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Tumor Angiogenesis Correlates with Metastasis in Invasive Prostate Carcinoma

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Tumor growth and metastasis require angiogenesis; and microvessel density, a measure of tumor angiogenesis, correlates with metastasis in breast and lung carcinoma. To determine how microvessel density correlated with metastasis in prostate carcinoma, we counted microvessels within the initial invasive carcinomas of 74 patients (29 with metastasis, 45 without). Microvessels were highlighted by immunostaining endothelial cells for factor VIII-related antigen. Without knowledge of the patient's cancer stage, microvessels were counted in a 200 field (0.739 mm^2) in the most active areas of neovascularization. The mean microvessel count in tumors from patients with metastases was 76.8 microvessels per 200 field (median, 66; standard deviation, 44.6). The counts within carcinomas from patients without metastasis were significantly lower, 39.2 (median, 36; standard deviation, 18.6) ($P < 0.0001$). Microvessel counts increased with increasing Gleason's score ($P < 0.0001$), but this increase was present predominantly in the poorly differentiated tumors. Although Gleason's score also correlated with metastasis ($P = 0.01$), multivariate analysis showed that Gleason's score added no additional information to that provided by microvessel count alone. Assay of microvessel density within invasive tumors may prove valuable in selecting patients for aggressive adjuvant therapies in early prostate carcinoma. (Am J Pathol 1993, 143:401–409)

Prostate carcinoma is currently the most common cancer in American men.[1] The incidence of prostate carcinoma increases with age, and in autopsy studies, 71% of men between 80 to 89 years have latent prostate carcinoma.[2] The fact that only 30,000 American men die of prostate carcinoma annually[1] suggests that the majority of prostate cancers do not progress. Moreover, thousands of latent cases are discovered incidentally in prostatectomy specimens performed for benign prostatic hyperplasia.[3] Thus, development of accurate prognostic indicators that correlate with outcome would help to determine which patients may require aggressive adjuvant therapy because of being at high risk for carcinoma recurrence and death.

A new prognostic indicator should possess a clear biological significance.[4] Much experimental evidence exists showing that tumor growth and metastasis are dependent upon tumor angiogenesis.[5–7] Tumor angiogenesis is the growth of new vessels toward and within a tumor.[8–10] Such neovascularization may be stimulated by factors released from the tumor cells, tumor-associated inflammatory cells, and/or from the extracellular matrix.[8–10]

The first evidence that the intensity of angiogenesis in a human tumor could predict the probability of metastasis was reported for cutaneous melanoma.[11–13] Subsequently, Weidner et al[14] demonstrated a statistically significant correlation ($P < 0.001$) between incidence of metastases and density of microvessels (a measure of angiogenesis) in histologic sections of 49 primary invasive breast carcinomas stained to detect factor VIII-related antigen (F8-RA). These initial findings have now been confirmed by a number of similar studies not only in breast carcinoma,[15–18] but also non-small-cell lung carcinoma.[19] Furthermore, in a follow-up study, Weidner et al[20] have found that microvessel density in the area of most intense neovascularization in invasive breast carcinoma is an independent, highly significant, and accurate prognostic indicator in predicting overall and relapse-free survival in patients with early stage breast carcinoma.

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This new indicator is likely to prove useful in selection of high-risk, node-negative patients with breast carcinoma for systemic adjuvant therapies.

To extend these initial studies to other cancer sites, we asked if the extent of angiogenesis in human prostate carcinoma correlated with metastasis. The hypothesis we wished to test is that those lesions that show little angiogenesis should have a relatively low metastatic rate, whereas those lesions that have a higher angiogenic state should have an increased metastatic potential. If true, such information might prove valuable in deciding whether or not to add adjuvant therapies to those patients having highly angiogenic prostate carcinomas.

Materials and Methods

Patients

Tumor specimens from 74 unselected patients with invasive prostate carcinoma (29 with metastasis and 45 without metastasis) were evaluated for this study. Specimens were retrieved from paraffin-embedded tissues of the radical prostatectomy specimens diagnosed at Moffitt-Long Hospital (24 cases) (San Francisco, CA), San Francisco Veterans Administration Hospital (25 cases), and the Brigham and Women's Hospital (25 cases) (Boston, MA).

Metastasis was defined as histologically proven carcinoma in pelvic lymph nodes from the radical prostatectomy specimens, positive bone scan indicative of metastatic prostate carcinoma, and/or abnormally elevated serum prostate-specific antigen (PSA) postoperatively. PSA elevations caused by local recurrence only were not considered evidence of metastasis. Nodal status was determined from the initial surgical pathology report, and follow-up was obtained by evaluating the patients' charts, and by collaboration with the Tumor Registry of the Brigham and Women's Hospital. Tumor grading (on hematoxylin and eosin (H&E) stained sections) followed the criteria of Gleason.[21]

Vessel Staining and Counting

Microvessel staining and counting followed procedures as previously published.[14] Briefly, microvessels were highlighted by staining endothelial cells for factor VIII-related antigen (F8-RA) (also known as von Willebrand factor), using a standard immunoperoxidase technique. Figure 1 illustrates how the staining technique highlights microvessels and shows how different tumors showed different microvessel densities. H&E-stained sections of the prostate carcinoma were used to choose invasive areas of the tumor (Figure 1A).

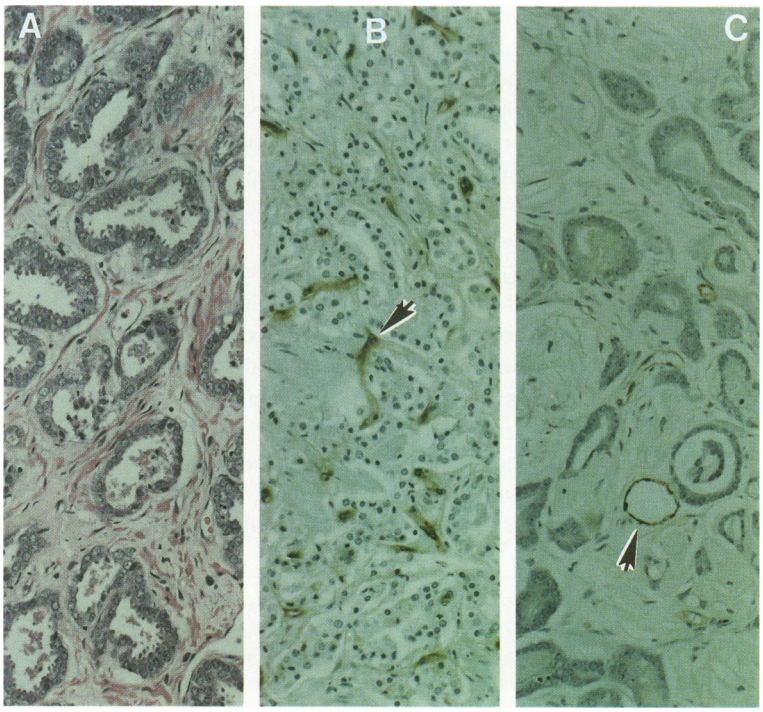

Figure 1. *Invasive prostate carcinomas stained for factor VIII-related antigen showing variable microvessel densities.* **(A, left)** *Invasive prostate carcinoma stained with H&E. Many microvessels are difficult to identify (original magnification [OM] × 50).* **(B, middle)** *From the same block as A, left is an invasive carcinoma immunostained for factor VIII-related antigen and showing relatively high angiogenesis (metastasizing tumor). Arrows indicate some representative microvessels; 22 microvessels were counted in the field shown in the photograph (Diaminobenzidine [DAB] immunoperoxidase stain with anti-factor VIII related antigen, OM × 50).* **(B, right)** *A second invasive carcinoma with relatively low angiogenesis (non-metastasizing tumor).* Arrows indicate some representative microvessels; 4 microvessels were counted in the field shown in the photograph. *(Diaminobenzidine [DAB] immunoperoxidase stain with anti-factor VIII related antigen, OM × 50).*

Microvessel density was assessed by light microscopy in areas of invasive tumor containing the highest numbers of capillaries and small venules per area (neovascular "hot spots"). Often, tumors were frequently heterogeneous in their microvessel content, but areas of highest neovascularization were found by scanning the tumor sections at low magnification (×40 and ×100) and identifying those areas of invasive carcinoma having the greatest numbers of distinct F8-RA staining (brown) microvessels per area. These high neovascular areas could occur anywhere within invasive tumor. Sclerotic areas within tumor, where microvessels were sparse, and areas immediately adjacent to benign prostate tissue were not considered in vessel counts. However, these microvessels served as internal controls for the quality of the F8-RA immunostaining.

After the area of highest neovascularization was identified, individual microvessel counts were made on a ×200 field (×20 objective and ×10 ocular, 0.739 mm^2 per field). Any brown-staining endothelial cell or endothelial cell cluster, clearly separate from adjacent microvessels, tumor cells, and other connective-tissue elements, was considered a single, countable microvessel (Figure 1B and 1C). Vessel lumens were not necessary for a structure to be defined as a microvessel, and red cells were not used to define a vessel lumen. Results were expressed as the highest number of microvessels identified within any single ×200 field.

Statistics

We used the Spearman rank correlation coefficient to examine the relationship between Gleason's score and microvessel count.[22] Since microvessel counts are not normally distributed, for univariate analysis we used the Wilcoxon rank sum test[23] to determine whether there were significant differences between the median microvessel counts in tumors with and without metastases in all patients, as well as in tumor categories formed by combining Gleason's scores 4 and 5 (well differentiated), 6 and 7 (moderately differentiated), and 8 through 10 (poorly differentiated). Lastly, for multivariate analysis we used logistic regression and the likelihood ratio ^{2} test to see if microvessel count contributed significant information about metastases above and beyond that of the Gleason's score.[24,25]

Results

Patients

Of the 74 unselected patients with invasive prostate carcinoma 29 showed metastases and 45 developed no metastases within the follow-up period. The mean follow-up for patients with metastases was 42.2 months (median, 34; standard deviation, 30.8 months; range, 2 to 102); whereas the mean follow-up for patients without metastases was 53.5 months (median, 50; standard deviation, 36.3 months; range, 2 to 144). The mean age for patients with metastases was 66.5 years (median, 67; standard deviation, 7.9 years; range, 44 to 80); whereas the mean age for patients without metastases was 66.6 years (median, 67; standard deviation, 6.9 years; range, 53 to 86). No statistically significant differences were found between the two groups for age at presentation (P = 0.89) and length of follow-up (P = 0.24) (Table 1).

Microvascular Density and Metastatic Disease

Within invasive prostate carcinomas from patients having metastases the mean microvessel count was 76.8 per ×200 field (median, 66; standard deviation, 44.6; range, 20 to 193) (Table 1). For those car-

Table 1. *Comparison of Prostate Carcinoma Patients with and Without Metastatic Disease*

	Patients with metastases n = 29	Patients without metastases n = 45	
	Mean (s.d., range)	Mean (s.d., range)	*P* Value
Microvessel count per ×200 field	**76.8 (44.6, 20–193)**	**39.2** (18.6, 10–110)	**<0.0001**
Tumor score (Gleason's)	**6.9** (1,8, 4–10)	**5.8** (1.9, 4–10)	**0.009**
Patient's ages (years)	66.5 (7.9, 44–80)	66.6 (6.9, 53–86)	0.89
Follow-up (months)	42.2 (30.8, 2–102)	53.5 (36.3, 2–144)	0.24

s.d. = standard deviation, statistically significant differences in bold type as calculated by the Wilcoxon rank sum test.

cinomas from patients without metastases the mean microvessel count was 39.2 per ×200 field (median, 36; standard deviation, 18.6, range, 10 to 110). The Wilcoxon rank sum test showed these differences to be statistically significant ($P < 0.0001$) (Table 1).

We plotted the percent of patients with metastatic disease in whom their vessel count fell within one of four distinct categories defined by progressive 33 vessel increments (Figure 2). This plot shows that the incidence of metastatic disease increases as vessel counts increase. Seventeen percent (4) of the 23 patients with microvessel counts up to 33 per ×200 microscopic field had or developed metastases. There was, however, a steady increase in the incidence of metastases as the microvessel count increased: 31% (10) of the 32 patients having microvessel counts of 34–67, 60% (6) of the 7 with microvessel counts of 68–100, and 100% (9) of the 9 with counts exceeding 100 per 200x microscopic field.

Histologic Grade (Gleason's Score) and Its Association with Microvessel Density

Within invasive prostate carcinomas from patients having metastases the mean Gleason's score was 6.9 (median, 7; standard deviation, 1.8; range, 4 to 10). For those carcinomas from patients without metastases the mean Gleason's score was 5.8 (median, 5; standard deviation, 1.9; range, 4 to 10). The Wilcoxon rank sum test showed these differences to be statistically significant $P = 0.009$ (Table 1).

Microvessel counts significantly increased with increasing Gleason's score with this increase being most apparent among the more poorly differentiated tumors (Spearman R = 0.60, $P < 0.0001$) (Figure 3). The mean microvessel count for well differentiatedcarcinomas (Gleason's scores of 4 or 5) was 34 (median, 30; standard deviation, 16.6; range, 10 to 73); for moderately differentiated carcinomas (Gleason's scores of 6 or 7) the mean microvessel count was 56 (median, 48; standard deviation, 28.6; range, 20 to 139); and for poorly differentiated carcinomas (Gleason's 8, 9, & 10) the mean microvessel count was 83 (median, 82; standard deviation, 44.6; range, 29 to 193).

Within the poorly differentiated group of tumors, the mean microvessel count in tumors from patients with metastases was 104.7 (median, 104; standard deviation, 43.6; range, 32 to 193); whereas the mean microvessel count within tumors from patients without metastases was 50.7 (median, 42; standard deviation, 20.8; range, 27 to 85)($P = 0.004$) (Figure 4). In contrast, within the well differentiated group, the mean microvessel count in tumors from patients with metastases was 45 (median, 43; standard deviation, 20.3; range, 20 to 70); whereas, the mean microvessel count within tumors from patients without

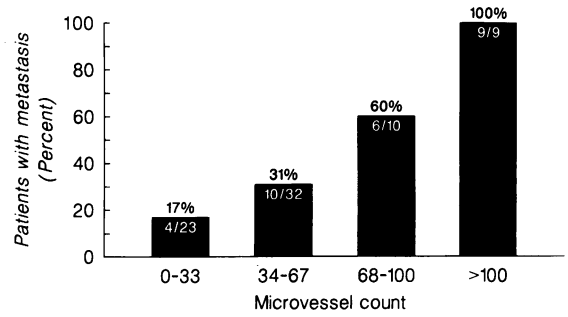

Figure 2. *Metastatic disease among 74 patients in relation to microvessel count in progressive 33 microvessel increments. This plot shows how the incidence of metastatic disease increased as vessel counts increase, becoming 100% for patients having invasive carcinomas containing microvessel counts of > 100 per ×200 field.*

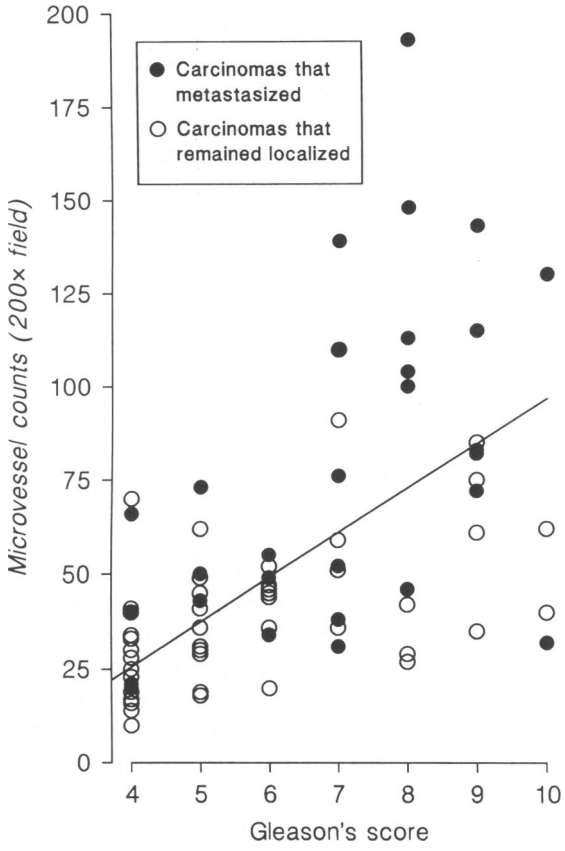

Figure 3. *Plot showing that microvessel counts increased with histology grade as determined by Gleason's score. n = 74. Spearman R = 0.60. P < 0.0001. A trend line is provided and represents a "best fit" as determined by simple linear regression.*

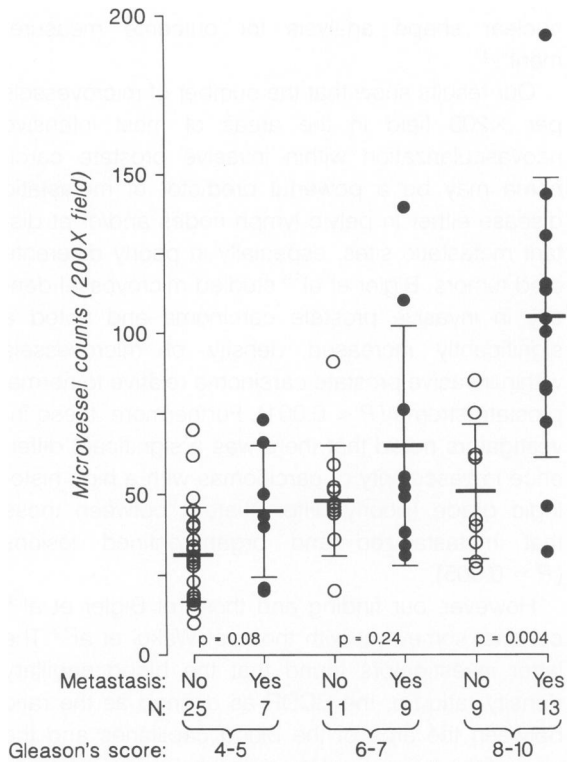

Figure 4. *Plot showing how microvessel counts varied with the presence or absence of metastasis within each Gleason's score grouping. Shown is the plot of the microvessel counts from the tumors of patients with or without metastatic disease according to the three Gleason's score groupings: 4 and 5 (well differentiated carcinoma), 6 and 7 (moderately differentiated carcinoma), and 8 through 10 (poorly differentiated carcinoma). Also provided are the numbers (N) within each category and the p values as determined by the Wilcoxon rank sum test.*

metastases was 31 (median, 30; standard deviation, 14.6; range, 10 to 70). Within the moderately differentiated group, the mean microvessel count in tumors from patients with metastases was 65 (median, 52; standard deviation, 37.1; range, 31 to 139); whereas the mean microvessel count within tumors from patients without metastases was 48 (median, 46; standard deviation, 17.6; range, 20 to 110). Although within these better differentiated groups microvessel counts were greater in metastasizing versus nonmetastasizing tumors, these differences did not achieve statistical significance (P = 0.08 and 0.24 respectively).

For multivariate analysis we used logistic regression and the likelihood ratio χ^2 test to see if some combination of variables provided a better estimate of the relative risk of metastases than any single variable. Logistic regression showed that there was a 1.39-fold risk of metastasis for each 1-point increase in Gleason's score (see Model 1; Table 2) (95% confidence interval, 1.07–1.82; P = 0.01). When microvessel count is also considered (see Model 2; Table 2), the likelihood ratio χ^2 showed that the microvessel count added significant additional information ($P < 0.0001$). Moreover, when microvessel count is considered, Gleason's score provided virtually no significant additional prognostic information when compared to that already provided by microvessel count alone (since the odds ratio for Gleason's score dropped to 0.98 from 1.39, and the 95% confidence interval then included 1.00). Logistic regression showed a 1.55-fold risk of metastasis for each 10-microvessel increase in count (95% confidence interval, 1.19 to 2.02, P = 0.002).

Discussion

These studies show a significant correlation between the density of microvessels in F8-RA stained histological sections of invasive prostate carcinoma and the incidence of metastases. These results are consistent with the known role of angiogenesis in the metastatic process. For a tumor cell to become a successful metastasis it must breach a series of barriers as well as produce and respond to several growth factors and cytokines. Indeed, metastasis is a multi-step process in which tumor cells gain access to the vasculature in the primary tumor, survive the circulation, arrest in the microvasculature of the target organ, exit from this microvasculature, and

Table 2. *Multivariate Analysis for Predicting Metastasis in Prostate Carcinoma Patients*

Model	Indicator	Odds ratio (± 95% CI)*	Model χ^2 and P	LR χ^2	LR P
1	Gleason's score	1.39 (1.07–1.82)	6.58 0.01		
2	Gleason's score plus microvessel count/10	0.98 (0.69–1.38) 1.55 (1.19–2.02)	23.37 0.002	16.79	<0.0001

Note: Table shows logistic regression models for predicting metastases in prostate carcinoma patients. Model 1 contains only Gleason's score. Model 2 adds microvessel count to Gleason's score (odds ratios determined at 10 microvessel increments). The likelihood ratio (LR) χ^2 (LR χ^2) compares the two models and tests whether adding microvessel count contributes significant additional information. The n for all models is 74.

*Odds ratio ± 95% confidence interval (CI).

proliferate in the target tissues.[7–10,26–29] In most primary tumors, fewer than one cell in 1000 has all of these capabilities.[30,31]

Tumor cells rarely shed into the circulation before the primary tumor is vascularized.[7–10,26–29] It has been shown that greater numbers of tumor vessels increase the opportunity for tumor cells to enter the circulation,[28,32,33] and it is likely that a primary tumor that contains a high proportion of angiogenic tumor cells will generate metastases that are already angiogenic. Because of the clonal origin of metastases,[26,34] a primary tumor containing a high proportion of angiogenic tumor cells will seed the blood stream with tumor cells that are already angiogenic. Indeed, tumors have been shown to be heterogeneous in the ability of their individual tumor cells to be angiogenic.[26,34] As with the primary tumor, angiogenesis will be necessary to establish a growing metastatic deposit, which will then give rise to additional metastatic deposits, thus amplifying the process of growth and dissemination. Clearly, angiogenesis is necessary at the beginning as well as at the end of the metastatic cascade.[7–10,26–29]

Moreover, tumor angiogenesis can facilitate metastatic spread in other ways. For example, newly proliferating capillaries have fragmented basement membranes and are leaky, making them more accessible to tumor cells than mature vessels.[29] Also, the invasive chemotactic behavior of endothelial cells at the tips of growing capillaries is facilitated by their secretion of collagenases and plasminogen activator.[35] These degradative enzymes may also facilitate the escape of tumor cells into the tumor neovasculature. However, it must be emphasized that neovascularization permits, but does not guarantee progressive tumor spread. For example, typical pulmonary carcinoid tumors are highly vascular but rarely metastasize to distant sites.[36] Thus, other factors besides angiogenesis are involved in metastasis.

An accurate and simple method for predicting the outcome of early stage prostate carcinoma is needed. A variety of tests have been tried including standard pathologic grading systems (Gleason's grading has enjoyed the most recent popularity) as well as a variety of morphometric, cytophotometric, flow cytometric, and immunohistochemical techniques.[37] Determination of the "random sample absolute" and "relative" nuclear roundness factors (NRF) has shown promise as a good prognosticator in prostate cancer; yet, "the tedious and time-consuming nature of NRF measurement continues to provide the primary obstacle to wide-scale use of nuclear shape analysis for outcome measurement".[37]

Our results show that the number of microvessels per ×200 field in the areas of most intensive neovascularization within invasive prostate carcinoma may be a powerful predictor of metastatic disease either in pelvic lymph nodes and/or at distant metastatic sites, especially in poorly differentiated tumors. Bigler et al[38] studied microvessel density in invasive prostate carcinoma and noted a significantly increased density of microvessels within invasive prostate carcinoma relative to normal prostate stroma ($P < 0.001$). Furthermore, these investigators noted that there was a significant difference in vascularity of carcinomas with a high histologic grade (poorly differentiated) between those that metastasized and organ-confined lesions ($P = 0.005$).

However, our finding and those of Bigler et al[38] contrast somewhat with those of Wakui et al[39] The latter investigators found that the blood capillary density ratio (ie, the BCDR as defined as the ratio between the area of the blood capillaries and the area of the tumor as determined by image analysis) was significantly higher in prostate carcinomas that developed bone marrow metastasis as compared to those that did not ($P < 0.001$), but only in the low and intermediate histologic grade tumors as defined by the Gleason's grading system. The authors concluded that "the BCDR may provide help in predicting tumor progression with regard to bone marrow metastasis of prostate carcinoma with low and intermediate Gleason's scores." Although all three studies demonstrated a correlation of tumor angiogenesis with metastasis, it is difficult to know for certain why Wakui et al[39] found a different association with Gleason's score. Possible reasons include that the latter investigators used a different end point (bone marrow metastasis only), quantitated vessels by image analysis over a larger tumor area (rather than the "hotspot"), and immunostained microvessels with vimentin (a less specific immunostain for capillaries than factor VIII-related antigen). Moreover, our results also showed that microvessel density was greater in those low and intermediate grade prostate carcinomas that metastasized, but the differences did not achieve statistical significance (ie, $P = 0.08$ and $P = 0.24$ respectively). Analysis of a larger group of tumors with more cases in the low to intermediate histologic categories may yield differences that achieve statistical significance.

The observation that microvessel density was greatest in the poorly differentiated prostate carcinomas suggests that the switch to a highly angiogenic phenotype occurs relatively late in the natural history of this disease, as relatively well differentiated tumors progress to "dedifferentiated" or poorly differentiated tumors.[40] Our observation that metastasizing poorly differentiated carcinomas had a significantly higher mean microvessel density (greater tumor angiogenesis) than those tumors that did not metastasize, suggests that poorly differentiated histology is not sufficient for metastases to occur. Indeed, a concomitant switch to a highly angiogenic phenotype appears to be as important, and by multivariate analysis possibly even more important, than development of poorly differentiated morphology.

Because counting microvessels is based on a standard immunohistochemical assay wherein endothelial cells (and thus microvessels) are highlighted with an antibody to F8-RA, the test is easy to perform using technology and reagents available worldwide in many pathology laboratories. Indeed, counting microvessels has been proven to be reproducible,[9,14,15,18,19] especially following a brief period of training.[9]

This test must be applied to a histologic section containing representative tumor present in sufficient amounts to allow counting of at least one ×200 field. This is best achieved on transurethral resection or radical prostatectomy specimens, and needle biopsy specimens are unlikely to contain enough invasive carcinoma to yield usable results. Moreover, it is true that other endothelial markers such as CD31 may mark more microvessels than F8-RA in some tumors.[18] CD31, as well as other endothelial markers such as CD34,[41] could be used in the same way as F8-RA was used in our current study. Maybe these other markers will produce even better results, and more studies are indicated, but this does not detract from the importance or significance of our findings using F8-RA, which show a clear correlation of tumor angiogenesis and metastasis in prostate carcinoma. Furthermore, F8-RA has been the most specific endothelial marker available (reacting only with endothelium, megakaryocytes, and platelets),[42] and F8-RA immunostaining provides very good contrast between microvessels and other tissue components. In our experience CD31 can cross-react with fibroblasts, some tumor cells (as does CD34), and plasma cells. Indeed, the cross reactivity of CD31 with plasma cells can significantly obscure the microvessels in those tumors with a prominent inflammatory background containing plasma cells, thus preventing accurate microvessel counts.

In summary, our findings suggest that assessment of tumor angiogenesis by counting microvessels in areas of invasive prostate carcinoma may be a clinically practical and valuable prognostic test in selecting patients for aggressive adjuvant therapies in early stage prostate carcinoma, especially in the group of poorly differentiated carcinomas. It remains to be seen whether the data reported here will continue to hold up as a predictor of metastasis, when utilized in a prospective manner by pathologists in different centers.

At the least, these results expand our understanding about the role of angiogenesis in the growth and metastasis of human tumors and provides additional evidence that tumor growth is angiogenesis-dependent. Recently, inhibitors of angiogenesis, which are not cytostatic to tumor cells *in vitro*, have been found to inhibit tumor growth *in vivo*.[43] For example, synthetic analogues of fumagillin, a naturally secreted antibiotic of *Aspergillus fumigatus* fresenius, inhibit endothelial proliferations *in vitro* and tumor-induced angiogenesis *in vivo*.[44] This "angioinhibin" (also known as AGM-1470), as well as closely related analogues, has been shown to suppress tumor growth with few side effects.[44] Such agents may prove to be valuable antitumor chemotherapeutic agents.

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