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RB1 Circulating Tumor DNA in the Blood of Patients with Unilateral Retinoblastoma

Before and after Intra-arterial Chemotherapy

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Purpose: Circulating tumor DNA (ctDNA) is released by many tumors into the plasma. Its analysis has minimal procedural risk and, in many cancers, has the potential for clinical applications. In retinoblastoma, the clinical correlations of ctDNA in eyes treated without enucleation have not been studied. This purpose of this study was to determine how the ctDNA *RB1* variant allele frequency (VAF) changes in patients with unilateral retinoblastoma after intra-arterial chemotherapy (IAC) treatment. Variant allele frequency is a proxy for tumor fraction.

Design: Case series from a single tertiary cancer referral center.

Participants: Five patients with retinoblastoma with at least 1 measurable ctDNA plasma specimen both at the time of active intraocular retinoblastoma before IAC and after at least 1 IAC cycle.

Methods: Circulating tumor DNA *RB1* was detected and VAF was measured before and after IAC treatment. Clinical correlations were made using clinical examination, fundus photography, ultrasound, and OCT.

Main Outcome Measures: Comparison of ctDNA *RB1* VAF before and after IAC treatment for retinoblastoma and concordance of ctDNA *RB1* detectability with activity of intraocular disease.

Results: Twenty-three ctDNA specimens were included from 5 patients. The 5 baseline *RB1* VAFs ranged from 0.27% to 4.23%. In all patients, the subsequent post−intra-arterial *RB1* VAF was lower than baseline (0.0%−0.17%). At 4 months (2 months after IAC completion), the ctDNA consistently was negative in the patients who demonstrated clinically inactive intraocular disease.

Conclusions: In this small cohort, a decremental decrease in ctDNA *RB1* VAF was found after IAC, suggesting that relative VAF changes could be a biomarker of treatment response. *Ophthalmology Science 2021;1:100042*

Liquid biopsies of blood have clinical applications for cancer[1,2] and, because of the minimal procedural risks, are particularly pertinent to retinoblastoma, for which direct tumor biopsy is avoided because of seeding potential. They allow for an analysis of exosomes, circulating tumor cells, microRNA, and circulating cell free DNA (cfDNA) that are released from malignant cells via necrosis and apoptosis.[1,2] Circulating tumor DNA (ctDNA) is a small component of cfDNA that specifically is sourced from tumor cells and is characterized by somatic variants.[1,2]

Intra-arterial chemotherapy (IAC) is now the first-line treatment for retinoblastoma at many centers worldwide, enabling eye salvage without compromising patient survival.[3] Given that IAC regresses tumors from their most active state, presumably through necrosis and apoptosis, we were interested to explore the dynamics of ctDNA throughout this treatment period. Therefore, this study investigated *RB1* ctDNA variant allele frequency (VAF), a proxy for tumor fraction, by comparing the values before IAC with those after treatment.

Methods

The study adhered to the tenets of the Declaration of Helsinki and was approved by the institutional review board of Memorial Sloan Kettering Cancer Center. This retrospective, single-center study included 5 eligible patients recruited from Memorial Sloan Kettering Cancer Center, New York, New York, between October 2019 and December 2020. The caregiver for each patient gave informed consent, and eligible patients had at least 1 measurable ctDNA plasma specimen both at the time of active intraocular disease before IAC and after at least 1 IAC cycle. Two patients with undetectable baseline cfDNA were excluded from the study.

The MSK-ACCESS liquid biopsy assay analyzed ctDNA plasma using deep sequencing and hybridization capture to detect very low−frequency somatic alterations in exons and introns from 129 cancer-related genes, including all exons of *RB1*. This assay was approved for clinical use by the New York State Department of Health on May 31, 2019. It can detect single nucleotide polymorphisms, insertions or deletions of bases, and copy number alterations. Variant allele frequencies (the proportion of allele bearing the variants divided by the total number of wild-type plus variant alleles at a given genomic location) at or more than 0.1%

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were recorded. Matched white blood cell sequencing was used (which identifies and filters out germline findings from the ctDNA); thus, mutations associated with clonal hematopoiesis were eliminated.[4]

The clinical status was evaluated under anesthesia with indirect ophthalmoscopy, RetCam fundus photography (Clarity), B-scan ultrasonography or ultrasonic biomicroscopy (Ellex), and OCT (Bioptigen, Inc). Patient and treatment data were collected. Tumor data included Reese-Ellsworth classification, Children's Oncology Group version of International Classification of Retinoblastoma and eighth edition American Joint Committee on Cancer retinoblastoma staging system. For each time point of ctDNA specimen collection, detectability, somatic *RB1* alterations, and VAF were recorded. The initial IAC cycle was administered at baseline, the second cycle at 1 month, and the third cycle at 2 months: all blood draws at these time points were obtained before the IAC procedure. Statistical analysis comparing VAF before and after IAC completion was performed with a 2-tailed Student *t* test using GraphPad software.

Results

Patient, disease, treatment, and ctDNA details are shown in Table 1. Five eyes from 5 patients (2 male, 3 female) with unilateral disease were included, and the median age at baseline ctDNA collection was 17.6 months. Eyes were all Reese-Ellsworth classification VB and American Joint Committee on Cancer classification cT2b and were classified as International Classification of Retinoblastoma class D in 3 eyes and class E in 2 eyes. Four patients were naïve to prior treatment and 1 patient (patient 15) had received 1 prior cycle of intra-arterial chemotherapy attempted at another institution.

Table 1 and Figure 1 show the ctDNA *RB1* VAFs. Twenty-three ctDNA specimens were included from 5 patients. The baseline *RB1* VAF ranged from 0.27% to 4.23%, and in all patients, the subsequent post−intra-arterial *RB1* VAF was lower than baseline, ranging from 0.0% to 0.5%. The highest baseline VAF occurred in a child with a unilateral International Classification of Retinoblastoma group E eye (patient 11). One eye (from patient 15) with detectable ctDNA (VAF, 0.13%) at 1 month after 3 IAC treatments and clinically inactive fish-flesh tumor regression pattern: the ctDNA from this patient became undetectable at 2 months after the third IAC treatment and an interim of no other treatment. For all eyes, 4 months from baseline (2 months after IAC completion), ctDNA levels consistently were negative. The VAF was significantly lower after treatment completion compared with time points before treatment completion ($P = 0.02$). In patient 56, cfDNA detected 2 alterations in *RB1* (exon 15 and exon 8), both of which exhibited decremental decline in VAF with subsequent intra-arterial cycles.

Discussion

Prior reports have demonstrated that liquid biopsies of both plasma and intraocular aqueous fluid of patients with retinoblastoma can detect somatic variants in *RB1*[5–7]; however, the potential clinical implications of this discovery are yet to be realized fully. Circulating tumor DNA has been shown to be capable of diagnosing a variety of cancers, detecting minimal residual disease, and monitoring treatment responses.[1,2,8] Changes in ctDNA levels during chemotherapy treatment can inform clinical disease in at least 2 ways. Elevations in ctDNA levels are associated with progression of disease and can even be detected before rises in tumor markers or radiographic confirmation with computed tomography.[9,10] Furthermore, early decreases in ctDNA are associated with a response to chemotherapy and have been recognized as a reliable predictive biomarker for early therapeutic response.[11] The relevance of shifting ctDNA levels in retinoblastoma are yet to be evaluated at this scale, and our study takes a step toward this. One group evaluated longitudinal copy number alteration amplitude and tumor fraction from aqueous humor of retinoblastoma and suggested that relative increase is correlated with tumor progression.[12]

To understand the dynamics of ctDNA levels in the context of retinoblastoma treatment, we evaluated the circulating tumor *RB1* VAF before and after treatment with IAC. We previously showed that after abrupt regression or removal of disease by enucleation, the ctDNA declines to zero rapidly (Abramson et al, unpublished data, 2021) and remains absent in all patients except when metastases develop and levels are high. Depending on the disease, IAC may cause variable responses: either a rapid regression with minimal residual active disease at 1 month after the initial cycle or a more gradual regression of active tumor over the (typically) 3-month IAC treatment course; rarely is tumor growth observed during the intra-arterial treatment course of a naïve eye. Herein, we showed that changes in ctDNA mimic these response patterns, and in our small cohort, ctDNA *RB1* VAF declined after intra-arterial treatment.

For instance, all tumors regressed after IAC, and likewise subsequent ctDNA *RB1* VAFs all were lower than prior measurements (Fig 1). At 1 month after the first IAC cycle, ctDNA was measurable but lower than baseline and was consistent with residual retinal disease. At 1 month after 3 IAC cycles, the ctDNA *RB1* VAF was 0 in all but 1 eye and corresponded to retinal tumor inactivity. At all follow-up visits 2 months or more after IAC completion, ctDNA *RB1* VAF measurements consistently were 0 and mirrored the sustained clinical inactivity of the tumors. These results are to be interpreted with caution, particularly with regard to undetectable cfDNA or VAF thresholds: false-negative results (i.e., a VAF of 0%) may exist in the presence of active intraocular tumor. To date, no objective threshold VAF exists that can be used to indicate active or inactive disease; however, given the relative decreases seen during treatment in this cohort, this is an active area of exploration.

Only 1 pathogenic RB1 allele was detected in the ctDNA of 4 of 5 patients; this is the expected result because of loss of heterozygosity (which occurs in 72% of retinoblastomas[13]) not being detectable by cfDNA analysis or a germline mutation being filtered out by the assay. One patient (patient 56) showed 2 *RB1* alterations (exon 15 and exon 8), both of which declined with treatment. Given that this

Table 1. Patient, Disease Treatment, Circulating Tumor DNA, and Germline Characteristics

Patient No.	Age at Baseline Circulating Tumor DNA Measurement (mos)	International Classification of Retinoblastoma Class*	Tumor Dimensions (mm)†	Treatment	Ophthalmic Artery Chemosurgery Dosage (mg) and Drugs	Circulating Tumor DNA Time Points (Variant Allele Frequency % Exon 1/Exon 2 [95% Confidence Interval])‡	MSK-ACCESS Somatic *RB1* Findings	Variant Allele Frequency at Baseline (%)	Germline *RB1* Findings
55	2.6	D	16 × 14	OAC × 3	2.5 M, 0.2 T, 30 C	BL (2.8 [1.8–4.2]), 12 mos (0), 15 mos (0)	RB1 exon14 p.R445* (c.1333C→T)	2.80	RB1 c.1695+2T →C variant
15	26.1	D	7 × 3	OAC × 3	5 M, 0.5 T, 50 C	BL (1.35 [0.9–2.0]), 3 mos (0.13 [0.02–0.5]), 4 mos (0), 5 mos (0), 6 mos (0)	RB1 (NM_000321) exon15 p. R467* (c. 1399C→T)	1.35 %	Negative
10	17.6	E	8 × 9	OAC × 3	4 M, 0.5 T, 40 C	BL (0.34 [0.1–0.8]), 3 mos (0), 5 mos (0), 6 mos (0)	RB1 (NM_000321) exon10 p.S318Nfs*13 (c.951_954delTTCT)	0.34	Negative
11	31.9	E	14 × 12	OAC × 3	5 M, 0.5 T, 40 C	BL (0.54 [0.2–1.0]), 2 mos (0), 3 mos (0), 4 mos (0), 5 mos (0), 6 mos (0)	RB1 (NM_000321) exon11 p.R358* (c.1072C→T)	4.23	Negative
56	6.0	D	14 × 9	OAC × 3	2.5 M, 0.3 T, 30 C	BL (0.27 [0.1–0.5]/0.5 [0.3 –0.9]), 1 mo (0.17 [0.05-0.4]/0.06 [0.03–0.4]), 2 mos (0/0), 3 mos (0/0), 6 mos (0/0)	1. RB1 exon15 p.R467* (c.1399C→T); 2. RB1 exon8 p.R262Gfs*2 (c.784delC)	1: 0.27; 2: 0.5	Negative

BL = baseline; C = carboplatin; ctDNA = circulating tumor DNA; M = melphalan; OAC = ophthalmic artery chemosurgery; T = topotecan.
*All eyes were Reese-Ellsworth class VB and American Joint Committee on Cancer class cT2.
†Largest basal diameter and height by magnetic coherence tomography.
‡Intra-arterial cycle 1 administered at baseline, cycle 2 administered at first month, and cycle 3 administered at second month.

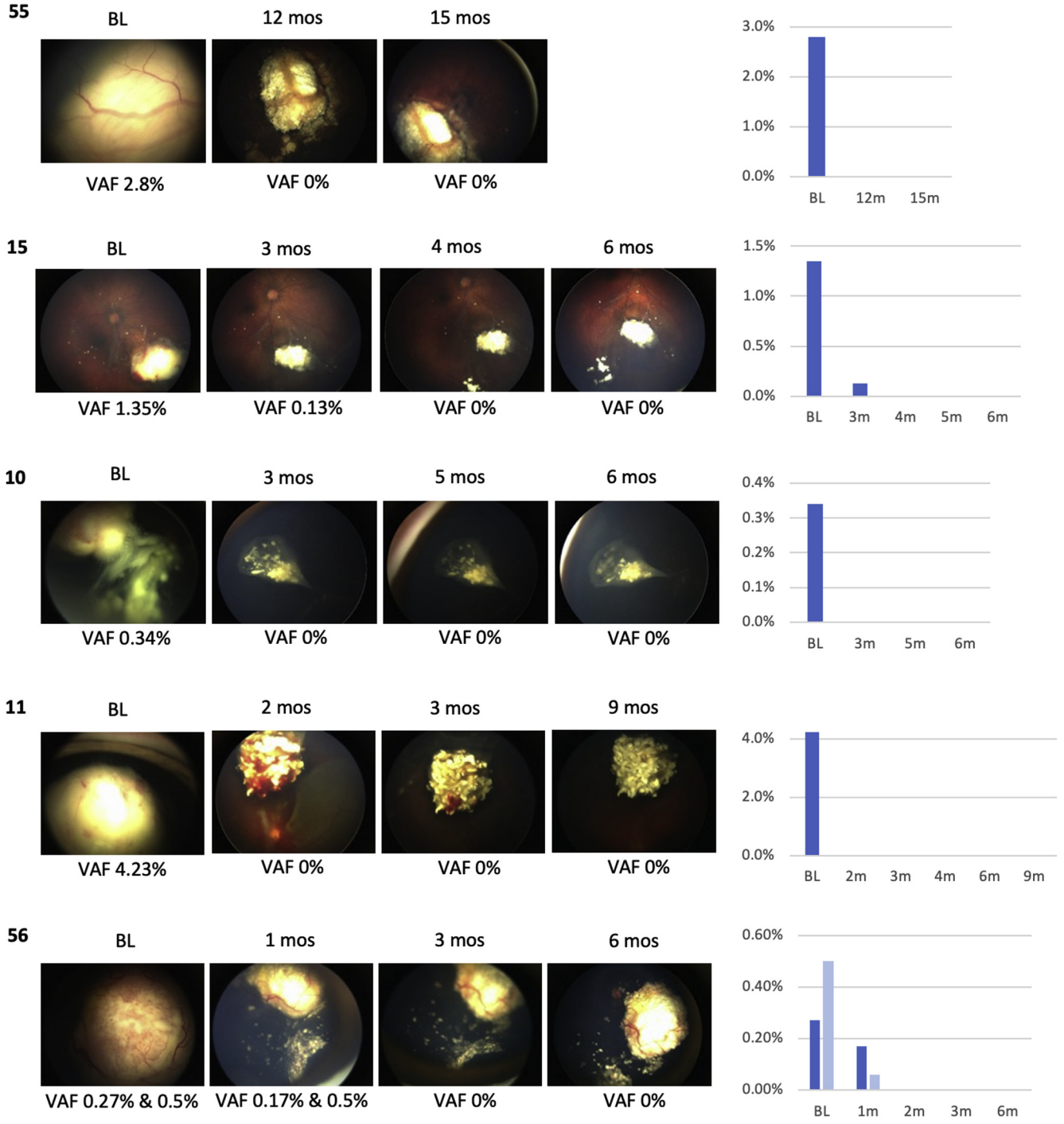

Figure 1. Fundus photographs and clustered bar graphs for 5 eyes. The left panels depict fundus imaging with the labeled time point and *RB1* variant allele frequency (VAF) for all 5 eyes as labeled by patient number. The right panels show bar graphs for each respective patient: the x-axis represents time points (BL = baseline, mos = months from baseline), and the y-axis shows plasma circulating tumor *RB1* VAF percentage. For patient 56, the clustered bar graph shows both *RB1* alterations: exon 15 (dark blue) and exon 8 (lighter blue). Intra-arterial cycle 1 was administered at baseline, cycle 2 was administered at the first month, and cycle 3 was administered at the second month.

patient had unilateral disease and without a germline *RB1* alteration, it is most likely these 2 *RB1* aberrations originated from the same eye. This demonstrated the capability of plasma cfDNA to detect multiple *RB1* alterations from a single eye.

In conclusion, in this cohort of patients with unilateral retinoblastoma undergoing IAC treatment, a decremental decrease in the levels of ctDNA *RB1* VAF was found corresponding with cycles of IAC, and although this was not evaluated formally (because of the retrospective nature of

the study and the variable and infrequent timing of ctDNA measurements), this decrease reflected the retinal tumoral response over the treatment course. These results are to be interpreted with caution particularly with regard to undetectable cfDNA or VAF thresholds: false-negative results may exist in the presence of active intraocular tumor.[14] An expanded cohort with more regimented collection time points will advance our knowledge further.

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Footnotes and Disclosures

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HUMAN SUBJECTS: Human subjects were included in this study. The Institutional Review Board at Memorial Sloan Kettering Cancer Center approved the study. All research adhered to the tenets of the Declaration of Helsinki. The caregivers of all participants provided informed consent. This retrospective, single-center study included eight eligible patients recruited from Memorial Sloan Kettering Cancer Center, New York between October 2019 and December 2020.

No animal subjects were included in this study.

Author Contributions:

Conception and design: Francis

Analysis and interpretation: Francis, Mandelker, Abramson

Data collection: Francis, Gobin, Brannon, Swartzwelder, Berger, Mandelker, Walsh, Abramson

Obtained funding: Francis, Abramson; Study was performed as part of regular employment duties at Memorial Sloan Kettering Cancer Center. No additional funding was provided.

Overall responsibility: Francis, Gobin, Brannon, Swartzwelder, Berger, Mandelker, Walsh, Dunkel, Abramson

Abbreviations and Acronyms:
cfDNA = circulating cell free DNA; **ctDNA** = circulating tumor DNA; **IAC** = intra-arterial chemotherapy; **VAF** = variant allele frequency.

Keywords:
Biomarker, Cell free DNA, Circulating tumor DNA, Intra-arterial chemotherapy, Retinoblastoma.

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References

1. Alix-Panabières C, Pantel K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. *Cancer Discov*. 2016;6:479−491.
2. Siravegna G, Marsoni S, Siena S, Bardelli A. Integrating liquid biopsies into the management of cancer. *Nat Rev Clin Oncol*. 2017;14:531−548.
3. Abramson DH, Shields CL, Munier FL, Chantada GL. Treatment of retinoblastoma in 2015: agreement and disagreement. *JAMA Ophthalmol*. 2015;133:1−7.
4. Ghiam BK, Xu L, Berry JL. Aqueous humor markers in retinoblastoma, a review. *Transl Vis Sci Technol*. 2019;8:13.
5. Berry JL, Xu L, Kooi I, et al. Genomic cfDNA analysis of aqueous humor in retinoblastoma predicts eye salvage: the surrogate tumor biopsy for retinoblastoma. *Mol Cancer Res*. 2018;16:1701−1712.
6. Gerrish A, Stone E, Clokie S, et al. Non-invasive diagnosis of retinoblastoma using cell-free DNA from aqueous humour. *Br J Ophthalmol*. 2019;103:721−724.
7. Kothari P, Marass F, Yang JL, et al. Cell-free DNA profiling in retinoblastoma patients with advanced intraocular disease: an MSKCC experience. *Cancer Med*. 2020;37:646−6101.
8. Corcoran RB, Chabner BA. Application of cell-free DNA analysis to cancer treatment. *N Engl J Med*. 2018;379:1754−1765.
9. Misale S, Yaeger R, Hobor S, et al. Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer. *Nature*. 2012;486:532−536.
10. Dawson S-J, Tsui DWY, Murtaza M, et al. Analysis of circulating tumor DNA to monitor metastatic breast cancer. *N Engl J Med*. 2013;368:1199−1209.
11. Osumi H, Shinozaki E, Yamaguchi K, Zembutsu H. Early change in circulating tumor DNA as a potential predictor of

response to chemotherapy in patients with metastatic colorectal cancer. *Sci Rep*. 2019;9:17358−17359.

12. Polski A, Xu L, Prabakar RK, et al. Cell-free DNA tumor fraction in the aqueous humor is associated with therapeutic response in retinoblastoma patients. *Transl Vis Sci Technol*. 2020;9:30.
13. Francis JH, Richards AL, Mandelker DL, et al. Molecular changes in retinoblastoma beyond RB1: findings from next-generation sequencing. *Cancers (Basel)*. 2021;13:149.
14. Abramson DH, Mandelker D, Francis JH. Retrospective evaluation of somatic alterations in cell-free DNA from blood in retinoblastoma. *Ophthalmol Sci*. 2021:1.