

Clinical and genetic characteristics of retinoblastoma patients in a single center with four novel *RB1* variants

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Abstract

• **AIM:** To assess the clinical and genetic characteristics of children diagnosed with retinoblastoma (RB) at Gazi University Faculty of Medicine's Department of Pediatric Oncology.

• **METHODS:** All cases diagnosed with RB and received treatment and follow-up in the Ophthalmology and Pediatric Oncology Department, October 2016 to May 2021 were evaluated retrospectively. The *RB1* gene was analyzed by next-generation sequencing (NGS) technique in DNAs obtained from peripheral blood samples of the patients.

• **RESULTS:** This study included 53 cases with 67 RB-affected eyes during the study period. The mean age was 24.6 (median: 18.5, range: 3–151)mo. There were 15 (22.3%) Group D eyes and 39 (58.2%) Group E eyes. The *RB1* gene was sequenced by the NGS method in 19 patients. Heterozygous *RB1*:NM_000321.3: c.54_76del (p.Glu19AlafsTer4) variant was detected in a 15-month-old female with bilateral RB. Heterozygous *RB1*:NM_000321.3: c.1814+3A>T variant was detected in a 5.5-month-old male with bilateral RB. The intronic *RB1*:NM_000321.3: c.1332+4A>G variant was detected in patient 14, a 13-month-old male with unilateral RB. The *RB1*:NM_000321.3: c.575_576del (p.Lys192SerfsTer10) variant was found in an 18-month-old female with an allele frequency of 37%. These variants have not been reported in the literature and mutation databases.

• **CONCLUSION:** Four novel variants are described and one of them is found in two different patients. This data

is crucial for assessing prognosis. It serves as a guide for estimating the long-term risk of secondary malignancy as well as the short-term risk of developing additional malignancies in the same eye and the other eye.

• **KEYWORDS:** retinoblastoma; *RB1* novel mutations; next generation sequencing

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INTRODUCTION

Retinoblastoma (RB) is the most common primary intraocular cancer in children, with a constant incidence worldwide of 1:15 000–1:20 000 live births^[1]. The majority of children with RB are diagnosed before they reach the age of five^[2].

Bilateral RBs constitute around a third of all RB cases, and are caused by germline abnormalities in the *RB1* gene^[3]. In young children, bilateral tumors are more common, indicating the existence of a germline mutation rather than a somatic mutation^[4].

Somatic mutations in the *RB1* gene produce non-hereditary (also called non-familial, sporadic, or somatic) RB, is characterized by unilateral, unifocal illness and is identified at a later age than hereditary RB. Patients with a positive family history of RB and those who have *de novo* mutations are thought to have hereditary (also called familial, genetic) RB caused by the *RB1* germline mutation, with a 50% chance of passing the mutation down to offspring, is characterized by bilateral, multifocal illness before one year of age.

Treatment goals include eradicating the disease to reduce mortality, preserving eye, vision to the greatest extent possible, preventing late complications, and detecting secondary cancers early^[5]. Treatment options are determined by tumor size and location, as well as the presence of vitreous or subretinal seeds^[6]. The goal of this study is to assess the clinical and genetic characteristics of children diagnosed with RB at Gazi

University Faculty of Medicine's Department of Pediatric Oncology.

SUBJECTS AND METHODS

Ethical Approval Informed consent was obtained. This study was approved by the local Ethical Committee of Gazi University Faculty of Medicine. All study procedures were performed according to the Declaration of Helsinki principles (08.09.2020.08).

All cases diagnosed with RB and received treatment in the Ophthalmology and Pediatric Oncology Departments, October 2016 to May 2021 were retrospectively evaluated. Analyses of risk factors for death, metastasis, and enucleation were conducted.

The ocular oncologist (Atalay HT) performed examinations on patients with RB while they were under general anesthesia at the beginning of the treatment and then every month during the treatment course. All cases were classified according to International Classification of RB (ICRB)^[5]. Bone marrow aspiration and lumbar puncture tests were performed in cases with Group D and Group E RB. Brain magnetic resonance imaging (MRI) studies were routinely performed at baseline, and at the end of the treatment.

Patients with bilateral RB had six cycles of intravenous chemotherapy with vincristine, etoposide, and carboplatin (VEC) at four weeks intervals. One patient received chemotherapy according to the ARET032 protocol consisting of four courses of vincristine, cisplatin, cyclophosphamide and etoposide^[7]. Both of these (VEC and ARET032 protocol) treatments are defined as chemoreduction. Patients who received chemoreduction also received transpupillary thermotherapy (TTT) and cryotherapy for tumor regression after second chemotherapy cycle. Unilateral Group A and B RBs underwent local therapies (TTT and cryotherapy). Groups B, C, and D RBs underwent intraarterial chemotherapy (IAC)^[8]. IAC is an endovascular procedure that involves catheterization of the ophthalmic artery. The procedure involves vascular access via the common femoral artery. IAC provides vascular access to the globe and allows for a chemotherapeutic dose delivery. Melphalan, topotecan, and carboplatin were the chemotherapy drugs utilized in IAC. Unilateral Group E RBs underwent enucleation. Unresponsive disease was defined as retinal tumors, vitreous seeds, or subretinal seeds that did not significantly regress with treatment. Secondary enucleation, IAC, and/or intravitreal chemotherapy were performed on eyes with resistant illness. Patients received intravitreal chemotherapy prepared in the operating room while the patient was under general anesthesia. The medicines were reconstituted in a sterile manner on a separate sterile tray. The injection site was carefully chosen to ensure there was no nearby tumor mass, vitreous seeds, or subretinal

fluid within 3 clock hours of the injection site. Drugs were separately injected through the pars plana (2–3 mm from the limbus, with a beveled or two-step approach) mostly superotemporally and infero-temporally using a 30-gauge (8 mm-length) needle. The needle pointing towards the center of the vitreous cavity and away from the anatomical location of the lens. Melphalan (3–7.5 mg), and topotecan (0.5–2 mg) were two of the chemotherapeutic drugs utilized in the intravitreal chemotherapy. We defined trilateral illness either unilateral or bilateral RB with pineal gland involvement.

Genetic Analysis

Sequencing analysis of the *RBI* gene The *RBI* gene was analyzed by next-generation sequencing (NGS) technique in DNAs obtained from peripheral blood samples of the patients. NGS was performed on an Illumina MiSeq system. All bioinformatics analyses were carried out on Sophia DDMTM platform. Pathogenicity classification of variants was made according to criteria of American College of Medical Genetics criteria^[9].

Chromosomal microarray Chromosomal microarray was performed from DNA obtained from the peripheral blood using the SurePrint G3 ISCA V2 CGH 8x60K Array (Agilent Technologies Santa Clara, CA, USA). The data were analyzed according to human genome 19 (Chr37) using Agilent CytoGenomics software. The interpretation of the copy number variations (CNVs) was performed according to the 2020 American College of Medical Genetics (ACMG) and Genomics and Clinical Genome Resource recommendations^[10].

RESULTS

This study included 53 cases with 67 RB-affected eyes during the study period from October 2016 to May 2021. In this study there were 36 male (67.9%) and 17 female (32%) patients. The mean age was 24.6 (median: 18.5, range: 3–151)mo. There were 45 (84.9%) patients diagnosed younger than three years of age and 34 (64.1%) of them were younger than two years of age. In 37 cases (69.8%), RB was unilateral, while it was bilateral in 14 cases (26.4%) and 2 cases (3.7 %) had trilateral RB. Both of the trilateral cases are unilateral RB with pineal gland involvement.

There were 2 (2.9%) Group A eyes, 6 (8.9%) Group B eyes, 5 (7.4%) Group C eyes, 15 (22.3%) Group D eyes, and 39 (58.2%) Group E eyes. There was vitreous seeding in 18 (26.8%) eyes and subretinal seeding in 23 (34.3%) eyes. Patients' characteristics are summarized in Table 1. There were 2 cases which were presented with both leucocoria and strabismus together.

Chemoreduction was administered to 27 cases in total, including those with bilateral involvement and those with unilateral Group D patients who were under 12mo of age at diagnosis due to the difficulties in administering IAC. Fifteen

Table 1 Patients' characteristics n (%)

Characteristics	
Age, mean (range)	24.6 (3-151)mo
Gender	
Female	17 (32)
Male	36 (68)
Laterality	
Unilateral	37 (69.8)
Bilateral	14 (26.4)
Trilateral	2 (3.7)
Initial symptoms	
Leucocoria	39 (73.5)
Strabismus	10 (18.8)
Other	6 (11.3)
ICRB Group	
A	2 (2.9)
B	6 (8.9)
C	5 (7.4)
D	15 (22.3)
E	39 (58.2)
Vitreous seeding	18 (26.8)
Subretinal seeding	23 (34.3)

ICRB: International classification of retinoblastoma.

Table 2 Treatment methods used in our patients with RB

Treatment methods	Number of eyes receiving treatment (%)
Primary enucleation	15 (22.3)
Secondary enucleation	14 (20.8)
Eye-conserving treatments	
TTT	21 (31.3)
Cryotherapy	23 (34.3)
Intraarterial chemotherapy	13 (19.4)
Intravitreal chemotherapy	5 (7.4)
Chemoreduction	27/53 patients

RB: Retinoblastoma; TTT: Transpupillary thermotherapy.

(22.3%) eyes underwent primary enucleation. The remaining 52 eyes (77.6%) had eye conserving treatments TTT in 21 (31.3%), cryotherapy in 23 (34.3%), IAC in 13 (19.4%), and intravitreal chemotherapy in 5 (7.4%) eyes. One of the thirteen eyes undergoing IAC had Group C, 4 had Group D, and 8 had Group E RB. The mean number of IAC applications were 3.2±1.6 (range: 1–7) courses. The age of the patient was also effective in giving IAC treatment, as it was postponed for patients younger than one year old. The mean number of TTT and cryotherapy applications were 4.8±4.6 (range: 1–17) and 3.6±2.9 (range: 1–10), respectively. The treatment methods used in RB were summarized in Table 2.

The mean follow-up was 38.5±34.7 (range: 1–149)mo. After eye-conserving treatments, additional measure for unresponsive, recurrent and new tumours included secondary

enucleation in 14 eyes (20.8%). The globe salvage rate was 86.6 % in Group D (13 of 15 eyes), and 30.7 % in Group E (12 of 39 eyes). One patient with central nervous system metastasis died.

Genetic Analysis The *RBI* gene was sequenced by NGS method in 19 patients (Table 3). Of these patients, 6 had bilateral, 12 had unilateral, and one had trilateral RB. One of the patients with unilateral RB had also developmental delay. Pathogenic variant was detected in the *RBI* gene in all patients with bilateral (patients No.2, 4, 12, 15, 19) and trilateral RB (patients No.1, 5), and in two of the patients with unilateral RB (patients No.9, 18). A variant of unknown clinical significance was detected in one patient (patient No.14). Sequence analysis of 9 patients was found to be normal. Variants detected in pt 15 with bilateral RB and pt 5 with trilateral RB were considered as mosaic because of their low allele frequencies.

Chromosomal microarray analysis was performed for deletion/duplication analysis in 6 of the patients whose sequencing analysis of the *RBI* gene was normal.

Among these patients, a 21 Mb deletion [arr[hg19] 13q14.11-q21.31 (41 717 956-62 568 113)×1] was detected in patient No.13 with unilateral RB and developmental delay, in the chromosome 13q14.11-q21.31 region including the *RBI* gene.

Patient 1 Heterozygous RB1:NM_000321.3: c.1891C>T (p.Gln631Ter) variant was detected in a 2-month-old male with trilateral RB. This variant, which is expected to cause premature termination of the protein, has been reported by a center in ClinVar (Variation ID:860313; <https://www.ncbi.nlm.nih.gov/clinvar>) and was interpreted as a pathogenic.

Patient 2 Heterozygous RB1:NM_000321.3: c.1294A>T (p.Lys432Ter) variant was detected in a 7-month-old male with bilateral RB. This nonsense truncating variant has been reported by a center in ClinVar (Variation ID: 590847) and was interpreted as a pathogenic.

Patient 4 Heterozygous RB1:NM_000321.3: c.54_76del (p.Glu19AlafsTer4) variant was detected in 15-month-old female with bilateral RB. This frameshift truncating variant was interpreted as a pathogenic. It has previously not been reported in the literature.

Patient 5 Patient No.5, was diagnosed with RB in the right eye and a pineal gland mass at the age of 16mo. The heterozygous RB1:NM_000321.3: c.54_76del (p.Glu19AlafsTer4) variant was found to be low mosaic (6%) in the NGS analysis of the *RBI* gene from blood. In RB1 sequencing analysis from the patient's formalin fixation and paraffin embedding (FFPE) tumor tissue to exclude false positivity, the allele frequency of this variant was 62%. This frameshift variant which was detected in patient No.4 too, was interpreted as pathogenic.

Table 3 Genetic characteristics of the patients

Patient No.	RB type	Age at diagnosis (mo)	RB1 sequencing result (NM_000321.3)	CMA results	Interpretation
1	Trilateral	2	c.1891C>T (p.Gln631Ter)	NA	Pathogenic
2	Bilateral	7	c.124G>A (p.Gly42Ser)	NA	Pathogenic
3	Unilateral	20	Normal	Normal	
4	Bilateral	15	c.54_76del (p.Glu19AlafsTer4)	NA	Pathogenic
5	Unilateral	16	c.54_76del (p.Glu19AlafsTer4) mosaic	NA	Pathogenic
6	Unilateral	22	Normal	Normal	
7	Unilateral	30	Normal	Normal	
8	Unilateral	8	Normal	Normal	
9	Unilateral	8	c.1981C>T (p.Arg661Trp)	NA	Pathogenic
10	Unilateral	10	Normal	Normal	
11	Unilateral	81	Normal	NA	
12	Bilateral	5.5	c.1814+3A>T	NA	Likely pathogenic
13	Unilateral	19	Normal	21 Mb deletion of 13q14.11-q21.31	Pathogenic
14	Unilateral	13	c.1332+4A>G	NA	Unknown significance
15	Bilateral	9	c.2520+3_2520+6del (mosaic?)	NA	Pathogenic
16	Unilateral	16	Normal	NA	
17	Unilateral	43	Normal	NA	
18	Unilateral	9	c.1154_1157del (p.Leu385)	NA	Likely pathogenic
19	Bilateral	18	c.575_576del (p.Lys192SerfsTer10)	NA	Likely pathogenic

CMA: Chromosomal microarray analysis; NA: Not available; RB: Retinoblastoma.

The c.763C>T (p.Arg255Ter) variant, which was evaluated as a second hit mutation, was also detected in the tissue with an allelic frequency of 42%. This variant was not found in blood analysis.

Patient 9 The RB1:NM_000321.3: c.1981C>T (p.Arg661Trp) variant was found to be heterozygous in a 8-month-old male with bilateral RB. This missense variant was the known pathogenic variant reported in patients with RB by multiple centers.

Patient 12 Heterozygous RB1:NM_000321.3: c.1814+3A>T variant was detected in a 5.5 month-old male with bilateral RB. This variant was not found in general population data (GnomAD; <https://gnomad.broadinstitute.org/>) and literature. However, A>C change in the same nucleotide position has been reported in the literature. Parental analysis with Sanger sequencing was normal. This *de novo* variant was interpreted as a likely pathogenic.

Patient 13 A 19 month-old male born at 34wk and 5d from a healthy parent, had a history of hydronephrosis and oligohydramnios in the prenatal period. The patient, whose hydronephrosis regressed in the postnatal period, was diagnosed as RB after a mass was detected in the cranial MRI performed due to motor developmental delay. The patient, who was able to sit with support at the 8th month, started walking at the 19th month. In the chromosomal microarray analysis of the patient, a 21 Mb deletion was detected in the 13q14.11-q21.31 region, including the *RB1* gene. Chromosome analysis of the parents was found to be normal.

Patient 14 The intronic RB1:NM_000321.3: c.1332+4A>G variant was detected in a 13 month-old male with unilateral RB. This variant has not been reported in the literature and mutation databases. This variant was detected in the father with no RB history, was interpreted as unknown clinical significance.

Patient 15 The c.2520+3_2520+6del variant with an allele frequency of 25% was found in a 9-month-old female patient. This pathogenic variant has been reported in the literature in patients with unilateral and bilateral RB (PMID: 7881418, 25754945, 8605116) and has been shown to cause exon 24 skipping in functional studies^[11]. Due to the low allele frequency, this variant was thought to be mosaic.

Patient 18 The RB1:NM_000321.3: c.1154_1157del (p.Leu385) variant with allele frequency of 25% was found in patient No.15, a 9-month-old female with bilateral RB. This variant has not been reported in the literature and mutation databases. This *de novo* variant was interpreted as a likely pathogenic.

Patient 19 The RB1:NM_000321.3: c.575_576del (p.Lys192SerfsTer10) variant was found in an 18-month-old female with allele frequency of 37%. This variant has not been reported in the literature and mutation databases.

In the families of 4 children with novel mutations, there were no history of RB. The parents' genes were examined for mutations, no mutations were discovered.

DISCUSSION

RB is the most common primary ocular malignancy in

childhood, originating from progenitor cells of retinal photoreceptors. Two-thirds of RB cases are diagnosed in the first two years of life and 95% before the 5-year of age. Shields *et al*^[12] reported a mean age of 18mo from the USA. The results of a study from Brazil with a mean age of 21.7mo were similar to those from our investigation in which the mean age of RB patients was 24.6mo^[13].

In a study from Thailand, Group E patients were reported as 66.5%^[14]. In our study 80% of the study patients were Group E and D. Currently, RBs are diagnosed in late stages in our region.

Hereditary RB is caused by germline mutations in the *RBI* gene. Patients with bilateral, multifocal illness, a positive family history, and known germline mutations are considered as having hereditary RB. Approximately 15% of unilateral RB cases are caused by germline mutations and are hence inherited. Furthermore, *de novo* mutations cause 75% of hereditary RB cases, with 25% having a favorable family history. As a result, the absence of a family history does not rule out the possibility of hereditary RB. Germline mutations in the *RBI* gene are found in roughly 40% of cases. Unilateral patients with hereditary RB are more likely to have a tumor in the other eye. In addition, there is a possibility of secondary malignancy in the long term^[15].

We were able to perform genetic testing on 35.8% of the patients who participated in the study. Of the detected variants, 4 (in patients No.1, 2, 9, 15) have previously been described in the literature, and three (in patients No.4, 5, 12, 18) were undescribed novel variants. Variant c.54_76del, one of the novel variants, detected in both patients No.4 and 5, was evaluated as a likely pathogenic because it was located in the 1st exon and was expected to cause a termination codon after 4 amino acids. And the other novel variant, c.1814+3A>T, in patient No.12 was not found in general population data (GnomAD; <https://gnomad.broadinstitute.org/>) and literature. However, A>C change in the same nucleotide position has been reported in patients with bilateral RB and has been shown to cause exon skipping in experimental studies^[11]. This *de novo* likely pathogenic variant, indicates that intronic variants close to the canonical splicing region should be evaluated more carefully.

In patient No.5, a very low level mosaic pathogenic variant was detected at a rate of 6%, which was confirmed by tissue analysis. This result shows that the NGS method is more successful in detecting mosaics that are too low to be detected by conventional sequence analysis methods, and confirmation by tissue analysis may be important in these patients.

In addition, the c.2520+3_2520+6del variant found in patient No.15 was detected with 25% allele frequency and evaluated as mosaic. Patients with normal *RBI* gene analysis in the blood

have a low risk of mosaicism. Therefore, it is recommended to perform regular clinical examinations of these patients, including ultrasound^[16].

Retinal examination and imaging should be performed every 1-2y in patients with a pathogenic variant of *RBI* in the blood or with bilateral RB. Because of the susceptibility to sarcoma in these patients, physicians and parents should also be vigilant in terms of bone pain or swelling.

In patient No.13 with unilateral RB and developmental delay, a 21 Mb deletion was detected in the chromosome 13q14.11-q21.31 region, including the *RBI* gene. Chromosome 13q14 deletions containing the *RBI* gene can be seen in 6%-8% of patients with RB^[17]. In deletions that are large enough to be seen cytogenetically, developmental delay and varying degrees of intellectual disability can be seen in addition to RB, as in patient No.13.

In our study, IAC was used in 24 (35.6%) eyes. IAC was used as the main form of treatment in 8 patients, and 5 eyes were saved. After chemoreduction and focused therapies failed in 19 eyes, IAC was utilized as a secondary salvage procedure, and 6 (31%) were saved. IAC has been used in other centers with noticeably improved outcomes, and Francis *et al*^[18] reported that none of the 64 naive bilateral RB eyes that received IAC were enucleated. According to Shields *et al*^[19], primary IAC could rescue 74% of unilateral RB eyes.

The small number of our patients, the inability to perform genetic testing on all patients, and the difficulties in administering intraarterial therapy in infants under one year of age are the limitations of our study.

We describe four novel variants and one of them was found in two different patients. In 3%-10% of patients with bilateral RB, the diagnosis cannot be made by conventional sequence analysis method. We mentioned that there may be low mosaic pathogen variants in these patients and that NGS method should be preferred for their detection. In addition, there may be pathogenic variants in the promoter, non-coding or deep intronic regions of the *RBI* gene, which cannot be detected even by NGS. There may also be balanced structural variants, such as translocations involving the *RBI* gene.

In conclusion, the diagnosis of RB can still be made at advanced stages in developing countries. We describe four novel variants and one of them was found in two different patients. This data is very important for assessing prognosis. It serves as a guide for estimating the long-term risk of secondary malignancy as well as the short-term risk of developing additional malignancies in the same eye and the other eye.

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