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Simultaneous identification of clinically relevant *RB1* mutations and copy number alterations in aqueous humor of retinoblastoma eyes

Liya Xu^{a,b}, Lishuang Shen^c, Ashley Polski^{a,d}, Rishvanth K. Prabakar^e, Rachana Shah^f, Rima Jubran^f, Jonathan W. Kim^{a,d}, Jacklyn Biegel^c, Peter Kuhn^{b,g,h,i}, David Cobrinik^{a,d,g,j}, James Hicks^{b,g,k}, Xiaowu Gai^c, Jesse L. Berry^{a,d,g,j}

^aThe Vision Center, Children's Hospital Los Angeles, Los Angeles, California, USA

^bDepartment of Biological Sciences, Dornsife College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, California, USA

^cCenter for Personalized Medicine, Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Los Angeles, California, USA

^dUSC Roski Eye Institute, Keck School of Medicine of USC, Los Angeles, California, USA

^eDepartment of Molecular and Computational Biology, University of Southern California, Los Angeles, California, USA

^fCenter for Blood Disorders, Children's Hospital Los Angeles, Los Angeles, California, USA

^gNorris Comprehensive Cancer Center, Keck School of Medicine of USC, Los Angeles, California, USA

^hDepartment of Aerospace and Mechanical Engineering, Viterbi School of Engineering, University of Southern California, Los Angeles, California, USA

ⁱDepartment of Biomedical Engineering, Viterbi School of Engineering, University of Southern California, Los Angeles, California, USA

^jThe Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, California, USA

^kDepartment of Biochemistry and Molecular Medicine, Keck School of Medicine of USC, Los Angeles, California, USA

Abstract

Background: Detection of germline *RB1* mutations is critical for risk assessment of retinoblastoma (RB) patients. Assessment of somatic copy number alterations (SCNAs) is also critically important because of their prognostic significance. Herein we present a refined approach

CONTACT Jesse L. Berry jesse.berry@med.usc.edu Children's Hospital Los Angeles and The USC Roski Eye Institute, Los Angeles, CA 90033, USA.

Declaration of interest

Drs. Berry, Xu, and Hicks have filed a provisional patent application entitled, Aqueous Humor Cell Free DNA for Diagnostic and Prognostic Evaluation of Ophthalmic Disease. Otherwise the authors report no potential conflict of interest.

for the simultaneous identification of *RB1* variants and SCNAs in the aqueous humor (AH) of RB eyes.

Materials and Methods: Subjects included 7 eyes of 6 RB patients that underwent AH extraction, and 4 matched tumor samples. Cell-free DNA (cfDNA) was isolated and sequenced to assess genome-wide SCNAs. The same sequencing libraries then underwent targeted resequencing and mutation detection using a custom hybridization panel that targets *RB1* and *MYCN*. Illumina paired-end 2×150bp sequencing was used to characterize single-nucleotide variants (SNVs) and loss of heterozygosity (LOH). Results were compared to peripheral blood *RB1* testing. Tumor fraction (TFx) was calculated using ichorCNA.

Results: Four of 7 AH samples contained clinically significant SCNAs. Of the 3 other samples, 1 showed focal *MYCN* amplification and 1 showed focal *RB1* deletion. All 4 enucleated tumors contained SCNAs. Mutational analysis of tumor DNA identified all first hits (2 germline *RB1* SNVs, 2 germline CNAs) and second hits (4 *RB1* SNVs). *RB1* variants in AH were concordant with those obtained from corresponding tumor tissue and blood. In AH samples without paired tumor, both *RB1* hits were identified with high variant allele frequency, even in the absence of SCNAs.

Conclusions: AH liquid biopsy is a minimally invasive, *in vivo* alternative to tissue analysis for the simultaneous identification of *RB1* variants and SCNAs in RB eyes.

Keywords

Aqueous humor; mutational analysis; *RB1* gene; Retinoblastoma; somatic copy number alterations

Introduction

Retinoblastoma (RB), a childhood intraocular cancer, is initiated by biallelic inactivation – usually from single-nucleotide variants (SNVs) or InDels – of the RB tumor suppressor gene (*RB1*) (1). *RB1* was the first cloned tumor suppressor gene and this discovery has dramatically altered our understanding of cancer genetics in general and RB tumorigenesis particularly (2–4). In approximately 40% of RB patients, the initial *RB1* inactivation event is due to a constitutional germline or mosaic mutation (i.e. hereditary RB) followed by a second somatic mutation which then leads to the tumor; in the other 60% of cases both *RB1* mutations are somatic in nature. Children with hereditary RB are at increased risk for 1) developing other ocular and non-ocular tumors over time and for 2) transmission of the mutated gene to future offspring (5). In current clinical practice, testing of peripheral blood leukocytes for the presence of a constitutional pathogenic *RB1* mutation is a crucial aspect of the management of children with RB and is used to determine surveillance strategies for RB patients and their families (6). While children with bilateral disease (e.g. retinoblastoma tumors in both eyes) can be presumed to have a constitutional mutation, upwards of 15% of patients with unilateral disease will also have a detectable mutation from peripheral blood which dramatically alters their care and prognosis (7). It is important to determine whether the detected mutation is a true germline mutation versus a mosaic mutation as the parents and siblings do not require screening in the setting of mosaicism (8); further, the percentage of mosaicism can also help to stratify the risk of second tumors to the child.

Somatic *RB1* mutations are not routinely identified in RB although there is some evidence that particular mutations, notably deletions, acceptor splice sites and frameshift mutations, may present with more aggressive forms of the disease (9). Beyond biallelic *RB1* inactivation, somatic copy number alterations (SCNAs) are thought to contribute to RB tumor growth and progression (10). Multiple studies have identified RB-related SCNAs from RB tumor tissue (10–13), and tumor-directed mutational analyses are also known to increase the accuracy of peripheral blood testing for *RB1*. However, access to this information is only available when RB tumor tissue is available from enucleated (surgically removed) eyes. This is because direct tissue biopsy can cause extraocular tumor spread and is therefore contraindicated in clinical practice (14–16). Only with our recent development of the AH liquid biopsy has *in vivo* detection of tumor derived cfDNA become a possibility for RB patients undergoing active, eye-salvaging therapy (17). In previous studies of the AH liquid biopsy, our group identified tumor-derived SCNAs with prognostic indications for therapeutic response and risk of enucleation (18). Independently, Gerrish et al. verified the presence of tumor derived cfDNA in the AH by identifying somatic *RB1* mutations (19). Detection and identification of tumor-derived *RB1* mutations in the AH has diagnostic significance, including the ability to more efficiently determine the presence of heritable germline *RB1* mutations; additionally there is prognostic significance in whole-genomic evaluation of RB tumors for SCNAs. However, a streamlined process for the simultaneous identification of both *RB1* mutations and largescale genomic alterations in an AH sample is yet to be described.

Herein, we demonstrate that the AH liquid biopsy is a minimally invasive, *in vivo* alternative to tissue analysis for the simultaneous identification of *RB1* mutations and SCNAs for RB. Using a novel, optimized in-house pipeline, we demonstrate that both RB SCNAs and *RB1* mutations can be interrogated from a single sample of <100ul of AH, and that the genetic and genomic alterations identified within AH match what is found by combined peripheral blood and tumor analyses with high accuracy.

Materials and methods

This research was conducted under Institutional Review Board approval and adhered to the tenets of the Declaration of Helsinki. Written informed consent was obtained from the parents of all participants prior to inclusion in the study.

Patient and specimen characteristics

This study included 7 eyes from 6 patients diagnosed with retinoblastoma between December 2014 and July 2019 at the Children's Hospital Los Angeles (CHLA) from whom written parental consent and AH sample(s) were obtained; genomic profiles from these cases have been previously published (18) and are presented here with additional mutation analyses.

Specimen collection and storage

A clear corneal paracentesis with a 32-gauge needle was performed to extract 0.1 mL of AH from retinoblastoma eyes (18). This procedure occurred either as part of the standard

intravitreal melphalan (IVM) injection protocol (20), immediately following enucleation of the eye, or at diagnosis after diagnostic examination under anesthesia. During sampling, needles entered only the anterior chamber via the clear cornea and remained bevel up over the pharmacologically dilated iris; they did not make contact with the iris, lens, vitreous cavity or tumor. While the anterior chamber shallowed slightly it remained formed during paracentesis, and the needle site was examined after AH extraction for any leakage. No patient had a complication from the extraction.

Immediately following specimen extraction, AH samples were stored at -80°C . All samples underwent cfDNA isolation and sequencing within 1 month of extraction, as described previously (18,21). If an enucleation was required for clinical care of the patient, tumor tissue was subsequently obtained from formalin-fixed and paraffin embedded (FFPE) blocks for further genetic and genomic analyses.

Concentration of cfDNA

DNA concentration in the AH before isolation has been reported previously (17); to preserve the minimal volume of the AH sample, this was not repeated until after amplification using the Qubit Fluorometer system (Thermo Fisher).

Genomic analysis of AH samples

Analysis of the cfDNA from AH samples was previously outlined in depth and based on established SCNA analysis methods (18,21–23). Briefly, isolated cfDNA was constructed into a whole genome library followed by shallow sequencing at 0.3x for copy number profiling. As before, SCNAs were considered to be present at 20% deflection from a baseline human genome, consistent with previously established liquid biopsy analyses (18,22,23). Genomic analyses were also performed on matched tumor samples, when available, as described previously (18).

RB1 mutational analysis of AH samples

The same constructed whole genome libraries were further amplified to 500ng each for capture-based targeted next generation sequencing (NGS) for mutation detection. The Agilent SureSelect hybridization capture panel covers the full length including introns and exons of the *RB1* gene, the *MYCN* gene and all exons of the *BCOR* and *CREBBP* genes (Agilent SureSelect Tier1). Illumina paired-end 2×150bp sequencing was carried out on the captured libraries to 100x.

Bioinformatics analysis

Bioinformatics analysis was performed to detect and to characterize single-nucleotide variant (SNV) using an in-house pipeline based on the bcbio pipeline at the CHLA Center for Personalized Medicine (24). The previously published pipeline was optimized for somatic mutational analyses. Briefly, the raw fastq data was trimmed off adaptors and low-quality bases with Atropos (25) and aligned to human GRCh37 reference with BWA-MEM and NovoAlign (v3) (26,27). Germline variants were called after marking duplicates with FreeBayes (28). The presence of somatic variants in the AH or tumor were called with VarDict without the paired normal blood sample (i.e. blinded to germline variant) (29).

Variant annotation was conducted with Ensembl VEP (v96) (30). LOH was called if a region's continuous variants had $\geq 3\%$ alternative alleles.

Determination of cfDNA TFx

The fraction of tumor DNA (TFx) for each sequenced AH cfDNA sample was estimated using ichorCNA software (available at <https://github.com/broadinstitute/ichorCNA>), which is a standard tool for determining cfDNA TFx in blood-based liquid biopsies (31). The algorithm employed by ichorCNA to determine TFx of cfDNA in the serum has been described in detail (31,32). In short, ichorCNA utilizes a hidden Markov model to predict large-scale SCNAs within sequenced cfDNA. TFx estimations based on the presence of large-scale SCNAs are derived while accounting for differences in ploidy and subclonality at each locus, and an optimal TFx solution (as well as numerous alternative solutions) is provided by ichorCNA (31). Per ichorCNA recommendations, genomic profiles and corresponding TFx solutions were visually inspected to confirm the TFx estimate for each sample (<https://github.com/broadinstitute/ichorCNA/wiki/Interpreting-ichorCNA-results>). If the selected solution appeared suboptimal based on ichorCNA guidelines (i.e. a large proportion of SCNAs were being called subclonal, the majority of datapoints were falsely amplified, or two distinct copy number levels were being called neutral) (31), then a more accurate alternative solution was manually selected from the ichorCNA output (available upon request).

Clinical demographics

A data set with clinical characteristics of patients was compiled including germline *RB1* status determined from peripheral blood cells as part of the routine clinical retinoblastoma work-up.

Results

AH samples were obtained from 7 eyes of 6 patients (labeled E1–7; Table 1). AH samples E1–4 were taken at the time of enucleation and matched tumor is available; 1 of the AH samples was taken during primary enucleation and 3 during secondary enucleation after failed attempts at eye salvaging therapy. AH samples E5, E6 and E7 were taken *in vivo*; E6 and E7 were from the right and left eyes of the same patient. There is no matched tumor available for samples E5, E6 or E7 as the eyes have not been enucleated. All patients underwent *RB1* mutational analysis of the peripheral blood as part of routine clinical testing from the CHLA CLIA lab.

Concentration of cfDNA in the AH

The DNA concentration in AH samples was reported previously (17); The DNA concentration before DNA isolation ranged from 0.084 to 56 ng/ μ L (median, 0.174 ng/ μ L). These results are within the range previously reported by Gerrish et al (19). To conserve the very limited AH sample volume, this was not repeated. After PCR amplification (14x) for whole genome library construction, the average final yield was 25.15 ng/ μ L (std dev $\pm$ 22.32). However, there is wide deviation with the highest yields in samples taken before therapy (diagnosis or primary enucleation) and the lowest yields from samples taken during

active treatment due to a decrease in tumor-derived cfDNA in the AH as the intraocular tumor responds to treatment.

Whole genome SCNA analysis on tumor derived cfDNA from AH

Whole genome analyses were performed on all blood, AH samples, and matched tumor samples when available (Figure 1). Large-scale SCNAs were identified in AH samples E1–4. These alterations were concordant with the SCNAs in the tumor tissue as has been previously shown with other AH-tumor pairs (18). For E5 and E7, there were no large-scale SCNA detected, but focal *MYCN* amplification was identified in AH sample from E5 and focal *RB1* gene loss was identified in AH from E7. These focal events were never detected in paired blood samples indicating they are somatic alterations.

RB1 variant detection in cfDNA from AH

From the same constructed whole genome libraries used in SCNA profiling, pathogenic mutations were identified in the AH cfDNA of all 7 eyes. These pathogenic mutations were identified in the AH in all 7 eyes by an customized bioinformatics pipeline at the CHLA Center for Personalized Medicine. The mutations were corroborated either by analyses of tumor tissue from enucleated eyes and/or from clinical testing of peripheral blood leukocytes. No pathogenic *BCOR* or *CREBBP* mutations were detected in these 7 eyes. *RB1* variant allele fraction (% VAF) in both the tumor and AH are presented in Table 1, followed with number in parentheses of unaltered and altered reads on the specific variant, and corresponding 95% CI range, respectively.

Tumor fraction

The tumor fraction (TFx) or percentage of tumor-derived cfDNA in the AH was calculated with ichorCNA software (31) which optimizes the calculation of the fraction based on the presence of large-scale chromosomal alterations. Focal alterations like *MYCN* amplification in E5 and *RB1* gene deletion in E7 have not been able to generate the TFx. The TFx ranged from 17.79–91.42% in tumor samples with +CNA for analysis (mean TFx 70.77%, Table 1).

In summary, our novel, optimized AH-based protocol demonstrates feasibility for the detection of both pathogenic *RB1* mutations, genomic copy number alterations and tumor fraction in retinoblastoma from a single sample of AH. This has potential for numerous, clinically significant applications including diagnostic (*RB1*), prognostic (SCNAs) and therapeutic (TFx) implications. This streamlined approach facilitates *in vivo* detection of tumor-derived DNA, making it possible to identify underlying somatic *RB1* mutations of RB – similar to Gerrish et al (19) – and SCNAs (18) from the same 100ul sample of AH. This can be done in the absence of a direct tumor biopsy or enucleation.

Discussion

Minimally invasive access to tumor-derived genetic and genomic information could be helpful not only for definitively diagnosing RB patients but also for prognostication – as identification of focal *MYCN* amplification (33) and gain of 6p (18) are poor prognostic indicators for overall globe salvage. We have demonstrated that our platform is sensitive for

the detection of single gene alterations, such as *MYCN* amplification (E5) and focal *RB1* loss (E7). Identification of somatic *RB1* mutations also allows for improved specificity of peripheral blood testing for germline *RB1* mutations, using tumor-directed mutational analyses. Such targeted genetic testing is especially important for identifying patients with low levels of germline *RB1* mosaicism, as low-level mosaics may be missed by the routine methods of peripheral blood testing for RB (34). Additionally, targeted *RB1* mutational analysis of the cerebrospinal fluid in patients with metastatic retinoblastoma has also shown promise in identifying minimal residual disease (35). Previously, identifications of these mutations would only be possible with tumor tissue in the setting of enucleation. Recent progress in the development of a liquid biopsy platform using the AH, however, has changed this paradigm (17–19). A larger prospective study of *RB1* somatic and germline mutations within AH and peripheral blood would be useful for clarifying the diagnostic role of AH evaluation in the clinical setting and whether there is prognostic value in identifying the specific somatic *RB1* mutations, or other pathogenic mutations such as *BCOR* or *CREBBP* (36), *in vivo* from every patient.

Another potential application of our approach is the determination of tumor fraction (TFx, i.e. the relative amount of tumor-derived DNA within a sample). Multiple studies of non-ocular cancers have shown a significant association between TFx and overall tumor burden, with increases in TFx reflecting progression of disease and vice versa (32,37). Although novel software, such as *ichorCNA* (31), allows for TFx estimation based on the detection of SCNAs within cfDNA, this approach is inherently limited for RB tumors that do not have any large-scale SCNAs, which account for nearly one-third of RB tumors, including samples E5, E6 and E7 here; in such tumors, accurate TFx estimation from current SCNA-based methods is not possible. However, with few exceptions (33), all RB tumors contain somatic mutations in *RB1*. Therefore, calculating TFx based on the presence of somatic *RB1* variants with VAF may be a useful alternative approach for estimating TFx in the absence of SCNAs. As demonstrated in Table 1, there is a high VAF for the *RB1* mutations identified in E6 and E7 which is on par with the tumor tissue, thus demonstrating the presence of tumor-derived DNA. Further investigation with implementing unique molecular identifiers (UMI) is necessary in order to develop a reliable *RB1*-based pipeline for TFx estimation in the setting of RB. The TFx can also be monitored overtime in the AH. Since TFx changes correlate with disease activity (32,37,38), this may be a novel way to monitor for minimal residual intraocular disease in retinoblastoma.

Given the potential prognostic significance of certain RB SCNAs, whole-genomic evaluation of RB tumors – in addition to *RB1* testing – is becoming an increasingly important area of investigation for ocular oncologists and their pediatric patients. The refined approach presented herein allows for diagnostic and prognostic data to be generated in an optimized fashion from the same prepared cfDNA sequencing library. This maximizes the use of a very limited sample of AH, which – while less invasive than tumor biopsy – remains a more invasive sample to obtain than blood. Additionally, the maximum volume that can be taken from an eye *in vivo* is approximately 100ul and it cannot be directly repeated. Thus, future applications of this novel liquid biopsy platform must optimize their testing to obtain clinically relevant and impactful information from this otherwise small biospecimen.

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Figure 1.
Whole genome copy number alteration profiling in AH cfDNA and tumor DNA.

Table 1.

Mutational and somatic copy number alteration (SCNA) analyses for aqueous humor samples from 7 retinoblastoma eyes (E1–E7), including corresponding germline *RB1* and tumor mutational analyses when available.

Rb Eye	Timing of Sampling	Tumor_CNA (TFs)	AH_CNA (TFs)	gDNA <i>RB1</i> mutation	<i>RB1</i> mutation	tDNA			AH_cDNA		
						%VAF	(unaltered, altered)	95% CI	%VAF	(unaltered, altered)	95% CI
E1	Secondary Enucleation	CNA ⁺ (17.79%)	CNA ⁺ (28.67%)	c.1758del, p.Glu587AsnfsTer24	c.1758del, p.Glu587AsnfsTer24 c.689 C>G, p.Ser230*	45.59% 29.10%	543, 455 609, 250	(42.47% – 48.74%) (26.08% – 32.27%)	65.71% 43.94%	36, 69 37, 29	(55.81% – 74.70%) (31.74% – 56.70%)
E2	Primary Enucleation	CNA ⁺ (83.03%)	CNA ⁺ (45.25%)	13qDel	13qDel c.256 G>T, p.Gly86*	13qDel/ NA 97.19%	... 5, 173	... (93.57% – 99.08%)	13qDel/ NA 95.56%	... 2, 43	... (84.85% – 99.46%)
E3	Secondary Enucleation	CNA ⁺ (91.42%)	CNA ⁺ (45.01%)	c.958 C>T, p.Arg320*	c.958 C>T, p.Arg320* c.751 C>T, p.Arg251*	30.74% 45.00%	196, 87 374, 306	(25.42% – 36.48%) (41.22% – 48.83%)	41.04% 55.56%	102, 71 112, 140	(33.63% – 48.76%) (49.19% – 61.79%)
E4	Secondary Enucleation	CNA ⁺ (90.82%)	CNA ⁺ (82.2%)	13q deletion	13q deletion c.1215 + 1 G>A, splice_donor_variant	13qDel/ NA 92.00%	... 4, 46	... (80.77% – 97.78%)	13qDel/ NA 92.45%	... 4, 49	... (81.79% – 97.91%)
E5	During Therapy	NA	CNA-with focal <i>MYCN</i> amp	c.2425del, p.Leu809*	c.2425del, p.Leu809* c.1024del, p.Thr342LeufsTer7	NA NA	NA NA	NA NA	32.14% 15.79%	19, 9 16, 3	(15.88% – 52.35%) (3.38% – 39.58%)
E6	Diagnosis	NA	CNA-	c.1666 C>T, p.Arg455*	c.1666 C>T, p.Arg455* LOH	NA NA	NA NA	NA NA	66.67% LOH	37, 74	(57.09% – 75.33%)
E7	Diagnosis	NA	CNA-with focal <i>RB1</i> deletion	c.1666 C>T, p.Arg455*	c.1666 C>T, p.Arg455* Focal <i>RB1</i> gene deletion	NA NA	NA NA	NA NA	100% Focal <i>RB1</i> gene deletion	0, 351	(98.95– 100%) [†]

[†] one-sided, 97.5% CI.

Abbreviations: AH, aqueous humor; amp, amplification; cDNA, cell-free DNA; CNA, copy number alteration; gDNA, genomic DNA; NA, not available; Rb, retinoblastoma; tDNA, tumor DNA; TFs, tumor fraction; VAF, variant allele frequency.