Utility of Immunohistochemistry for Endothelial Markers in Distinguishing Epithelioid Hemangioendothelioma From Carcinoma Metastatic to Bone

Ryan Gill, MD, PhD; Richard J. O’Donnell, MD; Andrew Horvai, MD, PhD

Context.—Epithelioid hemangioendothelioma (EHE) is a rare vascular neoplasm of intermediate malignancy. Epithelioid hemangioendothelioma often presents a difficult diagnostic problem, especially in bone, because the epithelioid morphology and radiographic features raise the possibility of metastatic carcinoma. The current trend of small biopsies obtained with computed tomography–guided techniques exacerbates the problem. The markedly different treatment for EHE and metastatic carcinoma underscores the need for specific markers that can differentiate between these 2 entities.

Objective.—To determine the relative utility of endothelial markers in differentiating EHE from metastatic carcinoma, with emphasis on bone biopsies.

Design.—We used immunohistochemistry in formalin-fixed paraffin-embedded tissue to compare the utility of Fli-1, CD34, CD31, podoplanin, and keratin cocktail in 13 EHEs and 13 morphologically similar carcinomas metastatic to bone. Immunohistochemical data were evaluated using Fisher exact test, and specificity and sensitivity were calculated.

Results.—Significant proportions of EHEs were positive for Fli-1 (100%), CD34 (85%), and CD31 (100%) compared with metastatic carcinoma (Fli-1, 15%; CD34, 15%; CD31, 38%) (P < .001, P = .005, and P = .01, respectively). However, these markers were not 100% specific for EHE. Cytokeratin cocktail stained significantly more metastatic carcinomas (100%) than EHEs (38%) (P = .01) but was not 100% specific. No significant difference was observed regarding immunostaining for podoplanin between the tumor types.

Conclusions.—Fli-1 is most helpful in distinguishing EHE from metastatic carcinoma. However, the absence of complete specificity of any of the endothelial markers for EHE, or of keratin cocktail for carcinoma, suggests that these markers are best used in combination.

Arch Pathol Lab Med. 2009;133:967–972

Epithelioid hemangioendothelioma (EHE) represents the most aggressive member of the hemangioendothelioma family, which is intermediate between hemangioma and angiosarcoma in terms of recurrence and metastatic potential.2 Epithelioid hemangioendothelioma can occur at any age and can arise in soft tissue, viscera, and bone.2–7 The tumor, especially in bone, is often multifocal. Histologically, EHE is characterized by strands or nests of rounded endothelial cells, some with intracellular lumina. The latter feature is often confused with mucin vacuoles on hematoxylin-eosin sections. Multifocality, together with the overall epithelioid appearance of the tumor cells, and the propensity for keratin positivity, render EHE particularly difficult to differentiate from metastatic carcinoma,8 especially on core biopsy.

The plain radiographic appearance of EHE of bone can also demonstrate overlapping features with metastatic carcinoma insofar as both can present as expansive, osteolytic, and poorly demarcated lesions. Computerized tomography and magnetic resonance scans are not entirely specific in this setting either; the differential diagnosis will likely include entities such as lymphoma, metastatic carcinoma, and fibrous dysplasia.

Epithelioid hemangioendothelioma reportedly has low recurrence (13%) and moderate metastasis risk (31%).13 In the absence of visceral involvement, EHE can thus have a relatively good prognosis.4,5 Consequently, for cytologically typical EHE, the treatment of choice is wide local excision, with possible regional lymph node dissection, but without adjuvant radiotherapy or chemotherapy.3 Multicentric tumors that are monomeric can sometimes be controlled with amputation, but otherwise, radiation therapy, with or without surgery, is the mainstay of management.4,5 Individualized approaches, including novel techniques such as radiofrequency thermal ablation, are most often necessary in cases of multifocal bone involvement.10 Chemotherapy is generally not a standard aspect of care plans.4,5 Given that carcinoma metastatic to bone may have a dismal prognosis, often warranting only palliative treatment, distinction between EHE and carcinoma on core biopsy is of critical importance in guiding therapy.

A variety of proteins, with varying degrees of specificity for endothelial cells, may be useful to identify EHE. Po-
Podoplanin (recognized by the monoclonal antibody designated D2-40) is specifically expressed on endothelial cells of lymphatic origin. Podoplanin was recently reported as a useful diagnostic marker in identifying EHE in liver.\textsuperscript{11} The FLI1 gene encodes a nuclear transcription factor that is critical for hematopoiesis and vessel development. Fli-1 protein is expressed in endothelial cells as well as in T cells and megakaryocytes.\textsuperscript{12} Fli-1 nuclear localization by immunohistochemistry has proven useful in identifying vascular neoplasms, including EHE,\textsuperscript{13,14} CD34 and CD31 are well-recognized endothelial cell markers, with CD34 (human hematopoietic progenitor cell antigen) also expressed in hematopoietic stem cells, interstitial cells of Cajal, and dermal dendritic cells. CD31 (platelet endothelial cell adhesion molecule 1) expression, on the other hand, is restricted to endothelial cells, macrophages, and platelets. CD34 is reportedly expressed in more than 90% of vascular tumors, but this marker has poor specificity, with expression in a variety of other soft tissue tumors noted.\textsuperscript{1} In contrast, CD31 is regarded as a relatively specific marker for vascular tumors, although interpretation of positive staining is complicated by tumor-associated macrophages.\textsuperscript{1} However, false-positive interpretations can generally be avoided by careful attention to whether staining is granular or linear, with the former associated with macrophage staining. Both CD34 and CD31 are recognized to be of some value in differentiating cytokeratin-positive epithelioid angiosarcomas from carcinomas.\textsuperscript{1}

Vascular tumors with epithelioid histologic features, such as EHE, are well documented to express cytokeratins; even angiosarcoma without epithelial features reportedly expresses some keratins in approximately 20% of cases.\textsuperscript{15} Clearly, when tumor morphology is compatible with poorly differentiated carcinoma, relying solely on cytokeratin staining to confirm the diagnosis will misidentify a significant number of vascular tumors.\textsuperscript{16–19} Therefore, we evaluated recently described markers of endothelial differentiation (podoplanin and Fli-1), along with proteins well described as endothelial cell markers (CD34 and CD31), for their ability to distinguish EHE from carcinoma metastatic to bone by immunohistochemical detection. We also evaluated keratin cocktail staining results for EHE and metastatic carcinoma to ascertain its specificity in these conditions.

### MATERIALS AND METHODS

#### Case Selection and Tissue Preparation

Following approval by the Institutional Committee on Human Research, 13 patients with EHE (8 primary to bone, 5 involving soft tissue) and 13 patients with metastatic carcinoma to bone were retrieved from the files at the Department of Pathology University of California, San Francisco. All histologic slides of tumors from all patients were reviewed by A.E.H. and R.M.G.; the diagnoses were confirmed according to most recent World Health Organization criteria.\textsuperscript{20} For patients who presented with recurrence(s), tumor from the first presentation was used. The metastatic carcinomas were chosen because of morphologic similarities to EHE and because the primary carcinoma had been histologically documented at our institution. All formalin-fixed paraffin-embedded tissue samples were routinely processed, and serial sections from representative paraffin blocks were used for hematoxylin-eosin staining and immunohistochemistry. The majority of both the EHE cases in bone (6/9) and the carcinoma cases (8/13) were decalcified in Easy\textsuperscript{®}Cut Decal (American Master Tech Scientific, Lodri, Calif). None of the 5 EHE cases arising in soft tissue required decalcification.

### Immunohistochemistry

Immunohistochemical analysis was performed using previously published techniques.\textsuperscript{22} Briefly, 4-μm paraffin-embedded sections were heat-treated, deparaffinized; heated in citrate buffer; blocked for endogenous peroxidase, avidin, and biotin; and incubated with antibodies, as summarized in Table 1. Sections were then washed and developed with the Vector Labs (Burlingame, Calif) ABC kit. For all antibodies, staining was scored as follows: 0 (negative), 1+ (1%–10% of cells positive), 2+ (11%–50% of cells positive), and 3+ (>50% of cells positive). Hyperplastic tonsil was used as a positive control for Fli-1, CD34, CD31, and D2-40. Skin with Kaposi sarcoma was also used as positive control for Fli-1. Breast carcinoma was used as a positive control for keratin cocktail. Sample tissues were incubated with nonspecific mouse serum (Cell Marque Corp, Rocklin, Calif) as negative controls. In evaluating Fli-1, only nuclear staining was considered positive. Slides were scored by both A.E.H. and R.M.G. who were blinded to the diagnosis with complete agreement in scoring.

### Statistical Analysis

Data were evaluated using Fisher exact test, and two-sided $P$ values were calculated, followed by a Bonferroni correction. $P < .05$ was considered significant.

### RESULTS

#### Demographics

There was no significant difference in the age or gender distribution of patients with a diagnosis of EHE compared with metastatic carcinoma in our study. The mean age for EHE diagnosis was 60 years (range, 19–92 years) versus 56 years for metastatic carcinoma (range, 39–84 years). Thirty-one percent of EHE diagnoses were in men versus 46% of metastatic carcinoma diagnoses.

#### Pathologic Features

Of the primary EHEs, 8 were located in bone (scapula, sternum, foot, radius, femur, sacrum, and multifocal) and 5 were found in the deep soft tissues of the extremities or trunk. All examples demonstrated vacuolated epithelioid cells growing in cords or sheets in a fibromyxoid stroma. All of the metastatic carcinomas were poorly differentiated. The most common sites of origin were lung and breast (5 and 4, respectively) (Table 2). All of the carcinomas demonstrated focally or diffusely univacuolated cells with well-circumscribed intracytoplasmic lumina. Images of EHE and metastatic carcinoma demonstrating similar radiographic and histologic features are represented in Figure 1, A through D.

### Immunohistochemical Results

The immunohistochemical results are summarized in Table 2. Nuclear Fli-1 was detected in 13 cases of EHE (100%) (Figure 2, A) and in 2 cases of metastatic carcinoma (15%) (Figure 3, A). CD34 was expressed in 11 cases

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### Table 1. Summary of Antibodies Used

<table>
<thead>
<tr>
<th>Human Antigen</th>
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<td>D2-40</td>
<td>Biocare</td>
<td>1:100</td>
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<td>Fli-1</td>
<td>G146-222</td>
<td>BD Pharmingen, San Jose, Calif</td>
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**Immunostaining of EHE Versus Metastatic Carcinoma—Gill et al**
Table 2. Summary of Immunohistochemical Results for Epithelioid Hemangioendothelioma and Metastatic Carcinoma*

<table>
<thead>
<tr>
<th></th>
<th>Fli-1</th>
<th>Cytokeratin</th>
<th>CD34</th>
<th>CD31</th>
<th>Podoplanin</th>
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<td>.005</td>
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<td>MCA (n = 13), %</td>
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<td>.85</td>
<td>100</td>
<td>54</td>
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<td>0</td>
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<tr>
<td>Bladder (n = 1), %</td>
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</table>

*Positive cytokeratin staining reflects cellular expression of cytokeratin recognized by a cocktail of monoclonal antibodies (AE1, AE3, and 5D3). EHE indicates epithelioid hemangioendothelioma; MCA, metastatic carcinoma.

of EHE (85%) (Figure 2, B) and in 2 cases of carcinoma (15%) (Figure 3, B). CD31 was expressed in 13 cases of EHE (100%) (Figure 2, C) and in 5 cases of carcinoma (38%) (Figure 3, C). For Fli-1, CD34, and CD31, most positive EHE cases demonstrated 3+ staining (11/13 [85%], 9/11 [82%], 7/13 [54%], respectively). Podoplanin was expressed in 7 cases of EHE (54%) (Figure 2, D) and in 4 cases of carcinoma (31%) (Figure 3, D); these data did not represent a significant difference. Both EHE and metastatic carcinoma tended to show focal, weak staining for podoplanin (typically 2+ intensity in positive cases). Pan-keratin was positive in significantly more cases of carcinoma (100%) than EHE (38%) (P = .01) (Figure 2, E; Figure 3, E). Positive cases typically demonstrated uniform, dark cytoplasmic staining (3+ intensity). Occasional metastatic carcinomas demonstrated patchy staining of usually low intensity (1–2+) for Fli-1 (Figure 3, F), CD34 (Figure 3, G), CD31 (Figure 3, H), and podoplanin (Figure 3, I).
Figure 2. Immunohistochemistry of epithelioid hemangioendothelioma. A, Epithelioid hemangioendothelioma demonstrates nuclear positivity for Fli-1 in all cases. B, Staining for CD34 is membranous and strong but is more prone to background. Staining for CD31 (C) and podoplanin (D) is often weaker and patchy. E, Patchy, but strong, cytoplasmic cytokeratin staining is present in more than one third of cases (original magnifications ×400).

I). Fourteen of 26 cases were decalcified prior to embedding, but this did not have a significant effect on immunolocalization of any of the markers in this study (data not shown).

The sensitivity and specificity of the antibodies used are summarized in Table 3. Fli-1 and CD31 had perfect sensitivity for EHE (100%), while Fli-1 and CD34 had moderately good specificity (85%) when comparing EHE with metastatic carcinoma. In comparison, CD31 specificity (62%) was significantly lower, which is interesting given earlier reports that CD31 was likely a completely specific marker for endothelial origin.22 In our experiments, this was not the case, despite careful evaluation for false-positive staining of tumor-associated macrophages. Importantly, CD31 staining in carcinomas was generally focal and weak (1–2+). However, high sensitivity and specificity (100% and 92%, respectively) was achieved in a panel combining both Fli-1 and CD31. Cytokeratin, as might be expected, was detected in all carcinomas but also in 38% of EHEs.

COMMENT

Despite advances in imaging it is not possible to consistently distinguish EHE from metastatic carcinoma. The distinction is especially challenging in bone tumors because both EHE and metastatic carcinoma can be multifocal. Given the recent trend for core biopsy evaluation, the pathologist is increasingly faced with small samples with which to make a definitive diagnosis. The morphologic overlap between EHE and carcinoma on routine hematoxylin-eosin sections can pose a diagnostic challenge. For the previously mentioned reasons, new markers that are both sensitive and specific are needed to help make this critical distinction. In the present study, we demonstrate that a recently described marker of endothelial cell differentiation, Fli-1, shows better combined sensitivity and specificity than established endothelial markers (CD31 and CD34) in helping to distinguish between these diagnoses. Our data reinforce the observed lack of specificity of cytokeratin immunostaining for making this distinction (Figure 2, E).3 Reportedly, some keratin subtypes can be detected in epithelioid angiosarcoma (keratin 8 and 18) and EHE (keratin 7 and 18), while a small subset of EHE can also express epithelial membrane antigen.15,17,23,24 In contrast, keratin 14 and high-molecular-weight keratins detected by the antibody 34ÆE12 expression is less common.15 These data suggest that epithelioid vascular neoplasms will not be readily distinguished from carcinoma through analysis of some cytokeratin subtypes or other epithelial proteins. Despite the relative rarity of EHE, we were able to identify a sufficient number of EHEs for our analysis, which was comparable to sample sizes reported in past series.4,25 Furthermore, our report specifically compares endothelial cell marker expression between EHEs and those poorly differentiated carcinomas that closely mimic EHE by hematoxylin-eosin staining.

Podoplanin was also evaluated as a marker of endothelial differentiation but was relatively nonspecific, expressed in 54% of EHE and 31% of carcinomas tested. Our results are in contrast to the recent report that podoplanin is a useful marker of EHE in liver11 but are in keeping with other reports.26,27 We also did not note any correlation between CD34 and podoplanin staining, as previously described.11 Therefore, our data suggest that differences in immunophenotype may be observed between EHE of bone and soft tissue and EHE of liver, although additional studies are necessary to address this specific question.

Because Fli-1 is reported to be of value in identifying
Figure 3. Immunohistochemistry of metastatic carcinoma. Most metastatic carcinomas are negative for Fli-1 (A), CD34 (B), CD31 (C), and podoplanin (D) although cytokeratin staining is diffusely strong (E). Occasional metastatic carcinomas can demonstrate patchy staining of variable intensity for Fli-1 (F), CD34 (G), CD31 (H), and podoplanin (I) (original magnifications ×400).

Table 3. Sensitivities and Specificities of Immunostains for Differentiating Epithelioid Hemangioendothelioma From Metastatic Carcinoma

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity, %</th>
<th>Specificity, %</th>
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<tbody>
<tr>
<td>For detecting epithelioid hemangioendothelioma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fli-1</td>
<td>100</td>
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</tr>
<tr>
<td>CD34</td>
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<td>CD31</td>
<td>100</td>
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<tr>
<td>Podoplanin</td>
<td>54</td>
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<tr>
<td>For detecting metastatic carcinoma</td>
<td></td>
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<tr>
<td>Keratin cocktail</td>
<td>100</td>
<td>62</td>
</tr>
</tbody>
</table>

endothelial derivation of tumors, we evaluated expression of this protein in EHE and in carcinoma. For the first time, we report the utility of this marker in distinguishing an epithelioid vascular neoplasm in bone from morphologically similar metastatic carcinoma. Fli-1 protein is a nuclear transcription factor involved in proliferation and tumorigenesis. It is implicated in the pathogenesis of Ewing sarcoma/primitive neuroectodermal tumor, of which more than 85% are characterized by fusion of EWS to FLI1.28,29 Folpe et al13 have reported that Fli-1 is a highly sensitive and specific marker of both benign and malignant vascular soft tissue tumors. Nuclear immunolocalization of Fli-1 was noted to be particularly useful in distinguishing epithelioid angiosarcoma from epithelioid sarcoma and carcinoma in soft tissue. Furthermore, Rossi et al14 demonstrated Fli-1 staining in 5 of 5 EHEs and in 0 of 10 colon as well as 1 of 10 breast and 6 of 10 lung carcinomas, respectively. Although our study was not powered to test the effect of primary site on vascular marker expression in primary carcinomas, the finding of Fli-1 in lung but not breast carcinomas in our series is in general agreement with the findings of Rossi et al.14 Our study adds to these findings by demonstrating that nuclear Fli-1 staining is very sensitive (100%) and reasonably specific (85%) in identifying EHE, including in decalcified bone specimens. Fli-1 immunolocalization, in our laboratory, demonstrated very little nonspecific background staining, consistent with previous reports.15 To some ex-
tent, the specific nuclear staining of Fli-1 also leads to more objective interpretation insofar as cytoplasmic markers are prone to artifact from background staining. CD31 if used alone, even when carefully evaluated to rule out misclassification based on tumor-associated macrophage staining, is less helpful in differentiating EHE from carcinoma than might be expected. CD34 immunostaining is also able to specifically differentiate EHE from carcinoma. However, CD34 is well known to stain other mesenchymal neoplasms, and a positive result must be interpreted in the appropriate morphologic context.

The relative lack of specificity of keratin for carcinoma (62%) is in keeping with previous reports. Similarly, the low specificity of podoplanin (69%) detection for EHE in bone and soft tissue suggests minimal diagnostic utility in the immunohistochemical evaluation of this protein's expression.

In summary, Fli-1 immunostaining demonstrates better sensitivity than CD34 and better specificity than CD31. When used in combination with these two endothelial markers, Fli-1 allows for both sensitive and specific discrimination between EHE and poorly differentiated carcinoma, regardless of cytokeratin expression. We propose that a combination of Fli-1 and CD31, which together achieve 100% sensitivity and 92% specificity for EHE, represents an ideal panel in this differential diagnosis. In contrast, podoplanin fails to demonstrate utility in differentiating between EHE (in soft tissue and bone) and poorly differentiated carcinoma. Furthermore, unlike the other markers, even positive cases show relatively weak, focal (typically 2+) staining, which can be challenging to interpret when dealing with small biopsies. Our data highlight the fact that endothelial markers are not 100% specific and that immunohistochemical workup of a tumor of questionable origin still requires evaluation of a number of markers in combination. This is especially germane when interpreting cytokeratin expression, which in this setting is not specific for epithelial origin. Therefore, we conclude that, in combination with other markers, Fli-1 immunostaining is a useful adjunct when facing a differential diagnosis of EHE versus metastatic carcinoma, especially for small bone biopsies.

We thank Mark Weinstein, BS, and the staff of the San Francisco General Hospital histology laboratory for their technical assistance.

References