Chromosome 6p Amplification in Aqueous Humor Cell-Free DNA Is a Prognostic Biomarker for Retinoblastoma Ocular Survival

Liya Xu[1,2,3], Ashley Polski[1,4], Rishvanth K. Prabakar[5], Mark W. Reid[1], Patricia Chevez-Barrios[6], Rima Jubran[7], Jonathan W. Kim[1,4], Peter Kuhn[2,3,8,9,10], David Cobrinik[1,3,4,8,11], James Hicks[2,8], and Jesse L. Berry[1,3,4,8]

ABSTRACT

Aqueous humor contains tumor-derived cell-free DNA (cfDNA) and can serve as a liquid biopsy for retinoblastoma. We previously associated somatic copy-number alteration (SCNA) 6p gain with a 10-fold increased risk of enucleation. Here we provide a 2-year update to further explore 6p gain as a prognostic biomarker for ocular survival. Patients diagnosed with retinoblastoma from December 2014 to July 2019 from whom aqueous humor was sampled were included. cfDNA was extracted and shallow whole-genome sequencing performed to identify highly recurrent retinoblastoma SCNAs (gain of 1q, 2p, 6p, loss of 13q, 16q). 116 aqueous humor samples from 50 eyes of 46 patients were included: 27 eyes were salvaged, 23 were enucleated. Highly recurrent retinoblastoma SCNAs were found in 66% eyes. 6p gain was the most prevalent SCNA (50% eyes). It was particularly more prevalent in enucleated eyes (73.9%) than in salvaged eyes (29.6%; $P = 0.004$). 6p gain in aqueous humor cfDNA portended nearly 10-fold increased odds of enucleation (OR = 9.87; 95% confidence interval = 1.75–55.65; $P = 0.009$). In the enucleated eyes, 6p gain was associated with aggressive histopathologic features, including necrosis, higher degrees of anaplasia, and focal invasion of ocular structures. With extended follow-up and nearly double the aqueous humor samples, we continue to demonstrate 6p gain as a potential prognostic biomarker for retinoblastoma.

Implications: Aqueous humor is a high-yield source of tumor-derived DNA in retinoblastoma eyes. Detection of 6p gain in the aqueous humor allows for targeted, patient-centered therapies based on this molecular prognostic marker. Prospective, multicenter studies with aqueous humor sampled from all eyes at diagnosis are warranted to validate these findings.

Introduction

Retinoblastoma is a primary intraocular malignancy that forms in the developing retina of young children. Although overall survival rates approach 98% in developed countries (1), ocular survival for advanced eyes (Group D/E or cT2b/3) is far lower. In the field of ocular oncology, there is no known molecular marker to inform the diagnosis, prognosis for eye survival, or treatment of patients with retinoblastoma. This is due, in part, to the inability to directly biopsy tumors for fear that globe entry could provoke tumor seeding outside the eye and lead to orbital relapse (2–8). Because direct tumor biopsy is prohibited in the setting of retinoblastoma, retrospective studies evaluating retinoblastoma treatment efficacy and corresponding tumor genetics have been limited to analysis of enucleated eyes (9–12). The lack of *in vivo* molecular data from patients with retinoblastoma limits our understanding of tumor biology as well as our ability to develop precision treatment plans and prognosticate treatment outcomes accurately.

In 2017, our group demonstrated that aqueous humor could be used to evaluate retinoblastoma *in vivo* at the molecular level (13). From a set of enucleated eyes, we also showed that the molecular findings in aqueous humor strongly correlate with genomics of enucleated tumors. With multiple studies now showing that aqueous humor can be safely extracted and used to analyze tumor-derived cell-free DNA (cfDNA) throughout the course of treatment, aqueous humor has been established as a novel source of surrogate tumor biopsy, or liquid biopsy, for retinoblastoma (13–15). In the context of this liquid biopsy research, we identified a specific highly recurrent somatic copy-number alteration (SCNA), 6p chromosomal gain, as being associated with a 10-fold increased risk of enucleation and significantly decreased rate of ocular salvage (14). This revealed that SCNAs in the aqueous humor correlate with clinical outcomes, and highlighted chromosome 6p gain in the aqueous humor as a potential prognostic biomarker for retinoblastoma. However, the study was limited by a relatively small cohort of patients, and it was unknown whether this relationship would persist over extended follow-up and with the addition of new patients.

[1]The Vision Center at Children's Hospital Los Angeles, Los Angeles, California. [2]Department of Biological Sciences, Dornsife College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, California. [3]The Saban Research Institute, Children's Hospital Los Angeles, Los Angeles, California. [4]USC Roski Eye Institute, Keck Medical School of the University of Southern California, Los Angeles, California. [5]Department of Molecular and Computational Biology, University of Southern California, Los Angeles, California. [6]Departments of Pathology and Genomic Medicine and Ophthalmology, Houston Methodist, Weill Cornell Medical College, Houston, Texas. [7]Department of Hematology-Oncology, Children's Hospital Los Angeles, Los Angeles, California. [8]Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, California. [9]Department of Aerospace and Mechanical Engineering, Viterbi School of Engineering, University of Southern California, Los Angeles, California. [10]Department of Biomedical Engineering, Viterbi School of Engineering, University of Southern California, Los Angeles, California. [11]Department of Biochemistry and Molecular Medicine, Keck School of Medicine, University of Southern California, Los Angeles, California.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

L. Xu and A. Polski contributed equally as co-first authors of this article.

Corresponding Author: Jesse L. Berry, Children's Hospital of Los Angeles, Los Angeles, CA 90027. Phone: 323-442-6335; E-mail: jesse.berry@med.usc.edu

Mol Cancer Res 2020;18:1166–75

doi: 10.1158/1541-7786.MCR-19-1262

Ultimately, research to establish an aqueous humor–based liquid biopsy for retinoblastoma is aimed at addressing the true clinical problems of (i) our inability to biopsy tumor tissue and (ii) the lack of prognostic biomarkers for retinoblastoma. Current management decisions for patients with retinoblastoma are made on the basis of clinical features that indicate the extent of tumor growth in the eye, the judgment and experience of the treating clinician, and collaboration with the parents. However, identifying biomarkers that inform the prognosis for ocular survival could revolutionize how we plan and care for these children. In this study, we provide a 2-year update—including our initial cohort with extended follow-up (14) and all newly diagnosed patients throughout this 2-year period—to more fully explore the relationship between highly recurrent retinoblastoma SCNAs [specifically gains on 1q, 2p, 6p; losses on 13q and 16q; and focal *MYCN* gains (16–19)] and ocular outcomes. More specifically, we explore the possibility of 6p gain as a clinically relevant prognostic biomarker for retinoblastoma.

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. The REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) guidelines for reporting tumor biomarkers were followed (20).

Patient and specimen characteristics

This study included all patients diagnosed with retinoblastoma between December 2014 and July 2019 at the Children's Hospital Los Angeles (CHLA) from whom written parental consent and aqueous humor sample(s) were obtained; previously published cases now with extended follow-up—in addition to cases that were diagnosed since the last study—are included (14). In all cases, samples consisted of approximately 0.1 mL of aqueous humor that was extracted via clear cornea paracentesis either (i) during routine clinical therapy with intravitreal melphalan (IVM), (ii) at primary or secondary enucleation, or (iii) at diagnosis. Consent from both parents was required for patients from whom aqueous humor was extracted at the time of diagnosis (i.e., prior to initiation of routine clinical therapy) as it was done as a research-only procedure. Patients with retinoblastoma who were treated but did not have aqueous humor extracted were excluded from the study. For all participants, retinoblastoma treatment was carried out in a nonrandomized manner per CHLA protocol (21, 22). As described previously (14), genomic testing results remained separate from clinical data until the final retrospective analysis, and thus did not influence patient treatment or the study endpoints.

The primary clinical endpoints were eye salvage (the ability to treat and save the eye using standard chemotherapeutic modalities) versus enucleation (surgical removal of the diseased eye).

Specimen collection and storage

A clear corneal paracentesis with a 32-guage needle was performed to extract 0.1 mL of aqueous humor from retinoblastoma eyes (14). This procedure occurred either as part of the standard IVM injection protocol (23), immediately following enucleation of the eye, or at the time of diagnosis following thorough 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 aqueous humor extraction for any leakage.

Immediately following specimen extraction, aqueous humor samples were stored at −80°C. Beginning on January 1, 2019, RNase inhibitor (Applied Bio N8080119) was added to newly extracted samples to a concentration of 1 U/μL. All samples underwent cfDNA isolation and sequencing within 1 month of extraction, as described previously (13, 14).

Genomic analysis of aqueous humor samples

Analysis of the cfDNA from aqueous humor samples was previously outlined in depth and based on established methods of SCNA analysis (13, 14, 24, 25). Briefly, isolated cfDNA was constructed into a whole-genome library followed by shallow whole-genome sequencing 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 (14, 24, 25).

Clinical demographics

To assess the relationship between the SCNAs present in aqueous humor samples and clinical features of retinoblastoma, we compiled a dataset with clinical characteristics of participants, germline *RB1* status (determined from peripheral blood cells as part of the routine retinoblastoma work-up; the results of this clinical test were recorded, it is not part of our research platform), SCNA profiles of aqueous humor samples, and matched tumor characteristics for enucleated eyes. Retrospective chart review was performed to determine the following clinical features: age at diagnosis, sex, laterality, IIRC group and tumor–node–metastasis (TNM) staging (26, 27), seeding morphology, therapy, clinical outcomes, histopathology when available and follow-up times for all participants.

Statistical analyses

Fisher exact test was used to evaluate associations between retinoblastoma SCNAs and clinical factors or outcomes. Kaplan–Meier survival analyses with log-rank tests were performed for comparisons of eye salvage in treated eyes based on the presence of retinoblastoma SCNAs. Single-variable analyses were performed using logistic regression, followed by the creation of a multivariable model wherein age and the presence of other retinoblastoma SCNAs were considered covariates; Hosmer–Lemeshow test was used to evaluate the fit of this multivariable model. To compare median amplitudes of 6p gain in enucleated versus salvaged eyes, Mann–Whitney *U* test was used (14). A Cox proportional hazard model was used to estimate HRs of retinoblastoma SCNAs. JMP Pro 13 (SAS Institute, Inc.) and Stata/SE 14.2 (StataCorp) were used for statistical analyses. We did not assess internal validation by split-sample or cross-validation due to the inherently small sample size of this rare disease.

Results

Clinical characteristics of participants

Forty-six patients were included in the study; 4 patients with bilateral disease had both eyes sampled, so a total of 50 eyes were included in the analysis. For the 26 patients that were included in the previous publication and followed for 2 additional years, case numbers (1–26) remained consistent with the prior study for comparison purposes (14). No patients who entered the study dropped out or withdrew consent over the study period. Demographics and clinical features of all participants are summarized in Supplementary Table S1. The median age at diagnosis for all patients was 13 months. Enucleated

eyes tended to be diagnosed at an older age than salvaged eyes, although this difference was not statistically significant (enucleated median, 19 months; range 0–59 months; salvaged median, 9 months; range 2–45 months; $P = 0.09$). Of all eyes, the most commonly diagnosed IIRC group was D (35/50, 70%) and the most common TNM stage was cT2b (36/50, 72%), although less advanced (Groups B and C) eyes were also present (see Supplementary Table S1). On the basis of the International Retinoblastoma Staging System (28), all salvaged (i.e., nonenucleated) eyes were stage 0 (intraocular disease only), and all enucleated eyes were stage I (intraocular disease only).

Eye-sparing treatment modalities were nonrandomized and included the following: 3-drug intravenous chemotherapy (CEV; refs. 21, 22), intraarterial chemotherapy, intravitreal chemotherapy for seeding (IVM; refs. 22, 29), laser consolidation, and cryotherapy. No patients were treated with external beam radiation therapy or plaque brachytherapy. Twenty-seven eyes were salvaged with one or more of the above treatments during the study period. Twenty-three eyes required enucleation either (i) primarily as initial treatment ($n = 8$ eyes), (ii) secondarily due to tumor recurrence ($n = 10$ eyes), or (iii) secondarily due to tumor persistence (i.e., poor response to initial chemotherapy; $n = 5$ eyes; **Fig. 1**). It should be noted that 2 eyes (cases 15 and 25) that were initially considered salvaged in the previous study (14) were both subsequently enucleated after 22 months of conservative treatment due to intraocular retinal recurrence; the recurrence was not associated with the paracentesis site and there was no orbital or metastatic disease for these or any patients in the study. No enucleated patient developed any subsequent tumor spread or recurrence throughout the study period.

Altogether, clinical follow-up from diagnosis to final evaluation ranged from 6 to 64 months (median, 28.5 months). There was no significant difference in length of follow-up between enucleated (median, 24 months; range, 6–64 months) and salvaged (median, 34 months; range, 7–63 months) eyes (T-test; $P = 0.93$).

Genomic changes in tumor-derived cfDNA of the aqueous humor

Genomic analysis included a total of 116 aqueous humor samples, of which 5 (4.3%) were removed for quality control due to poor reads alignment ratio (<2%). Of the remaining 111 aqueous humor samples, 91 were extracted at the time of IVM injection, 15 immediately after enucleation (primary or secondary), and 5 at the time of diagnosis in eyes that were treated conservatively for salvage. Ten eyes had a sample taken at some point during treatment and also at the time of enucleation. No participant had adverse effects or complications secondary to aqueous humor extraction—including no infection, bleeding, iris trauma, or cataract.

After genomic evaluation, 33 of 50 sampled eyes (66%) were positive for one or more of the highly recurrent retinoblastoma SCNAs (Supplementary Table S1), and 39 of 50 (78%) were positive for any measurable CNA. Gain of 6p was the most frequently observed retinoblastoma SCNA among all 50 eyes (50%), followed by 1q gain (38%), 16q loss (30%), 2p gain (including 3 eyes with focal *MYCN* gains; 18%), and 13q loss (10%). Of note, all 3 eyes with focal *MYCN* gains (cases 10, 28, and 31) had no other SCNAs and ultimately required enucleation, and all demonstrated biallelic *RB1* mutations on postenucleation tissue analysis. The frequency of 6p gain in the profiles was associated with 1q gain (Fisher exact, $P = 0.02$), and the presence of 1q gain was also associated with 16q loss (Fisher exact, $P \leq 0.0001$). 6p gain and 16q loss were not significantly associated; however, there was a trend toward significance (Fisher exact, $P = 0.062$). Age was

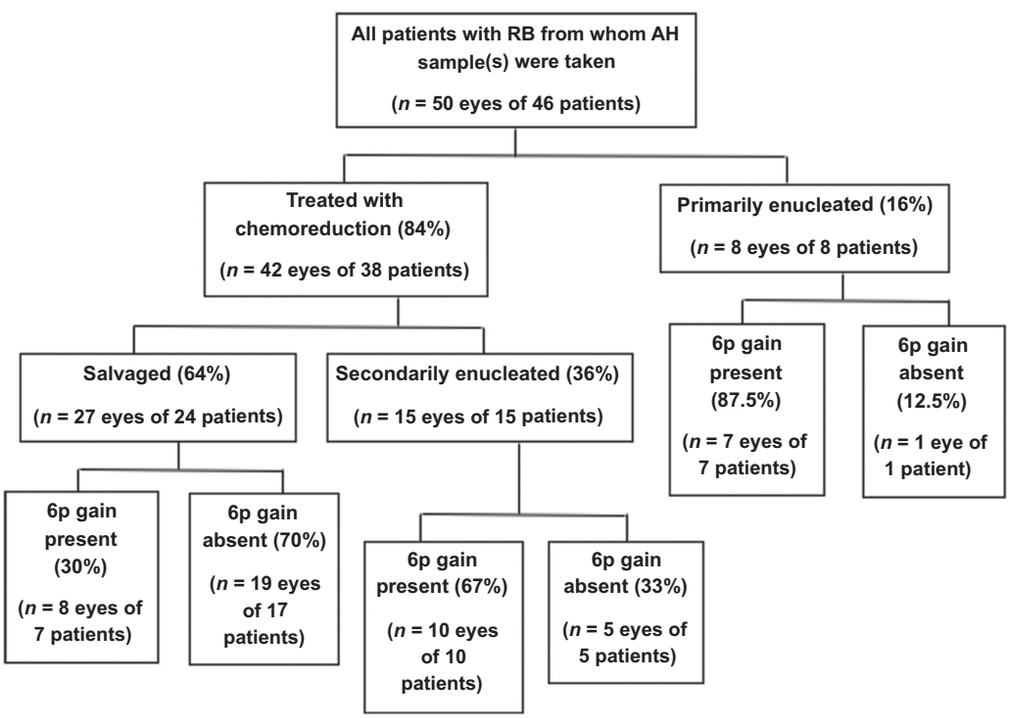


Figure 1.
Flow diagram summarizing the treatments given and the presence or absence of 6p gain in each treatment group.

positively associated with the frequency of SCNAs, as older patients had more SCNAs ($P < 0.0001$).

Retinoblastoma SCNAs and 6p gain in the aqueous humor predict enucleation

When the SCNA profiles for enucleated versus salvaged eyes were compared, the presence of any highly recurrent retinoblastoma SCNAs were significantly more common in enucleated eyes (21/23, 91.3%) than in salvaged eyes (12/27, 44.4%; Fisher exact, $P = 0.0007$). Altogether, the presence of any retinoblastoma SCNAs within the aqueous humor portended at least 13-fold greater odds of an eye requiring enucleation (OR = 13.13; 95% CI = 2.56–67.46; $P = 0.002$).

This association appears to be primarily driven by the presence of 6p gain. Of the retinoblastoma SCNAs, 6p gain in particular was more prevalent in enucleated eyes (17/23, 73.9%) than in salvaged eyes (8/27, 29.6%; Fisher exact, $P = 0.004$). There were no significant differences in the prevalence of other individual SCNAs (at 1q, 2p, 13q, or 16q) between salvaged and enucleated eyes (**Table 1**). Given that enucleation after a year of conservative therapy is less likely (21, 22), we also performed a more strict evaluation in which only eyes with ≥12 months of follow-up were considered salvaged. Overall, a total of 39 eyes (21 salvaged) had ≥12 months of follow-up in our study. Using this stricter criterion for the salvaged group, 6p gain was still significantly more prevalent in the enucleated group (17/23, 73.9%) than in the modified salvaged group (7/21, 33.3%; Fisher exact, $P = 0.014$).

Considered as a single variable, the presence of 6p gain in the aqueous humor was associated with a nearly 7-fold increased odds of enucleation (OR = 6.73; 95% CI = 1.94–23.36; $P = 0.003$); no other retinoblastoma SCNA demonstrated a statistically significant association. On the basis of univariable Kaplan–Meier analysis (**Fig. 2**), the risk of enucleation in eyes with 6p gain was 3.8 times greater after one year than in eyes without this chromosomal alteration [82.8% of eyes without 6p gain remain, vs. 34.1% of eyes with 6p gain; $\chi^2(1) = 11.90$; $P = 0.0006$]. After 3 years, 68.3% of eyes without 6p were salvaged, versus only 27.3% of eyes with 6p gain in the aqueous humor. Overall, the hazard of enucleation over time is 4.27 times greater in those patients with 6p gain (95% CI = 1.65–11.07; $P = 0.003$).

Overall, enucleated eyes also demonstrated a significantly greater median amplitude of 6p gain compared with salvaged eyes (1.46 in enucleated eyes vs. 1.02 in salvaged eyes; $P = 0.005$; **Fig. 3**).To determine whether greater median amplitudes of 6p gain are more predictive of enucleation, we compared all eyes with median amplitudes at 6p of ≥1.5 to eyes with median amplitudes at 6p of <1.5. Gains of 6p with median amplitudes of ≥1.5 were significantly more prevalent in enucleated eyes (14/23, 60.9%) than in salvaged eyes (4/27, 14.8%; Fisher exact, $P = 0.001$). Considered as a single variable, the presence of 6p gain with a median amplitude of ≥1.5 in the aqueous humor was associated with a nearly 9-fold increased odds of enucleation (OR = 8.94; 95% CI = 2.31–34.58; $P = 0.001$).

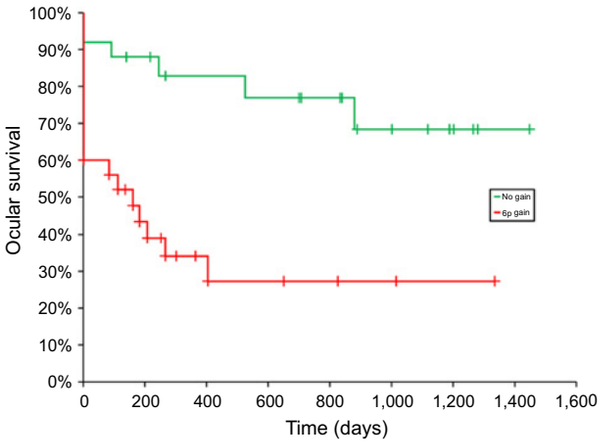
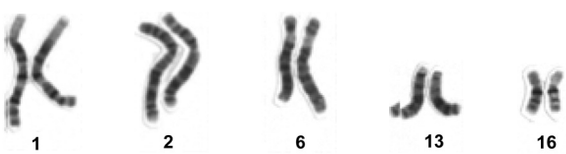


Figure 2.
Kaplan–Meier analysis demonstrating a 3.8 times greater risk of enucleation in eyes with 6p gain compared with eyes without a 6p gain after 1 year.

Utility of SCNAs in the prediction of enucleation

Previously univariate analyses were done to evaluate the association between retinoblastoma SCNAs and ocular outcomes (14). With the addition of new patients, samples, and longer overall follow-up, a more accurate model can be constructed. As shown in **Table 2**, 6p gain is most strongly predictive of enucleation, after controlling for age, sex, IIRC group, and the presence of other SCNAs ($P = 0.01$). Notably, 2p gain (including the 3 patients with focal *MYCN* gain) is marginally predictive of enucleation in this model ($P = 0.08$). Evaluated together, this model correctly classifies 74% of patients, with 87% sensitivity for predicting enucleation and 70% specificity for predxicting salvage.

Table 1. Frequencies of highly recurrent retinoblastoma SCNAs, including gain of 1q, 2p, and 6p, and loss of 13q and 16q.



	1q	2p	6p	13q	16q	Any retinoblastoma SCNA	Totals
Salvaged eyes	29.6%	11.1%	29.6%	7.4%	25.9%	44.4%	27
Enucleated eyes	47.8%	21.7%	73.9%	13.0%	34.8%	91.3%	23
P	0.2465	0.4442	**0.0041**	0.6507	0.548	**0.0007**	
IVM during CTx for persistent seeds	57.1%	28.6%	57.1%	14.3%	50.0%	92.9%	14
IVM after CTx for recurrent seeds	23.8%	9.5%	33.3%	14.3%	23.8%	38.1%	21
P	0.075	0.1907	0.1871	1	0.1534	**0.0015**	

Note: Frequencies were compared using Fisher exact test. Statistically significant *P*-values (<0.05) are bolded.
Abbreviations: CTx, chemotherapy; IVM, intravitreal melphalan; RB, retinoblastoma; SCNA, somatic copy-number alteration.

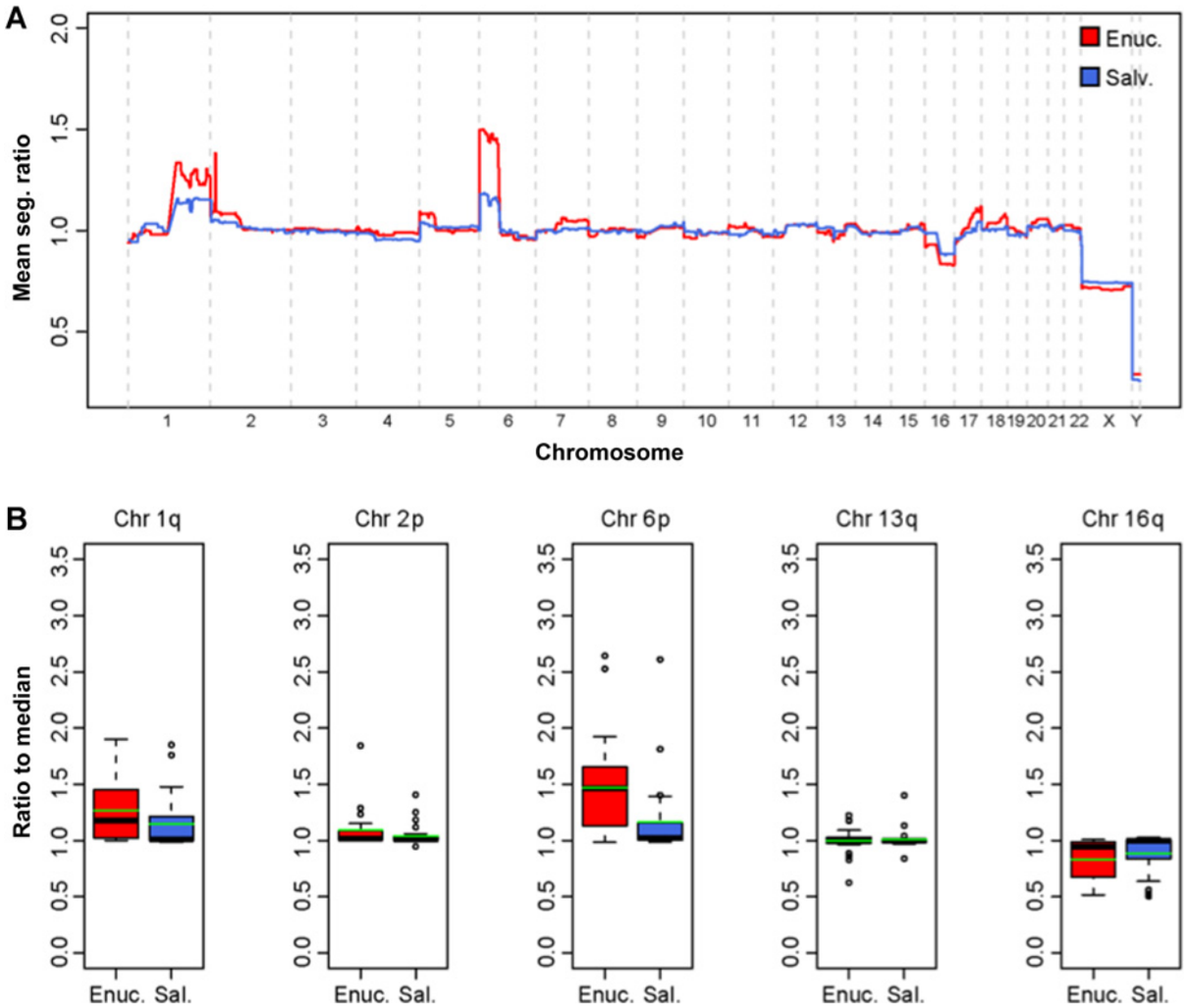


Figure 3.

A, Composite SCNA profile of cfDNA sampled from the aqueous humor (AH) of enucleated (Enuc., red) and salvaged (Salv., blue) eyes. **B,** Box plots demonstrating the range of ratio to median amplitudes for SCNAs in enucleated (Enuc.) and salvaged (Salv.) eyes (median, black bar; mean, green bar). The median of the ratio to the median amplitude of chromosome 6p gain is significantly greater in enucleated eyes compared with salvaged eyes ($P = 0.005$).

This model cannot be rejected for issues related to goodness of fit, as evaluated using the Hosmer–Lemeshow test ($P = 0.21$).

When the criterion for 6p gain is made stricter (≥1.5 times normal values), a trade-off of greater specificity for lower sensitivity emerges. More patients are correctly classified (80%). Furthermore, this model is 78% sensitive and 81% specific for the prediction of enucleation or salvage, respectively. This model also cannot be rejected for fit issues (Hosmer–Lemeshow goodness-of-fit, $P = 0.36$).

Other clinical associations with retinoblastoma SCNAs

Retinoblastoma SCNAs in the aqueous humor are associated with persistent tumor seeding

Over the course of the study, 35 eyes required IVM injections for seeding; 21 of these eyes received IVM for *recurrent* seeds (i.e., seeding that resolved with treatment and then subsequently returned) and 14 received IVM for *persistent* seeds (i.e., seeding that never fully responded to systemic or intraarterial chemotherapy). retinoblastoma SCNAs were significantly more prevalent among eyes with persistent seeds (13/14, 92.9%) than in eyes with recurrent seeds that initially responded to treatment (8/21, 38.1%; Fisher exact, $P = 0.001$). There was also a trend toward an association between 1q gain and eyes with persistent seeding compared with eyes with recurrent seeding, although this difference was not statistically significant (Fisher exact, $P = 0.075$; **Table 1**). Each increase in seeding class (from none to dust, from dust to sphere, and from sphere to cloud) was associated with 2-fold increased odds of having a 6p gain present in the aqueous humor ($P = 0.04$).

6p gain is associated with aggressive histopathologic features

Seven of the 23 enucleated eyes (30.4%) had classic higher risk features on histopathologic analysis (30); 6p gain was present in 5 of these eyes (4 eyes had >3 mm of choroidal invasion and 1 eye demonstrated postlaminar optic nerve invasion; see Supplementary Table S1). None of the eyes in this study had massive extrascleral or anterior segment invasion. All of the enucleated tumors with 6p gain had mild to severe nuclear anaplasia ranging from 3% to 60% of the

Table 2. Odds of enucleation given SCNA variations.

SCNA	OR (95% CI)	*P*
1q Gain	1.06 (0.18–7.11)	0.90
2p Gain	5.12 (0.82–32.11)	0.08
6p Gain (≥1.15)	9.61 (1.62–57.07)	0.01
13q Loss	1.12 (0.14–9.22)	0.92
16q Loss	0.62 (0.07–5.27)	0.66
Age (covariate)	1.00 (0.93–1.06)	0.93
Sex (covariate)	1.22 (0.28–5.27)	0.79
Group (covariate)	2.10 (0.60–7.33)	0.66

tumor area studied, while the tumors without 6p gain ranged from none to moderate anaplasia. Eighteen of the enucleated eyes had vitreous and/or subretinal tumor seeds present on histopathologic analysis, and 16 of these (88.9%) had 6p gain. The tumors with 6p gain had more necrosis (range 1%–70% of the tumor) than the tumors without 6p gain (none—25% of the tumor). Finally, only cases with 6p gain (8 eyes) had *focal* invasion of the optic nerve head/lamina cribrosa, choroid (<3 mm), or anterior segment. None of the eyes without 6p gain had *focal* invasion of any ocular structures. *MYCN* amplification was seen in three aqueous humor samples; two of these associated tumors were poorly differentiated with scant rosettes, and one had massive (7 mm) choroidal invasion. No specific nuclear features (round bland nuclei with nucleoli and no neuroendocrine-type chromatin) typically associated with *MYCN* amplification in wild-type (18) retinoblastoma were seen in these tumors.

Minimum region of 6p gain in the aqueous humor

We evaluated the boundaries of 6p gain in all aqueous humor samples to determine the minimum region of gain (MRG) for this alteration. 6p gain was seen in 50 aqueous humor samples from 25 eyes. Of these, 35 (70%) demonstrated gain of the entire 6p arm of the chromosome, consistent with an isochromosome 6p. In a single sample (case 11), the MRG was localized to a 19 Mbp region of the distal 6p arm that included *DEK* but not *E2F3*. This 19 Mbp gain was present in only one of the 50 aqueous humor samples; in all other samples, *DEK* and *E2F3* were both included within the regions of 6p gain regardless of the specific lengths of the gains (**Fig. 4**).

Discussion

Herein, we present an analysis of retinoblastoma tumor-derived DNA from 111 separate aqueous humor samples that were extracted from 50 eyes either at the time of diagnosis, postdiagnosis but prior to IVM therapy, and/or after primary or secondary enucleation. The goal of this study is to refine our understanding of molecular biomarkers in the aqueous humor and identify clinically impactful targets for prognostication of tumor response to therapy and ocular survival. Although we previously identified chromosomal gain of 6p as a potential indicator of aggressive retinoblastoma, the study included a smaller cohort of patients and limited follow-up period (14). Now with extended follow-up and nearly double the number of eyes and aqueous humor samples, the data continue to demonstrate that (i) aqueous humor sampling is safe (i.e., the aqueous humor extraction procedure caused no complications in any of our study participants), (ii) aqueous humor is a reliable source of tumor cfDNA, and (iii) the presence of retinoblastoma-specific SCNAs—specifically gain of 6p—has the potential to serve as a prognostic biomarker for retinoblastoma. Historically, clinicians have been very limited in their ability to provide targeted prognostic information to parents of children with retinoblastoma, given the lack of eye-specific molecular biomarkers due to the contraindication to direct tumor biopsy (2–8).

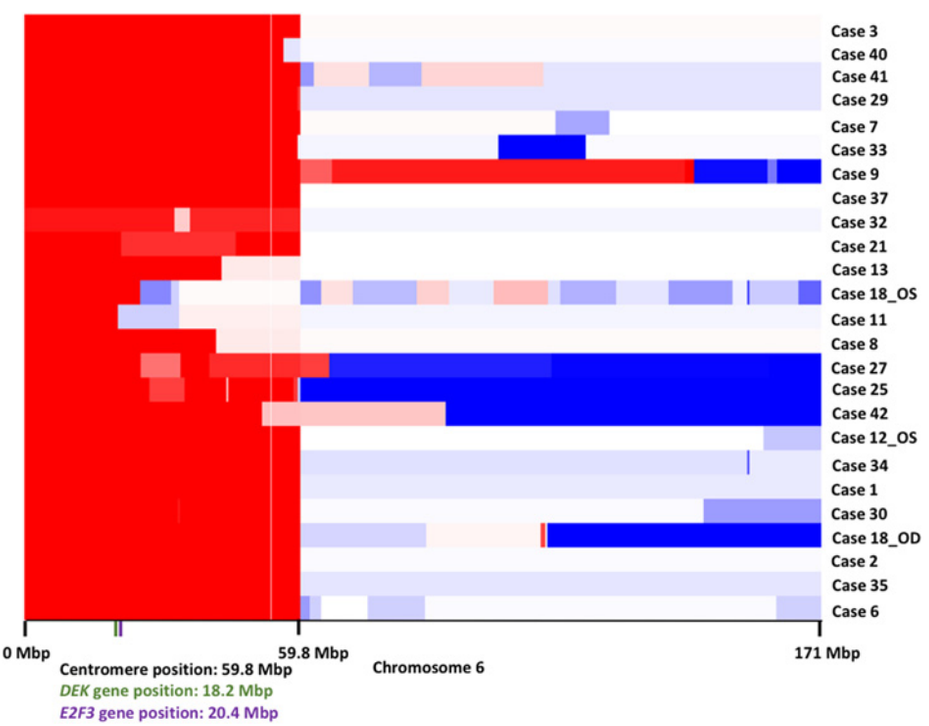


Figure 4.
Chromosome 6 heatmap showing the lengths of 6p gain (red) in 25 eyes with RB. Most eyes demonstrated a gain of the entire 6p arm. The MRG was localized to a 19 Mbp region that included a gain of *DEK* (Case 11).

With a larger cohort of patients and increased follow-up, we have demonstrated additional findings that are clinically impactful. First, we have demonstrated that the aqueous humor harbors detectable levels of tumor-derived cfDNA not only in advanced (Groups D and E) eyes but also in less advanced (Groups B and C) eyes. While the majority of sampled eyes in our study were relatively advanced with seeding (because most aqueous humor samples were extracted during IVM treatment for intravitreal seeding), the presence of cfDNA in less advanced eyes suggests that advanced intraocular disease is not required for meaningful aqueous humor cfDNA analyses. This is an important factor to consider so that these findings can be applied to all patients regardless of extent of intraocular disease.

In a prior analysis, we demonstrated a high concordance between SCNAs in tumor tissue and in the aqueous humor for 13 eyes (please see **Fig. 2** from Berry and colleagues 2018; ref. 14). Now we continue to show with nearly double the number of total aqueous humor samples that aqueous humor cfDNA can be used to reliably generate profiles of SCNAs that are consistent with the expected genomic landscape of retinoblastoma tumors. In addition, a recent study from an independent group demonstrated the feasibility of evaluating tumor DNA in the aqueous humor *in vivo* to identify tumor *RB1* mutations (15). In our study, the majority (33/50 eyes; 66%) of our sampled eyes contained at least one of the highly recurrent retinoblastoma SCNAs, and these alterations were particularly prevalent in enucleated eyes compared with salvaged eyes ($P = 0.0007$). Retinoblastoma SCNAs have been hypothesized to confer a growth advantage to cells by activating oncogenes involved in tumorigenesis and/or inactivating tumor suppressor genes that would normally protect tissues from dysregulated proliferation (31, 32). These genomic alterations are widely accepted as crucial events in the progression of retinoblastoma (13, 14, 16, 17, 31–34). However, it is important to note that the exact molecular roles and whether there is a stepwise progression of SCNAs in retinoblastoma development are not yet known. In addition, 17 of 50 (34%) eyes in our study displayed SCNAs but none of the highly recurrent ones associated with retinoblastoma, and 11 of 50 (22%) had a complete absence of any SCNAs throughout the genome. Similar to tissue-based studies, these findings suggest that although SCNAs seem to influence the progression or advancement of retinoblastoma, they are not required for tumor formation (31). It should be noted that in the 22% of eyes without any SCNAs, the lack of detectable alterations in aqueous humor could be (i) due to a true absence of SCNAs in the tumor or (ii) theoretically confounded by a lower tumor burden leading to lower tumor-derived cfDNA levels (i.e., tumor fraction) in the aqueous humor. Research is ongoing to develop a reliable method of tumor fraction estimation for the aqueous humor, in addition to the SCNA analyses that we currently employ.

Although we have demonstrated a significant correlation between 6p gain and increased risk of enucleation, the specific molecular pathways that influence this relationship are uncertain. The ultimate remaining question is what genes and/or corresponding molecular processes are involved to make 6p gain a viable biomarker for retinoblastoma in the first place. Previous studies have mapped the common region of 6p gain to band 6p22, a central region of the short arm of chromosome 6 that contains numerous known genes (16, 31, 32, 35). Of these, the oncogenes *DEK* and *E2F3* have been identified as possible drivers of retinoblastoma, as they demonstrate both RNA and protein overexpression in the setting of 6p gains and promote abnormal cell proliferation when overexpressed (31, 32, 34). Using cfDNA from aqueous humor samples, our study localized the minimum region of 6p gain to a 19 Mbp region of the distal 6p arm, within the much larger MRG identified in previous studies (31, 32, 35). We also found that *E2F3* and *DEK* are almost always included within the region of 6p gain, regardless of the width of the alteration. While *DEK* and *E2F3* remain promising retinoblastoma candidate genes, 6p gains are highly nonfocal in nature and can cover tens to hundreds of different genes in addition to *DEK* and *E2F3*—making it difficult to distinguish between true driver genes in this region versus passenger genes that are not affecting cancer development (16, 17, 31). We and others have shown that 6p gains often span the entire region of 6p—consistent with the formation of an isochromosome, in which misdivision at the centromere leads to abnormal gain of the entire 6p arm (32). This isochromosome is the most common underlying cause of 6p gain in retinoblastoma and is rarely seen in other ocular malignancies (32). From our research and the body of research on retinoblastoma SCNAs (31, 34, 35), it remains unclear whether a particular gene (or genes) on 6p is driving aggressive tumor activity, or whether 6p gain is simply a measurable molecular marker of other underlying processes within the tumor genome. In conjunction with aqueous humor sampling, expression analyses as well as functional assays with cultured retinoblastoma cell lines may be useful for further elucidation of potential 6p candidate genes and their possible roles in retinoblastoma progression (31, 36).

While the localization of genomic changes is crucial for identifying potential driver genes involved, elucidating the relative sequence of these changes would be a useful step in better understanding retinoblastoma progression. Prior to the development of aqueous humor sampling for retinoblastoma, Bowles and colleagues (33) proposed a molecular model for the progression of SCNA events using frequency studies of chromosomal gains and losses in enucleated eyes. On the basis of the assumption that more prevalent SCNAs likely occur earlier in tumor development, they inferred that 1q gain (which was the most common SCNA in their study and was closely associated with 6p gain) was one of the earliest genomic events in the progression of retinoblastoma, followed subsequently by gain of 6p. While we, too, demonstrated a significant association between 1q and 6p gains in sampled eyes, 6p gain (rather than 1q gain) was by far the most common SCNA both in our study and in others (14, 16, 17, 31, 35). Interestingly, loss of 16q was also associated with a 1q gain—consistent with studies of breast carcinoma that have identified simultaneous 1q gains and 16q losses as early tumor changes (37). Unlike the Bowles and colleagues model, our findings suggest that 6p gain may be a relatively earlier event than 1q gain given its predominance in sampled eyes. While the exact chronology of SCNAs in retinoblastoma is still poorly understood, our novel liquid biopsy provides the first approach in which the relative frequencies of chromosomal changes could be studied in both enucleated *and* salvaged eyes.

Because a specific prognostic marker for retinoblastoma in the aqueous humor could change how clinicians and parents make decisions regarding retinoblastoma management, our interest was especially focused on the somatic gain of chromosome 6p and its potential predictive value for tumor outcomes. As demonstrated previously (14), we found that 6p gain is not only the most common chromosomal alteration in the aqueous humor (present in 50% of all sampled eyes); but it is also the only individual SCNA that is significantly more prevalent in enucleated eyes compared with salvaged eyes ($P = 0.004$). With both univariate and multivariate analyses we demonstrated that the presence of 6p gain in the aqueous humor portends between a 7- and 10-fold increased odds of enucleation (univariate OR = 6.73; 95% CI = 1.94–23.36; $P = 0.003$, multivariate analyses controlling for age and the presence of other SCNAs OR = 9.87; 95% CI = 1.75–55.65; $P = 0.009$). These ORs are highly clinically

impactful; as a point of reference, the OR for developing breast cancer in the setting of a positive BRCA mutation is 5.91 (CI = 5.25–6.67; ref. 38). While our findings strongly suggest that 6p gain—when present—is a reliable predictive biomarker of ocular survival, we emphasize that the majority of aqueous humor samples with 6p gain were extracted either during active IVM treatment or immediately following enucleation. Therefore, many of the eyes were already at fairly advanced stages of disease at the time a 6p gain was discovered. While we have now started to collect aqueous humor at diagnosis (before any treatment is initiated) under a novel IRB-approved research protocol, a larger prospective study of aqueous humor evaluation at diagnosis is required to elucidate whether 6p gain is present and prognostic at the time of initial diagnosis and not just later in the disease process.

In addition to the correlation between retinoblastoma SCNAs and enucleated eyes, we also found that 6p gain was associated with other features of retinoblastoma that are clinically associated with aggressive disease. On histopathologic analysis, 6p gain was associated with aggressive and high-risk findings, including higher levels of necrosis and focal invasion of intraocular structures. 6p gain was also significantly more prevalent in eyes with persistent tumor seeding that did not respond to therapy (Fisher exact, $P = 0.001$). Intraocular seeding of tumor cells is a well-known clinical indicator of advanced disease and a common cause of treatment failure and loss of the eye, even with targeted intravitreal chemotherapy (23, 39). In separate studies, Berry and others found that seeds with a cloud-like morphology were particularly difficult to eradicate due to suboptimal response to IVM injections, compared with eyes with seeding spheres or dust (39–41). In our study, we found that an eye with cloud-type seeding was two times more likely to have gain of 6p than eyes with dust or sphere morphology of seeding, or no seeding (OR = 2.09; 95% CI = 1.02–4.31; $P = 0.04$). The association between 6p gain and more aggressive tumor activity is not unique to retinoblastoma. Gains at 6p have been associated with advanced and even metastatic disease in multiple nonretinal cancers, including colorectal, hepatocellular, bladder, and ovarian carcinomas; although the exact molecular influence of 6p gain on these malignancies is not entirely understood (32, 42–45). While a better understanding of the specific mechanism for this biomarker in retinoblastoma would be ideal, it does not diminish the value of 6p as a reliable indicator of aggressive disease.

Future studies are ongoing to explore the possibility of additional biomarkers, besides 6p gain, within the aqueous humor of retinoblastoma eyes. A possible indicator of aggressive retinoblastoma that deserves further investigation is the focal *MYCN* gain. Numerous studies have identified primary focal *MYCN* amplifications (i.e., in patients without any *RB1* mutations) as being independent drivers of high-risk disease (18, 46). However, focal *MYCN* gains can also occur secondarily (i.e., in patients with known *RB1* mutations; ref. 47). In our study, we identified a total of three 2p gains that were confined to the *MYCN* region [case 10 was reported previously (14), cases 28 and 31 were recruited since the previous publication]. Cases 10 and 28 were both positive for blood *RB1* mutations and all 3 cases demonstrated biallelic *RB1* mutations in tumor tissue, suggesting that their *MYCN* gains were secondary in nature rather than the primary *MYCN*-amplified subtype of retinoblastoma that was described previously (18, 46). In all three of these cases, focal *MYCN* gain was the only SCNA present in tumor-derived aqueous humor cfDNA, and all 3 eyes ultimately required enucleation due to either severity, persistence, and/or recurrence of disease. This is consistent with studies in pediatric neuroblastoma that found an association between *MYCN* overexpression and more aggressive disease (48, 49). Given that 2p alterations are relatively rare in retinoblastoma—and focal *MYCN* gains are rarer still—we encourage larger-scale studies of this chromosomal site within aqueous humor cfDNA in the future.

The novel use of aqueous humor as a liquid biopsy is a promising yet restricted approach to retinoblastoma evaluation. At this time, there is no accepted clinical protocol for using genomic information obtained from the aqueous humor to influence diagnostic or treatment decision-making. However, the ability to sample aqueous humor *in vivo* and detect biomarkers like 6p gain at diagnosis and throughout the course of a patients' disease—including at the end of conservative therapy or in the setting of a clinical recurrence—allows for the possibility of an improved therapeutic approach in the future. In this study, we established 6p as a viable prognostic biomarker of retinoblastoma tumors. A prospective, multicenter study with aqueous humor sampled at diagnosis and longitudinally throughout therapy is warranted to determine whether aqueous humor analysis can become a standard component of retinoblastoma prognostication and management.

Disclosure of Potential Conflicts of Interest

L. Xu reports a patent for Aqueous humor cell-free DNA and ophthalmic disease pending. P. Chevez-Barrios reports grants from NASA outside the submitted work. J. Hicks reports grants from Carol Vassiliadis Research Fund and grants from Vicky Joseph Research Fund during the conduct of the study; other from Epic Sciences, Inc outside the submitted work; and provisional patent application entitled Aqueous humor cell free DNA for diagnostic and Prognostic evaluation of Ophthalmic Disease. J.L. Berry reports grants from National Cancer Institute of the National Institute of Health Award Number K08CA232344, grants from Hyundai Hope on Wheels, grants from Childhood Eye Cancer Trust, grants from American Cancer Society #IRG-16-181-57, grants from Wright Foundation, grants from Knights Templar Eye Foundation, grants from The Larry and Celia Moh Foundation, grants from The Institute for Families, Inc., Children's Hospital Los Angeles, grants from Research to Prevent Blindness, grants from The National Institute of Health P30EY029220, and grants from The National Cancer Institute P30CA014089 during the conduct of the study; in addition, J.L. Berry has a provisional patent application entitled Aqueous Humor Cell Free DNA for Diagnostic and Prognostic Evaluation of Ophthalmic Disease pending. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

L. Xu: Conceptualization, data curation, formal analysis, investigation, visualization, methodology, writing-review, and editing. **A. Polski:** Conceptualization, data curation, formal analysis, investigation, visualization, methodology, writing-original draft, writing-review, and editing. **R.K. Prabakar:** Conceptualization, formal analysis, investigation, visualization, methodology, writing-review, and editing. **M.W. Reid:** Formal analysis, writing-review, and editing. **P. Chevez-Barrios:** Investigation, writing-review, and editing. **R. Jubran:** Writing-review, and editing. **J.W. Kim:** Data curation, writing-review, and editing. **P. Kuhn:** Conceptualization, visualization, writing-review, and editing. **D. Cobrinik:** Conceptualization, visualization, writing-review, and editing. **J. Hicks:** Conceptualization, visualization, writing-review, and editing. **J.L. Berry:** Conceptualization, data curation, formal analysis, supervision, funding acquisition, investigation, visualization, methodology, writing-original draft, writing-review, and editing.

Acknowledgments

The authors would like to thank Subramanian Krishnan, Dilshad Contractor, Brianne Brown, Mitali Singh, Kayla Stepanian, and Mark Reid for their technical support and expertise. This research was also supported by the following sources: NCI of the NIH Award K08CA232344 (to J.L. Berry); Hyundai Hope on Wheels RGA012351 (to J.L. Berry); Childhood Eye Cancer Trust (to J.L. Berry); American Cancer Society IRG-16-181-57 (to J.L. Berry); Wright Foundation (to J.L. Berry and A. Polski); Knights Templar Eye Foundation (to J.L. Berry); The Larry and Celia Moh Foundation (to J.L. Berry); The Institute for Families, Inc., Children's Hospital Los Angeles (J.L. Berry); an unrestricted departmental grant from Research to Prevent Blindness (all); The NIH P30EY029220 (all); The NCI P30CA014089 (all); Vicky

Joseph Research Fund (to P. Kuhn); Carol Vassiliadis Research Fund (to P. Kuhn); and USC Dornsife College of Letters, Arts and Sciences (to P. Kuhn).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 9, 2020; revised April 10, 2020; accepted May 18, 2020; published first May 20, 2020.

References

1. Fernandes AG, Pollock BD, Rabito FA. Retinoblastoma in the United States: a 40-year incidence and survival analysis. J Pediatr Ophthalmol Strabismus 2018;55:182–8.
2. Shields JA, Shields CL, Ehya H, Eagle RC, De Potter P. Fine-needle aspiration biopsy of suspected intraocular tumors. The 1992 Urwick Lecture. Ophthalmology 1993;100:1677–84.
3. Karcioglu ZA, Gordon RA, Karcioglu GL. Tumor seeding in ocular fine needle aspiration biopsy. Ophthalmology 1985;92:1763–7.
4. Karcioglu ZA. Fine needle aspiration biopsy (FNAB) for retinoblastoma. Retina 2002;22:707–10.
5. Eide N, Syrdalen P, Walaas L, Hagmar B. Fine needle aspiration biopsy in selecting treatment for inconclusive intraocular disease. Acta Ophthalmol Scand 1999;77:448–52.
6. Eide N, Walaas L. Fine-needle aspiration biopsy and other biopsies in suspected intraocular malignant disease: a review. Acta Ophthalmol 2009;87:588–601.
7. Eriksson O, Hagmar B, Ryd W. Effects of fine-needle aspiration and other biopsy procedures on tumor dissemination in mice. Cancer 1984;54:73–8.
8. Ali MJ, Honavar SG, Vemuganti GK, Singh AD. Fine needle aspiration biopsy of retinal tumors. Monogr Clin Cytol 2012;21:72–81.
9. Di Nicolantonio F, Neale M, Onadim Z, Hungerford JL, Kingston JL, Cree IA. The chemosensitivity profile of retinoblastoma. Recent Results Cancer Res 2003;161:73–80.
10. Francis JH, Abramson DH, Gobin YP, Marr BP, Tendler I, Brodie SE, et al. Efficacy and toxicity of second-course ophthalmic artery chemosurgery for retinoblastoma. Ophthalmology 2015;122:1016–22.
11. Francis JH, Brodie SE, Marr B, Zabor EC, Mondesire-Crump I, Abramson DH. Efficacy and toxicity of intravitreous chemotherapy for retinoblastoma: four-year experience. Ophthalmology 2017;124:488–95.
12. Francis JH, Roosipu N, Levin AM, Brodie SE, Dunkel IJ, Gobin YP, et al. Current treatment of bilateral retinoblastoma: the impact of intraarterial and intravitreous chemotherapy. Neoplasia 2018;20:757–63.
13. Berry JL, Xu L, Murphree AL, Krishnan S, Stachelek K, Zolfaghari E, et al. Potential of aqueous humor as a surrogate tumor biopsy for retinoblastoma. JAMA Ophthalmol 2017;135:1221–30.
14. Berry JL, Xu L, Kooi I, Murphree AL, Prabakar RK, Reid M, et al. Genomic cfDNA analysis of aqueous humor in retinoblastoma predicts eye salvage: the surrogate tumor biopsy for retinoblastoma. Mol Cancer Res 2018;16:1701–12.
15. Gerrish A, Stone E, Clokie S, Ainsworth JR, Jenkinson H, McCalla M, et al. Non-invasive diagnosis of retinoblastoma using cell-free DNA from aqueous humour. Br J Ophthalmol 2019;103:721–4.
16. Kooi IE, Mol BM, Massink MP, de Jong MC, de Graaf P, van der Valk P, et al. A meta-analysis of retinoblastoma copy numbers refines the list of possible driver genes involved in tumor progression. PLoS One 2016;11:e0153323.
17. Kooi IE, Mol BM, Massink MP, Ameziane N, Meijers-Heijboer H, Dommering CJ, et al. Somatic genomic alterations in retinoblastoma beyond RB1 are rare and limited to copy number changes. Sci Rep 2016;6:25264.
18. Rushlow DE, Mol BM, Kennett JY, Yee S, Pajovic S, Thériault BL, et al. Characterisation of retinoblastomas without RB1 mutations: genomic, gene expression, and clinical studies. Lancet Oncol 2013;14:327–34.
19. Thériault BL, Dimaras H, Gallie BL, Corson TW. The genomic landscape of retinoblastoma: a review. Clin Exp Ophthalmol 2014;42:33–52.
20. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM, et al. REporting recommendations for tumour MARKer prognostic studies (REMARK). Br J Cancer 2005;93:387–91.
21. Berry JL, Jubran R, Kim JW, Wong K, Bababeygy SR, Almarzouki H, et al. Long-term outcomes of Group D eyes in bilateral retinoblastoma patients treated with chemoreduction and low-dose IMRT salvage. Pediatr Blood Cancer 2013;60:688–93.
22. Berry JL, Shah S, Bechtold M, Zolfaghari E, Jubran R, Kim JW. Long-term outcomes of Group D retinoblastoma eyes during the intravitreal melphalan era. Pediatr Blood Cancer 2017;64:e26696.
23. Munier FL, Soliman S, Moulin AP, Gaillard MC, Balmer A, Beck-Popovic M. Profiling safety of intravitreal injections for retinoblastoma using an anti-reflux procedure and sterilisation of the needle track. Br J Ophthalmol 2012;96:1084–7.
24. Baslan T, Kendall J, Rodgers L, Cox H, Riggs M, Stepansky A, et al. Genome-wide copy number analysis of single cells. Nat Protoc 2012;7:1024–41.
25. Baslan T, Kendall J, Rodgers L, Cox H, Riggs M, Stepansky A, et al. Corrigendum: Genome-wide copy number analysis of single cells. Nat Protoc 2016;11:616.
26. Linn Murphree A. Intraocular retinoblastoma: the case for a new group classification. Ophthalmol Clin North Am 2005;18:41–53.
27. Mallipatna AC, Gallie BL, Chevez-Barrios P, Lumbroso-Le Rouic L, Chantada G, Doz F, et al. AJCC Cancer Staging Manual. 8th ed. In: Amin MB, Edge SB, Greene FL, editors. New York: Springer; 2017. p. 819–31.
28. Chantada G, Doz F, Antoneli CB, Grundy R, Clare Stannard FF, Dunkel IJ, et al. A proposal for an international retinoblastoma staging system. Pediatr Blood Cancer 2006;47:801–5.
29. Munier FL, Gaillard MC, Balmer A, Beck-Popovic M. Intravitreal chemotherapy for vitreous seeding in retinoblastoma: recent advances and perspectives. Saudi J Ophthalmol 2013;27:147–50.
30. Chévez-Barrios P, Eagle RC, Krailo M, Piao J, Albert DM, Gao Y, et al. Study of unilateral retinoblastoma with and without histopathologic high-risk features and the role of adjuvant chemotherapy: a children's oncology group study. J Clin Oncol 2019;37:2883–91.
31. Corson TW, Gallie BL. One hit, two hits, three hits, more? Genomic changes in the development of retinoblastoma. Genes Chromosomes Cancer 2007;46:617–34.
32. Santos GC, Zielenska M, Prasad M, Squire JA. Chromosome 6p amplification and cancer progression. J Clin Pathol 2007;60:1–7.
33. Bowles E, Corson TW, Bayani J, Squire JA, Wong N, Lai PB, et al. Profiling genomic copy number changes in retinoblastoma beyond loss of RB1. Genes Chromosomes Cancer 2007;46:118–29.
34. Grasemann C, Gratias S, Stephan H, Schüler A, Schramm A, Klein-Hitpass L, et al. Gains and overexpression identify DEK and E2F3 as targets of chromosome 6p gains in retinoblastoma. Oncogene 2005;24:6441–9.
35. Chen D, Gallie BL, Squire JA. Minimal regions of chromosomal imbalance in retinoblastoma detected by comparative genomic hybridization. Cancer Genet Cytogenet 2001;129:57–63.
36. Menyhárt O, Harami-Papp H, Sukumar S, Schäfer R, Magnani L, de Barrios O, et al. Guidelines for the selection of functional assays to evaluate the hallmarks of cancer. Biochim Biophys Acta 2016;1866:300–19.
37. Farabegoli F, Hermsen MA, Ceccarelli C, Santini D, Weiss MM, Meijer GA, et al. Simultaneous chromosome 1q gain and 16q loss is associated with steroid receptor presence and low proliferation in breast carcinoma. Mod Pathol 2004;17:449–55.
38. Kurian AW, Hughes E, Handorf EA, Gutin A, Allen B, Hartman A-R, et al. Breast and ovarian cancer penetrance estimates derived from germline multiple-gene sequencing results in women. JCO Precis Oncol 2017;1:1–12.
39. Berry JL, Bechtold M, Shah S, Zolfaghari E, Reid M, Jubran R, et al. Not all seeds are created equal: seed classification is predictive of outcomes in retinoblastoma. Ophthalmology 2017;124:1817–25.
40. Francis JH, Iyer S, Gobin YP, Brodie SE, Abramson DH. Retinoblastoma vitreous seed clouds (Class 3): a comparison of treatment with ophthalmic artery chemosurgery with or without intravitreous and periocular chemotherapy. Ophthalmology 2017;124:1548–55.
41. Munier FL. Classification and management of seeds in retinoblastoma. Ellsworth Lecture Ghent August 24th 2013. Ophthalmic Genet 2014;35:193–207.
42. Richter J, Wagner U, Schraml P, Maurer R, Alund G, Knönagel H, et al. Chromosomal imbalances are associated with a high risk of progression in early invasive (pT1) urinary bladder cancer. Cancer Res 1999;59:5687–91.
43. Al-Mulla F, Keith WN, Pickford IR, Going JJ, Birnie GD. Comparative genomic hybridization analysis of primary colorectal carcinomas and their synchronous metastases. Genes Chromosomes Cancer 1999;24:306–14.

44. Pang A, Ng IO, Fan ST, Kwong YL. Clinicopathologic significance of genetic alterations in hepatocellular carcinoma. Cancer Genet Cytogenet 2003;146: 8–15.
45. Bernardini M, Lee CH, Beheshti B, Prasad M, Albert M, Marrano P, et al. High-resolution mapping of genomic imbalance and identification of gene expression profiles associated with differential chemotherapy response in serous epithelial ovarian cancer. Neoplasia 2005;7:603–13.
46. Ewens KG, Bhatti TR, Moran KA, Richards-Yutz J, Shields CL, Eagle RC, et al. Phosphorylation of pRb: mechanism for RB pathway inactivation in MYCN-amplified retinoblastoma. Cancer Med 2017;6:619–30.
47. Stenfelt S, Blixt MKE, All-Ericsson C, Hallböök F, Boije H. Heterogeneity in retinoblastoma: a tale of molecules and models. Clin Transl Med 2017;6:42.
48. Lee JW, Son MH, Cho HW, Ma YE, Yoo KH, Sung KW, et al. Clinical significance of MYCN amplification in patients with high-risk neuroblastoma. Pediatr Blood Cancer 2018;65:e27257.
49. Dzieran J, Rodriguez Garcia A, Westermark UK, Henley AB, Eyre Sánchez E, Träger C, et al. MYCN-amplified neuroblastoma maintains an aggressive and undifferentiated phenotype by deregulation of estrogen and NGF signaling. Proc Natl Acad Sci U S A 2018;115:E1229–38.