Expression of Angiogenic Factors in Invasive Retinoblastoma Tumors Is Associated With Increase in Tumor Cells Expressing Stem Cell Marker Sox2

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- **Context.**—Progression of retinoblastoma is associated with increased tumor angiogenesis. However, a clear relationship between the expression of angiogenic markers in specific regions of the tumor and tumor progression has not been established. This study investigates the association between angiogenic factors in retinoblastomas with choroidal and/or optic nerve invasion (high-risk/invasive retinoblastoma) and expression of Sox2, a stem cell marker.

**Objective.**—To investigate the association between the expression of angiogenic factors and markers of tumor invasiveness, such as the stem cell marker Sox2, in retinoblastoma tissues.

**Design.**—Immunohistochemistry was used to evaluate coexpression of the angiogenic growth factors vascular endothelial growth factor A (VEGF-A), VEGF receptor 2 (VEGFR-2), and endoglin (CD105); markers of glial differentiation (vimentin and glial fibrillary acidic protein); and a neural stem cell marker (Sox2). Expression was assessed in nonneoplastic and neoplastic ocular tissues collected from enucleated eyes of patients with retinoblastoma. During qualitative data interpretation, evaluating pathologists were masked to patient grouping.

**Results.**—Expression of VEGF-A and VEGFR-2 in noninvasive (non–high-risk feature) retinoblastoma tumors was lower than in the invasive, or high-risk feature tumors. Moreover, our data indicate that the tumor cells, and not the surrounding stroma, secrete VEGF-A and that angiogenesis is mostly localized to the iris. Finally, our data showed that the expression of the neural stem cell marker Sox2 is associated with eyes with increased VEGF-A expression and tumor invasiveness.

**Conclusions.**—Increased expression of angiogenic factors, with a concomitant increase in expression of the stem cell marker Sox2 observed in retinoblastoma tissues, may partially explain the aggressiveness of these tumors. The complex interaction of angiogenic and stem cell–related pathways in these tumors, especially in high-risk feature retinoblastoma, suggests that targeting tumor cells capable of secreting vasculogenic factors, as well as proangiogenic genes and signaling pathways, may be necessary for development of effective antimetastatic retinoblastoma drugs.


**Retinoblastoma** is the most common intraocular malignant neoplasm in children, with most cases initiated by a mutation of the tumor-suppressor gene RB1. Despite extensive research efforts, retinoblastoma-associated mortality rate remains around 70%, especially in lower-income countries. These poor outcomes are, in part, due to delayed tumor detection and lack of effective therapies targeting late-stage disease. Consequently, although retinoblastoma can be effectively treated through surgery and/or chemotherapy, untreated tumors can lead to patient death within 1 to 2 years. As with other malignancies, retinoblastoma mortality rates increase with higher pathologic grade. Specifically, a localized intraocular disease has a greater than 95% cure rate after enucleation, which sharply...

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contrasts with a very poor prognosis for children with extraocular disease.² Combined, these factors underscore the importance of correlating disease biology, tumor location, and invasiveness in establishing patient prognosis and assigning therapeutic regimens.

The first retinoblastoma histopathologic findings were described in 1960 by Parks and Zimmerman, followed by the establishment of the Reese-Ellsworth clinical tumor classification aimed at determining the likelihood of salvaging the affected eye, and a more recent international retinoblastoma classification that stratifies tumors by their response to chemotherapy.¹⁰–¹¹ Studies based on these classifications have shown that histopathologic risk factors for metastasis are most often associated with group 5 of the Reese-Ellsworth classification and groups D and E of the international classification.¹² There are also several clinicopathologic features of retinoblastoma that should be considered at diagnosis and during treatment. The genetic changes contributing to invasion and metastasis are still poorly understood and cannot predict prognosis. However, recent evidence collected from enucleated eyes revealed a correlation between metastasis, the plane of tumor invasion, and the degree of invasion into the optic nerve and choroid.¹³,¹⁴ Moreover, neovascular glaucoma in enucleated eyes, caused by iris neovascularization (NVI) or secondary peripheral anterior synchiae, has also been shown to correlate with tumor recurrence.¹⁵,¹⁶ Specifically, clinically identified NVI directly correlates with retinoblastoma histopathologic features, such as tumor size, orbital invasión, choroidal invasion, and scleral invasion, among others.¹⁷ Together, these findings emphasize the importance of establishing a better understanding of the relationship between the retinoblastoma clinicopathologic features and tumor recurrence or metastasis, and the clinical significance of NVI as one of the factors associated with high-risk (tumor) features (HRF) and metastasis.

Tumor neovascularization is one of the first steps in the progression of retinoblastina and results from tumor hypoxia with subsequent expression of several angiogenic growth factors, mainly the vascular endothelial growth factor (VEGF).¹⁹ Of all VEGF isotypes, VEGF-A is the most studied and clinically important. Expression of VEGF-A is detected in many disorders involving vascularization of the eye, as well as in aberrant angiogenesis and intravitreal neovascularization associated with retinopathy of prematurity. Moreover, VEGF-A also regulates retinal vascular development and functions as a retinal neural survival factor.²⁰ For example, Müller cells, principal glial cells present in the retina, medullate vascular proliferation in the ischemic retina through secretion of VEGF.²¹ However, it is important to note that VEGF-A and its receptors are not always expressed concurrently during retinal vascularization, which suggests the presence of additional mechanisms regulating vascular patterning. Specifically, in a developing retina, cell proliferation, migration, and survival are regulated by spatially restricted VEGF receptor expression and ligand availability.²² Neovascularization of the retina tends to follow the preexisting astrocytic meshwork, suggesting a very important role for glial cells.²³–²⁵ Some studies have addressed the importance of VEGF in the development of retinal vasculature²⁶ and in survival of the newly formed retinal vessels.²⁷ Other studies²⁸ have found correlations between VEGF staining intensity and time to retinoblastoma progression and its mitotic and apoptotic indexes. Although these studies described VEGF expression in the tumor, they did not elaborate on its spatiotemporal expression or coexpression with other important angiogenic markers.

In addition to VEGF and its receptors, it is important to consider other factors expressed in the retinal microenvironment that also play important roles in neovascularization and tumor invasion. For example, activated Müller cells express high levels of vimentin and glial fibrillary acidic protein (GFAP). Vimentin is also expressed by myofibroblasts and many other cells in the iris vascular endothelial stroma, while GFAP is expressed in microglia surrounding retinal vessels. Consequently, GFAP immunohistochemistry can identify both glial and Müller cells.²⁹,³⁰ Endoglin (CD105) is a recognized marker of activated endothelium, with its expression limited to proliferating vascular cells³¹; hence, it was recently used to correlate the expression of VEGF receptor 2 (VEGFR-2) with neovascularization. Lastly, Sox2 is expressed on uncommitted dividing stem and progenitor cells of the developing nervous system, and on adult neural stem cells.³² Importantly, cancer stem cells in brain and eye tumors have also been shown to express Sox2. As with other cancer types, these cancer stem cells promote tumor replication, progression, drug resistance, and recurrence.³³–³⁵

In the current study, we analyzed the expression of several angiogenic factors in different areas of retinoblastoma tumors that concurrently exhibited neovascularization of the iris and compared it to the pattern of Sox2 expression. We also investigated whether any of these tumor characteristics correlated with high-risk features of retinoblastoma invasiveness.

MATERIALS AND METHODS

Patients and Tumors

The study was approved by the Institutional Review Board of the Houston Methodist Research Institute (IRB0209-0017). It was conducted by using enucleated eyes of patients with retinoblastoma that had neovascularization of the iris. Twelve eyes were selected, of which 6 had HRF by histopathology while 6 exhibited a non-HRF profile (Table 1). These samples were selected from a total of 212 eyes because they presented adequate amounts of tumor tissue for all representative areas to be studied (retinal invasion, center of tumor, areas of necrosis, and sites of invasive tumor). Moreover, these specimens were enucleated within the past 5 years and had adequately preserved antigens. The required criteria for HRF were choroidal invasion greater than 3 mm in diameter, postlaminar optic nerve invasion, and/or optic nerve invasion concomitant with choroidal invasion, according to the Children’s Oncology Group and the International Retinoblastoma Staging Group.¹³

Histopathology

The enucleated eyes were processed for routine histopathologic examination (formalin fixed, paraffin embedded). Hematoxylin-eosin–stained slides were reviewed to confirm the presence of neovascularization of the iris and the presence or absence of HRFs. Unstained slides directly adjacent to the hematoxylin-eosin sections were used for subsequent double immunostaining.

Angiogenic and Differentiation Markers

We performed double immunostaining by using differentiation and angiogenesis markers to evaluate cellular localization of the angiogenic factors in the eye and tumor. Sox2 was used as a single stain. The following double stains were used: VEGF-A and vimentin; VEGF and GFAP; VEGFR-2 and CD105.
**Table 1. High-Risk Features**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Unilateral/Bilateral</th>
<th>Optic Nerve Invasion</th>
<th>Choroidal Invasion</th>
<th>Tumor Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>(R) Retinoblastoma (B)</td>
<td>Prelaminar</td>
<td>Focal</td>
<td>HRF</td>
</tr>
<tr>
<td>2</td>
<td>(L) Retinoblastoma (U)</td>
<td>Retrolaminar</td>
<td>No</td>
<td>HRF</td>
</tr>
<tr>
<td>3</td>
<td>(R) Retinoblastoma (B)</td>
<td>Retrolaminar</td>
<td>Focal</td>
<td>HRF</td>
</tr>
<tr>
<td>4</td>
<td>(R) Retinoblastoma (B)</td>
<td>Retrolaminar</td>
<td>Massive</td>
<td>HRF</td>
</tr>
<tr>
<td>5</td>
<td>(L) Retinoblastoma (U)</td>
<td>Retrolaminar</td>
<td>Massive</td>
<td>HRF</td>
</tr>
<tr>
<td>6</td>
<td>(L) Retinoblastoma (U)</td>
<td>Retrolaminar</td>
<td>Focal</td>
<td>HRF</td>
</tr>
<tr>
<td>7</td>
<td>(L) Retinoblastoma (U)</td>
<td>No</td>
<td>No</td>
<td>Non-HRF</td>
</tr>
<tr>
<td>8</td>
<td>(L) Retinoblastoma (B)</td>
<td>No</td>
<td>No</td>
<td>Non-HRF</td>
</tr>
<tr>
<td>9</td>
<td>(L) Retinoblastoma (U)</td>
<td>Prelaminar</td>
<td>No</td>
<td>Non-HRF</td>
</tr>
<tr>
<td>10</td>
<td>(L) Retinoblastoma (U)</td>
<td>Prelaminar</td>
<td>No</td>
<td>Non-HRF</td>
</tr>
<tr>
<td>11</td>
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<td>Prelaminar</td>
<td>No</td>
<td>Non-HRF</td>
</tr>
<tr>
<td>12</td>
<td>(L) Retinoblastoma (B)</td>
<td>Prelaminar</td>
<td>No</td>
<td>Non-HRF</td>
</tr>
</tbody>
</table>

Abbreviations: B, bilateral; HRF, high-risk feature; L, left; R, right; U, unilateral.

* List of high-risk features in patient samples used for the study, specifying side (left or right), bilaterality or unilaterality, the type of optic nerve invasion, and the presence of choroidal invasion (focal: <3 mm; massive: >3 mm).

**Immunohistochemistry**

Slides were deparaffinized, rinsed with deionized water, and placed in Dako 1X wash buffer (Carpinteria, California). All reagents were prepared according to manufacturer’s instructions, and incubations were performed in a humidified chamber at room temperature. Antigens were unmasked with Dako target retrieval solution at 99°C, peroxidase activity was blocked with 3% hydrogen peroxide, and biotin activity was blocked with Vector Avidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, California). Dako serum-free protein block was used to prevent nonspecific protein binding.

Slides were incubated for 30 minutes with a goat polyclonal antibody to human VEGF-165 (VEGF-A) (R&D Systems, Minneapolis, Minnesota) diluted 1:10, or a polyclonal antibody to human VEGFR-2 (KDR) (R&D Systems) diluted 1:15 in Dako antibody diluent. Slides were washed with buffer and incubated for 30 minutes with biotinylated anti-goat immunoglobulin G (IgG; Dako, Carpinteria, California) diluted 1:500. After washing, slides were incubated with biotinylated anti-goat immunoglobulin G (IgG; Dako, Carpinteria, California) diluted 1:10, or a goat polyclonal antibody to human VEGF-165 (VEGF-A) (R&D Systems, Minneapolis, Minnesota) diluted 1:5000, or a rabbit polyclonal antibody to human vimentin (Abcam, Cambridge, Massachusetts) diluted 1:1500, or a rabbit polyclonal antibody to human vimentin (Abcam, Cambridge, Massachusetts) diluted 1:5000, or a rabbit polyclonal antibody to human vimentin (Abcam, Cambridge, Massachusetts) diluted 1:1500 in antibody diluent. Slides were washed, incubated for 30 minutes with a goat anti-rabbit IgG (Vector Laboratories) diluted 1:500, washed again, and incubated for 30 minutes with phosphatase-labeled streptavidin (KPL, Gaithersburg, Maryland). After a wash step, slides were incubated with Dako Liquid Permanent Red for 5 minutes, washed in distilled water, counterstained with Hematoxylin 2 (Thermo Scientific), air dried, and coverslipped by using Dako Faramount Aqueous Mounting Medium.

**Qualitative Interpretation and Expression Quantification**

All tissues and specific tumor areas were reviewed systematically and included the iris, the nonneoplastic retina, and 3 different regions of the tumor: center of the tumor, perinecrotic tumor, and tumor infiltrating into the retina. Expression patterns of angiogenic markers detected in individual cell types were recorded, and staining intensity per surface area was assigned as follows: no expression was designated 0, 25% of positive tissue was designated 1+, 25% to 75% of positive tissue was designated 2+, and greater than 75% of positive tissue was designated 3+. The evaluation also included morphologic identification of cellular components of the retina that may have expressed the same differentiation markers but that were actually of different cell type. ImageJ 1.47 software package (http://image.nih.gov/ij/; National Institutes of Health, Washington, DC) was used to quantify staining localization and colocalization. Digital micrographs were obtained by using the same magnification (×40) and camera settings, and data were analyzed by area. To determine staining colocalization, images were obtained by using the appropriate color channels (1 for each marker), and the images were then overlaid to determine signal colocalization.

**Statistical Analysis**

Data were analyzed by using a nonparametric t test, and P < .05 was considered statistically significant (GraphPad, La Jolla, California).

**RESULTS**

**Coexpression of Angiogenic and Differentiation Markers**

**Vascular Endothelial Growth Factor A and Vimentin.—**Qualitative evaluation of VEGF-A/vimentin coexpression in the HRF tumors and the retina revealed that vimentin was predominantly expressed in the Müller cells of the retina, with focal expression in vessels and retinal pigment epithelial cells. Vascular endothelial growth factor A was expressed in both vimentin-positive Müller cells and vessels and in vimentin-negative non-Müller cells in the retina and tumor. The vimentin-positive non-Müller cells were predominantly present in the inner retinal layers surrounding the blood vessels; some neurons also stained positively (Figure 1, A). Vascular endothelial growth factor A was most frequently found in cancer cells near the retina of HRF tumors (Table 2), and high VEGF levels (2–3+) were detected proximally to areas exhibiting signs of necrosis (Figure 1, B). In the iris, both blood vessels and stromal cells stained positively for vimentin and VEGF-A, which were coexpressed in the vessels of the neovascular membrane (Figure 1, C).

Staining quantitation revealed that VEGF expression was similar throughout the different regions of HRF samples. In contrast, VEGF expression differed in various regions of non-HRF samples, with higher VEGF levels observed in the retina than in the tumor. Overall, VEGF expression was significantly higher in HRF versus non-HRF samples (Figure 1, D). Likewise, vimentin expression was significantly higher in HRF tumors but displayed no spatial expression differences in nonneoplastic tissues (Figure 1, E). Surprisingly, although VEGF and vimentin colocalized, there was...
no significant difference between the HRF and non-HRF samples. From our analysis, we believe that most of this colocalization takes place in the Müller cells of the retina (Figure 1, F).

**Vascular Endothelial Growth Factor and GFAP.**—We then examined glial cells present in the retina and tumor for expression and colocalization of GFAP and VEGF. Our data showed that GFAP-positive cells were present in the inner portions of the retina and in the perivascular tumor regions, which represent areas of high astrocyte density (Figure 2, A). However, there was no statistical difference in the expression and colocalization of VEGF and GFAP, with VEGF expression detected mostly in GFAP-negative tissues (Figure 2, B through F).

**Vascular Endothelial Growth Factor Receptor 2 and CD105.**—CD105 and VEGFR-2 were mostly coexpressed in tumor vessels (Figure 3, A), epiretinal neovascular membranes (Figure 3, B), and iris neovascular membranes (Figure 3, C); VEGFR-2 was also expressed in the retina and tumor cells. Quantitative evaluation revealed higher VEGFR-2 expression in the nonneoplastic retina than the iris, in HRF versus non-HRF tumors, and in the retina versus non-HRF tumor (Figure 3, D through F).

**Expression of the Neural Stem Cell Marker Sox2**

To correlate the expression of angiogenic markers with tumor invasiveness and likelihood of relapse, we analyzed coexpression of several angiogenic factors with Sox2, a marker of tumor stem cells and progenitor cells. Qualitative evaluation of Sox2 expression in nuclei of neural stem cells revealed clusters of positive cells located near the edge of the tumor, invading into the retina and in the perinecrotic tumor areas. However, central portions of the tumor and tumor-invasive edges near the choroid or optic nerve exhibited virtually no Sox2positivity (Figure 4, A through F). Finally, quantitative analysis of Sox2 expression in the near retina and perinecrotic areas demonstrated a significant difference in Sox2 positivity between HRF and non-HRF tumors (Figure 4, G).

![Figure 1](image-url)  
**Figure 1.** Vascular endothelial growth factor A (VEGF-A) (brown) and vimentin (red) qualitative and quantitative expression in eyes with retinoblastoma. A, Nonneoplastic retina. Müller cells expressing vimentin (arrowhead) and perivascular cells expressing VEGF-A (arrows). B, Perinecrotic tumor (arrows) expressing VEGF-A and vessels (*) are vimentin positive. C, Neovascular iris membrane (arrows) with double expression of VEGF-A and vimentin. Quantification of high-risk feature (HRF) versus non-HRF tumors’ expression for VEGF-A (D), vimentin (E), and VEGF-A/vimentin (F) (VEGF-A [3,3’,3-diaminobenzidine] and vimentin [permanent red], original magnification ×40 [A through C]).

![Table 2](image-url)  
**Table 2. Vascular Endothelial Growth Factor (VEGF) Qualitative Interpretation**

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Tumor Type</th>
<th>Central Tumor</th>
<th>Necrotic Tumor</th>
<th>Tumor Near Retina</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HRF</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>HRF</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>HRF</td>
<td>2</td>
<td>1</td>
<td>3</td>
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<tr>
<td>4</td>
<td>HRF</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>HRF</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>HRF</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Non-HRF</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>Non-HRF</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>Non-HRF</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
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<td>Non-HRF</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>11</td>
<td>Non-HRF</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>Non-HRF</td>
<td>0</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

Abbreviation: HRF, high-risk feature.

*a List of the subjective quantification of VEGF expression in the center of the tumor, in the tumoral perinecrotic areas, and in the tumor areas that are close to the preserved retina.

*P = .02 between HRF and non-HRF.
Figure 2. Qualitative and quantitative expression of glial fibrillary acidic protein (GFAP) (red) and vascular endothelial growth factor A (VEGFA) (brown) in retinoblastoma tissues. A, Expression of GFAP/VEGFA in retina, vessels (*) showing perivascular glia, and neurons (arrows) mostly expressing VEGFA. B, Expression of GFAP/VEGFA in the tumor localized to the retina. Müller cells are identified by arrows and coexpress GFAP and VEGFA. Perivascular (vessel [*]) glial cells also express both markers. C, Expression of GFAP/VEGFA in the tumor cells located near a necrotic area (arrows). Quantification of high-risk feature (HRF) versus non-HRF tumors’ expression with GFAP (D), VEGF-A (E), and VEGF-A/GFAP (F) (VEGFA 3,3'-diaminobenzidine and GFAP [permanent red], original magnification ×40 [A through C]).

Figure 3. Qualitative and quantitative expression of vascular endothelial growth factor receptor-2 (VEGFR2) (brown) and endoglin (CD105) (red) in eyes with retinoblastoma tumors. A, Note that some of the tumor (T) cells near new vessels (*) are expressing VEGFR2. B, Nonneoplastic retina expressing VEGFR2 and epiretinal neovascular membrane with many new vessels (*). C, Iris with neovascular membrane and neovessels (*). Quantification of high-risk feature (HRF) versus non-HRF tumors’ expression with VEGFR2 (D), VEGFR2/CD105 (E), and CD105 (F) (VEGFR2 3,3'-diaminobenzidine and CD105 [permanent red], original magnification ×40 [A through C]).
The prognosis for patients with retinoblastoma primarily depends on the presence of tumor invading beyond the sclera. Local spread of tumor cells into the neighboring tissues occurs through the invasion of the choroid or postlamellar invasion of the optic nerve. Although relatively rare, retinoblastoma also metastasizes to distant sites via the lymphatic system, and several clinical factors have been identified that predict the likelihood of retinoblastoma recurrence and metastasis. For example, secondary neovascular glaucoma is highly predictive of optic nerve invasion. Histopathologic studies have also shown that tumor dissemination may be predicted by the number of tumor blood vessels. Moreover, it is well known that pathologic angiogenesis may result in peripheral anterior synechiae and secondary glaucoma, which are both associated with worse retinoblastoma prognosis. Our study aimed to further elucidate localization of the tumor neovasculature and angiogenic factors expressed within the different regions of the eye of patients with retinoblastoma. Furthermore, we compared clinical characteristics of HRF and non-HRF tumor samples collected from enucleated eyes. Lastly, we correlated our findings related to tumor angiogenesis with the expression profile of a differentiation marker and Sox2 stem cell marker.

Vascular endothelial growth factor is one of the most important and potent angiogenic factors expressed in the eye, and its expression is prevalent during tumorigenesis and tumor progression. Our results show that the HRF tumors express more VEGF than non-HRF tumors. Consistent with previously published literature describing nonneoplastic ocular pathology, our data confirm that most VEGF-expressing cells localize to the retina. The importance of Müller cells in retinal vascularization and secretion of VEGF has been previously suggested. We now show that although many vimentin-positive cells in the retina express VEGF, there are other vimentin-negative cells that also express this growth factor. The morphologic features and localization of these VEGF-expressing cells suggest that they may be part of nerve fibers and are thus neuronal cells. Importantly, these areas of VEGF expression coincide with those areas where VEGF is expressed in the developing retina during normal vasculogenesis. This finding suggests that these inner retinal cells conserve mechanisms that allow them to reexpress VEGF under stress conditions. Moreover, we found a significantly higher

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**Figure 4.** Qualitative and quantitative expression of Sox2 expression in retinoblastoma tumors by area. High-risk feature (HRF) tumor: tumor near the retina; notice the increase in positive cells (arrows) near retina (A); tumor adjacent to a necrotic region (B); and central tumor with scarce positive cells (C). Non-HRF tumor: tumor near the retina (D); tumor adjacent to a necrotic region (E); and central tumor (F). G, Sox2-positive cell count by region comparing HRF (n = 4) and non-HRF (n = 5) tumors (Sox2 [3,3′-diaminobenzidine], original magnification ×40 [A through F]).
VEGF expression in the HRF tumors but not in their retinas, as compared to the non-HRF tumors, which may indicate that the non-HRF tumors allow for a more physiologic response of the retina than the HRF tumors. Further morphologic analysis of different tumor regions revealed that higher VEGF levels were expressed in the tumor cells located at the retina/tumor interface and in the tumor cells adjacent to areas of necrosis, but not near the choroidal or postlaminar optic nerve invasion. Although the degree of vimentin expression in HRF tumors was also higher when compared to non-HRF tumors, there was no colocalization with VEGF. This finding indicated that the HRF tumor cells, and not the stromal cells, express VEGF. Finally, we assayed the role of Müller cells and astrocytes in VEGF expression by examining patterns of GFAP expression. Our results clearly demonstrated that in our samples, there were no significant differences in colocalization of VEGF and GFAP between the HRF and non-HRF retinoblastoma cases.

In addition to evaluating the expression pattern of VEGF, we also analyzed the expression of its cognate receptor, VEGFR-2. Our results showed that VEGFR-2 was expressed at high levels in the retina of both HRF and non-HRF tumor types. However, its expression in HRF tumor cells was similar to that in the retina, whereas its expression in the non-HRF tumors was lower. We also found that HRF tumors displayed a higher level of both VEGF and VEGFR-2. Although CD105, a marker of neovascularization, indicated that angiogenesis occurs to a higher degree in the iris of HRF tissues, we did not observe a significant level of coexpression between VEGFR-2 and CD105. This unexpected result may be explained by the previously described pattern of temporal VEGFR-2 expression, which suggests that these receptors may be expressed at initiation, but not during the later stages of angiogenesis.

The collective findings presented in this report support the hypothesis that HRF tumors elicit more angiogenesis than non-HRF tumors, especially in the tumor and iris. Although the mechanisms underlying tumor cell–mediated vascularity as they relate to tumor progression and cancer metastasis remain unclear, data have shown that various intrinsic and extrinsic factors activate oncogenes and vasculogenic genes, enhance vasculogenic signaling pathways, and trigger tumor neovascularization. As previously described by Pe’er et al., our study confirms that VEGF might play a crucial role in NVI of retinoblastoma. Our study, however, used a standardized measure of tumor invasiveness to further study the possible origin, role, and mechanism of VEGF actions in retinoblastoma.

Our current report describes the almost insignificant role of glial cells present in retinoblastoma tumors in secreting VEGF. There is a possibility that retinoblastoma cells disrupt a physiologic VEGF signaling pathway within the eye by simultaneously secreting high levels of this growth factor and expressing large numbers of its cognate receptor; temporal VEGF/VEGFR-2 expression patterns might explain the occurrence of NVI in the anterior chamber of the eye. To explain these findings, we must consider the etiology of retinoblastoma. As explained earlier, most retinoblastoma cases are associated with a mutation of the tumor-suppressor gene RB1. Normally, RB1 functions as a main regulator of the G1 checkpoint, but several recent studies also defined the role for RB1/p105 in a wide range of cellular processes, including maintenance of chromosome stability, induction and maintenance of senescence, apoptosis, differentiation, and angiogenesis. Although more experimental evidence is needed, the main role RB1 plays in angiogenesis seems to be through p21/E2F/Rb pathway, which indirectly represses VEGF expression. Normally, this pathway is activated by continued hypoxia and is mechanically distinct from the classic role of the p21/E2F/Rb pathway plays in cell-cycle regulation. Because in most retinoblastoma tumors the main event is a mutation of the RB1 gene, resulting in an absent or nonfunctional RB1 protein, one of the indirect VEGF repressors may be absent, thereby increasing tumor neovascularization.

Recently, Beck et al demonstrated that in squamous cell carcinomas, skin cancer stem cells (CSCs) are localized in a perivascular niche and in the immediate vicinity of endothelial cells. Blockage of VEGFR-2 caused tumor regression by decreasing the microvascular density, reducing the CSC pool size, and impairing CSC renewal properties. Conditional deletion of VEGF-A in the epithelial tumor cells caused tumors to regress, whereas a reciprocal VEGF overexpression accelerated tumor growth. In addition to its well-known effect on angiogenesis, VEGF affected skin tumor growth by promoting cancer stemness and symmetric CSC division, leading to CSC expansion. In the case of retinoblastoma, our study provides further evidence linking the tumor VEGF expression with its invasiveness, possibly by increasing the CSC pool. We demonstrate that there is not only a higher expression of VEGF in the HRF tumors, but also a coinciding higher Sox2 expression in cells located in the same tumor region. Although these results are compelling, there is also a discrepancy that must be addressed in subsequent studies. Specifically, Sox2-expressing cells are absent in the invasive tumor present in the choroid or the optic nerve, which may be the result of adverse conditions for the Sox2-expressing cells in this milieu encompassing the presence of metalloproteinases and other enzymes present at the sites of active invasion. At the same time, an increased number of Sox2-expressing cells can be observed at the leading edge of an invasive retinal tumor located just before the invasive site. Thus, the more aggressive tumors have more Sox2-expressing cells probably providing more tumor cells adjacent to the leading edge of invasion.

In summary, Sox2-expressing cells play important roles in tumor replication, progression, drug resistance, and recurrence. Our results, for the first time, show that in retinoblastoma tumors with HRF, there is an overexpression of VEGF-A that correlates to an increase in Sox2-positive stem/progenitor cells present in the tumor. This association may partially explain the aggressiveness of HRF tumors, as stem cells have been implicated in metastasis, drug resistance, and regression. Further biological studies are needed to confirm this association and to provide possible venues for targeting this unique retinoblastoma feature. Our data also suggest that the HRF retinoblastoma tumor cells may serve as the initiators of neovascularization. Therefore, targeting vasculogenic tumor cells, genes, and signaling pathways may be necessary for development of effective antimetastatic retinoblastoma drugs. Concurrent targeting of tumorigenesis, angiogenesis, and tumor cell–mediated neovascularization may overcome current clinical challenges in the treatment of aggressive and recurrent retinoblastoma tumors. However, caution should be exercised when designing antiangiogenic treatments, as nonneoplastic retina needs to preserve its vasculogenic function, especially in the setting of a developing child.
References