

Retrospective Evaluation of Somatic Alterations in Cell-Free DNA from Blood in Retinoblastoma

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Purpose: Analysis of circulating tumor DNA (ctDNA) in the plasma of patients with retinoblastoma and simulating lesions.

Design: Retrospective cross-sectional study of the association of plasma ctDNA from retinoblastoma and simulating lesions with disease course.

Participants: Fifty-eight Memorial Sloan Kettering Cancer Center patients with retinoblastoma comprising 68 plasma ctDNA samples and 5 with retinoblastoma-simulating lesions.

Methods: The ctDNA analyzed with hybridization capture and next-generation sequencing in blood (plasma) of patients who had retinoblastoma or simulating lesions were evaluated for association with clinical course of the disease.

Main Outcome Measures: Presence or absence of molecular aberrations in the *RB1* gene and correlations with clinical features.

Results: *RB1* cell-free DNA (cfDNA) was detected in 16 of 19 patients with newly diagnosed, untreated intraocular retinoblastoma and in 3 of 3 patients with newly diagnosed, untreated metastatic disease. It was also present in 3 patients with recurrent intraocular disease before therapy, but was not present in patients with recurrent disease who received intra-arterial chemotherapy, nor in 21 patients who had undergone enucleation for unilateral disease. In 1 patient who had delayed treatment (insurance reasons) and showed rapid growth of the intraocular tumor, the variant allele frequency increased in 1 month from 0.34% to 2.48%. No *RB1* mutations were detected in the cfDNA from plasma of patients with simulating lesions (3 with Coats disease and 1 with persistent fetal vasculature [PFV]). In 2 patients, we identified 2 independent *RB1* mutations in plasma.

Conclusions: Mutations in *RB1* were found in the cfDNA from blood of patients with newly diagnosed, untreated retinoblastoma and in patients who showed disease recurrence in the eye after prior treatment, but not in unilateral retinoblastoma after enucleation. Levels of ctDNA increase in patients with progressive disease who did not receive any treatment. High plasma cfDNA levels were detected in patients with newly diagnosed metastatic disease, and these levels decreased after systemic chemotherapy was administered. Further validation is needed for measuring the somatic alterations in cfDNA from blood in retinoblastoma that could provide a promising method of monitoring patients in the future. *Ophthalmology Science* 2021;1:100015 © 2021 by the American Academy of Ophthalmology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).



Supplemental material available at www.ophtalmologyscience.org/.

Both circulating tumor cells and many noncellular substances from human cancers commonly are present in peripheral blood.¹ In addition to circulating tumor cells, smaller packets of membrane-encased vesicles, or exosomes, are common and play a complex role in the life cycle of cancer cells.² Exosomes may contain DNA, RNA, and protein. Cell-free RNA, and both single- and double-stranded circulating tumor DNA (ctDNA) can be found in blood, pleural fluid, cerebral spinal fluid, ascites, stool, saliva, and urine.³ Recent studies have identified

ctDNA in the aqueous of eyes with retinoblastoma,^{4–6} and the somatic alterations seen in aqueous ctDNA correspond well to the same alterations found in the tumor tissue itself.⁷ MicroRNAs also have been described recently in the blood of patients with retinoblastoma.⁸

Little is known about ctDNA in the blood of patients with retinoblastoma. In 2017, 4 patients were studied, and ultrashort (50–150 n) DNA fragments (ultrashort fragments are characteristic of ctDNA from cancer) were identified in

all patients.⁶ However, another study compared whole genome sequencing of aqueous humor with plasma from 20 patients and identified no plasma *RBI* abnormalities, leading the authors to state that “aqueous humor is superior to blood as a liquid biopsy for retinoblastoma.”⁹

We recently identified somatic *RBI* mutations from ctDNA in the blood of 8 of 10 patients with retinoblastoma who were treated with primary enucleation.¹⁰ Although it seemed that those children with the highest level of ctDNA in blood demonstrated metastatic disease, this requires confirmation from additional studies because the number of patients who had samples and metastatic disease was small. We have been analyzing the ctDNA from the plasma of patients with retinoblastoma for 4 years, and herein we present the results and observations with the goal of encouraging colleagues to explore this evolving technology further and to build on the clinical associations we outline in this article.

Methods

Circulating tumor DNA was analyzed in blood (plasma) by the Analysis of Circulating cfDNA to Evaluate Somatic Status (MSK-ACCESS) liquid biopsy assay. The details of this test have been published previously.¹¹ Briefly, this assay uses hybridization capture and deep sequencing to detect very low-frequency somatic alterations and to identify multiple classes of genomic abnormalities including single nucleotide polymorphisms, insertions or deletions of bases, and copy number alterations. Select exons and introns from 129 genes recognized to be aberrant in human cancers are analyzed. Notably, the assay routinely includes all exons of *RBI* and detects variant allele frequencies (VAFs; i.e., the proportion of allele bearing the variants over the total number of wild-type plus variant alleles at a given genomic location) down to 0.1%. This assay uses matched white blood cell sequencing, which identifies and filters out germline findings from the ctDNA results and can distinguish mutations associated with clonal hematopoiesis.¹² The test was approved for clinical use by the New York State Department of Health on May 31, 2019. Ten of these patients were reported previously and are included here for further correlate analyses. The methodology used in that cohort¹⁰ covered only the exons of *RBI* and followed a standard library preparation without unique molecular identifiers.

This study was approved by the Memorial Sloan Kettering Cancer Center Institutional Review Board/Privacy Board and conformed to the tenets of the Declaration of Helsinki. Consent was obtained from parents or guardians of all patients before blood was drawn for cell-free DNA (cfDNA).

Results

Circulating tumor DNA from 58 patients (68 samples) was analyzed and is presented in [Tables 1, 2, and 3](#).

After Enucleation

The ctDNA of 21 patients was analyzed 3 months to 21 years after treatment for retinoblastoma. In each patient, no ctDNA in *RBI* was detectable.

Progressive Intraocular Disease

One patient was referred with progression of intraocular disease in both eyes after multiple treatments at another

center, and ctDNA VAF was 0.34%. An enucleation of 1 eye was performed and intraarterial chemotherapy was planned for the fellow eye, but for financial reasons, the intraarterial chemotherapy was not performed. One month later, the tumor in the remaining eye had more than doubled in size and *RBI* mutation VAF in cfDNA increased to 2.48%.

Simulating Lesions

Three patients with Coats disease, 1 patient with PFV, and 1 patient with an iris tumor showed negative results for *RBI* ctDNA. One of the Coats patients is demonstrated in [Figure 1](#).

Discussion

MSK-ACCESS is a next-generation sequencing technique that has been used to investigate somatic alterations in a diverse group of cancer types. Multiple somatic alterations are common in solid cancers. In a survey of 681 blood samples from 31 solid cancers of 617 patients studied at Memorial Sloan Kettering Cancer Center using MSK-ACCESS, 73% of the samples showed either somatic mutations, structural variants, copy number alterations, or a combination thereof.¹¹

MSK-ACCESS uses molecular barcoding to tag replicate sequence reads originating from the same double-stranded template cfDNA molecule. These replicate reads are collapsed to consensus sequences to eliminate background sequencer errors, thereby considerably reducing false-positive results. Although excellent concordance exists with the alterations seen in cfDNA and tissue (biopsy) specimens, clear reasons exist regarding why cfDNA from blood has advantages over classical surgical specimens. In some cases, obtaining an adequate surgical specimen is impossible; in almost 9% of surgical biopsies, tumor tissue is insufficient for analysis, and needle biopsy specimens may not reflect the biological heterogeneity of a tumor accurately. Finally, repeated biopsies during and after therapy are not always practical.¹

Little work has been published on ctDNA in children with retinoblastoma. Four patients were reported to have detectable levels of plasma ctDNA in 2017 and 1 in 2019¹³; since then, investigators have focused on ctDNA in aqueous humor.^{4,5} In 1 additional study, no peripheral ctDNA was detected in any of the 20 samples tested, and that led the authors to comment that “aqueous humor is superior to blood as a liquid biopsy for retinoblastoma.”⁹ In another study, 1 of 3 patients with retinoblastoma (all with metastatic disease) showed detectable ctDNA in peripheral blood.¹³ It is striking that, despite the small size of these children, the small size of the involved organ (the eye), and the even smaller size of the intraocular tumor, ctDNA was detectable in the blood of untreated eyes.

The previous largest collection of positive ctDNA retinoblastoma specimens from blood were reported by our group in 2020.¹⁰ Eight of 10 patients showed detectable ctDNA in blood specimens, and those with the highest levels went on to demonstrate metastatic disease. Here are the highlights of our clinical correlates and suggestions for

Table 1. *RB1* Circulating Tumor DNA Results of Patients with Retinoblastoma before Treatment (Treatment Naïve)

Sample Identification	Laterality	Circulating Tumor DNA MSK-ACCESS <i>RB1</i> Results	Variant Allele Frequency	95% Confidence Interval	Cell-Free DNA Input (ng)
8_1	B	Negative			3.0
9_1	U	Negative			19.1
53	U	Negative			20.0
1	B	<i>RB1</i> intron16 splicing variant p.X500_splice (c.1498+2T→C)	1.71%	1.06–2.71	20.0
2_1	U	<i>RB1</i> exon8 p.E287* (c.859G>T)	2.45%	1.76–3.38	8.75
3_1	U	<i>RB1</i> exon17 p.R556* (c.1666C→T); <i>RB1</i> exon8 p.N258Kfs*2 (c.774_786delCAGGAGTGCACGG)	R556*, 1.63%; N258Kfs*2. 2.0%	1.21–2.20, 1.48–2.69	11.30
4	B	<i>RB1</i> exon18 p.E580* (c.1738G→T)	0.68%	0.28–1.55	9.05
5_1	U	<i>RB1</i> exon17 p.W516* (c.1547G→A)	2.92%	2.27–3.75	14.85
6_1	U	<i>RB1</i> exon10 p.Q344* (c.1030C→T); <i>RB1</i> exon14 p.R445* (c.1333C→T)	Q344*, 2.72%; R445*, 3.22%	2.02–3.64 2.39–4.33	7.75
10	U	<i>RB1</i> exon10 p.S318Nfs*13 (c.951_954delTTCT)	0.34%	0.13–0.84	7.7
11	U	<i>RB1</i> exon11 p.R358* (c.1072C→T)	4.23%	3.43–5.21	17.25
P01	B	<i>RB1</i> exon18 p.R579*	1.37%		
P02	U	<i>RB1</i> exon19 p.N623fs; <i>RB1</i> exon8 p.R255*	N623fs, 7.46%; R255*, 7.69%		
P03	U	<i>RB1</i> exon10 p.R320*	8.11%		
P04	U	<i>RB1</i> exon20 splicing variant; <i>RB1</i> exon21 p.Q736*	x702_splice, 0.09%; Q736*, 0.09%		
P05	U	<i>RB1</i> exon14 p.R445*	6.77%		
P06	U	<i>RB1</i> exon17 p.R556*; <i>RB1</i> exon10 p.E315*	R556*, 0.7%; E315*, 3.04%		
P07	U	<i>RB1</i> exon15 p.X474_splicing variant	12.60%		
P08	U	<i>RB1</i> exon16 p.N480del	0.48%		
			Mean 3.35%		
			Median 2.45%		

B = bilateral; MSK-ACCESS = Analysis of Circulating cfDNA to Evaluate Somatic Status; U = unilateral.

future avenues of exploration with ctDNA from the blood of patients with retinoblastoma.

Naïve Patients

The plasma ctDNA of 19 patients was analyzed before any treatment was administered, and 18 of these showed detectable ctDNA with VAFs ranging from 0.09% to 12.6% (Table 1). Variant allele frequency is a surrogate measurement of the proportion of the sample carrying the variant (the percentage

of the specific DNA variant divided by the overall coverage of that locus). The 2 patients who showed negative results included 1 eye with small putative retinal tumors, but extensive vitreous seeding, and 1 eye without retinal or vitreous disease, but extensive subretinal seeding. This suggests that most newly diagnosed patients with retinoblastoma have detectable ctDNA in plasma and that eyes with mostly vitreous disease or subretinal disease do not shed into blood, nor do they have detectable plasma ctDNA for *RB1*.

Table 2. *RB1* Circulating Tumor DNA Results of Retinoblastoma Patients Who Have Active Disease

Sample Identification	Laterality	Circulating Tumor DNA MSK-ACCESS <i>RB1</i> Results	Variant Allele Frequency	Comment
12	B	Negative		On treatment
13	B	Negative		On treatment
14	B	Negative		On treatment
16	U	Negative		On treatment
17	B	Negative		On treatment
8_2	B	Negative		On treatment
15_1	U	<i>RB1</i> exon15 p. R467* (c.1399C→T)	1.35%	Before treatment for recurrence
15_2	U	<i>RB1</i> exon15 p.R467* (c.1399C→T)	0.13%	Receiving treatment
18_1	B	<i>RB1</i> exon14 p.R455* (c.1363C→T)	0.34%	Before treatment for recurrence
18_2	B	<i>RB1</i> exon14 p.R455* (c.1363C→T)	2.48%	After enucleation, no treatment to other eye

B = bilateral; MSK-ACCESS = Analysis of Circulating cfDNA to Evaluate Somatic Status; U = unilateral.

Table 3. *RBI* Circulating Tumor DNA Results of Retinoblastoma Patients Who Developed Metastatic Disease

Sample Identification	Laterality	Circulating Tumor DNA MSK-ACCESS <i>RBI</i> Results	Variant Allele Frequency	Comment
21	U	Negative		After successful treatment
22_1	U	Negative		After successful treatment
23_1	U	<i>RBI</i> exon13 p.R418Sfs*9 (c.1251_1252delAA)	94.53%	At metastasis diagnosis before treatment
23_2	U	Negative		After successful treatment
24	U	Negative		After successful treatment
20	B	<i>RBI</i> exon9 p.F296Lfs*5 (c.888delT)	17.03%	At metastasis diagnosis before treatment
25_1	U	<i>RBI</i> exon8 p.R255* (c.763C>T)	0.93%	At metastasis diagnosis before treatment
25_2	U	Negative		After successful treatment

B = bilateral; MSK-ACCESS = Analysis of Circulating cfDNA to Evaluate Somatic Status; U = unilateral.

Active Disease with Treatment

Nine patients showed active intraocular disease and were being treated when ctDNA was drawn (Table 2). Five had received intraarterial chemotherapy within 30 days, and all of these patients' samples showed negative results (patients 12, 13, 14, 16, and 17). Two patients had blood drawn after coming to Memorial Sloan Kettering Cancer Center with active disease, despite prior systemic chemotherapy within 30 days, and both showed positive results for *RBI* ctDNA (patients 15 and 18). For patient 15, the VAF dropped from 1.35% to 0.13% after treatment with intraarterial chemotherapy. One patient experienced recurrent subretinal disease, was receiving treatment, and showed negative results (patient 16). These data suggest that repeated ctDNA in children with retinoblastoma who are receiving treatment can be used as a guide for tumor activity or volume in the ocular tumor. This was

highlighted further by the patient (patient 18) in whom VAF was 0.34%, a child who had been managed elsewhere and had been referred to us with active disease in both eyes. In that patient, enucleation was carried out and intraarterial chemotherapy was planned for the remaining eye, but because of insurance issues, additional treatment was delayed for 1 month, when repeat VAF was 2.48%.

Patients with Metastatic Disease

Only 4% of patients in the United States experience metastases.¹⁴ Six patients in this series demonstrated metastatic disease (Table 3). Three patients had ctDNA drawn on the day they sought treatment with metastases (before it was proven or treated), and all showed positive results (patients 20, 23_1, and 25). Two of these patients had orbital and marrow disease with VAFs of 17.03% and 94.53%. One patient had orbital disease only and had a

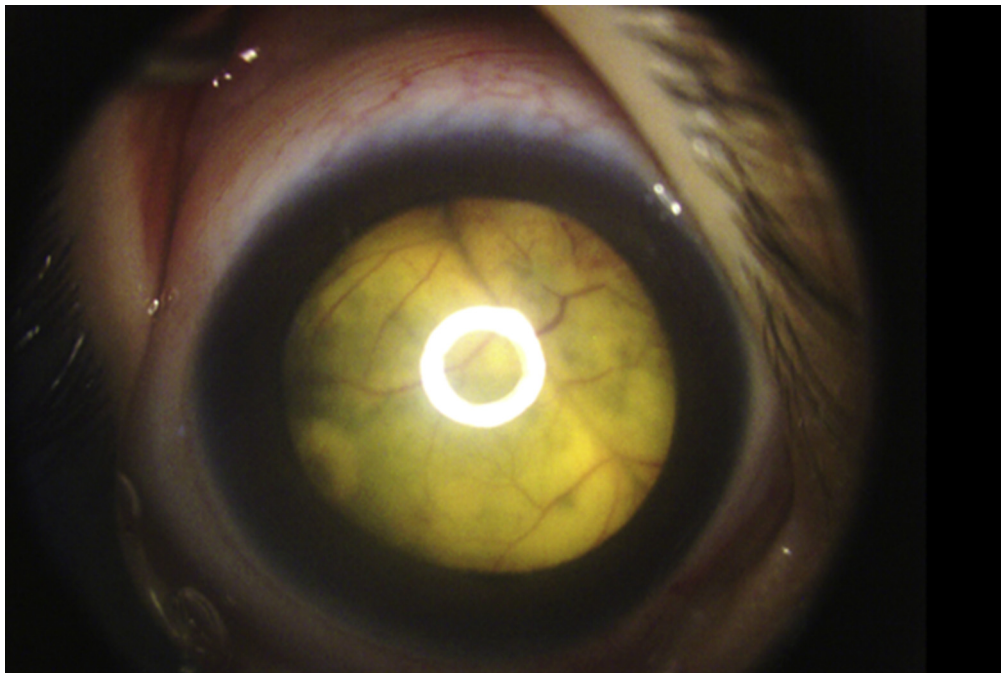


Figure 1. Photograph of eye with leukocoria suspicious for retinoblastoma. Circulating tumor DNA results were negative for *RBI* mutations, but positive for *TINF2* on Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT), which assisted in making the diagnosis of Coats plus disease.

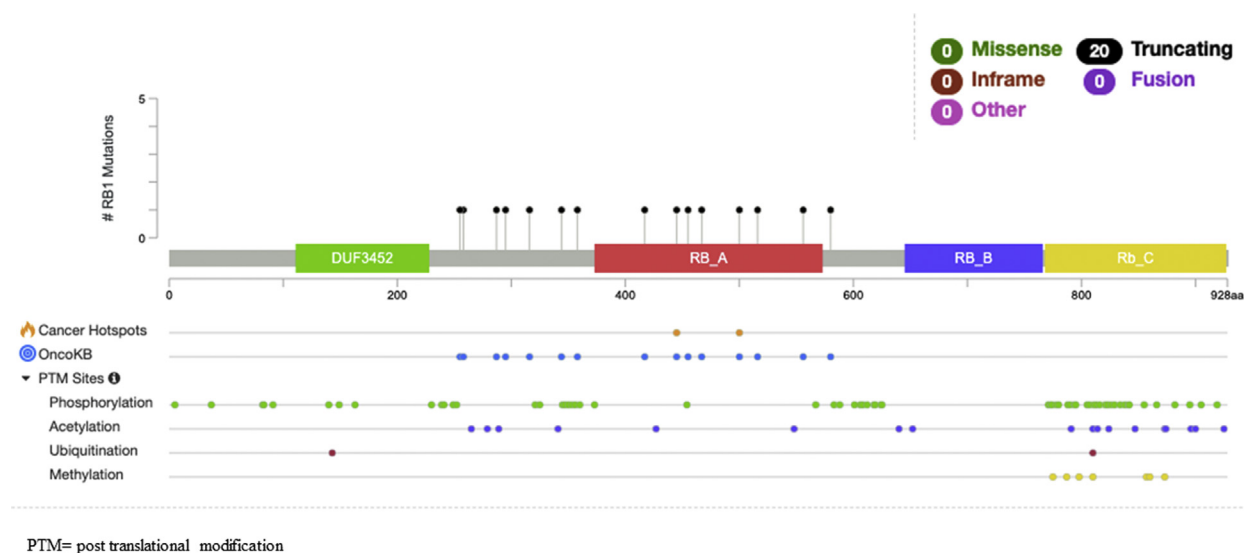


Figure 2. Graphic representation of location of all the *RB1* mutations detected in patients. PTM = posttranslational modification.

VAF of 0.93%. After 3 ophthalmic artery intraarterial sessions, the VAF was 0. Three patients had ctDNA analyzed after high-dose systemic chemotherapy (all 3 patients) and bone marrow transplantation (2 of 3 patients), and at that point (6 months, 1 year, and 10 years), all showed negative results (patients 21, 23_2, and 24). One patient had ctDNA analyzed after 3 cycles of high-dose systemic chemotherapy (in the ARET0321 study) and immediately after stem cell harvest, but before transplantation, and that specimen also showed negative results (patient 22). These results suggest that ctDNA can be used to monitor success of treatment for metastatic disease and response to high-dose chemotherapy before transplantation and to detect metastasis after enucleation.

Patients Who Have Undergone Treatment and Show Stable Disease

Twenty-one patients had ctDNA analyzed 3 months to 21 years after treatment for retinoblastoma. In all patients, no ctDNA in *RB1* was detectable. Elevated *RB1* ctDNA after enucleation may indicate active disease in the fellow eye if bilateral or may indicate metastatic disease if unilateral (especially if levels are high).

Diversity of *RB1* Mutations

Using MSK-ACCESS, 22 patients showed identifiable somatic alterations in ctDNA for *RB1*, but no 2 patients showed the same mutation, emphasizing the well-known diversity of *RB1* mutations in retinoblastoma (Fig 2). A representation of the mutations identified is presented in Table 3. It has been suggested that the clinical presentation, age at diagnosis, and multiplicity of tumors, are related to the specific mutation, so knowledge of the exact mutation detected¹⁵ by ctDNA may impact management and outcome in the future.

Sensitivity of Mutation Detection

In 1 patient (patient 26), conventional genetic counseling had been carried out and the family was informed that no mutation was detected; however, after using MSK-ACCESS (buffy coat) for ctDNA, that same patient showed mosaicism at the rate of 4.8%. This implies that ctDNA can detect very low levels of mosaicism in retinoblastoma that has been missed by performing conventional genetic testing. It suggests that unilateral patients who show negative results on conventional genetic testing should undergo ctDNA testing, which may be more sensitive for the detection of low-level mosaicism. Mosaicism influences the age at detection of retinoblastoma, the laterality, the number of tumors, and the development of new intraocular tumors and is related to the degree of mosaicism,¹⁶ so knowledge of very low levels of mosaicism initially may influence frequency of examinations, decisions for treatment, and the advice physicians can give to families about outcomes. We are conducting additional studies to determine the prevalence of this finding.

Detection of Both Somatic Hits

The initiating event for retinoblastoma development is the inactivation of the *RB1* gene on both chromosomes caused by a variety of well-described phenomena,¹⁷ including loss of heterozygosity, large deletions, translocations, promoter hypermethylation, chromothripsis, single nucleotide variants, and insertions and deletions. Of this range of genomic alterations, cfDNA analysis can detect single nucleotide variants, insertions and deletions, and multiexon deletions when tumor-derived cfDNA content is sufficient for analysis.

One of the less common mechanisms is 2 independent and different mutations (usually 1 on each chromosome). We were able to detect this in 4 patients who showed 2 mutations at different exons (patients 3, 6, P02, and P06). In patients with bilateral disease, it is impossible to know which molecular abnormality is from which eye.

Diagnosis

Three patients with Coats disease, 1 patient with PFV, and 1 patient with a small, round, blue-cell iris tumor were tested and found to have had no *RB1* alterations in their ctDNA.

Opaque Media

One patient who underwent enucleation of 1 eye and demonstrated phthisis in the other eye showed no detectable *RB1* alterations in the blood (patient 30). This may indicate no metastatic disease or activity in the eye. Future studies of these patients may confirm this impression, and ctDNA may help to guide decisions about eyes with opaque media.

Second Cancers

One patient who had demonstrated widespread metastatic disease after primary enucleation (disease free for > 10 years after high-dose chemotherapy and bone marrow transplantation) demonstrated a second cancer (kidney; patient 24). No *RB1* alterations were found in the ctDNA at that time. A second patient who survived bilateral retinoblastoma and 30 years later an osteosarcoma of the sinus showed no *RB1* alterations by ctDNA (patient 43). Perhaps using ctDNA may assist in the differential diagnosis of small, round, blue tumors that develop in patients with retinoblastoma because at times, it is impossible to be certain if the second cancer is metastatic retinoblastoma or a second cancer on clinical and pathologic grounds alone.

Footnotes and Disclosures

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Concordance of *RB1* Mutations

Fourteen patients who showed identifiable *RB1* somatic mutations with ctDNA also underwent analysis of *RB1* status by Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) on tumor tissue. In each patient, the exact same mutation was found. This includes 2 patients who each had 2 mutations identified in blood and tumor (patients 1, 2, 3, 5, P01, P02, P03, P04, P06, P07, P08, P09, 20, and 23).

In conclusion, plasma ctDNA from eyes with active retinoblastoma is commonly detectable, but is not present in patients who have undergone enucleation or have stable, treated disease. Future studies will help to determine if *RB1* mutations missed on conventional genetic testing may help in differentiating metastases from second cancers, and ultimately may aid in the accurate differential diagnosis of retinoblastoma (because biopsy is rarely—if ever—performed). Sequential elevation of VAF may indicate progression of intraocular disease. No 2 patients showed the exact same *RB1* mutation. Detectable levels of *RB1* alterations in the blood weeks or months after enucleation for unilateral retinoblastoma may indicate the development of metastatic disease and may be used routinely for patient monitoring in the future. However, these observational findings will need to be validated in future prospective studies using algorithms as designed in [Supplemental Table S4](#). Based on these findings, we will continue to collect ctDNA from patients with retinoblastoma for research, but we modified our clinical algorithm, as presented in [Supplemental Table S4](#).

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 Abbreviations and Acronyms:
cfDNA = cell-free DNA; **ctDNA** = circulating tumor DNA; **MSK-ACCESS** = Analysis of Circulating cfDNA to Evaluate Somatic Status; **MSK-IMPACT** = Integrated Mutation Profiling of Actionable Cancer Targets; **VAF** = variant allele frequency.

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