.

Methods

Patients

Two hundred and sixty-three unrelated patients with unilateral retinoblastoma and some of their parents, who referred to the Beijing Children's Hospital and Beijing Tongren Hospital, China between February 2014 and August 2020, were included. All experimental protocols were approved by the Institutional Review

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Board (IRB) of the Beijing Children's Hospital of Capital Medical University (2020-Z-126) and all procedures complied with the relevant ethical standards and the Declaration of Helsinki. Retinoblastoma was diagnosed through standard ophthalmologic and histological criteria by two experts in ocular oncology. Signed written informed consent for the genetic analysis was obtained from the patients or their legal custodians. *RB1* genetic testing for all retinoblastoma patients in the cohort was performed at Nanjing Geneseeq Technology Inc (Nanjing, China). Peripheral blood samples (5 mL) were collected from patients and their parents, and stored at −20°C until DNA extraction. If a germline mutation was identified in a retinoblastoma patient, the parental blood sample was requested to test for the identified mutation. Detailed information on *RB1* genetic testing, clinical presentation, International Intraocular Retinoblastoma Classification (IIRC) stage, family history, treatments, and survival were obtained. In addition, the time of retinoblastoma diagnosis, age at diagnosis, and survival time were recorded.

Sequencing

Genomic DNA was isolated from peripheral blood leukocytes using the QIAamp Blood & Tissue kit (Qiagen, Düsseldorf, Germany) according to the manufacturer's protocol. The quality and quantity of the extracted genomic DNA were measured using a NanoDrop 2000 (Thermo Fisher Scientific) and the Qubit 3.0 dsDNA HS kit (Thermo Fisher Scientific), respectively, according to the manufacturer's protocol. Sequencing libraries were prepared using the KAPA Hyper Prep kit (KAPA Biosystems) with optimization of the manufacturer's protocol. Briefly, 600 ng whole-blood genomic DNA was sheared into ~350 bp fragments using a Covaris M220 instrument (Covaris). The fragmented DNA sample was then processed by end-repair, A-tailing, ligation to sequencing adapters, size selection, and PCR amplification. The size selection and purification steps were performed using Agencourt AMPure XP beads (Beckman Coulter). The prepared library was then pooled up to 2 μg. Target enrichment was performed using a hybridization capture protocol with Dynabeads M-270 (Thermo Fisher Scientific) and xGen Lockdown Reagent (Integrated DNA Technologies). Human Cot-1 DNA (Thermo Fisher Scientific) and xGen Universal blocking oligos (Integrated DNA Technologies) were added as blocking reagents. A customized next-generation sequencing (NGS) panel covering the core promoter region and 27 exons of the *RB1* gene, including flanking intronic regions, was used. Captured libraries were amplified by on-bead PCR with Illumina p5 (5′ AAT GAT ACG GCG ACC ACC GA 3′) and p7 (5′ CAA GCA GAA GAC GGC ATA CGA GAT 3′) primers in KAPA HiFi HotStart ReadyMix (KAPA Biosystems), and purified using Agencourt AMPure XP beads. Libraries were quantified by qPCR using the KAPA Library Quantification kit (KAPA Biosystems) and sequenced on HiSeq 4000 NGS platforms (Illumina) to a coverage depth of ~2500 × .

Analysis of *RB1* variants

FASTQ file quality control was performed using Trimmomatic. Reads from each sample were mapped to the reference sequence hg19 (Human Genome version 19) using Burrows-Wheeler Aligner (BWA-mem, v0.7.12). Local realignment around insertions/deletions (indels) and base quality score recalibration were applied with the Genome Analysis Toolkit (GATK 3.4.0). Single nucleotide polymorphism (SNP) and short Indel calling were performed using a customized bioinformatics algorithm. Exon-level copy number variant (CNV) calling was performed using an independently developed pipeline and compared to a pool of normal samples. Variant annotation was performed using VEP. Additional information on mutations and polymorphisms in the *RB1* gene was obtained from the LOVD database (http://RB1-lovd.dlohmann.de), ClinVar, and the Exome Aggregation Consortium. Human Splicing Finder and MaxEntScan were used to predict the pathogenicity of splice variants, and PolyPhen-2, SIFT, PROVEAN, Mutation Taster, and ClinPred (Alirezaie et al., 2018) were used to predict the pathogenicity of missense variants. Detected variants were classified according to the American College of Medical Genetics and Genomics guidelines (4).

Additional validation of RB1 variants

RB1 SNPs, splice site variants, and Indels were validated using Sanger sequencing of PCR-amplified exons. Mosaic mutations with low allelic frequencies were validated by PCR amplification of the corresponding exons, followed by library preparation and NGS of the PCR products. The PCR primer sequences used in this study to amplify the targeted *RB1* region are listed in Table 1.

RB1 CNV or large genomic rearrangements were validated using QX200 droplet digital (dd)PCR (Bio-Rad). Briefly, each ddPCR reaction contained 450 nM of each primer, 250 nM of each hydrolysable probe, and 50 ng of genomic DNA input. The PCR program was run with the manufacturer's recommended protocol of 95°C for 10 min, 40 cycles of 94°C for 30 s and 60°C for 1 min, and 98°C for 10 min, with 2°C/min ramp rate. *RPP30* was selected as the internal copy number control.

Statistics analysis

The relevant data were subjected to statistical analyses using SPSS software (version 15.0, SPSS Inc., Chicago, IL) to explore the relationships between clinical features and genetic information in retinoblastomas. The geographical distribution map was drawn using JMP software (version 14.0). The medians and quartiles were calculated for abnormal distribution of age. Differences between groups were evaluated by using the Mann–Whitney U-test. Differences were considered statistically significant at *P*-values less than 0.05.

Result

Clinical characteristics of patients

A total of 263 Chinese patients with unilateral retinoblastoma including 115 females (43.7%) and 148 males (56.3%) were included for genetic testing of germline mutations in the *RB1* gene. The clinical features and outcomes of the 263 patients with retinoblastoma were summarized in Table 2. Patients were geographically distributed, representing 21 provinces and four municipalities, four economic regions, seven ethnic groups, and rural and urban areas (Figure 1). The age at diagnosis ranged from 0.07 to

Table 1. PCR primer sequences used to amplify the targeted *RB1* region.

Exon	Primer Direction	Sequence (5′ to 3′)
Exon 1	Forward	ACAGTCACCCACCAGACTCTTT
	Reverse	ACCCCTCGCCCAAGAACCCA
Exon 2	Forward	TGTTATGTGCAAACTATTGAAACAAG
	Reverse	AAATTTCCTCTGGGTAATGGA
Exon 7	Forward	ACTCTACCCTGCGATTTTCTC
	Reverse	TGACCCAAACCACTTTCTACC
Exon 8	Forward	TGTTACCAAGATTATTTTTGACC
	Reverse	TACATCTAAATCTACTTTAACTG
Exon 9	Forward	ATGGGGGATTGACACCTCTAAC
	Reverse	ACCACAATTCTACTTGGCTAG
Exon 10	Forward	TATATTGCATGCGAACTCAG
	Reverse	TGATATCTAAAGGTCACTAAGCTAAA
Exon 12	Forward	CCACAGTCTTATTTGAGGGA
	Reverse	GCAAGAAAAGATTATGGATAACTACA
Exon 13	Forward	CTCTAGCCTAGTGGCAGAAA
	Reverse	TAGTACCACGAATTACAATG
Exon 14	Forward	TGAGCCCAGGAGTGTGAAG
	Reverse	GATGATCTTGATGCCTTGAC
Exon 16	Forward	CTGGCAACAGAGCAAGACAC
	Reverse	GATCTAAAATAAGCATTCCTTCTCC
Exon 17	Forward	TGAGTCCGTAGACTTCAAAA
	Reverse	TTCCCTATTTGTTCTTGAGGT
Exon 19	Forward	TGATGACAAGCAGTTTTCCT
	Reverse	CGCAACATTATCATTTCCAT
Exon 20	Forward	CTCTGGGGGAAAGAAAAGA
	Reverse	ATCAGTTAACAAGTAAGTAG
Exon 21	Forward	TGATCAGTCCTGGATAATTG
	Reverse	GTCAGACAGAATATATGATCTC
Exon 22/23	Forward	TGCTGCCTGGCTATTTCTCT
	Reverse	AATGCAGAAATCACCCGTCT
Exon 25	Forward	CTTGAGGTTGCTAACTATGAAACAC
	Reverse	TCAGCTACTGGAAAACATTC

95.5 months, with a median of 20.8 months. The median age at the first sign was 18.8 months, ranging from 0.03 to 94.6 months. A total of 68 (25.8%) of the 263 patients were diagnosed before 1 year of age, 150 (82.6%) were before 3 years of age, and only six patients (9.4%) were diagnosed after the age of five. The most common symptoms of retinoblastoma in patients were leukocoria (185/263, 70.3%) and strabismus (30/263, 11.4%). Four (1.1%) of the 263 patients had a family history of retinoblastoma and three of these four patients had *RB1* germline mutations. The median lag time between the first sign and treatment was 1.13 months (range: 0.07 to 18.9 months). According to IIRC staging, 10 patients were in the early stage (group A, B, or C), 234 were in the advanced stage (150 in group D and 84 in group E), and 19 patients were unclear.

Genetic analysis

In total, *RB1* mutations were identified in 39 of the 263 patients (14.8%). Four patients were positive for family history, and the rate of *RB1* gene mutation was 13.9% (36/259) for patients without a family history of retinoblastoma. Among the 39 mutations identified by NGS and Sanger sequencing, 27 (69.2%) patients had point mutation, 11 (28.2%) had deletion and 1 (2.6%) had insertion. From the genetic standpoint, 11 (28.2%) had a missense mutation, 10 (25.6%) had nonsense mutations, 2 (5.1%) had frameshifts, 1 (2.6%) had synonymous mutation, and 7 (17.9%) had a large deletion, 2 (5.1%) had splice site mutations, 6 (15.4%) had variant of uncertain significance (VUS). Importantly, 17 (43.6%) of the 39 mutations were mosaic mutations including nonsense mutations (n = 10), a splice site mutation (n = 1), deletions (n = 4), and frameshifts (n = 2). Table 3 shows detailed information about specific germline *RB1* mutations identified in Chinese retinoblastoma patients. Among the seven patients with large detections, two (RB-101, RB-1206) had deletions encompassing the entire *RB1* gene, three were CNV in 1–27 exons, and two were CNV in exon 4 or exon 27. In addition, 33 unique mutations were found in only one retinoblastoma patient or family in our cohort and three mutations were identified in two or more patients/families.

Moreover, 12 of 39 (30.8%) patients inherited the mutation from their parents (7 were paternal and 5 were maternal transmissions) and the rest were *de novo* mutations. All these 12 patients of the identified germline mutation also detected in one parent were all in a homogeneous state. In one case, the mutation was inherited from the father with unilateral retinoblastoma but other parents were unaffected. Six novel mutations were found: c.1961–9 T > G in intron 19; c.2324 G > T (p.Arg775Lys) in exon 22; c.39delC (p.Ala14ProfsTer51) in exon 1; c.719–34A>G in intron 7; c.1695 + 84 T > G in intron 17; and c.830 T > C (p.Leu277Pro), which resided within a highly conserved nucleotide and amino acid residue.

Mutation and clinical features

Most patients (97.7%, 257/263) in this cohort were followed up for more than 1 year. The median follow-up time was 68.4 months, ranging from 0.3 to 193.6 months. Two (0.8%) patients with IIRC stage D died of blood or brain metastasis after a series of treatments. One patient received three cycles of vincristine + etoposide + carboplatin (VEC) and Pars Plana Vitrectomy (PPV), and died on the last date of follow-up, 16.5 months after being diagnosed with retinoblastoma while the other patient received three cycles of VEC, enucleation (pT3b) and three cycles of VEC after enucleation but developed brain metastasis 27.6 months after retinoblastoma diagnosis.

The overall rate of enucleation was 73% (192/264). According to the tumor IIRC staging, only one of the patients with stages A, B, and C was enucleated (10 patients). The eye salvage rate was 38.7% (58/150) in stage D and 2.4% (2/84) in stage E. Patients with stage D or E disease had significantly lower rates of eye salvage than those with stages A, B, or C disease (60/234 vs. 9/10, $P < .001$).

Moreover, 27 (69.2%) of 39 patients identified *RB1* mutations were predicted to be pathogenic mutation. The age at diagnosis of patients with identified *RB1* pathogenic mutations ranged from 0.1 to 95.5 months, with a median of 16.9 months and the median age at diagnosis of patients with the wild-type allele was 21.1 months ($P = .323$, Figure 2). In addition, the age at sign of patients with pathogenic *RB1* mutation and wild-type was 16.8 months and 19.6 months, respectively ($P = .601$).

Discussion

In this study, we analyzed all *RB1* mutations detected in a long-term follow-up multicenter cohort of 263 unilateral retinoblastoma patients referred to the Beijing Children's Hospital and Beijing Tongren Hospital, China, between 2014 and 2020. We found that the rate of *RB1* mutation was 14.8% in unilateral retinoblastoma patients, which was consistent with previous reports (5,16). This high incidence suggested that *RB1* genetic testing was also important for unilateral retinoblastoma patients.

Table 2. Demographic Characteristics of retinoblastomas in the study cohort.

	Total		Mutation group (n = 39)		Wild type group (n = 224)		
Variables	N	%	N	%	N	%	P value
Sex							0.473
Female	115	43.7	15	38.5	100	44.6	
Male	148	56.3	24	61.5	124	55.4	
Age at presentation (mons, median)	18.7 (0.03–94.6)		15.9 (1.4–55.2)		19.6 (0.03–94.6)		0.207
Age at diagnosis (mons, median)	20.8 (0.07–95.5)		17.6 (1.5–55.9)		21.1 (0.07–95.5)		0.107
<12	68						
12–36	150						
36–60	39						
>60	6						
Main complaints							
Leukocoria	185	70.3	22	56.4	163	72.8	
Strabismus	30	11.4	7	17.9	23	10.3	
Impaired vision	7	2.7	3	7.7	4	1.8	
Red eye	21	8.0	4	10.3	17	7.6	
Screening	6	2.3	1	2.6	5	2.2	
Trauma	5	1.9	1	2.6	4	1.8	
Cornea opacity	3	1.1	0	0	3	1.3	
Others	6	2.2	1	2.6	5	2.2	
IIRC stage							
A	2	0.8	0	0	2	0.9	
B	3	1.1	2	5.1	1	0.4	
C	5	1.9	3	7.7	2	0.9	
D	150	57.0	21	53.8	129	57.6	
E	84	32.0	10	25.6	74	33.0	
N/A	19	7.2	3	7.7	16	7.1	
Lag time (mons)	1.1 (0.07–18.9)		1.0 (0.07–8.3)		1.2 (0.13–18.9)		0.528
≥2 weeks	214	81.3					
≥2 mons	78	29.7					
≥6 mons	23	8.7					
Outcomes							
No enucleation	71	27.0	17		54		
Enucleation	192	73.0	22		170		
Overall survival							0.554
Alive	261	99.2	39		222		
Died	2	0.8	0		2		

Mon, month; Lag time: Lag time between presentation and treatment; *, $P < 0.05$

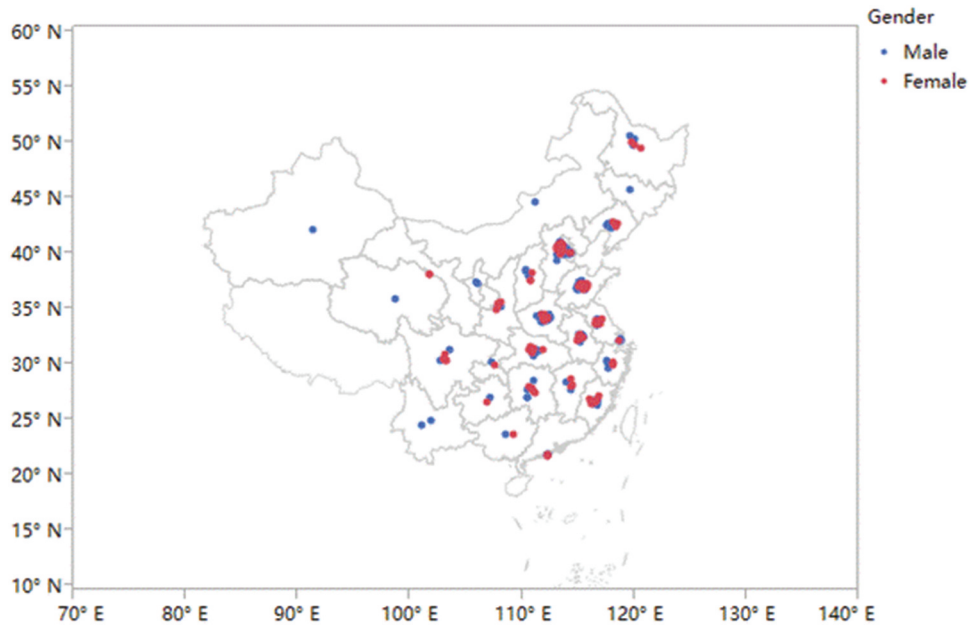

Figure 1. The residence distribution map of children with unilateral retinoblastoma in our cohort.

Table 3. Summary of *RB1* mutation identified in 39 Chinese patients with retinoblastoma using Sanger sequencing and Next-Generation Sequencing (NGS).

Patient ID	Gender	IIRC stage	Age at Dx (mons)	Mutation type	Exon/intron	Change in cDNA	Change in Protein	Pathogenic mutation(Predicted)	Present in mother/father	Status	Survival time (mons)
RB-077	M	C	13.9	Synonymous/splice	Exon 21	c.2211 G > A	p.Glu737Glu	n/a	No	Alive	88.3
RB-019	M	E	19.7	Missense/VUS	Exon 19	c.1861 C > A	p.Arg621Ser	n/a	Mother	Alive	124.3
RB-049	M	N/A	27.2	Missense	Exon 13	c.1332 G > C	p.Gln444His	Yes	No	Alive	128.5
RB-012	F	D	24.8	Missense/VUS (NEW)	Exon 22	c.2324 G > T	p.Arg775Lys	n/a	Father	Alive	17.6
RB-069	F	N/A	25.7	Splice (NEW)	Intron 19	c.1961–9 T > G	N/A	No	Father, grandmother	Alive	77.6
RB-082	F	D	34.4	Nonsense(Mosaic)	Exon 14	c.1333 C > T	p.Arg445Ter	Yes	No	Alive	72.1
RB-008	M	E	2.4	Nonsense(Mosaic)	Exon 8	c.751 C > T	p.Arg251Ter	Yes	No	Alive	27.1
RB-080	M	D	13.9	Nonsense(Mosaic)	Exon 7	c.689 C > A	p.Ser230Ter	Yes	No	Alive	128.2
RB-101	F	D	55.9	Large deletion	Whole gene	del P→27		Yes	No	Alive	69.5
RB-066	F	D	12.2	Large deletion	Exon1-Exon27	CNV		Yes	No	Alive	131.9
RB-065	M	C	3.7	Large deletion	Exon1-Exon27	CNV		Yes	No	Alive	102.7
RB-054	M	E	13.9	Large deletion	Exon1-Exon27	CNV		Yes	No	Alive	85.9
RB-021	M	D	32.8	Large deletion	Exon 4	Copy number loss		Yes	Father, brother	Alive	77.9
RB-1261	M	D	21.2	Large deletion(Mosaic)	Exon 17	Del17		Yes	No	Alive	70.5
RB-1206	F	B	15.0	Large deletion(Mosaic)	Exon1-Exon27	Deletion		Yes	No	Alive	73.4
RB-1268	M	B	1.5	Point mutation/VUS (NEW)	Intron 7	c.719–34A>G	N/A	No	Mother	Alive	46.4
RB-1250	F	D	21.4	Point mutation/VUS (NEW)	Intron 17	c.1695 + 84 T > G	N/A	No	Father	Alive	38.2
RB-1290	F	N/A	10.8	Point mutation/VUS	N/A	c.137 + 5 G > A	N/A	n/a	N/A	Alive	31.2
RB-1246	M	C	20.8	Splice(Mosaic)	Intron 23	c.2489 + 1 G > A	N/A	Yes	No	Alive	68.1
RB-1288	M	D	38.2	Deletion/VUS(Mosaic)	N/A	c.62del	p.Pro21ArgfsTer44	Yes	No	Alive	33.0
RB-1245	F	E	17.8	Deletion/VUS(Mosaic)	Exon 16	c.1485_1494del	p.Met495IlefsTer21	Yes	No	Alive	65.6
RB-1111	M	E	17.0	Deletion/VUS (NEW)	Exon 1	c.39delC	p.Ala14ProfsTer51	Yes	Cousin	Alive	65.4
RB-1135	M	D	15.3	Nonsense(Mosaic)	Exon 23	c.2359 C > T	p.Arg787Ter	Yes	No	Alive	105.3
RB-1150	M	D	9.27	Nonsense(Mosaic)	Exon 17	c.1666 C > T	p.Arg556Ter	Yes	No	Alive	70.6
RB-1267	F	E	14.8	Nonsense(Mosaic)	Exon 14	c.1363 C > T	p.Arg455Ter	Yes	No	Alive	55.9
RB-1151	M	D	3.3	Nonsense(Mosaic)	Exon 13	c.1306 C > T	p.Gln436Ter	Yes	No	Alive	72.4
RB-1174	F	D	6.23	Nonsense(Mosaic)	Exon 12	c.1147 C > T	p.Gln383Ter	Yes	No	Alive	64.1
RB-1131	F	E	13.9	Nonsense (Mosaic)	Exon 10	c.958 C > T	p.Arg320Ter	Yes	No	Alive	69.1
RB-1190	M	D	26.7	Nonsense(Mosaic)	Exon 10	c.958 C > T	p.Arg320Ter	Yes	No	Alive	88.5
RB-1264	M	D	2.8	Missense (NEW)	N/A	c.830 T > C	p.Leu277Pro	n/a	Mother	Alive	44.2
RB-1252	M	D	44.1	Missense	Exon 25	c.2663 G > A	p.Ser888Asn	Yes	No	Alive	61.3
RB-1158	F	E	7.3	Missense	Exon 9	c.938A>G	p.Glu313Gly	n/a	Father	Alive	51.5
RB-1119	M	D	33.1	Missense	Exon 21	c.2134 T > C	p.Cys712Arg	Yes	No	Alive	41.3
RB-1109	M	D	19.9	Missense	Exon 20	c.1981 C > T	p.Arg663Trp	n/a	No	Alive	51.8
RB-1116	M	D	18.0	Missense	Exon 20	c.1981 C > T	p.Arg663Trp	n/a	No	Alive	87.2
RB-1124	M	E	17.6	Missense	Exon 20	c.1981 C > T	p.Arg663Trp	n/a	Father	Alive	57.8
RB-1208	F	E	39.1	Missense	Exon 16	c.1468 G > A	p.Ala490Thr	n/a	No	Alive	65.4
RB-1283	M	D	8.5	Insertion/Frameshift(Mosaic)	N/A	c.219_220dup	p.Ala74GlufsTer4	Yes	No	Alive	116.1
RB-1207	F	D	21.0	Deletion/Frameshift(Mosaic)	Exon 2	c.371_372del	p.Ile124ArgfsTer6	Yes	No	Alive	100.2

Rb, retinoblastoma; *RB1*, Retinoblastoma transcriptional corepressor 1; pRB, protein of retinoblastoma; F, female; M, male; FH, family history; Dx, diagnosis; mons, months; VUS, Variant of Uncertain Significance; CNV, copy number variation.

Figure 2. The age at diagnosis in retinoblastoma cohort grouped by genetic testing. The left shows the mutation group and the right shows the wild-type group. The blue dots represent males and the red dots represent females. mons—months.

Mutational analysis of the *RB1* gene can help clinicians administer appropriate treatment and long-term follow-up. In addition, this analysis can facilitate early screening of at-risk relatives of retinoblastoma patients who test positive for certain *RB1* mutations, and eliminate unnecessary screening of relatives who are not at risk. To our knowledge, the present study involved the largest cohort of unilateral retinoblastomas for which germline mutations in the *RB1* gene have been comprehensively analyzed.

Our study concluded that *RB1* germline mutation can be affected by a heterogeneous spectrum of genetic abnormalities ranging from varied point mutation, intragenic exons loss or duplication to whole-gene deletion, and the most predominant mutations in unilateral patients were missense, nonsense, and large deletions. The type of *RB1* mutations is related to the laterality of retinoblastoma (8). The reported predominant mutations in unilateral patients were missense and large deletions whereas the most common mutations in bilateral patients were nonsense, frameshift and less frequently splice-site mutations (8,10,12,14,18). The whole *RB1* deletions are more common in unilateral patients (13).

The NGS combined with Sanger sequencing analysis of the *RB1* exons and flanking intronic splice regions is a standard method for detecting germline mutations in retinoblastoma patients (10). NGS analysis of the *RB1* gene can detect low-level mosaic variants with a frequency between 8% and 24% in blood DNA (19). Therefore, NGS is an effective method to identify mosaic variants that cannot be identified using Sanger sequencing. The assay developed in this study is, thus, significantly more comprehensive in its ability to detect *RB1* germline defects than previous tests. The rate of large deletion in our study was 17.9%, consistent with those in previous literature reports (20), proving the sensitivity of detection of our method. In 17 of the 39 patients with mutant *RB1* alleles in peripheral blood, DNA existed in a mosaic state, and even low-level *RB1* mosaicism was identified in our test.

Patients with hereditary retinoblastoma are usually diagnosed earlier than non-hereditary retinoblastoma patients (19,21,22). Moreover, the mean age of retinoblastoma diagnosis was significantly different between patients with detected *RB1* mutations and those with no genetic findings in bilateral patients (12). However, this rule remains controversial for children with unilateral disorders (23) and previous studies have shown that the distribution of age at diagnosis is often not different between patients with and without a constitutional *RB1* mutation (20,24). Our study suggests that patients with pathogenic *RB1* mutations are diagnosed or presented earlier than wild-type patients but had no statistic difference. This suggested that genetic testing should not be restricted to children diagnosed at an early age. Moreover, *RB1* mutations might not be a main factor of early onset in unilateral patients (20). Specifically, we posit that it is inaccurate to assess the risk of genetic disease only based on age at diagnosis in unilateral retinoblastomas.

The relationship between genotype and phenotype in retinoblastomas should be discussed further based on larger sample and longer follow-up with the further understanding of the underlying molecular pathogenesis of the disease. Moreover, the mosaic mutation in retinoblastoma will be explored more in our further study.

Conclusion

In this study, the rate of germline *RB1* mutations was 14.8% in patients with unilateral retinoblastomas. This is one of the few study which detailed described the comprehensive spectrum of germline mutations in unilateral retinoblastomas. In addition, we report the clinical features of individuals with or without

RB1 mutation. The high incidence of germline mutations in unilateral retinoblastoma patients indicates that genetic testing and counseling for families of retinoblastoma patients would be beneficial.

Acknowledgments

We thank for the Impact Genetics in assisting with pathogenic allele interpretation.

Disclosure statement

The authors have no conflict of interest to declare.

Funding

This work was supported by Beijing Hospitals Authority' Ascent Plan (DFL20191201); Capital Funds for Health Improvement and Research (2018-1-2091).

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