PLOS ONE

RESEARCH ARTICLE

Mutant-*RB1* circulating tumor DNA in the blood of unilateral retinoblastoma patients: What happens during enucleation surgery: A pilot study

David H. Abramson[1,2]*, **Diana L. Mandelker**[3], **A. Rose Brannon**[3], **Ira J. Dunkel**[4,5], **Ryma Benayed**[3], **Michael F. Berger**[3], **Maria E. Arcila**[3], **Marc Ladanyi**[3], **Danielle Novetsky Friedman**[4], **Gowtham Jayakumaran**[3], **Monica S. Diosdado**[3], **Melissa A. Robbins**[1], **Dianna Haggag-Lindgren**[1], **Neerav Shukla**[4], **Michael F. Walsh**[4,6], **Prachi Kothari**[4], **Dana W. Y. Tsui**[3,7¤], **Jasmine H. Francis**[1,2]

1 Department of Surgery, Ophthalmic Oncology Service, Memorial Sloan Kettering Cancer Center, New York, New York, United States of America, 2 Department of Ophthalmology, Weill Cornell Medical Center, New York, New York, United States of America, 3 Department of Pathology, Memorial Sloan Kettering Cancer Center, New York, New York, United States of America, 4 Department of Pediatrics, Memorial Sloan Kettering Cancer Center, New York, New York, United States of America, 5 Department of Pediatrics, Weill Cornell Medical Center, New York, New York, United States of America, 6 Department of Medicine, Memorial Sloan Kettering Cancer Center, New York, New York, United States of America, 7 Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, New York, United States of America

¤ Current address: PetDX, Inc., San Diego, California, United States of America
* abramsod@mskcc.org

Abstract

Cell free DNA (cfDNA) and circulating tumor cell free DNA (ctDNA) from blood (plasma) are increasingly being used in oncology for diagnosis, monitoring response, identifying cancer causing mutations and detecting recurrences. Circulating tumor *RB1* DNA (ctDNA) is found in the blood (plasma) of retinoblastoma patients at diagnosis before instituting treatment (naïve). We investigated ctDNA in naïve unilateral patients before enucleation and during enucleation (6 patients/ 8 mutations with specimens collected 5–40 minutes from severing the optic nerve) In our cohort, following transection the optic nerve, ctDNA *RB1* VAF was measurably lower than pre-enucleation levels within five minutes, 50% less within 15 minutes and 90% less by 40 minutes.

Introduction

Ultrashort fragments of DNA of the retinoblastoma gene are commonly present in the peripheral blood (plasma) of retinoblastoma patients prior to treatment [1–3]. Because the DNA is extracellular it is referred to as "cell free DNA" or **cf**DNA and because the tumor derived cfDNA is from a cancer-causing mutation (*RB1*) it is designated **ct**DNA.

In adult solid cancers, the fates of both cfDNA and ctDNA have been studied following surgery, radiation, chemotherapy and immunotherapy [4–8]. Persistence of ctDNA after any of

these treatments is associated with a higher incidence of recurrence and progression of disease [9, 10]. cfDNA increases with lung, kidney and bladder surgery (thought to be related to surgical trauma) and may remain elevated for days to weeks after surgery [11, 12]. ctDNA usually decreases after surgery but elevated levels after surgery suggest ongoing local or metastatic disease. There is little information about the fate of circulating cfDNA during surgery so we explored plasma cfDNA at different time points during enucleation and compared them to pre-enucleation levels in children with advanced unilateral retinoblastoma undergoing enucleation surgery.

Materials and methods

Plasma ctDNA was analyzed using the MSK-ACCESS liquid biopsy assay with deep sequencing and hybridization capture to detect very low frequency somatic alterations in coding exons from 129 cancer related genes including all exons of *RB1* [1, 3]. This assay was approved by the New York State Department of Health and can detect point mutations (single nucleotide variants/SNV's), insertions or deletions (Indels), and copy number alterations. Two 10cc Streck tubes of peripheral venous blood were used for each assay. Variant allele frequencies were reported (VAF: the proportion of allele bearing the variants divided by the total number of wild-type plus variant alleles at a given genomic location). Our criteria for a call on MSK-ACCESS for de novo specimens were as follows: only duplex reads are used and 3 (hotspot) or 5 (non-hotspot) are required. For copy number variations (CNV) we use a fold change of -1.5 to call a deletion. Testing was performed using white blood cells (WBC) as a matched normal control which facilitates filtering of germline variants as well as accurate dissemination of mutations associated with clonal hematopoiesis.

Eligible patients for this study were children with unilateral retinoblastoma treated at MSKCC who had measurable *RB1* ctDNA assayed prior to enucleation and also at any time-point during enucleation. Patients who did not have measurable levels before surgery or did not have blood collected during surgery were not included. Bilateral patients were excluded because the remaining eye could have contributed to the remaining VAF making analysis of the impact of enucleation impossible.

MSKCC Institutional Review Board (IRB) approval was obtained for the study and all parents/guardians signed consent for cfDNA analysis and blood draw.

Results

A total of 8 *RB1* gene alterations in 6 different patients were identified in this cohort and had VAF's measured prior to and during enucleation surgery. All enucleated eyes were classified Reese-Ellsworth group Vb and International Classification of Retinoblastoma group E. Table 1 summarizes the mutations detected, patient and tumor characteristics.

The left panels of Fig 1 depict baseline fundus imaging and mutant *RB1* variant allele frequency (VAF) for all eight mutations as labeled by patient number. The right panels show bar graphs for each respective ctDNA *RB1* alteration: the x-axis represents timepoints (BL = baseline, mos = months from baseline), and the y-axis shows plasma circulating tumor *RB1* variant allele frequency percentage. For patients 3 and 6, ctDNA detected two *RB1* alterations shown in separate clustered bar graphs: first exon (dark blue columns) and second exon (lighter blue columns).

Fig 2 demonstrates the normalized plasma levels of ctDNA at different time periods after severing the optic nerve (during the enucleation surgery) for each exon that had been detected prior to the surgery.

Table 1. Cell free *RB1* alteration results of unilateral retinoblastoma patients: Pre-enucleation and post-enucleation.

Pt	Age at BL ctDNA (mos)	ctDNA VAF % pre-enuc [95%CI]	Time post-enuc (mins)	ctDNA VAF % post-enuc [95%CI]	ctDNA Somatic RB1 alteration	F/u (mos)	Tumor LBD x ht (mm)	IOP (mmHg)	High risk histopathological features*
3	112	1. 1.63 [1.2–2.2]; 2. 2.0 [1.48–2.69]	5	1. 1.26 [0.9–1.64]; 2. 1.6 [1.24–2.06]	1. RB1 **exon17** p.R556* (c.1666C>T); 2. RB1 **exon8** p.N258Kfs*2 (c.774_786delCAGGAGTGCACGG)	16.6	12 x 19.5	12	massive choroidal & ciliary body invasion; prelaminar optic nerve
5	10	2.92 [2.27–3.75]	20	0.42 [0.22–0.77]	RB1 (NM_000321) **exon17** p.VV516* (c. 1547G>A)	14.9	15 x 13.5	14	none
6	41	1. 2.72 [2.02–3.64]; 2. 3.22 [2.39–4.33]	40	1. 0.2 [.11-.61]; 2. 0.27 [0.11–0.61]	1. RB1 (NM_000321) exon10 p.Q344* (c.1030C>T); 2. RB1 (NM_000321) exon14 p. R445* (c.1333C>T)	19.2	16 x 8	12	none
29	7	3.04 [1.92–4.75]	30	0.2 [0.05–0.64]	RB1 (NM_000321) exon19 splicing variant p. X654_splice (c.1960+1G>A)	3.72	15 x 13	uk	none
30	18	26.25 [24.49–28.10]	30	10.75 [9.57–12.06]	RB1 (NM_000321) exon15 p.R467* (c.1399C>T)	3.68	20 x 6	14	none
31	7	4.88 [4.17–5.69]	12	4.97 [4.18–5.90]	RB1 (NM_000321) exon17 p.W563* (c.1689G>A)	5.0	17 x 8	45	none

BL = baseline, mos = months, ctDNA = circulating tumor DNA, post-enuc = post-enucleation, mins = minutes, VAF = variant allele frequency, 95%CI = 95% confidence interval, LBD = largest basal diameter, ht = height, IOP = intraocular pressure.

https://doi.org/10.1371/journal.pone.0271505.t001

Discussion

Cell free DNA in cancer patients has been reported in blood, saliva, pleural fluid, cerebral spinal fluid, ascites, stool, and urine [11]. It has also been identified in the aqueous humor and blood of retinoblastoma patients at the time of diagnosis [1–3, 13, 14]. Cell free DNA in the plasma of patients with cancer is common and cancer patients have higher levels of cfDNA than non-cancer patients [15]. In a previously reported study from MSKCC on 681 blood samples from 31 solid cancers using MSK-ACCESS, 73% of the samples had structural variants, somatic mutations, and/or copy number alterations [16].

The fate of cfDNA and ctDNA after cancer treatment has been studied. Immediately after surgery in adults for colon, bladder and kidney cancer for example, cfDNA increases for 3–30 days while ctDNA decreases [4, 17]. Persistent elevation of ctDNA is associated with a worse prognosis [15]; the situation with radiation is somewhat different. For example, in lung cancer (non-small cell lung cancer) cfDNA and ctDNA are stable for two hours after radiation and then both increase till the 22nd fraction when it begins to decrease [5]. The pattern with systemic chemotherapy is interesting. In breast cancer, cfDNA decreased after the completion of adjuvant chemotherapy [6]. In ovarian cancer, patients whose ctDNA rose after the first cycle of chemotherapy had improved disease-free survival [18]. The impact of modern immunomodulation on cfDNA in cancer has also been studied. In metastatic melanoma CTLA-4 and PD-1 antibody therapy caused a decrease in ctDNA when measured at 3 weeks and overall survival correlated with this decrease in ctDNA [8]. In lung cancer, immune checkpoint blockage patients with complete disappearance of ctDNA was associated with better outcome than those who had an increase in ctDNA [7].

We recently reported that following intrarterial chemotherapy for intraocular retinoblastoma ctDNA diminishes quickly (90% of patients had none identifiable at 1 month) and in all cases there was no measurable ctDNA in plasma at 3 months [19].

We did not find any elevation of ctDNA after enucleation for unilateral retinoblastoma patients in the current study and all patients but one had decreased plasma levels following severing of the optic nerve.
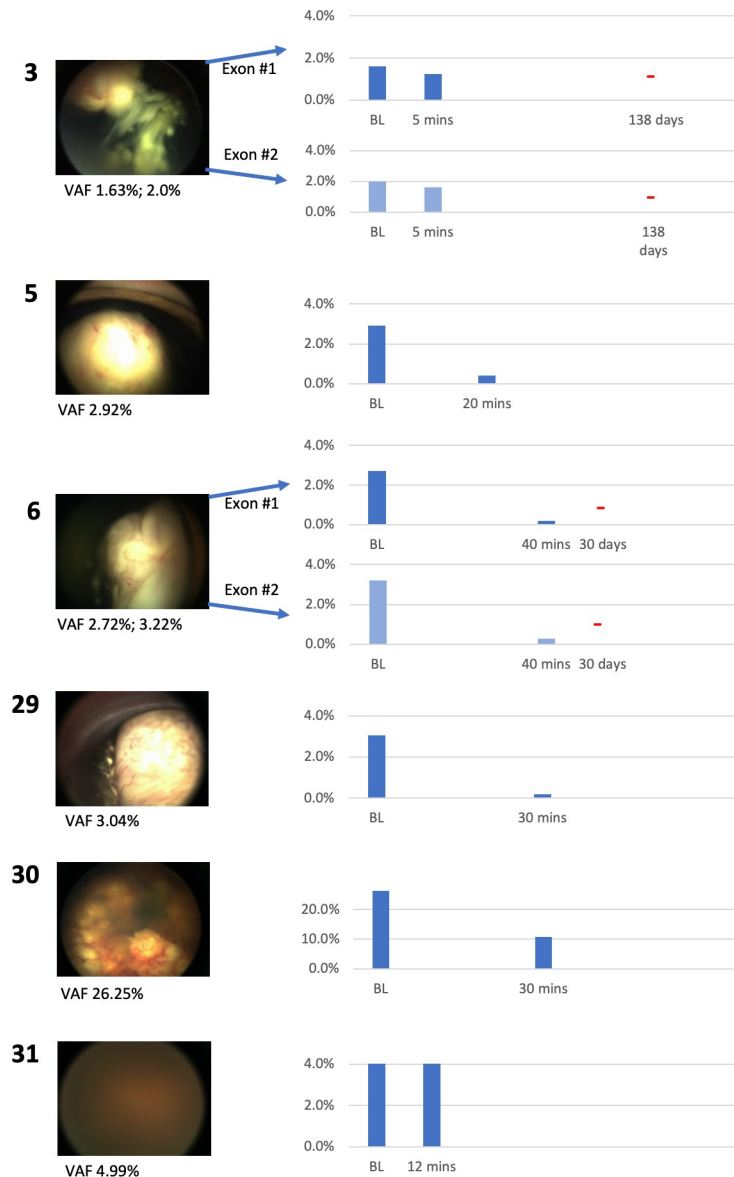
Fig 1. Fundus photographs and clustered bar graphs for four eyes.

https://doi.org/10.1371/journal.pone.0271505.g001

The half-life of plasma ctDNA in retinoblastoma has not been studied and most of the information about half-life of cfDNA comes from animals without cancer [9, 15, 20–22]. In general, half-life of plasma cfDNA is short (a few minutes to several hours). Although better studied rigorously with multiple time points from the same patient that is impossible in children because (at present) 10–20 cc of blood are needed for each specimen analysis. When we compared post-enucleation *RB1* VAF to pre-enucleation values, the VAF decreased by 13–20% within 5 minutes, 86% by 20 minutes and more than 90% by 40 minutes after transecting the optic nerve. Enucleation did not cause an increase in plasma ctDNA.

The curve depicts baseline and post-transection of the optic nerve *RB1* variant allele frequency (VAF) for three eyes as labeled by patient number. The x-axis represents time in minutes and the y-axis shows plasma circulating tumor *RB1* variant allele frequency percentage. Six patients had eight *RB1* mutations measured prior to enucleation (baseline) and a second
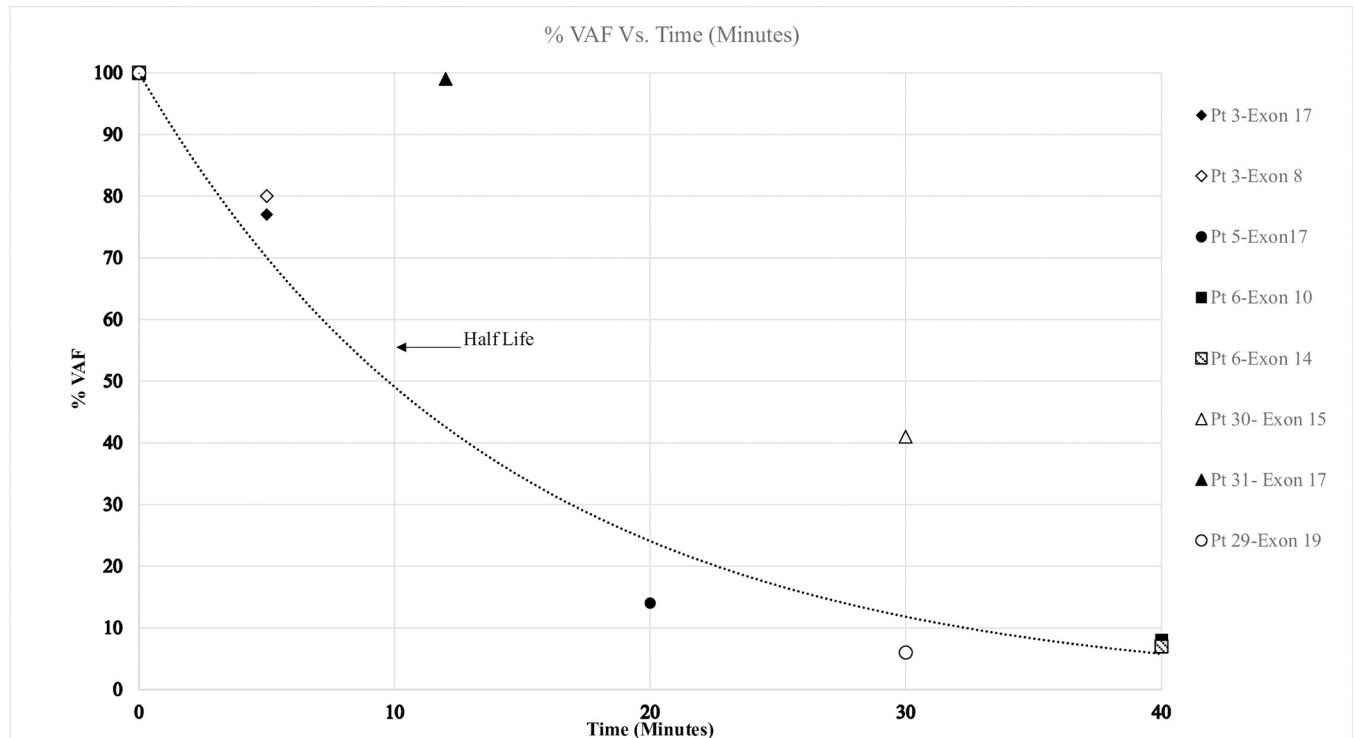
Fig 2. VAF (%) vs. time (minutes).

measurement within one-hour post-surgery (5 minutes, 12 minutes, 20 minutes, 30 minutes, 40 minutes). In two patients, two distinct mutations were identified and in both cases the decrease in VAF was similar for both exons of the same patient.

Interestingly, it has been shown that cfDNA gets incorporated into the genome of cells and that it can alter the biology of those cells; this phenomenon is thought to influence the subsequent development of metastases in some other cancers [23].

Conclusions

In this small cohort of naïve unilateral retinoblastoma patients receiving enucleation, the ctDNA *RB1* VAF decreased quickly *during* the enucleation. This decrease was evident within minutes of severing the optic nerve in surgery. By 40 minutes following enucleation, cfDNA VAF had declined to 7–8% of prenucleation VAF. This suggests that the half-life of *RB1* ctDNA after enucleation is short; although this question is best answered by rigorous multiple time point specimen collection from the same patient. These results suggest that detection of ct*RB1* alterations may be a clinically meaningful tool to monitor response to treatment. This also raises the question as to whether post enucleation elevation of cfDNA in blood of unilateral patients after enucleation may indicate ongoing disease outside the eye.

Author Contributions

Conceptualization: David H. Abramson, Diana L. Mandelker, A. Rose Brannon, Ryma Benayed, Maria E. Arcila, Danielle Novetsky Friedman, Michael F. Walsh, Prachi Kothari, Dana W. Y. Tsui, Jasmine H. Francis.

Data curation: David H. Abramson, Diana L. Mandelker, A. Rose Brannon, Ira J. Dunkel, Ryma Benayed, Melissa A. Robbins, Dianna Haggag-Lindgren, Neerav Shukla, Michael F. Walsh, Prachi Kothari, Jasmine H. Francis.

Formal analysis: David H. Abramson, Diana L. Mandelker, A. Rose Brannon, Ryma Benayed, Maria E. Arcila, Danielle Novetsky Friedman, Gowtham Jayakumaran, Monica S. Diosdado, Melissa A. Robbins, Dianna Haggag-Lindgren, Neerav Shukla, Michael F. Walsh, Prachi Kothari, Dana W. Y. Tsui, Jasmine H. Francis.

Funding acquisition: David H. Abramson, Ira J. Dunkel, Neerav Shukla, Jasmine H. Francis.

Investigation: David H. Abramson, A. Rose Brannon, Ira J. Dunkel, Michael F. Berger, Maria E. Arcila, Marc Ladanyi, Danielle Novetsky Friedman, Melissa A. Robbins, Prachi Kothari, Dana W. Y. Tsui, Jasmine H. Francis.

Methodology: David H. Abramson, Diana L. Mandelker, Ira J. Dunkel, Michael F. Berger, Marc Ladanyi, Danielle Novetsky Friedman, Melissa A. Robbins, Dianna Haggag-Lindgren, Prachi Kothari, Dana W. Y. Tsui, Jasmine H. Francis.

Project administration: Diana L. Mandelker, A. Rose Brannon, Ryma Benayed, Michael F. Berger, Maria E. Arcila, Marc Ladanyi, Monica S. Diosdado, Melissa A. Robbins, Dianna Haggag-Lindgren, Neerav Shukla, Prachi Kothari, Dana W. Y. Tsui, Jasmine H. Francis.

Resources: David H. Abramson, A. Rose Brannon, Ira J. Dunkel, Ryma Benayed, Michael F. Berger, Maria E. Arcila, Marc Ladanyi, Gowtham Jayakumaran, Monica S. Diosdado, Neerav Shukla, Prachi Kothari, Dana W. Y. Tsui.

Software: Ryma Benayed, Michael F. Berger, Maria E. Arcila, Marc Ladanyi, Gowtham Jayakumaran, Monica S. Diosdado, Michael F. Walsh.

Supervision: David H. Abramson, Ira J. Dunkel, Michael F. Berger, Maria E. Arcila, Marc Ladanyi, Michael F. Walsh, Dana W. Y. Tsui.

Validation: Diana L. Mandelker, A. Rose Brannon, Ryma Benayed, Michael F. Berger, Maria E. Arcila, Marc Ladanyi, Gowtham Jayakumaran, Monica S. Diosdado, Michael F. Walsh, Dana W. Y. Tsui.

Visualization: Gowtham Jayakumaran, Michael F. Walsh.

Writing – original draft: David H. Abramson, Melissa A. Robbins, Dianna Haggag-Lindgren, Jasmine H. Francis.

Writing – review & editing: David H. Abramson, Diana L. Mandelker, A. Rose Brannon, Ira J. Dunkel, Ryma Benayed, Michael F. Berger, Maria E. Arcila, Marc Ladanyi, Danielle Novetsky Friedman, Gowtham Jayakumaran, Monica S. Diosdado, Melissa A. Robbins, Dianna Haggag-Lindgren, Neerav Shukla, Michael F. Walsh, Prachi Kothari, Dana W. Y. Tsui, Jasmine H. Francis.

References

1. Abramson DH, Mandelker D, Brannon AR, et al. Retrospective Evaluation of Somatic Alterations in Cell Free DNA from Blood in Retinoblastoma, Ophthalmology Science, 2021; 1(1). https://doi.org/10.1016/j.xops.2021.100015 PMID: 36246006

2. Gerrish A, Jenkinson H, Cole T. The Impact of Cell-Free DNA Analysis on the Management of Retinoblastoma. Cancers (Basel). Multidisciplinary Digital Publishing Institute; 2021 Mar 29; 13(7):1570.

3. 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. https://doi.org/10.1002/cam4.3144 PMID: 32633890

4. Henriksen TV, Reinert T, Christensen E, et al. The effect of surgical trauma on circulating free DNA levels in cancer patients-implications for studies of circulating tumor DNA. Mol Oncol 2020; 14(8):1670–9. https://doi.org/10.1002/1878-0261.12729 PMID: 32471011

5. Nygård L, Ahlborn LB, Persson GF, et al. Circulating cell free DNA during definitive chemo-radiotherapy in non-small cell lung cancer patients—initial observations. PLoS One 2020; 15(4):e0231884. https://doi.org/10.1371/journal.pone.0231884 PMID: 32343749

6. Peled M, Agassi R, Czeiger D, et al. Cell-free DNA concentration in patients with clinical or mammographic suspicion of breast cancer. Sci Rep 2020; 10(1):14601. https://doi.org/10.1038/s41598-020-71357-4 PMID: 32884019

7. Anagnostou V, Forde PM, White JR, et al. Dynamics of Tumor and Immune Responses during Immune Checkpoint Blockade in Non-Small Cell Lung Cancer. Cancer Res 2019; 79(6):1214–25. https://doi.org/10.1158/0008-5472.CAN-18-1127 PMID: 30541742

8. Forschner A, Battke F, Hadaschik D, et al. Tumor mutation burden and circulating tumor DNA in combined CTLA-4 and PD-1 antibody therapy in metastatic melanoma—results of a prospective biomarker study. J Immunother Cancer 2019; 7(1):180. https://doi.org/10.1186/s40425-019-0659-0 PMID: 31300034

9. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008; 14(9):985–90. https://doi.org/10.1038/nm.1789 PMID: 18670422

10. Stewart CM, Tsui DWY. Circulating cell-free DNA for non-invasive cancer management. Cancer Genet 2018; 228–229:169–79. https://doi.org/10.1016/j.cancergen.2018.02.005 PMID: 29625863

11. Corcoran RB, Chabner BA. Application of Cell-free DNA Analysis to Cancer Treatment. N Engl J Med 2018; 379(18):1754–65. https://doi.org/10.1056/NEJMra1706174 PMID: 30380390

12. Hu W, Yang Y, Zhang L, et al. Post surgery circulating free tumor DNA is a predictive biomarker for relapse of lung cancer. Cancer Med 2017; 6(5):962–74. https://doi.org/10.1002/cam4.980 PMID: 28382702

13. Berry JL, Xu L, Murphree AL, et al. Potential of Aqueous Humor as a Surrogate Tumor Biopsy for Retinoblastoma. JAMA Ophthalmol 2017; 135(11):1221–30. https://doi.org/10.1001/jamaophthalmol.2017.4097 PMID: 29049475

14. Abramson DA. Cell free DNA (cfDNA) in the blood of retinoblastoma patients: the Robert M. Ellsworth lecture. Oph Gen 2022; https://doi.org/10.1080/13816810.2021.2004433:1–5

15. Bronkhorst AJ, Ungerer V, Holdenrieder S. The emerging role of cell-free DNA as a molecular marker for cancer management. Biomol Detect Quantif 2019; 17:100087. https://doi.org/10.1016/j.bdq.2019.100087 PMID: 30923679

16. Brannon AR, Jayakumaran G, Diosdado M, et al. Enhanced specificity of high sensitivity somatic variant profiling in cell-free DNA via paired normal sequencing: design, validation, and clinical experience of the MSK-ACCESS liquid biopsy assay. bioRxiv 2020:2020.06.27.175471.

17. Guo N, Lou F, Ma Y, et al. Circulating tumor DNA detection in lung cancer patients before and after surgery. Sci Rep 2016; 6:33519. https://doi.org/10.1038/srep33519 PMID: 27641744

18. Alves MC, Fonseca FLA, Yamada A, et al. Increased circulating tumor DNA as a noninvasive biomarker of early treatment response in patients with metastatic ovarian carcinoma: A pilot study. Tumour Biol 2020; 42(5):1010428320919198. https://doi.org/10.1177/1010428320919198 PMID: 32364828

19. Francis JH, Gobin YG, Brannon AR, et al. RB1 circulating Tumor DNA in the Blood of Unilateral Retinoblastoma Patients: Before and After Intraarterial Chemotherapy, Ophthalmology Science 2021; 1(3):100042.

20. Yao W, Mei C, Nan X, Hui L. Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study. Gene 2016; 590(1):142–8. https://doi.org/10.1016/j.gene.2016.06.033 PMID: 27317895

21. Yu SC, Lee SW, Jiang P, et al. High-resolution profiling of fetal DNA clearance from maternal plasma by massively parallel sequencing. Clin Chem 2013; 59(8):1228–37. https://doi.org/10.1373/clinchem.2013.203679 PMID: 23603797

22. Mittra I, Khare NK, Raghuram GV, et al. Circulating nucleic acids damage DNA of healthy cells by integrating into their genomes. J Biosci 2015; 40(1):91–111. https://doi.org/10.1007/s12038-015-9508-6 PMID: 25740145

23. Wang W, Kong P, Ma G, et al. Characterization of the release and biological significance of cell-free DNA from breast cancer cell lines. Oncotarget 2017; 8(26):43180–91. https://doi.org/10.18632/oncotarget.17858 PMID: 28574818