EpCAM Expression in Retinoblastoma: A Novel Molecular Target for Therapy

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PURPOSE. This study was conducted to investigate the potential of targeting epithelial cell adhesion molecules (EpCAMs) in the treatment of retinoblastoma. It was first determined whether EP-CAM is expressed in retinoblastoma and then whether EpCAM reactivity correlates with tumor aggressiveness.

METHODS. EpCAM reactivity was evaluated by immunohistochemistry in 43 retinoblastoma specimens from 43 patients, using the monoclonal antibody GA733-2. The tumors were divided into two groups. There were 20 tumors with no invasion of the choroid and optic nerve (group A) and 23 tumors with invasion of the choroid, optic nerve, and orbit (group B). EpCAM reactivity was correlated with invasion and differentiation of the tumors.

RESULTS. Among the 43 tumors, EpCAM reactivity was observed in 100% (43/43) tumors. EpCAM reactivity was significantly higher in the invasive than the noninvasive tumors (P < 0.05) and in poorly differentiated than in well-differentiated tumors (P < 0.055). Non-neoplastic retina also expressed EpCAM.

CONCLUSIONS. The results confirm that EpCAM is vastly expressed in retinoblastoma and point to its use as a target for therapy in the future. (Invest Ophthalmol Vis Sci. 2004;45: 4247–4250) DOI:10.1167/iovs.04-0591

Retinoblastoma is the most common intraocular malignancy in children.1 However, it is an uncommon tumor accounting for 3% of all childhood malignancies in developed countries.2 There is indirect evidence that it may be more frequent in some developing areas, such as Latin America, Africa, and India.3 In these areas, retinoblastoma is usually the most frequent solid tumor encountered in patients in pediatric oncology units. In this setting, retinoblastoma is diagnosed late, usually when extraocular dissemination has occurred and the prognosis is poor.4,5 Current management modalities for retinoblastoma include enucleation, external beam radiotherapy, plaque radiotherapy, laser photocoagulation and hyperthermia, and cryotherapy. Recently, neoadjuvant chemotherapy has been introduced for retinoblastoma, to avoid external-beam radiotherapy. New treatment modalities, such as subconjunctival injection, selective ophthalmic artery injection, and vitreous injection, are being investigated and have achieved favorable results. Although many modalities are used, almost half of eyes with retinoblastoma have to be enucleated. New treatment modalities are expected.6

EpCAM has gained interest as a potential therapeutic target and an attractive candidate tumor-associated antigen (TAA) to serve as a target for antibody-based immunotherapy.8–10 Chimeric and humanized mAbs have been generated, such as chimeric mAb 323/A3 and 17-1A, or humanized mAb huNR-LU-1317 and MT201.11 Immunotherapy with the mAb 17-1A (edrecolomab, Panorex; Glaxo Wellcome GmbH, Hamburg, Germany) decreases the frequency of distant metastasis in patients with colorectal cancer12–14 and eliminates disseminated breast cancer tumor cells in the bone marrow.15 EpCAM is overexpressed in carcinomas of various origins, including colon and rectum, prostate, liver, esophagus, lung, head and neck, pancreas, breast, and kidney.8–11,16 There is no information available on the expression of EpCAM in retinoblastoma. The purpose of this study was to investigate the potential of targeting EpCAM in the treatment of retinoblastoma. We first determined whether EpCAM is expressed in retinoblastoma. Moreover, the correlation of EpCAM expression with tumor aggressiveness and differentiation was determined.

MATERIALS AND METHODS

Forty-three tumors were available from 43 eyes for the study. Among them were tumors from 22 males and 21 females. The age ranged from 4 months to 21 years (median, 1 year). There were 25 unilateral retinoblastomas and 18 bilateral retinoblastomas.
Laboratories, Burlingame, CA). by the avidin biotin complex method (Vectastain ABC Kit; Vector (DakoCytomation, Glostrup, Denmark), and the reaction was amplified the secondary antibody used was biotinylated rabbit anti-mouse which was a generous gift from one of the authors (L.R.-H.).

expression of EpCAM protein by using the GA733.2 murine mAb, Retinoblastoma cells were examined immunohistochemically for the proteins expressed in both choroidal invasion: 9 with diffuse and 9 with focal choroidal invasion. There were 20 tumors with no invasion of the choroid or optic nerve: 10 with focal invasion or diffuse invasion of the choroid. All tumor slides were reviewed and examined for invasion of choroid, optic nerve, and orbital invasion. Choroidal invasion was classified as either focal invasion or diffuse invasion of the choroid. For optic nerve invasion, prelaminar and postlaminar invasion and invasion of the optic nerve alone. The information is summarized in Table 1.

Histopathologic Features
All tumor slides were reviewed and examined for invasion of choroid, optic nerve, and orbital invasion. Choroidal invasion was classified as either focal invasion or diffuse invasion of the choroid. For optic nerve invasion, prelaminar and postlaminar invasion and invasion of the surgical end of the optic nerve were analyzed.17 There were 20 tumors with no invasion of the choroid or optic nerve (group A) and 23 tumors with invasion of the choroid, optic nerve, and orbit (group B). Among the 23 tumors with invasion, 18 had choroidal invasion: 9 with diffuse and 9 with focal choroidal invasion. There were 15 tumors with invasion of the optic nerve: 10 with invasion of both choroid and optic nerve and 5 with invasion of the optic nerve alone. The information is summarized in Table 1. Retinoblastomas were graded microscopically into three groups according to the predominant pattern of differentiation.1 There were 9 well-differentiated, 5 moderately differentiated, and 29 poorly differentiated tumors.

mAb and Chemicals
Retinoblastoma cells were examined immunohistochemically for the expression of EpCAM protein by using the GA733.2 murine mAb, which was a generous gift from one of the authors (L.R.-H.). The secondary antibody used was biotinylated rabbit anti-mouse (DakoCytomation, Glostrup, Denmark), and the reaction was amplified by the avidin biotin complex method (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA).

### Table 1. Clinical and Immunohistochemical Information on Invasive Retinoblastomas

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Age (y)/Sex</th>
<th>Clinicopathologic Features</th>
<th>EpCAM Expression (% of cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2/F</td>
<td>OD: PD; invasion of surgical end of ON</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>1/F</td>
<td>OD: PD; diffuse Ch invasion</td>
<td>90</td>
</tr>
<tr>
<td>3</td>
<td>2/F</td>
<td>OS: MD; focal Ch; pre-lam ON invasion</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>3/F</td>
<td>OS: PD; diffuse Ch invasion</td>
<td>70</td>
</tr>
<tr>
<td>5</td>
<td>2/F</td>
<td>OS: PD; focal Ch invasion</td>
<td>70</td>
</tr>
<tr>
<td>6</td>
<td>7/M</td>
<td>OD: PD; diffuse Ch invasion; orbital invasion</td>
<td>80</td>
</tr>
<tr>
<td>7</td>
<td>13 mo/F</td>
<td>OD: PD; focal Ch; post-lam ON invasion</td>
<td>80</td>
</tr>
<tr>
<td>8</td>
<td>4/F</td>
<td>OS: PD; post-lam ON invasion</td>
<td>70</td>
</tr>
<tr>
<td>9</td>
<td>2/M</td>
<td>OS: PD; diffuse Ch invasion; post-lam ON invasion</td>
<td>80</td>
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<tr>
<td>10</td>
<td>2/M</td>
<td>OD: PD; pre-lam ON invasion</td>
<td>70</td>
</tr>
<tr>
<td>11</td>
<td>21/F</td>
<td>OS: PD; diffuse Ch invasion; post-lam ON invasion</td>
<td>80</td>
</tr>
<tr>
<td>12</td>
<td>3/M</td>
<td>OS: PD; diffuse Ch invasion; post-lam ON invasion</td>
<td>70</td>
</tr>
<tr>
<td>13</td>
<td>4/M</td>
<td>OS: PD; focal Ch invasion</td>
<td>60</td>
</tr>
<tr>
<td>14</td>
<td>3/M</td>
<td>OS: PD; diffuse Ch invasion; pre-lam ON invasion</td>
<td>70</td>
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<td>15</td>
<td>4/F</td>
<td>OD: PD; pre-lam ON invasion</td>
<td>70</td>
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<tr>
<td>16</td>
<td>3/M</td>
<td>OS: PD; diffuse Ch invasion</td>
<td>80</td>
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<tr>
<td>17</td>
<td>3/M</td>
<td>OD: PD; focal Ch invasion</td>
<td>60</td>
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<tr>
<td>18</td>
<td>2/M</td>
<td>OS: PD; focal Ch invasion; pre-lam ON invasion</td>
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<tr>
<td>19</td>
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<td>20</td>
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<td>OS: PD; focal Ch invasion; post-lam ON invasion</td>
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<tr>
<td>21</td>
<td>4/F</td>
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<tr>
<td>22</td>
<td>2/F</td>
<td>OD: PD; focal Ch invasion; pre-lam ON invasion</td>
<td>60</td>
</tr>
<tr>
<td>23</td>
<td>4/F</td>
<td>OS: PD; pre-lam ON invasion</td>
<td>40</td>
</tr>
</tbody>
</table>

MD, moderately differentiated tumor; PD, poorly differentiated tumor; WD, well-differentiated tumor; diff Ch, diffuse choroidal invasion; focal Ch, focal choroidal invasion; pre-lam ON, prelaminar portion of optic nerve; post-lam ON, postlaminar portion of optic nerve.

### Tumor Specimens
The study was reviewed and approved by the local ethics committee at Vision Research Foundation, Sankara Nethralaya, and the committee deemed that it conformed to the generally accepted principles of research, in accordance with the Helsinki Declaration. Tumors enucleated between 1997 and 2002 with no preoperative chemotherapy and with a minimum follow-up of at least 24 months were included in the study. Paraﬃn-embedded blocks from 45 cases derived from enucleation of retinoblastomas were used for immunohistochemistry.

### Immunohistochemistry
The immunostaining procedures were then performed. In brief, 5-μm-thick parafﬁn-embedded sections were dewaxed and rehydrated. Antigen retrieval was performed by trypsinization. Endogenous peroxidase activity in the investigated specimens was blocked with 3% H₂O₂ in H₂O for 10 minutes, and the slides were incubated with monoclonal mouse anti-human EpCAM (1:10 dilution) for 2 hours at room temperature. Immunostaining was performed with the ABC kit (Vector Laboratories). The reaction was revealed by 3,3'-diaminobenzidine and counterstained with hematoxylin. For the positive control, basal cell carcinoma and adenocarcinomas, which express EpCAM, were included. For the negative control, the primary antibody was omitted and immunostaining was performed.

### Evaluation of Slides
Tissue sections were read independently by two investigators (SK, JB) using light microscopy, each without knowledge of the results obtained by the other investigator. Furthermore, each investigator read all the slides twice without knowledge of the results obtained in the previous reading. The interobserver reproducibility according to the test was 0.786 for EpCAM. Antigen expression was defined as the presence of specific staining on the surface membranes of tumor cells. All stained cells were considered positive, irrespective of staining intensity. Because EpCAM was expressed heterogeneously, 20 vital tumor fields were evaluated (under 20× magnification) and a final mean score for each tumor was obtained.10 The staining was scored as the percentage of positively stained cells. EpCAM immunoreactivity was correlated with the invasiveness and differentiation of the tumors.

### Statistical Analysis
The Mann-Whitney test was used to analyze the relation between the percentage of EpCAM-positive tumor cells and invasion and differentiation of the tumors. For statistical purposes, we grouped moderately differentiated and well-differentiated retinoblastomas and compared them with poorly differentiated retinoblastomas. Statistical analysis was performed on computer (SPSS ver. 10.0; SPSS, Chicago, IL).
RESULTS

Table 1 summarizes the immunohistochemical information of retinoblastomas with invasion. Figure 1 shows a plot of the percentage of EpCAM-positive cells in invasive and noninvasive tumors.

EpCAM Reactivity in Nonneoplastic Retina

EpCAM was expressed in the inner- and outer nuclear layers and in the ganglion cell layer of the retina. The photoreceptor layer, retinal pigment epithelial (RPE) cells, and the optic nerve tissues did not express EpCAM. The corneal epithelium also did not express EpCAM.

EpCAM Reactivity in Tumors with No Invasion

Among the 20 tumors with no choroid and optic nerve invasion, EpCAM reactivity was observed in 100% (20/20) tumors. There was 1 tumor with 30% positively stained cells, 7 tumors with 31% to 50% positively stained cells, and 12 tumors with >50% positively stained cells.

EpCAM Reactivity in Tumors with Invasion

All 18 tumors with choroidal invasion (9 tumors with focal and 9 with diffuse choroidal invasion) had >50% positively stained cells. Among the 10 tumors with both choroid and optic nerve invasion, all had >50% positively stained cells, and, among the 5 tumors with only optic nerve invasion, 4 had >50% positively stained cells and a single tumor with prelaminar optic nerve invasion had 40% positively stained cells. The difference in the percentage of tumor cells with EpCAM reactivity between the tumors with no invasion and invasion was significant (P < 0.05). The plot in Figure 1 shows the distribution of the percentage of EpCAM-positive cells in noninvasive and invasive retinoblastomas.

EpCAM Reactivity and Differentiation of Tumors

Among the nine well-differentiated retinoblastomas, there were four tumors with 31% to 50% positively stained cells and five with >50% positively stained cells. Among the five moderately differentiated tumors, there were two tumors with 31% to 50% positively stained cells and three with >50% positively stained cells. Among the 29 poorly differentiated tumors, I had 30% positively stained cells, 2 had 31% to 50% positively stained cells, and 26 had >50% positively stained cells. The difference in the EpCAM reactivity between the poorly differentiated tumors and the well- and moderately differentiated tumors (that were grouped together) was significant (P < 0.005), with higher reactivity in the former group of tumors.

DISCUSSION

Among the 43 retinoblastomas included in the study, EpCAM reactivity was observed in 100% (43/43) tumors. Among the 23 tumors with invasion of the choroid and optic nerve, >50% positively stained cells were observed in 22 (95%) tumors. EpCAM reactivity was greater in retinoblastomas with invasion of the choroid and optic nerve (P < 0.05; Fig. 1). EpCAM reactivity was also significantly higher in poorly differentiated retinoblastomas (P < 0.005). Thus, retinoblastoma joins the list of the tumors that express EpCAM.

EpCAM was also expressed in the nuclear layers of the retinal tissue. EpCAM is reported to be a molecule with adhesion properties similar to the family of cell adhesion molecules (CAMs). It is a type I transmembrane glycoprotein, not structurally related to any of the four major CAM families. The ability of EpCAM to regulate cadherin-mediated adhesions, tissue morphogenesis, and the transcription of genes indicates that the molecule plays a morphoregulatory role necessary for normal embryonic development and homeostasis of mature tissues. Thus, EpCAM may also play a role in retinal tissue development.

The expectation that EpCAM, like other adhesion molecules, provides invasion-suppressor properties to epithelia through cell-cell aggregation has been demonstrated in vitro and in clinical models. Normally nonadhesive cell lines have been induced to aggregate through transfection of EpCAM and, in addition, have shown reduced mobility and invasive behavior. EpCAM-transfected tumor cells have shown reduced metastases in vivo mouse models. However, this finding is not consistent across all studies and tumor types, as elevated EpCAM expression has been linked to increasing lymph node metastases, recurrence, and mortality in breast cancer. In those tissues with preexisting EpCAM expression, EpCAM positivity is enhanced during neoplastic development. In normal tissues where EpCAM is absent, its de novo expression indicates dysplasia or malignancy. The overexpression of EpCAM correlates with both benign and malignant proliferation of tumor cells. EpCAM-mediated cell-cell adhesion prevents cell scattering, suggesting that the molecule may prevent metastasis. However, the negative effect of EpCAM on cadherin-mediated adhesions may actually promote invasion and metastasis from tumor nodules. Thus, the dualistic role of EpCAM in tumor development requires further investigation.

In our study, we observed greater EpCAM reactivity in poorly differentiated tumors, which were associated with invasion of the choroid and optic nerve. Elevated EpCAM has a negative effect on E-cadherin-mediated adhesion by decreasing the association of the cadherin-catenin-cytoskeleton complex. Some studies report that loss of E-cadherin expression leads to a decrease in differentiation and an increase in both invasive tumor behavior and lymph node metastases, resulting in overall poorer prognosis. Thus, further studies are needed to understand the complex multifunctionality of EpCAM and its interactions with other molecules.

Our findings are of particular interest because of their clinical relevance. First, EpCAM may be a therapeutic target. Anti-EpCAM antibodies may be included in the treatment of retinoblastoma. Because its expression increases with tumor aggressiveness, its inhibition could represent an alternative treatment strategy in advanced and resistant retinoblastomas. A drawback of this would be that EpCAM is also expressed in normal retinal tissue and anti-EpCAM treatment may cause damage. Second, EpCAM as a TAA may offer a target in immunotherapy with bispecific antibodies (BiAb). This may be important to overcome the problems with respect to major histocompatibility complex (MHC)-restricted target recognition by T cells and downregulation of MHC molecules ex-
pressed by the tumor cells in many cancers, including retinoblastoma. The concept of using a bispecific antibody (BiAb) is for redirecting T cells toward tumor cells in a non-MHC-restricted manner by cross-linking cell surface antigens on tumor cells (i.e., TAA) and the CD3-T-cell receptor (TCR) complex on cytotoxic T lymphocytes (CTLs) for targeting tumor cells. When a BiAb bridges a T cell and a TAA on the tumor cell, the BiAb triggers this T cell to become a specific CTL that bypasses the MHC restrictions and destroys the tumor cell directly. The BiAb approach has improved survival rates in animal cancer models and is being tried in human cancers.

Thus, the results presented herein give an insight into Ep-CAM expression in retinoblastoma and open new possibilities for antibody-based therapy. Because all of the invasive retinoblastomas expressed higher EpCAM, EpCAM targeting or the use of BiAb-mediated immunotherapy offers a possibility for treatment of retinoblastoma in the future.

References


