Comparative Evaluation of Angiogenesis Assessment with Anti-Factor-VIII and Anti-CD31 Immunostaining in Non-Small Cell Lung Cancer

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ABSTRACT

Anti-Factor VIII vessel immunostaining has been widely used in the detection of angiogenesis in non-small cell lung cancer and other tumors. Several new antibodies have shown a higher sensitivity, and anti-CD31 has recently been proposed to be the standard for microvessel study. In the present study, we comparatively evaluated the two antibodies in 134 cases of early operable non-small cell lung cancer. The F8/86 (anti-Factor VIII-associated antigen) and JC70 (anti-CD31) MoAbs were used in paraffin-embedded material. Eye appraisal of vascular grade (VG) and microvessel score (MS) was performed by three experienced pathologists. Different cutoff points were used for the analysis of VG and MS correlation with nodal involvement, overall survival, and thymidine phosphorylase expression. Intra- and interobserver variability was minimal for both antibodies. MS and VG were significantly correlated with each other. However, 54 and 22% of cases with high anti-CD31 VG or high MS, respectively, had low vascularization on anti-Factor VIII assessment. Anti-CD31 scoring was significantly associated with nodal involvement and overall survival for all cutoff points considered, which was not verified for anti-Factor VIII staining. VG was the most significant indicator of nodal involvement and survival for both antibodies. Tumors with high VG by anti-CD31 but low or medium VG by anti-Factor VIII behaved as tumors of high neoangiogenesis, defining a poor prognosis (P = 0.005) despite the failure of anti-Factor VIII antibody to highlight intense neoangiogenesis. Anti-CD31 MS significantly associated with thymidine phosphorylase overexpression (P = 0.01), whereas no correlation was found for anti-Factor VIII counting. It was concluded that anti-CD31 microvessel immunostaining has several advantages over anti-Factor VIII, being a more sensitive method for highlighting small, immature microvessels or single endothelial cells. This could be of importance in revealing possible correlation of tumor angiogenesis with metastatic behavior, prognosis, or angiogenic factor overexpression. Vascular grading was the best method for neovascularization assessment, efficiently defining groups of tumors with aggressive clinical course.

INTRODUCTION

Tumor neoangiogenesis as a metastasis-defining mechanism and also as a prognostic marker is under intense investigation (1). A large body of studies on immunohistochemical staining of microvessels and subsequent assessment of microvessel density shows an association of the degree of intratumoral neovascularization with metastatic disease and poor prognosis in a variety of human cancers, although several studies disagree (2). This may be due to methodology differences. Anti-Factor VIII antibody was one of the first used for microvessel staining and the most popular in the published clinicopathological studies. However, new antibodies with a higher specificity, such as anti-CD31, anti-CD34, and PAL-E, have recently been introduced (3–5).

The first study correlating microvessel density with prognosis in non-small cell lung cancer was reported by Macchiarini et al. in 1992 (6). The anti-Factor VIII immunostaining used in that study was subsequently followed by four other studies (7–10) confirming the prognostic significance of microvessel density. The first study of non-small cell lung cancer using the anti-CD34 antibody was reported by Giatromanolaki et al. (11), showing a strong correlation of VG with node involvement and overall survival. Anti-CD34 MoAb was used by Fontanini et al. (12) to show a significant increase of microvessel density during disease progression form hyperplasia to in situ and invasive carcinoma.

An international consensus on the methodology and criteria of evaluation of microvessel density proposed that anti-CD31 MoAb immunostaining should be the standard for microvessel assessment (13), although it is stressed that unresolved technical aspects require a comparative confirmation. In the present study,

1 The abbreviations used are: MoAb, monoclonal antibody; PECAM, platelet/endothelial cell adhesion molecule; MS, microvessel score; CI, confidence interval; VG, vascular grade; HVG, high VG; MVG, medium VG; LVG, low VG; HLI, high lymphocytic infiltration; LLI, low lymphocytic infiltration; TP, thymidine phosphorylase.

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we comparatively evaluated microvessel staining using anti-CD31 and anti-Factor VIII-associated antigen antibodies in non-small cell lung cancer. Differences in microvessel counting and their role in predicting metastatic potential and overall survival, as well as in the assessment of potential correlation of microvessel density with angiogenic factor overexpression, are reported.

PATIENTS AND METHODS

Patients and Tissues. We examined a series of 134 tumor samples from patients with operable (stages T1-2N0-1; Ref. 14) non-small cell lung cancer. Tumors other than adenocarcinomas and squamous cell carcinomas were excluded. All patients were treated with surgery alone and survived at least 60 days after operation (to exclude perioperative mortality related bias). The median follow-up period was 32 months (maximum, 7 years).

Histological diagnosis, grading and N-stage was performed on H&E-stained sections. Eighty-eight cases were squamous cell carcinomas, and 46 were adenocarcinomas. Lymph node involvement was present in 48 of 134 cases. Histological grade I/II was confirmed in 62 cases, and grade III was confirmed in 72 cases.

Assessment of VG. The JC70 MoAb (DAKO) recognizing CD31 (PECAM-1; Ref. 3) was used for microvessel staining on 5-μm paraffin-embedded sections using the alkaline phosphatase/anti-alkaline phosphatase procedure. Sections were dewaxed, rehydrated, and predigested with protease type XXIV for 20 min at 37°C. JC70 as undiluted supernatant was applied at room temperature for 30 min and washed in TBS. Rabbit anti-mouse antibody (1:50) was applied for 30 min, followed by application of mouse alkaline phosphatase/anti-alkaline phosphatase complex (1:1) for 30 min. After washing in TBS, the last two steps were repeated for 10 min each. The color was developed by a 20-min incubation with New Fuchsin solution.

The F8/86 mouse antihuman MoAb (DAKO) recognizing the Factor VIII-related antigen was used to highlight microvessels. Sections were dewaxed, rehydrated, and predigested with protease type XXIV for 20 min at 37°C. A 60-min incubation with F8/86 MoAb was followed by TBS washing, and thereafter, sections were incubated for 30 min after applying the secondary antibody (biotinylated goat antimouse diluted in TBS 1:200). Afterward, sections were incubated with 100 μl of ABC/AP kit (DAKO) at room temperature for 30 min and washed in TBS for 3 min. The color was developed with New Fuchsin substrate for 20 min, and counterstaining with hematoxylin was subsequently performed.

Eye appraisal of immunohistochemically stained microvessels and microvessel counting were used for angiogenesis assessment. For eye appraisal, sections were scanned at low power (×40 and ×100); afterward they were scanned at ×250 to group cases in three VG categories (LVG, MVG, and HVG). The areas of the highest vascularity were chosen at low power (×100), and microvessel counting followed on three chosen ×250 fields of the highest density. The MS was the sum of the vessel counts obtained in these three fields. Microvessels adjacent to normal lung were excluded from the appraisal. Vessels with a clearly defined lumen or well-defined linear vessel shape, but not single endothelial cells, were taken into account for microvessel counting. Because endothelial cells were also counted in most of the studies, we separately assessed the endothelial cell score by counting single endothelial cells in the same fields in which microvessel counting was performed.

Definition of Cutoff Points for MS. Several cutoff points for MS were considered to test MS correlation with nodal involvement and overall survival. Three statistical points were the median, the mean MS obtained for anti-CD31 and anti-Factor VIII staining, and the MS suggested by the upper 95% CI. The MS corresponding to the 50% survival rate at the time of analysis was also considered. Eye appraisal defined two cutoff points that grouped cases in LVG, MVG, and HVG groups.

To allow objective comparison of a MS obtained using the two antibodies, we also used the retrospectively derived "adjusted" cutoff point, which was the MS that defined the group of patients with the highest percentage of death events at the time of analysis (Fig. 1 and Table 1). This group included more than 30% (38 of 134 cases) of the analyzed cases. Although the adjusted cutoff point defined a different percentage of high angiogenesis cases when anti-CD31 was considered than when anti-Factor VIII staining was considered, we thought this was the point at which each antibody might show the best prognostic correlation.

TP Immunohistochemistry. To test the validity of the two antibodies in correlation with angiogenesis, TP expression

Table 1 Percentage of death events observed using sequential MSs obtained with anti-CD31- and anti-Factor VIII-associated antigen staining.

<table>
<thead>
<tr>
<th>Anti-CD31</th>
<th>Anti-Factor VIII</th>
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<tbody>
<tr>
<td>MS</td>
<td>Death events</td>
</tr>
<tr>
<td>&gt;75 &amp;</td>
<td>23/38 (61%)</td>
</tr>
<tr>
<td>&gt;65 &amp;</td>
<td>24/44 (54%)</td>
</tr>
<tr>
<td>&gt;55 &amp;</td>
<td>29/52 (56%)</td>
</tr>
<tr>
<td>&gt;45 &amp;</td>
<td>33/65 (51%)</td>
</tr>
<tr>
<td>&gt;25 &amp;</td>
<td>46/98 (47%)</td>
</tr>
</tbody>
</table>

* Death events, group of patients with MS > 1.
* Adjusted cutoff point.
was assessed with the P-GF.44C MoAb (15). This enzyme has shown in vitro angiogenic properties, and its overexpression associates with high neovascularization in breast, gastric, colon, and non-small cell lung cancer (16–19). Staining was performed with the streptavidin-biotin-peroxidase (DAKO) technique that has been described previously (20). Tumors were assessed for TP expression by the intensity and extent of staining. Two staining groups were considered: low reactivity (0–50% of cells stained) and high reactivity (strong staining in more than 50% of cells).

Statistical Analysis. Statistical analysis and graphs were performed using the Stata 3.1 package (Stata Corp., College Station, TX) and the GraphPad Prism 2.01 package. Survival curves were plotted using Kaplan-Meier analysis, and the log-rank test was used to determine statistical differences between life tables. A Fisher’s exact test or a paired or unpaired two-tailed t test was used for testing relationships between categorical tumor variables, as appropriate. Nonparametric analysis was used to assess intra- and interobserver variability and correlation between microvessel counts.

RESULTS

VG and MS. Normal lung vasculature was equally well delineated in both JC70- and F8/86-stained slides. In tumors, however, a substantially higher number of vessels, and particularly small vessels, were clearly better seen with anti-CD31 staining. Cross-reactivity with CD31-positive lymphocytes was seen with the anti-CD31 staining. This did not complicate vessel assessment and could also be used in quantifying CD31-positive infiltration, a parameter that could be of pathogenetic and prognostic importance (21). Fig. 2, a and b, shows two HVG cases, one with LLI and one with HLI. Anti-Factor VIII antibodies very often cross-reacted with tumor cells and RBC tissue contamination. Large or medium-sized vessels were well stained, but small vessels, clearly defined in the anti-CD31-stained slides, were less frequently identified with anti-factor VIII immunostaining (Fig. 3, a and b).

Using the anti-CD31 staining, HVG, MVG, and LVG by eye appraisal was observed in 39, 24, and 71 patients, respectively. Using the anti-Factor VIII antibody, HVG, MVG, and LVG was observed in 24, 31, and 79 patients, respectively. MS assessed with anti-CD31 ranged between 9 and 213 (median, 45; mean 60.5 ± 46.1; 95% CI, 52–68), whereas a significantly lower score (P < 0.00001) was obtained with anti-factor VIII staining (range, 8–23; median, 19; mean, 26.8 ± 21.3; 95% CI, 23–30). The mean number of microvessels detected with JC70 was 2.26 times as high as F8/86. A significant association of MS with eye appraisal for anti-CD31 (P < 0.0001; r = 0.82) and anti-factor-VIII (P < 0.0001; r = 0.63) staining was also observed.

Single endothelial cells were clearly stained with JC70, whereas stained single cells with anti-Factor VIII often raised doubts about cell origin (RBC contamination, etc.; Fig. 3). The MS for 31 cases with high MS (in both JC70- and F8/86-stained slides) was 120 ± 37 and 55 ± 24, respectively, showing a microvessel number 2.18 times as high as that obtained with anti-CD31 staining in high angiogenesis cases. The number of single endothelial cells detected with JC70 was 3.95 times higher (77 ± 25 versus 20 ± 11), showing a severe deficiency of anti-Factor VIII staining to detect immature endothelial component (Fig. 4).

Intra- and Interobserver Variability. Both VG (eye appraisal) and MS were examined for intra- and interobserver variability. Three experienced pathologists assessed the slides separately and repeated the assessment 10–30 days later. The second assessment highly correlated with the first for all observers (r > 0.90; P < 0.0001 for VG; r = 0.93 and P < 0.0001 for MS). Similarly, the three investigators’ vascular grading and MS highly correlated to each other (r > 0.90; P < 0.0001). The final decision was made at the conference microscope.

MS and Probability of Death. In Fig. 1, the probability of death is plotted with linear regression analysis. Death probability refers to the group of patients defined by a MS score equal to or higher than the value reported on the X axis. Both anti-CD31 and anti-Factor VIII gave a line with a slope (0.2 ± 0.01 and 95% CI, 0.18–0.24 for anti-CD31; 0.50 ± 0.09 and 95% CI, 0.29–0.72 for anti-Factor VIII) that significantly differed from zero (P < 0.0001 and P = 0.0006, respectively). Using these curves, we defined the adjusted cutoff point (maximum % of death events) for MS: it was 75 for anti-CD31 and
Fig. 3 High vascularization and intense endothelial cell aggregation observed with JC70 MoAb staining (a and c; ×250). F8/86 vessel staining revealed a low number of vessels in the same tumor area (b and d; ×250).

Table 2 Comparative analysis of VG (eye appraisal) and of microvessel counts obtained with anti-CD31- and anti-Factor VIII-associated antigen MoAbs

<table>
<thead>
<tr>
<th>Anti-CD31</th>
<th>Anti-Factor VIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>VG</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>18</td>
</tr>
<tr>
<td>Medium</td>
<td>4</td>
</tr>
<tr>
<td>Low</td>
<td>2</td>
</tr>
<tr>
<td>Microvessel counting</td>
<td></td>
</tr>
<tr>
<td>MS &gt;74</td>
<td>9</td>
</tr>
<tr>
<td>MS &lt;75</td>
<td>75</td>
</tr>
</tbody>
</table>

The Spearman correlation test showed a significant association between MS assessed with anti-CD31 and anti-Factor VIII antibodies ($P < 0.0001$) but a relatively low r factor ($r = 0.68$). Taking into account the adjusted cutoff point (Table 2), 9 of 40 (22%) cases with a high anti-CD31 MS (>74) had a low score (<25) with anti-Factor VIII assessment. Moreover, 19 of 50 (38%) cases with a high anti-Factor VIII MS (>24) had a low (<75) score with anti-CD31 assessment. Thus, a wide discrepancy (up to 54%) between the two antibodies in defining

25 for anti-Factor VIII staining. The 50% survival probability was pointed at MSs of 40 and 19 for anti-CD31 and anti-Factor VIII assessment, respectively.

VG and MS Assessment Comparison. Table 2 shows the correlation between VG as assessed with the two antibodies. Although a significant association was confirmed ($P < 0.0001$), 21 of 39 (54%) cases with HVG on anti-CD31-stained slides were of LVG or MVG on anti-Factor VIII-stained ones.

Fig. 4 MS (clearly defined vessels), endothelial cell score (ECS; endothelial cell counting), and total score (TS) obtained with JC70 and F8/86 MoAbs.
groups of patients with high neoangiogenesis was observed for both eye appraisal and microvessel counting.

**Nodal Involvement Analysis.** Eye appraisal VG categories significantly correlated with nodal involvement for both anti-CD31 (P < 0.0001) and anti-factor VIII staining (P = 0.0004). High anti-CD31 MS significantly correlated with nodal involvement for every cutoff point considered (P < 0.0004). However, this was not the case for anti-Factor VIII staining. The eye appraisal was the most significant predictor of nodal involvement (Table 3).

**Overall Survival Analysis.** In Table 3, the impact of high neoangiogenesis on the overall survival was analyzed using different cutoff points for the definition of the group of tumors with angiogenic phenotype. A significant prognostic role of neoangiogenesis assessed with anti-CD31 antibody was observed using the eye appraisal (P = 0.005), the adjusted cutoff point (P = 0.004), and the upper 95% CI (P = 0.02). High vascularization assessed with anti-Factor VIII was marginally significant using eye appraisal (P = 0.04) and significant only for the adjusted cutoff point (P = 0.01).

Fig. 5, a and b, shows the Kaplan-Meier survival curves obtained by stratifying for VG (eye appraisal) using anti-CD31 and anti-Factor VIII immunostaining. Stratifying for both anti-CD31 and anti-factor VIII, cases with HVG by anti-CD31 assessment but LVG by anti-Factor VIII assessment had a significantly poorer survival as compared to LVG cases (P = 0.005; Fig. 5c). VG analysis in node-negative patients did not reveal any significant prognostic impact on survival for either anti-CD31 or anti-Factor VIII MS assessment.

Survival analysis for MS (adjusted cutoff point) did not show any difference between anti-CD31- and anti-Factor VIII-defined groups. The small number of cases with high MS on anti-CD31 but low MS on anti-FVIII did not permit analysis. Nineteen cases with low anti-CD31 MS and high anti-Factor VIII MS had a prognosis similar to low MS cases (P = 0.45) but not a significantly better prognosis than cases with high anti-CD31 MS (P = 0.21), probably because of the small number of cases. Nor anti-CD31 not anti-Factor VIII MS defined a group with significantly poorer prognosis in node-negative patients.

**CD31-positive Lymphocytic Infiltration Analysis.** Anti-CD31 MoAb stained an inflammatory cell component that morphologically is plasma cell. Cases could subjectively be grouped in two categories with LLI and HLI (LLI, 75 cases; HLI, 59 cases). HLI was marginally associated with HVG as assessed with anti-CD31 (P = 0.04) but not with anti-Factor VIII (P = 0.80). The anti-CD31 MS was not significantly higher in the HLI cases (63 ± 48 versus 58 ± 44; P = 0.57). Overall survival analysis did not reveal any prognostic difference between HLI and LLI (Fig. 6a). However, stratifying for lymphocytic infiltration within the HVG (anti-CD31 eye appraisal) group, a significantly better prognosis was seen in cases with HLI (P = 0.002; Fig. 6b). Repeated slide evaluation of the HVG/HLI subgroup confirmed that vascular grading was definitely not biased by intense CD31-positive lymphocytic infiltration. Lymphocytes were often aggregated around the vessel walls and into the vessel lumen (Fig. 2b).

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**Table 3** Significance of high angiogenesis association with nodal involvement and overall survival using different cutoff points

<table>
<thead>
<tr>
<th>Cutoff point</th>
<th>Anti-CD31 (P)</th>
<th>Anti-Factor VIII (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean MS</td>
<td>0.0002</td>
<td>0.01</td>
</tr>
<tr>
<td>Median MS</td>
<td>0.0004</td>
<td>0.14</td>
</tr>
<tr>
<td>Upper 95% CI</td>
<td>0.0004</td>
<td>0.03</td>
</tr>
<tr>
<td>50% survival</td>
<td>0.0009</td>
<td>0.14</td>
</tr>
<tr>
<td>VG</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
</tr>
<tr>
<td>Adjusted</td>
<td>&lt;0.0001</td>
<td>0.01</td>
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</tbody>
</table>

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**Fig. 5** Overall survival analysis for 134 non-small cell lung cancer cases treated with surgery alone. a, stratification for anti-CD31-assessed VG; b, stratification for anti-Factor VIII-assessed VG; c, the group of patients with HVG on anti-CD31 and LVG on anti-Factor VIII staining had poor prognosis.
Correlation with TP Expression. Nineteen of 39 (49%) of cases with high TP reactivity were of HVG in anti-CD31 assessment versus 20 of 95 (21%) cases with low TP expression ($P = 0.006$). However, no correlation between VG and TP reactivity was shown using the anti-Factor VIII staining, because 8 of 39 (20.5%) and 16 of 95 (17%) of cases with high and low TP reactivity, respectively were of HVG ($P = 0.87$).

Fig. 7 shows the MS obtained with anti-CD31 and anti-Factor VIII antibodies for cases with high and low TP reactivity. Although MS assessed with anti-CD31 was significantly higher in the high-TP reactivity group, no significant difference was confirmed by analyzing for anti-Factor VIII MS. The mean score assessed with anti-CD31 staining was $54 \pm 40$ and $76 \pm 54$ in low- and high-TP reactivity cases, respectively ($P = 0.01$). The mean MSs assessed with anti-Factor VIII staining were $25 \pm 18$ and $30 \pm 26$, respectively ($P = 0.16$).

DISCUSSION

The aim of the present study was to test the use of anti-CD31 microvessel staining as a more sensitive and useful marker for clinicopathological studies compared to the more widely used anti-Factor VIII antibody for non-small cell lung cancer. Physiological properties of normal vasculature are quite different from tumoral vasculature. Tumor vessels are permeable to macromolecules (22), and lack of differentiation features, such as alkaline phosphatase and S'-nucleotidase endothelial cell activity, has been reported (23). The lack of tumor vasculature endothelial cell differentiation is believed to be the reason why markers of normal endothelium, such as Factor VIII-related antigen, are not always reliable (24, 25). On the other hand, PECAM-1 (CD31) not only is highly expressed in mature and immature endothelium (>10^6 molecules/cell) but its localization at the endothelial cell junctions suggests an important role in transendothelial cell migration (26, 27). In our study, eye appraisal clearly showed that anti-CD31 staining highlights smaller vessels occasionally detected by anti-Factor VIII staining. Although normal lung vasculature was well shown by both antibodies, tumor microvessel counting showed a significant discrepancy between the number of stained microvessels per visual field; the mean value was 2.3 times as high for anti-CD31 staining. Of interest, single endothelial cells were only occasionally stained with anti-Factor VIII; the anti-CD31 staining detected 4 times as many endothelial cells in high-angiogenesis cases.

Counting of any single endothelial cell or very small clusters results in a lower interobserver variability (13). However, intra- and interobserver variability using the criteria reported in “Patients and Methods” was minimal. We chose to base our analysis on vessel counting and to count single endothelial cells separately because tumor ability to form functional microvessels (tube formation) could be a step in the disease pathogenesis distinct from endothelial cell migration and/or proliferation (28–30). Counting of single endothelial cells could well be indirect evidence of angiogenic factor activity that could confer aggressive tumor behavior through mechanisms in addition to angiogenesis (e.g., stimulation of cancer cell migration). We recently showed that TP overexpression, although it correlates with angiogenesis, defines a group of LVG tumors with poorer prognosis (19). Similarly, TP overexpression in bladder cancer, although it did not correlate with microvessel count, was significantly associated with aggressive behavior (31). Paracrine effects of endothelial cells are well established, and active endothelial cells may interact with tumor stroma and infiltrating inflammatory components or even tumor cells (32). Microvessel density counting single endothelial cells may be an indirect evaluation of complex angiogenesis-related phenomena that could be of major prognostic importance but could not be assessed with anti-Factor VIII antibodies.

The probability of death using MS as serial cutoff point gave a line with a slope that was significantly non-zero, showing that the quantity of functional microvessels is proportionally associated with the clinical outcome. The incidence of metastases to the lymph nodes was uniformly highly associated with the anti-CD31 MS no matter what the cutoff point was, which was not the case for anti-Factor VIII MS. Of interest, VG assessed with eye appraisal was the most important cutoff point that defined HVG association with nodal involvement and survival.
for both anti-CD31 and anti-Factor VIII antibodies. This could be explained by the fact that eye appraisal VG is based on the scanning of all assessable visual fields, allowing the experienced pathologist to reliably estimate the overall vessel component. Two highly angiogenic areas are enough to give a high MS, but eye appraisal would classify the case in the MVG group if the remaining 20 (or more) fields show a low vascularization. Given, also, the high variability of microvessel count reported in the literature (mean value per field ranging from 8 to 75; Refs. 6 and 7), as well as the significant association of vascular grading with microvessel count, it is suggested that eye appraisal performed by an expert pathologist should safely be the standard technique for angiogenesis assessment in the clinical practice (33).

Platelet-derived endothelial cell growth factor (TP) is a protein with a wide range of activities, including DNA synthesis (34), stimulation of angiogenesis, and endothelial cell migration (35). A significant association of TP expression with neoangiogenesis has been reported in several human tumors (16–19). In the present study, MS assessed with anti-CD31 but not with anti-Factor VIII correlated with TP overexpression. This should be attributed to the higher sensitivity of the anti-CD31 antibody in staining small vessels with immature endothelium, which is the main evidence of active neoangiogenic process within the tumor. The results suggest that erroneous results concerning correlation of angiogenic factor expression with microvessel density may be obtained using anti-Factor VIII antibody. The marginally significant association (P = 0.03 and 0.05) of microvessel density (anti-Factor VIII antibody) with TP expression in colon (18) and breast (16) cancer might have been better interpreted after anti-CD31 vessel immunostaining.

CD31-positive lymphocytes are definitely and clearly stained with JC-70 antibody (not occasionally, as reported in Ref. 13), and intense lymphocytic infiltration is observed in about 45% of non-small cell lung cancer cases (21). However, this is far from being a confusing factor because endothelial cells are easily differentiated from the inflammatory stained component. Although CD31-stained lymphocytes morphologically resemble plasma cells, several studies show that CD31 is a differentiation antigen of a human CD4 T-cell subpopulation lost during maturation into T-helper effector cells (36). We observed that CD31-positive lymphocytes were aggregated in close proximity to the vessel walls or even into the vessel lumen, which may show a role of PECAM in transendothelial lymphocyte migration. This is supported by the study of Pilia et al. (37) showing that CD31/PECAM-1 is involved in the adhesion of leukocytes to endothelium. In our study, intense CD31-positive lymph cell infiltration defined a group of HVG cases with a better prognosis. This observation is in accordance with a previous study by Pupa et al. (38), in which intense lymphocytic infiltration correlated with better outcome in breast cancer cases with poor prognostic features, such as high histological grade and c-erbB-2 overexpression. Moreover, Anastassiou et al. (39) showed that CD31-positive lymphocytic infiltration in renal tumor was associated with better prognosis. The ability of JC70 to stain CD31-positive lymphocytes should therefore be considered an advantage over anti-Factor VIII staining because it permits the evaluation of tumor infiltration by a pathogenetically and prognostically important inflammatory component.

It is concluded that anti-CD31 microvessel immunostaining has several advantages over anti-Factor VIII; it is a more sensitive method for small microvessels and results in a more reliable MS or eye appraisal VG. This could be of importance in accurately evaluating the possible correlations between tumor vasculature, metastatic behavior, prognosis, and angiogenic factor overexpression. Eye appraisal has been shown to be the best method for neovascularization assessment, efficiently defining groups of tumors with aggressive behavior and thus limiting the necessity of artificial cutoff points. The advantage of lymphocytic infiltration grading after anti-CD31 immunostaining should not be underestimated because intense lymphocytic infiltration may prove to be of particular importance in highly angiogenic tumors.

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