Aqueous Humor Markers in Retinoblastoma, a Review

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Purpose: Retinoblastoma (Rb) is the most common primary intraocular cancer in children. Unlike with most solid tumors, direct biopsy is contraindicated due to risk of tumor dissemination. However, recent therapeutic techniques have allowed for the safe extraction of aqueous humor (AH) from eyes undergoing therapy, providing the unique opportunity to use AH as a liquid biopsy for Rb. Although the extraction of AH in Rb eyes undergoing therapy is new, the consideration of whether there are tumor biomarkers in the AH is not. The current manuscript is a systematic review of all studies that have examined biomarkers in the AH of Rb eyes. The authors hypothesized that AH sampling and analysis of tumor biomarkers may have new clinical relevance for the diagnosis, prognosis, and/or management of Rb.

Methods: A comprehensive database search (PubMed, Web of Science, Embase, and Cochrane Databases) was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement to identify articles on AH markers in Rb eyes. Inclusion criteria included English language articles with original reports on AH markers in the eyes of patients with confirmed Rb. Data on marker type, number of eyes, marker means and ranges, and when available, control values and clinicopathological correlations were collected. Articles were stratified based on marker type, and assessed quantitatively and qualitatively.

Results: An initial database search produced 325 articles, and an additional 11 articles were identified through searching citations. After removing duplicates and applying the eligibility criteria, we selected 27 articles to be included in the current review. A total of 463 eyes with histologically confirmed Rb were included in this review. The various markers and their values, with comparison to controls and clinicopathological correlations, are discussed.

Conclusions: AH sampling and tumor biomarker analysis in eyes without undergoing enucleation have the potential to revolutionize the management of Rb.

Translational Relevance: Although previous studies evaluated markers in the AH only after enucleation and not at diagnosis or during therapy, the clinical relevance of these markers was limited. However, recent changes in the management of Rb have allowed for safe sampling of the aqueous during therapy and, thus, correlation of tumor biomarkers with disease course. Thus, the authors felt it important to revisit previous research to evaluate whether these markers may now be applicable for the diagnosis, prognosis, or management of Rb.

Introduction

Retinoblastoma (Rb) is the most common primary intraocular cancer in children, comprising 4% of all pediatric malignancies.¹,² Intraocular disease often goes undiagnosed until the tumor is advanced and threatens the globe; although survival rates are in excess of 90% in developed countries, a critical focus of Rb therapy is globe (and vision) preservation.³,⁴ Historically, any attempt to biopsy or obtain fluid from Rb eyes had been contraindicated for risk of
tumor seeding and dissemination. However, aqueous humor (AH) paracentesis is now a standard part of the protocol for intravitreal chemotherapy (melphalan and/or topotecan) injections for Rb. As a safety measure, a volume of 0.1 mL of aqueous fluid is aspirated to induce transient hypotony prior to the intravitreal injection to prevent reflux to the injection site. Current clinical indications for intravitreal chemotherapy and, therefore, aqueous paracentesis include active seeding requiring further therapy and the eye must meet the following conditions to ensure safety: (1) presence of clear medium, (2) absence of invasion of the anterior and posterior chamber on ultrasound biomicroscopy, (3) absence of tumor at planned entry site, (4) absence of vitreous seeds at entry site, and (5) the absence of retinal detachment at the entry site. These features are aimed at protecting the safety of the pars plana injection site, which includes the aspiration of AH. Intravitreal injection of chemotherapy for seeding has now been widely adopted, and the risk of extraocular spread is considered extremely low (0 reported cases with the safety enhanced procedure). With this demonstrated safety record, we can now access the AH in eyes with Rb undergoing active therapy and evaluate for the presence of biomarkers that may correlate with features of the intraocular tumor and provide diagnostic and prognostic value. It should be noted that aside from active research protocols, there are no clinical tests commercially available at this time that are indicated for diagnostic or prognostic evaluation in Rb.

AH is the clear intraocular fluid produced by the ciliary processes that fills the anterior and posterior chambers of the eye. AH functions to maintain intraocular pressure; provide nutrients for the lens, cornea, and trabecular meshwork; remove waste products of metabolism; and protect against oxidative damage. AH has been shown to be a rich source of products of metabolism; and protect against oxidative damage. It is a source of biomarkers that may correlate with disease activity; the authors suggested this may aid in diagnosis. Following that initial report, further studies explored the various clinicopathological correlations associated with LDH as an AH marker of ocular disease. Over the past few decades, numerous studies have explored AH in enucleated Rb eyes and have uncovered various markers that may demonstrate clinical value for diagnosis, clinicopathological associations, monitoring response to treatment, and possibly serve as targets for therapy. However, because all previous studies were done on enucleated eyes, clinical correlations were difficult if impossible to make and the role of the AH in management of Rb remained limited. A recent study by the authors demonstrated that Rb tumor DNA is present in the AH in sufficient concentrations for subsequent sequencing and analysis. This study suggested the AH has the potential to serve as a surrogate to direct tumor biopsy, thus providing tumor genetic information in eyes undergoing salvage therapy (e.g., without enucleation of the eye). A broader evaluation demonstrated that genomic evaluation of the cell-free tumor DNA (cfDNA) in the AH may be predictive of aggressive tumor activity and the need for enucleation. AH is now routinely extracted during intravitreal chemotherapy injections for eyes undergoing salvage therapy; thus, the authors hypothesized that AH sampling and analysis, without enucleation, may now be clinically applicable for the diagnosis, prognosis, and/or management of Rb. The current systematic review summarized all studies that have explored AH markers that may be of value in the diagnosis, prognosis, or characterization of Rb.

Methods

Search Strategy

This study was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Supplementary File S1). A comprehensive electronic search of databases, including PubMed, Web of Science, Embase, and Cochrane Databases, was conducted on August 1, 2018 with keywords “aqueous humor” or “aqueous humour” and “retinoblastoma”. The search strategy was developed for PubMed and then applied to other databases. The search results were imported into a reference manager (Paperpile), and duplicates were excluded. Abstracts of all related studies were evaluated, and full texts were obtained through PubMed, Google Scholar, and other related databases. The reference list of the included articles was also examined to identify additional studies that were not previously found with the electronic search. Full texts of articles were then evaluated by two authors (B.K.G. and J.L.B.) to be included in the review. Disagreements among the reviewers were resolved by discussion and subsequent consensus.

2 TVST | 2019 | Vol. 8 | No. 2 | Article 13
Selection Criteria

Eligible studies included all original research articles that were published in English up to 2018, which examined markers within the AH of eyes with histologically confirmed Rb.

Excluded were (1) nonresearch articles such as brief reports and case reports; (2) studies examining markers in the serum (or other non-AH fluid compartments; i.e., tears) only; (3) studies examining markers in non-Rb eyes only; and (4) studies that were not written in English, or where an English translation was not available. The search was not limited to specific years. Studies that quantitatively measured markers and compared their levels against control groups were sought out, although studies that did not perform control group comparisons were also discussed in this review.

Quantitative and Qualitative Data Extraction

The characteristics extracted from the studies included type of marker, number of eyes, and, when possible, means and ranges of markers examined from each study. If the study compared Rb marker levels to control groups, the control means and ranges were also extracted, along with the $P$ values (if available). Quantitative data on the number of eyes examined from each study were combined to determine the total number of eyes examined for each marker type, and when available, ranges of markers for Rb and control groups were processed and combined to create unified ranges for each marker. The narrative synthesis of qualitative findings for each marker was performed to discuss clinicopathological correlations of each marker. Studies were stratified based on the marker type evaluated.

Quality Assessment

The review was conducted in accordance with the PRISMA approach\textsuperscript{30} to ensure the transparent and complete report of our sensitive search, systematic screening, and independent quality assessment. Quality assessment was performed for all studies by independently using the protocols of the National Institute of Health Quality Assessment Tool checklist (Supplementary File S2). The checklist consists of 12 different items that evaluate various aspects of the case-control methodology, including sampling methods, control status, and adjustment of confounders. Studies were grouped in three categories (good, fair, and poor). This information was used to assess the quality and strength of markers in clinical care.

Results

An initial database search produced 325 articles, and an additional 11 articles were identified through searching citations. After removing duplicates and applying the eligibility criteria, we selected 27 articles to be included in the current review. The PRISMA flowchart representing information on the selection process of articles is illustrated in the Figure.

Data from the 27 included articles were extracted into a table with core characteristics of these studies (author[s] and year, type of marker, number of eyes, mean value of marker in AH for Rb eyes [if available], range of marker values in AH for Rb eyes [if available], number of control eyes [if available], mean value of markers in AH for control eyes [if available], range of marker values in AH for control eyes [if available], $P$ value [if available], and quality assessment rating) (Table 1).

Table 2 includes quantitative and qualitative data from the included articles, stratified based on the different markers. Quantitative data analysis from the included articles has been performed to arrive at a total number of Rb and control eyes. The values of the biomarkers from different studies have been processed and combined to produce unified ranges for both Rb and control eyes. Also, a narrative synthesis of qualitative findings for each marker has been performed to include clinicopathological correlations (Table 2).

A total of 463 eyes with histologically confirmed Rb were included in this review, and 302 eyes in which the diagnosis of Rb was excluded were used as controls. Twelve studies examined levels of LDH in AH.\textsuperscript{21–24,31–38} Five studies examined neuron-specific enolase.\textsuperscript{26,39–42} Two studies examined levels of survivin,\textsuperscript{43,24} and one of which additionally examined transforming growth factor beta (TGF-\textbeta) and LDH.\textsuperscript{24} One study examined levels of uric acid.\textsuperscript{44} Three studies analyzed proteins in AH, including protein concentrations\textsuperscript{25,31} and protein analysis.\textsuperscript{27} One study examined the cytokines, chemokines, matrix metalloproteinases, and acute-phase protein concentrations.\textsuperscript{45} Two studies examined levels of aromatic amino acids and catecholamine metabolites.\textsuperscript{46,47} One study performed nucleic acid analysis\textsuperscript{28} and another performed genomic evaluation\textsuperscript{29} (Table 1).

Discussion

Lactate Dehydrogenase

LDH is an enzyme found in nearly all cells as a regulator of metabolism. Clinically, LDH has been
used as a nonspecific marker found within body fluids in various pathological conditions, including malignant tumors.\textsuperscript{48} Our search found 12 studies that have examined LDH activity in the AH of children with Rb. A total of 245 eyes with histologically confirmed Rb were included, compared to 206 patients without Rb as controls.

Dias et al.\textsuperscript{21} were among the first to examine LDH levels in the AH, obtaining a 0.1-mL sample of AH from enucleated eyes from children with Rb. Early reports demonstrated a significant increase in the levels of LDH within the AH of enucleated eyes with Rb when compared to patients without Rb, such that levels of >1000 U/L strongly support the diagnosis of Rb.\textsuperscript{21–24,33–38,49} Additionally, Rb AH demonstrates a characteristic pattern of the LDH isoenzyme pattern in which isoenzymes LDH4 and LDH5 are characteristically elevated, which may be useful information in the diagnosis of Rb.\textsuperscript{34,37} Clinicopathological parameters that significantly correlate to AH LDH levels include tumor cell necrosis,\textsuperscript{49} presence of tumor in the anterior chamber,\textsuperscript{33} and increasing duration of untreated tumor.\textsuperscript{22,49} It was demonstrated that children with later classifications of the disease (Reese Ellsworth classification IV and V) had significantly higher levels than children with early classification (I to III).\textsuperscript{36} These studies suggest that further elevations of AH LDH may correspond to a greater severity of disease.

Shehata et al.\textsuperscript{24} examined serum LDH levels before and after treatment with chemoreduction with focal consolidation therapies (cryotherapy, laser thermo-
therapy, and brachytherapy), external beam radiotherapy, and/or enucleation. Shehata et al. demonstrated a significant reduction of serum LDH concentrations after treatment, suggesting a possible role in monitoring serum LDH as an index of therapeutic response and tumor regression in children with Rb. However, serum and AH LDH levels do not appear to correlate with each other, and AH LDH levels have not been shown to correlate with prior therapy, nor tumor or patient demographics. Moreover, one study from Dias in 1979 showed persistent elevation in AH LDH after irradiation of the eye, despite no visible Rb tissue ophthalmoscopically. This argues against the use of AH LDH as a marker for tumor regression. Additionally, tumor regression is best monitored via indirect ophthalmoscopy by an ocular oncologist; however, an accurate test for early recurrence or recurrence under a calcified otherwise regressed scar would have clinical utility. This would also be clinically applicable if levels correlated with high-risk histopathologic disease (e.g., suggesting some metastatic risk to attempted salvage of the globe), but this has not yet been shown.

In summary, multiple studies on LDH levels in the AH from enucleated eyes were done between the years 1971–2008, which found that LDH levels were significantly elevated compared to controls and more elevated in advanced eyes with delayed diagnosis; however, these levels did not correlate with other clinical features or outcomes and did not correlate...
Elevations in AH LDH levels have also been described in patients with other ocular conditions, including primary open angle glaucoma and Coats’ disease. Additionally, 6 out of the 12 studies that examined AH LDH have a fair quality assessment rating (the remaining studies are graded good) largely due to the absence of statistical evidence and calculated \( P \) value. Although LDH was the first described marker of tumor activity in the AH, the lack of specificity and correlation with patient or tumor features limits its use clinically. Due to this lack of correlation as well as inability to extract AH from eyes undergoing treatment, this research was mostly abandoned.

### Table 1. Extended

<table>
<thead>
<tr>
<th>Author(s), Date</th>
<th>Ctl Eyes</th>
<th>Ctl AH Mean</th>
<th>Ctl AH Range</th>
<th>( P ) Value</th>
<th>Quality Rating</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dias et al. 1971(^{21})</td>
<td>33</td>
<td>-</td>
<td>0–350</td>
<td>-</td>
<td>Fair</td>
</tr>
<tr>
<td>Kabak and Romano 1975(^{32})</td>
<td>7</td>
<td>24</td>
<td>0–99</td>
<td>-</td>
<td>Fair</td>
</tr>
<tr>
<td>Piro et al. 1978(^{33})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Fair</td>
</tr>
<tr>
<td>Abramson et al. 1979(^{34})</td>
<td>34</td>
<td>47</td>
<td>10–167</td>
<td>&lt;0.02</td>
<td>Good</td>
</tr>
<tr>
<td>Dias 1979(^{22})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Fair</td>
</tr>
<tr>
<td>Dias 1979(^{23})</td>
<td>6</td>
<td>-</td>
<td>180–420</td>
<td>-</td>
<td>Good</td>
</tr>
<tr>
<td>Das et al. 1983(^{35})</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>&lt;0.01</td>
<td>Fair</td>
</tr>
<tr>
<td>Dias 1985(^{49})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Fair</td>
</tr>
<tr>
<td>Dayal et al. 1985(^{36})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Good</td>
</tr>
<tr>
<td>Singh et al. 1991(^{37})</td>
<td>15</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Good</td>
</tr>
<tr>
<td>Mukhopadhyay et al. 2008(^{38})</td>
<td>21</td>
<td>90.6</td>
<td>48–146</td>
<td>&lt;0.001</td>
<td>Good</td>
</tr>
<tr>
<td>Shehata et al. 2016(^{24})</td>
<td>80</td>
<td>62.18</td>
<td>19.38–181.25</td>
<td>&lt;0.01</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.2</td>
<td>6.5–38</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.07</td>
<td>11.22–52.14</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Abramson et al. 1989(^{41})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Fair</td>
</tr>
<tr>
<td>Comoy et al. 1990(^{26})</td>
<td>19</td>
<td>5.0</td>
<td>-</td>
<td>-</td>
<td>Fair</td>
</tr>
<tr>
<td>Nakajima et al. 1986(^{42})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Good</td>
</tr>
<tr>
<td>Shine et al. 1990(^{40})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Good</td>
</tr>
<tr>
<td>Wu et al. 1997(^{39})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Good</td>
</tr>
<tr>
<td>Shehata et al. 2010(^{43})</td>
<td>17</td>
<td>20.57</td>
<td>6.5–38</td>
<td>&lt;0.05</td>
<td>Good</td>
</tr>
<tr>
<td>Mendelsohn et al. 1998(^{44})</td>
<td>17</td>
<td>7.67</td>
<td>6.91–8.43</td>
<td>&lt;0.05</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.43</td>
<td>0.37–0.49</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>Dias 1979(^{31})</td>
<td>10</td>
<td>-</td>
<td>0–0.17</td>
<td>-</td>
<td>Good</td>
</tr>
<tr>
<td>Hadjistilianou et al. 2012(^{25})</td>
<td>10</td>
<td>0.19</td>
<td>0.16–0.24</td>
<td>&lt;0.01</td>
<td>Good</td>
</tr>
<tr>
<td>Cheng et al. 2017(^{27})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Good</td>
</tr>
<tr>
<td>Cheng et al. 2017(^{45})</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>&lt;0.05</td>
<td>Fair</td>
</tr>
<tr>
<td>Abramson et al. 1978(^{47})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Fair</td>
</tr>
<tr>
<td>Abramson et al. 1993(^{46})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Fair</td>
</tr>
<tr>
<td>Berry et al. 2017(^{28})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Good</td>
</tr>
<tr>
<td>Berry et al. 2018(^{29})</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Good</td>
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</tbody>
</table>

with serum LDH levels. Elevations in AH LDH levels have also been described in patients with other ocular conditions, including primary open angle glaucoma and Coats’ disease. Additionally, 6 out of the 12 studies that examined AH LDH have a fair quality assessment rating (the remaining studies are graded good) largely due to the absence of statistical evidence and calculated \( P \) value. Although LDH was the first described marker of tumor activity in the AH, the lack of specificity and correlation with patient or tumor features limits its use clinically. Due to this lack of correlation as well as inability to extract AH from eyes undergoing treatment, this research was mostly abandoned.

### Enolase/Neuron-Specific Enolase

Neuron-specific enolase (NSE) is an isoenzyme of the glycolytic enzyme enolase that occurs as gamma and alpha gamma dimers and is highly specific for neurons and peripheral neuroendocrine cells. The findings of increased body fluid levels of NSE have been found to occur with malignant proliferation and, thus, have been of value in the diagnosis and characterization of neuroendocrine tumors, including small cell lung cancer and, recently, Rb. Five studies in our review examined NSE in the AH of Rb patients that include 67 enucleated Rb eyes compared to 65 normal eye controls. Three high-quality studies used an enzyme immunoassay...
Table 2. Quantitative Aqueous Humor Marker Data with Clinocpathological Correlations

<table>
<thead>
<tr>
<th>Marker</th>
<th>Studies</th>
<th>Rb, n</th>
<th>Ctl, n</th>
<th>Rb Range</th>
<th>Control Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH</td>
<td>Dias et al. 1971&lt;sup&gt;31&lt;/sup&gt;</td>
<td>245</td>
<td>206</td>
<td>10–15,920 U/L</td>
<td>0–420 U/L</td>
</tr>
<tr>
<td></td>
<td>Kabak and Romano 1975&lt;sup&gt;32&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Piro et al. 1978&lt;sup&gt;33&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Abramson et al. 1979&lt;sup&gt;34&lt;/sup&gt;</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Dias 1979&lt;sup&gt;22&lt;/sup&gt;</td>
<td></td>
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<tr>
<td></td>
<td>Dias 1985&lt;sup&gt;35&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Das et al. 1983&lt;sup&gt;36&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Dayal et al. 1985&lt;sup&gt;37&lt;/sup&gt;</td>
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<td></td>
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<tr>
<td></td>
<td>Singh et al. 1991&lt;sup&gt;38&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Mukhopadhyay et al. 2008&lt;sup&gt;39&lt;/sup&gt;</td>
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</tr>
<tr>
<td></td>
<td>Shehata et al. 2016&lt;sup&gt;40&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSE</td>
<td>Abramson et al. 1989&lt;sup&gt;41&lt;/sup&gt;</td>
<td>67</td>
<td>19</td>
<td>83–60,000 ng/mL</td>
<td>5 ± 7.7</td>
</tr>
<tr>
<td></td>
<td>Comoy et al. 1990&lt;sup&gt;42&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nakajima et al. 1986&lt;sup&gt;43&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Shine et al. 1990&lt;sup&gt;44&lt;/sup&gt;</td>
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<tr>
<td></td>
<td>Wu et al. 1997&lt;sup&gt;45&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Survivin</td>
<td>Shehata et al. 2010&lt;sup&gt;46&lt;/sup&gt;</td>
<td>109</td>
<td>97</td>
<td>17.63–52.70 pg/mg</td>
<td>6.5–38 pg/mg</td>
</tr>
<tr>
<td></td>
<td>Shehata et al. 2016&lt;sup&gt;47&lt;/sup&gt;</td>
<td></td>
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</tr>
<tr>
<td>TGF-β</td>
<td>Shehata et al. 2016&lt;sup&gt;48&lt;/sup&gt;</td>
<td>88</td>
<td>80</td>
<td>40.54–166.86 pg/mg</td>
<td>11.22–52.14 pg/mg</td>
</tr>
<tr>
<td>Uric acid</td>
<td>Mendelsohn et al. 1998&lt;sup&gt;49&lt;/sup&gt;</td>
<td>38</td>
<td>17</td>
<td>4.72–31 μg/mL</td>
<td>6.91–8.43 μg/mL</td>
</tr>
<tr>
<td>Xanthine</td>
<td>Mendelsohn et al. 1998&lt;sup&gt;49&lt;/sup&gt;</td>
<td>38</td>
<td>17</td>
<td>0.27–8.03 μg/mL</td>
<td>0.37–0.49 μg/mL</td>
</tr>
<tr>
<td>Protein content</td>
<td>Dias 1979&lt;sup&gt;50&lt;/sup&gt;</td>
<td>55</td>
<td>40</td>
<td>0.89–6.9 mg/mL</td>
<td>0–0.24 mg/mL</td>
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<td>Hadjistilianou et al. 2012&lt;sup&gt;51&lt;/sup&gt;</td>
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<td>Cheng et al. 2017&lt;sup&gt;52&lt;/sup&gt;</td>
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<td>Cheng et al. 2017&lt;sup&gt;53&lt;/sup&gt;</td>
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<td>Nucleic acid</td>
<td>Berry et al. 2017&lt;sup&gt;54&lt;/sup&gt;</td>
<td>32</td>
<td>3</td>
<td>0.084–56 ng/μL</td>
<td>0.05–0.16 ng/μL</td>
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<td>(cfDNA)</td>
<td>Berry et al. 2018&lt;sup&gt;55&lt;/sup&gt;</td>
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<sup>a</sup> RE, Reese Ellsworth classification; IFN-γ, interferon gamma; PIGF-1, placenta growth factor 1; VEGF-A, vascular endothelial growth factor A; β-NGF, nerve growth factor beta; HGF, hepatocyte growth factor; EGF, epidermal growth factor; FGF-2, fibroblast growth factor 2; OR, odds ratio; CI, confidence interval.
and electrophoresis to examine the isoenzyme patterns of enolase in the AH of enucleated eyes of children with Rb and demonstrated that the gamma subunits, indicating NSE, were elevated in AH Rb, whereas enolase was not detectable in the AH from controls. Two fair quality studies quantified NSE levels in AH of Rb eyes. Abramson et al. found elevations in NSE significantly correlated with inflammation and tumor invasion into the anterior chamber. NSE levels did not correlate, however, with

<table>
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<th>Marker</th>
<th>Take-Home Points and Clinicopathological Correlations</th>
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| **LDH** | - Nonspecific indicator of Rb tumor presence  
- Possible correlations with tumor invasion in anterior chamber, increased duration of tumor, and late stage (RE IV and V) |
| **NSE** | - Significant histopathological correlation with tumor invasion in anterior chamber ($t = 2.5, P = 0.05$) and inflammation ($t = 2.7; P = 0.05$)  
- Possible index of remission |
| **Survivin** | - Significantly elevated levels in Rb AH, compared to control AH ($P < 0.01$)  
- Higher levels in late stages (RE III, IV, and V; ($n = 15$) $31.302 \pm 10.97$) than (early stages I and II; ($n = 5$) $20.495 \pm 1.86$) ($P = 0.002$)  
- Significant correlation with optic nerve invasion ($P = 0.003$)  
- Significant reduction of serum concentrations after treatment ($P < 0.01$).  
- Proposed cutoff value of AH survivin $= 25.2 \text{ pg/mg}$ (62% sensitivity, 100% specificity) |
| **TGF-β** | - Significantly elevated levels in Rb AH, compared to controls AH ($P < 0.01$).  
- Statistically significant higher concentration of AH TGF-β1 in undifferentiated tumors ($P < 0.0006$)  
- Proposed cutoff value of AH TGF-β1 $= 39.8 \text{ pg/mg}$ (100% sensitivity, 90% specificity) |
| **Uric acid** | - Significantly elevated AH concentrations compared to controls ($P < 0.05$) |
| **Xanthine** | - Significantly elevated AH concentrations compared to controls ($P < 0.05$) |
| **Protein content** | - Significantly decreased protein concentration in Rb eyes following treatment with intra-arterial melphalan, compared to nontreated Rb eyes ($P < 0.01$) |
| **IL-6, IL-7, IL-8, IFN-γ, PIGF-1, VEGF-A, β-NGF, HGF, EGF, and FGF-2** | - Significantly higher in the Rb AH, compared to controls ($P < 0.05$) |
| **Nucleic acid (cfDNA)** | - PEDF (downregulated) and STRA6 (upregulated) as possible candidates in targeted therapy for Rb.  
- Tumor-specific DNA is present in the AH of Rb eyes and can be safely extracted and evaluated without undergoing enucleation  
- The AH cfDNA concentration in 8 Rb eyes ranged from 0.084 to 56 ng/μL (eyes receiving melphalan treatment, mean: 0.2 ng/μL; Eyes with large untreated tumors that underwent primary enucleation, mean: 43.6 ng/μL)  
- Median concentration of DNA in control AH was 0.15 ng/μL (mean, 0.12; range, 0.05–0.16 ng/μL).  
- Genomic evaluation of Rb AH samples demonstrated the presence of any RB SCNA in enucleated eyes was 12/13 (92%), whereas the fraction in salvaged eyes was 6/16 (38%) ($P = 0.006$)  
- Presence of 6p gain in the AH was associated with a ten-fold increased odds of an eye requiring enucleation ($P = 0.0092$) (OR, 10; 95%CI, 1.8–55.6)
survivin is a bifunctional inhibitor of an apoptosis protein that has been implicated in the regulation of mitosis and protection from apoptosis.\textsuperscript{54} It has garnered significant interest as a diagnostic and prognostic factor in human neoplasms, including Rb. Elevated survivin levels are found in most human neoplasms,\textsuperscript{55} and it is used as a prognostic factor in several human neoplasms, including lung and colorectal cancers.\textsuperscript{56}

Survivin expression in enucleated eyes of children with Rb was examined by Shehata et al.\textsuperscript{43} in 2010 and found to be significantly elevated in both AH and serum, when compared to patients with non-malignant ophthalmic disease, such as congenital cataracts and glaucoma ($P < 0.05$).\textsuperscript{24} This high-quality study found that within AH, survivin levels significantly correlated with tumor stage and histopathologic postlaminar optic nerve involvement. A follow-up study by Shehata et al.\textsuperscript{43} similarly examined survivin and TGF-\beta1 expression in the AH of enucleated Rb eyes and found that both markers were significantly higher than the corresponding control group. The study also examined the expression of both markers in the serum of children with Rb before and after 6 months of treatment with focal consolidation therapies (cryotherapy, laser thermotherapy, and brachytherapy), external beam radiotherapy, and/or enucleation and found a significant reduction of serum concentrations after treatment ($P < 0.01$; aqueous was not assayed).\textsuperscript{24} The study also reported a significant correlation between AH TGF-\beta1 levels and poor differentiation of the tumor. Proposed optimal cutoff values of AH survivin of 25.2 pg/mg corresponded with 62% sensitivity and 100% specificity, and cutoff values of AH TGF-\beta1 of 39.8 pg/mg corresponded with 100% sensitivity and 90% specificity.\textsuperscript{24,43} The high sensitivity, specificity, and accuracy of serum and AH survivin and TGF-\beta1 proteins make them promising markers for the early detection and follow up of Rb patients. The authors of that study proposed that the roles of these markers in their pathogenesis of Rb should be further investigated, as they may be described as potential future targets for Rb therapy.

Uric Acid and Xanthine

During cell turnover, nucleic acids and nucleotides are degraded into xanthine and uric acid. Elevated levels of serum uric acid have been associated with many malignancies, as well as after the rapid destruction of malignant tissues after treatment with chemotherapy or radiation. Mendelsohn et al.\textsuperscript{44} examined the levels of uric acid and xanthine in the AH and tears of 38 eyes of children with Rb compared to 17 eyes of patients with senile and congenital cataracts as controls. The study demonstrated significantly elevated concentrations of uric acid and xanthine in the AH of Rb patients compared with control eyes including senile and congenital cataracts ($P < 0.05$). No significant difference was found in the concentrations of uric acid in the tears of patients with Rb and those of normal patients. The mean concentrations of uric acid and xanthine in Rb AH were found to be 18.3 and 2.43 \textmu g/mL, respectively, compared to control values of 7.67 and 0.43 \textmu g/mL, respectively.\textsuperscript{44} Elevated levels of xanthine and uric acid in AH may support the diagnosis of Rb in suspected children; however, further studies are necessary to establish optimal cutoffs, explore clinicopathological correlations, and compare Rb levels to lesions simulating Rb (Coats’ disease and persistent fetal vasculature).

Protein Content

In nonpathologic states, AH is virtually protein free. Protein concentration in the AH is known to be low due to the need for a clear optical region between the cornea and the lens for vision.\textsuperscript{57,58} However, early studies demonstrated that Rb has been associated with an increase in globulin content and an albumin/globulin of $<1$.\textsuperscript{51} To investigate the AH protein content in Rb patients, Hadjistilianou et al.\textsuperscript{25} performed gel electrophoresis on the AH from
enucleated eyes with Rb and found that the AH protein concentration was significantly higher in Rb patients compared to controls with cataracts (P < 0.01); in addition, significantly decreased protein concentration was demonstrated in eyes following treatment with selective intra-arterial chemotherapy (melphalan injection in the ophthalmic artery) that were subsequently enucleated after attempts at salvage compared to nontreated eyes (P < 0.01).25 Another study examined 45 cytokines/chemokines, matrix metalloproteinases, and acute-phase proteins in the AH of 35 enucleated eyes with Rb and 20 eyes with cataracts as controls.45 The study found that the concentrations of interleukin 6 (IL-6), IL-7, IL-8, interferon γ, placenta growth factor 1, vascular endothelial growth factor A, nerve growth factor β, hepatocyte growth factor, epidermal growth factor, and fibroblast growth factor 2 were significantly higher in the AH of patients with Rb than those in the control group (P < 0.05).45 Two additional studies, with fair quality-assessment ratings due to the lack of control group comparisons, assayed AH from Rb patients postenucleation with electrochemical liquid chromatography and identified more than 20 metabolites of tyrosine, tryptophan metabolic pathways, catecholamine degradation pathways, and ascorbate.46,47 Thus, although the technique is not currently used clinically, the determination of protein concentration in AH may be of value to monitor response to treatment.

One study further assessed AH proteins by using the comparative proteomic technique of isobaric tags for relative and absolute quantitation coupled with offline two-dimensional liquid chromatography-tandem mass spectrometry.27 A total of 83 proteins that were expressed differently between the controls and patients’ AH samples were identified using isobaric tags for relative and absolute quantitation analysis, including pigment epithelium-derived factor (PEDF) and retinoic acid 6 (STRA6).27 The study revealed that the expression level of PEDF was lower in Rb than that in control samples. PEDF has been demonstrated to be an inhibitor of tumor cell invasion, migration, and metastasis and has been identified as a potent inhibitor of angiogenesis in the eye.39–61 The antiangiogenic effect of PEDF makes it a possible candidate as a therapy target for Rb. The study also demonstrated upregulated STRA6 in the AH of children with Rb. Overexpression of STRA6 has been observed in numerous types of human cancer, including Wilm kidney tumors; melanomas; and colorectal, ovarian, and endometrial cancer.62 This suggests a possible role for STRA6 in targeted therapy for Rb, although further research is required to identify the function of STRA6 in the development of Rb.

Nucleic Acids

Nucleic acid levels (DNA, RNA, and microRNA) have recently been studied in AH samples from three Rb patients, namely, two post primary enucleation and one from a patient undergoing active therapy.28 The AH samples from the latter patient were obtained through paracentesis with an extraction of 0.1 mL of AH, which was performed routinely as part of the procedure for intravitreal injection of melphalan.11,15 The DNA concentration found in the AH was significantly higher in the eyes with primary enucleation (median, 30 ng/μL) than that in the treated eye (median, 0.2 ng/μL). Despite the low concentration, the DNA could be amplified and sequenced for chromosomal copy number alterations (gains and loss of regions of chromosomes). The genomic profiles from the DNA in the AH were consistent with those obtained from the tumor tissue postenucleation, demonstrating proof of principle that Rb tumor DNA can be found in the AH of advanced eyes.28 These novel findings suggest that AH may serve as a surrogate tumor biopsy (a liquid biopsy) that could allow for an analyses of tumor-derived DNA in children with Rb, without undergoing enucleation, unlike all the previous studies included in this review. A subsequent analysis by Berry et al.29 in 2018 showed that the genomic evaluation of cfDNA in the AH has significant prognostic potential. In the study, concentrations of cfDNA in the AH of eight Rb eyes ranged from 0.084 to 56 ng/μL, with a mean of 0.2 ng/μL for eyes receiving melphalan treatment, and much higher concentrations for large untreated tumors that underwent primary enucleation (mean, 43.6 ng/μL) than that of control eyes from children with congenital glaucoma (two patients) and pediatric cataracts (one patient), which demonstrated median cfDNA concentrations of 0.15 ng/μL (mean, 0.12 ng/μL; range, 0.05–0.16 ng/μL). Additionally, AH from 13 enucleated Rb eyes were compared against the AH extracted via paracentesis from 16 salvaged (saved) eyes. The presence of detectable somatic chromosomal copy number alterations was 92% in enucleated eyes versus 38% in salvaged eyes (P = 0.006), with a gain of chromosome 6p being the most common. There were significantly increased odds of an eye failing therapy and requiring enucleation due to persistent or progressive disease activity if a gain of chromosome 6p was demonstrated in the AH cfDNA.
Further research is required before this information can be applied clinically, including AH sampling at diagnosis; however, this was the first time that tumor chromosomal changes could be compared between eyes that responded to therapy and those that failed therapy due to the ability to safely access AH in Rb eyes undergoing salvage therapy.

**Conclusion**

Despite significant investigation into tumor markers for Rb spanning more than 4 decades, currently there are no active uses for the AH in a clinical setting. Diagnosis is made on the basis of examination, and imaging findings without a biopsy and molecular tumor markers are not useful for diagnosis, prognosis, or to monitor therapeutic response (from any fluid including AH, serum, or tumor). This is due in large part to the contraindication to biopsy in Rb; therefore, previously neither tumor nor AH was evaluated outside of specimens from enucleated eyes, which clearly limited significant clinical correlations. However, with recent advances in local therapy for Rb, paracentesis with extraction of the AH has now been shown to be safe. This opens the door to use the AH as a liquid biopsy or surrogate to tumor biopsy for Rb.

This review highlights the various markers within AH that can now be safely examined in children with Rb without undergoing enucleation. Of specific interest are tumor biomarkers that may be associated with findings that cannot be determined by clinical examination and imaging alone; this includes associations with poorly differentiated tumors and post-laminar optic nerve invasion that are associated with higher risk disease and a small but present increased risk of metastatic disease for the child. Survivin and TGF-β1 may be valuable markers to associate with these features. High-quality studies from Shehata et al. 24,43 demonstrated the prognostic implications of AH survivin and TGF-β1, and the high sensitivity, specificity, and accuracy of serum and AH survivin and TGF-β1 make them promising markers for early detection and follow up of Rb patients. The gain of chromosome 6p is another marker associated with poor therapeutic outcomes and an aggressive tumor phenotype. 29 Taken together, these markers may better guide the clinician to understanding which eyes are likely to respond to therapy and benefit from attempts at salvage.

Other markers of clinical utility include those that correlate with treated disease and tumor status. In general, this can be determined by direct ophthalmic examination; however, whether or not there are lurking vitreous seeds or tumor under calcified Rb scars can be difficult to determine on examination or imaging alone. Findings by Comoy et al. 26 have suggested that serial AH NSE measurements may have a significant role in determining tumor status in Rb patients, and future studies may further clinically substantiate the use of this tumor marker. Additionally, a high-quality study from Hadjistilianou et al. 25 demonstrated significantly decreased protein concentration in eyes following treatment with selective intra-arterial chemotherapy. If AH markers such as NSE, protein concentration, PEDF, or STRA6 could reliably indicate remission in treated eyes, this would be a significant clinical benefit to both the patients and the practitioners. Furthermore, these authors have suggested that PEDF and STRA6 should be further examined for future directions in targeted therapy in Rb. 52 The remaining biochemical markers that have been previously investigated, including xanthine and uric acid, have shown potential in diagnostic utility; however, further studies would be necessary to elucidate their significant clinical utility, including prognostic, clinicopathological, and therapeutic implications.

In the late 20th century, biochemical markers, including LDH, were widely investigated within body fluids in various pathological and malignant conditions. Although LDH was the first described marker of tumor activity in the AH, the lack of specificity and correlation with patient or tumor features limited its use clinically. Trends in the choice of biomarker surveillance have evolved over time and reflect the limits of technology during the era in which they were identified. Accordingly, a shift from biochemical toward genomic markers has also occurred. The 2017 study from Berry et al. 28 was the first to describe AH as a surrogate tumor biopsy, allowing clinicians to safely examine tumor DNA in children with Rb during therapy (e.g., in an eye that has not been enucleated). A follow-up study by Berry et al. 29 demonstrated significantly increased odds of an eye failing therapy and requiring enucleation due to persistent or progressive disease activity if a gain of chromosome 6p was demonstrated in the AH cfDNA, suggesting prognostic potential. Furthermore, this suggests a possible marker for targeted therapy by evaluation of chromosome 6p. 29 Expression analysis has identified genes targeted by 6p genomic gain, in which the specific genes DEK and E2F3 showed developmental regulation. 63 The study further showed that E2F3 and DEK mRNA overexpression was...
associated with protein overexpression, which was determined by immunoblotting or immunofluorescent staining of primary tumors, relative to the adjacent normal retina. Both DEK and E2F3 have important oncogenic roles in multiple cancers. DEK, first discovered in acute myeloid leukemia, is overexpressed in multiple cancers, including melanoma, hepatocellular carcinomas, brain tumors, and breast cancers, and evidence has suggested that DEK overexpression may facilitate tumor progression and perhaps chemoresistence. Additionally, E2F3 overexpression has been implicated in bladder, prostate, lung, and breast cancers, and small interfering RNA-mediated knock down of this gene has significantly reduced its proliferative capacity. These results suggest that inactivation of DEK and E2F3 remains an attractive therapeutic target in multiple cancers, including Rbs.

With increased access to AH in Rb eyes, future investigation of these genes, as well as the previously mentioned biochemical markers, is possible. Although AH evaluation is currently not used clinically for patients with Rb, with further investigation into specific biochemical and genomic markers and their associations with treatment response and potential as therapeutic targets, AH sampling and analysis has the potential to revolutionize the way Rb is managed.

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References


