Vitreous and Subretinal Seeds in Retinoblastoma: Clinico-pathologic Correlation

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**Purpose:** Vitreous seeding has been recognized as the strongest predictor of retinoblastoma treatment failure. Recently, intravitreal chemotherapy injection has led to improved outcomes for these patients. Munier et al recently proposed a new clinical classification scheme for retinoblastoma with vitreous seeds; Francis et al was then able to demonstrate a significant difference in regression between seed classes. Here we describe the first correlation of this clinical classification scheme with histopathological features.

**Methods:** We reviewed enucleated eyes with retinoblastoma from the Retinoblastoma Center of Houston that had clinical and macroscopic photographs and routinely processed, hematoxylin and eosin stained slides from 2010-2015 to identify those with vitreous and subretinal seeds. Immunohistochemistry with CD68 was used to evaluate for the presence of macrophages. Eyes were classified by clinical and macroscopic tumor seed type and cellular components were correlated within each category.

**Results:** 14 of 138 eyes reviewed had adequate vitreous or subretinal seeds and clinical/macroscopic photos. Clinically identified “dust” seeds (Type 1) represent individual viable tumor cells and macrophages. Clinically “sphere” seeds (Type 2) represent two histological types: 1. Macroscopically gray/translucent spheres are composed of non-necrotic, mitotically active retinoblastoma cells. 2. Macroscopically gray spheres with a white/yellow center are composed of an outer rim of viable cells with a center of necrotic material. Both sphere types contain a pole of dispersing single, viable cells. “Cloud” seeds (Type 3) are composed of necrotic debris with few scattered macrophages and rare viable cells.

**Conclusions:** Spheres with translucent centers may represent the most aggressive vitreous seed subtype as they contain multiple layers of viable tumor cells and shed single cells. In contrast, the overall lack of response to treatment seen in “cloud” seeds is likely due to an absence of replicating tumor cells. Knowledge of the composition of retinoblastoma seed types should help guide treatment and anticipate clinical response in trials that focus on the safety, efficacy, and outcomes of novel retinoblastoma therapy.

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Aqueous Humor as a Surrogate Liquid Tumor Biopsy in Retinoblastoma

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**Purpose:** The aim of this study is to determine whether tumor-derived nucleic acids and tumor DNA copy number alterations can be detected in the aqueous humor (AH) of retinoblastoma (RB) eyes.

**Methods:** AH was analyzed for DNA, RNA and miRNA using Qubit HS kits. Circulating cell-free DNA (cfDNA) isolation and sequencing library protocols were optimized to retain cfDNAs from the AH and these optimized methods were applied to AH samples from RB patients. Shallow whole genome sequencing was performed on Illumina platform followed by genome-wide copy number variation (CNV) profiling to assess the presence of tumor DNA fractions in AH cfDNA.

**Result:** Eighteen AH samples from 8 patients (6 patients pre-intravitreal injection and 2 post-enucleation) were examined. All had measurable DNA, RNA and miRNA, with miRNA having the highest concentrations. Whole genome sequencing of AH cfDNA from 2 primarily enucleated eyes and sequential AH cfDNAs obtained from 2 eyes undergoing intravitreal melphalan injections for treatment of tumor seeding revealed tumor-derived cfDNA based on CNV profiles that demonstrated tumor copy number alterations.

**Conclusions:** This is the first study to evaluate AH from RB eyes undergoing salvage therapy with intravitreal injection of melphalan. We were able to assay quantifiable levels of nucleic acids in treated and untreated eyes and found that AH cfDNA had retinoblastoma-related DNA copy number alterations in all four tested RB patients. This suggests that AH can serve as a ‘surrogate tumor biopsy’ when tumor tissue is not available.

**Commercial Relationships:** Jesse L. Berry, None; Liya Xu, None; Kevin Stachelek, None; A. Linn Murphee, None; Emily Zolfaghari, None; Kathleen McGovern, None; Thomas Lee, None; Anders Carlsson, None; Peter Kuhn, None; Jonathan Kim, None; David Cobrinik, None; James Hicks, None

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Identifying Invasion-Promoting Molecular Pathways In Retinoblastoma

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Laurna Asnaghi1, Alka Mahale2, Hind Alkatan3, Deepak P. Edward2, Wayne Yu4, Saleh Al Mesfer2, Azza Maktabi2, Leen Abu Safihe2, Charles Eberhart4,1, USC Roski Eye Institute, Los Angeles, CA; 1Ophthalmology, Children’s Hospital Los Angeles, Los Angeles, CA; 2University of Southern California, Department of Biological Sciences, Dornsife College of Letters, Arts, and Sciences, Los Angeles, CA; 3University of Southern California, Norris Comprehensive Cancer Center, Keck School of Medicine, Los Angeles, CA.

**Purpose:** Our previous studies demonstrated that STAT3 inhibition could be a treatment option for retinoblastoma and chemicals with a Michael acceptor from our in-house library exerted inhibitory action on STAT3 activation. This study aims to develop potent STAT3 inhibitors from a library of 27 different derivatives with a Michael acceptor for the treatment of retinoblastoma.

**Methods:** To figure out cellular toxicity of 27 different chemicals with a Michael acceptor, SNUOT-Rb1 and Y79 cells from 2 different retinoblastoma cell lines were treated with STAT3 inhibitors. Cell viability was measured using WST-1 assay and the level of STAT3 phosphorylation was estimated by ELISA measurement. Quantitative

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Novel STAT3 Inhibitors with Michael Acceptor from In-House Chemical Library as Therapeutics for Retinoblastoma

Eunoo Bak1,2, Dong Hyun Ju3, Jin Hyoung Kim1,3, Kyoejin Kim4, Seungbeom Lee5, Young-Ger Suh5, Jeong Hun Kim1,6,1, Fight against Angiogenesis-Related Blindness (FARB) Laboratory, Clinical Research Institute, Seoul National University Hospital, Seoul, Korea (the Republic of); 2Department of Ophthalmology, Seoul National University College of Medicine, Seoul, Korea (the Republic of); 3Tumor Microenvironment Research Center, Global Core Research Center, Seoul National University, Seoul, Korea (the Republic of); 4College of Pharmacy, Seoul National University, Seoul, Korea (the Republic of); 5College of Pharmacy, Chon University, Seoul, Korea (the Republic of); 6Department of Biomedical Sciences, Seoul National University College of Medicine, Seoul, Korea (the Republic of).

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real-time polymerase chain reaction was performed to measure the expression levels of target genes of STAT3 upon treatment with STAT3 inhibitors. From a library of 27 STAT3 inhibitors, the 2 most potent STAT3 inhibitors were selected. Then, in vivo therapeutic efficacy of them was investigated in the mouse orthotopic transplantation model. The toxicity of STAT3 inhibitors on normal cells and tissues was investigated at the levels of gene expression, cellular viability, and histologic integrity.

**Results:** Differential cellular toxicity, STAT3 phosphorylation, and expression of target genes were investigated in retinoblastoma cells upon treatment with 27 different chemicals with a Michael acceptor. The 2 most potent STAT3 inhibitors effectively inhibited the formation of tumors in the mouse orthotopic transplantation model. Interestingly, these STAT3 inhibitors did not significantly affect the cellular viability, the expression of genes regarding cell survival, and histologic integrity of the retina with a sufficient range of therapeutic window.

**Conclusions:** Novel STAT3 inhibitors identified from screening of an in-house library of chemicals with a Michael acceptor demonstrated potent in vitro and in vivo efficacy without definite toxicity on normal tissues. We expect that these STAT3 inhibitors can be utilized for the treatment of retinoblastoma.

**Commercial Relationships:** Eunooh Bak, None; Dong Hyun Jo, None; Jin Hyoung Kim, None; Kyeojin Kim, None; Seungbeom Lee, None; Young-Ger Suh, None; Jeong Hun Kim, None

**Methods:** Ocular vascular supply was determined angiographically in 79 eyes of 47 3.0kg-New Zealand white rabbits. The dominant ophthalmic artery (OA) of each eye was selectively catheterized. Melphalan 0.4mg/mL (up to 1.2mg/kg) was infused in pulsatile fashion. For pharmacokinetic studies, 18 rabbits were sacrificed at serial time-points. Retina, bilateral vitreous, and blood were collected. Toxicity was assessed by fluorescein angiography, electroretinography, and histopathology, prior to and 5-weeks post-treatment. Complete blood counts were obtained weekly.

**Results:** The OA was successfully catheterized for 79/79(100%) eyes in 47/47(100%) rabbits. Melphalan was delivered in 31/31(100%) eyes. External OA-dominant vascular variation was present in >75% of eyes, and dual internal/external supply in ~5%, with no correlation between a rabbit’s two eyes. In treated eyes, maximum melphalan concentration (Cmax) in retina was 4.95µM (30-minutes post-infusion) vitreous Cmax was 2.24µM (1-hour), and areas-under-the-curve (AUC0-∞) were 5.26µM*hr for retina and 4.19µM*hr for vitreous. Peripheral blood Cmax was 1.04µM. Drug half-life was ~1 hour. Treated eye vitreous Cmax was >100-fold higher, and AUC0-∞ was ~50-fold higher, than untreated eye. No angiographic or histopathologic evidence of vascular occlusion, emboli, or retinal damage were seen, even with 1.2mg/kg melphanal. Electroretinographic reductions were not seen 5 weeks following IAC melphanal treatment. With 0.8-1.2mg/kg melphalan, transient neutropenia occurred at 1-week, which was not seen with 0.4mg/kg doses.

**Conclusions:** This is the first small animal model of IAC. Ocular vascular supply in the rabbit is variable, and is independent for each eye. IAC melphanal delivery in rabbits leads to excellent ocular penetration and pharmacokinetics, with peak vitreous drug concentrations and areas-under-the-curve that are significantly better than in previous large animal models of IAC. IAC melphanal did not lead to significant ocular, vascular, or systemic toxicities in our rabbit model system.

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