High Tumor Angiogenesis Is Associated with Poorer Survival in Carcinoma of the Cervix Treated with Radiotherapy[1]

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ABSTRACT

The purpose of this study was to examine the relationship between tumor angiogenesis and prognosis in carcinoma of the cervix treated with radiotherapy with a median follow-up time of 55 months. A retrospective study was carried out on 111 patients. Formalin-fixed, paraffin-embedded tumor biopsies were stained with anti-factor VIII using immunohistochemistry. Tumor angiogenesis was assessed by scoring the distance to the closest microvessel from random points within the tumor and the intratumor microvessel density (IMD) in the areas of highest neovascularization. High vascularity, as measured by both methods, was associated with a poor prognosis but was only significant for IMD. The 5-year survival rates for tumors with high *versus* low values were 50 and 65%, respectively. IMD was a significant prognostic factor within a Cox multivariate analysis. Higher tumor vascularity was associated with lower overall survival and locoregional control, but this association was not significant in the case of metastasis-free survival. The method used to assess tumor vascularity is important. The level of angiogenesis in carcinoma of the cervix is an independent prognostic parameter.

INTRODUCTION

The growth of a tumor is dependent upon angiogenesis (1). Initial tumor growth is associated with the passive diffusion of nutrients and waste products, but subsequent growth must be accompanied by the development of blood vessels. This has led to interest in studying the relationship between tumor angiogenesis and prognosis in cancer patients (2). It is generally assessed by measuring the degree of tumor vascularity using a variety of methods (3), and several groups have reported a correlation between increased tumor vascularity and poor prognosis. This has been shown in a number of solid tumors including breast (2, 4), prostate (5), bladder (6), melanoma (7), and head and neck malignancies (8).

In carcinoma of the cervix, the association between tumor vascularity and prognosis is less clear. One of the earliest studies reported a correlation between large intercapillary distance, which was studied by colpophotography, and an increased rate of local recurrence (9). The findings were related to the level of tumor hypoxia, which was measured directly by polarographic microelectrode, and showed that larger intercapillary distances were associated with poorly oxygenated tumors. Two subsequent reports also confirmed an association between decreased tumor vascularity measured as either intercapillary distance (10) or the proportion of vascular elements in relation to stromal and parenchymal components (11) and poor prognosis. However, these earlier studies have been criticized for using staining and morphological techniques that are likely to underestimate the extent of vascularization (8). With the introduction of more specific stains for endothelial cells, in particular factor VIII, and the standardization of the measuring technique, there is increasing evidence that, as in other tumor groups, higher vascular density is associated with a poorer prognosis (12–14). Nevertheless, using the same staining and counting techniques, other investigators have found either no association between prognosis and vascular density (15) or a poorer survival associated with low microvessel density (16).

In view of the equivocal findings in carcinoma of the cervix, the present study was set up to further investigate the association between tumor vascularity and prognosis. We studied a large series of 111 patients who had all been treated with radiotherapy alone with a uniformity of management and 5 years of follow-up. Vascularity was assessed as IMD[3] in vascular hot spots and the DTCMV.

MATERIALS AND METHODS

Patients. A total of 111 patients with stage I–III disease were included in the study, all of whom gave prior informed consent. Random cervical tumor punch biopsies were taken immediately before the initiation of treatment. The histology was squamous cell carcinoma in 103 patients and adenocarcinoma in the remaining 8 patients. All patients received radical radiotherapy given according to the standard techniques and dosage of the Manchester School (17). The median time of follow-up was 55 months, with a range of 28–117 months.

[3] The abbreviations used are: IMD, intratumor microvessel density; DTCMV, distance to the closest microvessel; CV, coefficient of variation.

Patients were routinely reviewed. Local and distant recurrence was identified by clinical and radiological investigation, and histological confirmation of recurrence was documented where possible.

Staining and Morphometric Analysis. Formalin-fixed, paraffin-embedded, 5-μm-thick sections were prepared from the pretreatment biopsy specimens. All samples were stained with anti-factor VIII (DAKO) as described previously (18). Briefly, the sections were trypsinized for 20 min at 37°C in 0.05 M Tris-HCl containing 0.01% trypsin (Sigma) and 0.01% calcium chloride. After washing, the samples were incubated in 10% normal swine serum for 10 min, followed by rabbit antihuman factor VIII (1:500), biotinylated swine antirabbit antibody (1:4000) for 30 min, and avidin biotin complex/horseradish peroxidase for 30 min. The sections were then treated with 0.01% 3,3'-diaminobenzidine tetrahydrochloride and 0.015% hydrogen peroxide for 5–8 min, rinsed in water, and counterstained with Mayer's hematoxylin. To check for the absence of nonspecific staining, parallel sections were stained with normal rabbit IgG (1:2000) for 1 h at 37°C.

Tumor vascularity was assessed without prior knowledge of patient outcome using two methods. First, tumor sections were analyzed using the vascular hot spot technique (2) to obtain IMD. Using a Leitz microscope, the tumor sections were scanned at ×120 to determine the areas of highest vascular density. Within this region, individual microvessels were counted in three separate random fields at ×300 (×25 objective and ×12 ocular; field size, 0.142 mm²). The mean vessel count from these three fields was used in the analysis. A single countable microvessel was defined as any endothelial cell or group of cells that was clearly separate from other vessels, stroma, or tumor cells without the necessity of a vessel lumen or RBC within the lumen. Areas of gross hemorrhage and necrosis were avoided.

In the second method, the DTCMV from random points was measured using an image analysis system (12). A sidearm projection device was fitted to the same microscope used for the first method. This allowed the superimposition of a light source onto the microscope field, which enabled the tracing of the distance between each of the random 25 dots in the Chalkley grid and the nearest microvessel onto a magnetic tablet. These measurements were then fed directly into a computer, allowing further basic analyses to be made. Before measurement, the system was calibrated using a stage micrometer. The whole of the tumor section (apart from areas of gross hemorrhage and necrosis) was scanned at ×120 magnification (×10 objective and ×12 ocular; 0.785 mm²/field). This resulted in a median of 175 measurements (range, 50–775 measurements) for the 111 tumor biopsies. Inaccurate measurements at the edge of the field were avoided, because the dots within the Chalkley grid are set within a circle 150 μm from the edge of the field. Microvessels were defined as described previously.

For both methods, all scoring was performed by a single observer; however, intraobserver error and interobserver error was assessed by repeat scoring of 10 and 20 randomly selected sections, respectively.

Statistical Analysis. The relationships between variables were investigated using Spearman's rank correlation. The probabilities of overall survival, locoregional control, and metasta-

Table 1 Summary of intra- and interobserver variation for IMD and DTCMV values

	IMD			DTCMV	
	n	r	P	r	P
Intraobserver variation	10	0.84	0.04	0.91	<0.001
Interobserver variation	20	0.88	<0.0001	0.74	<0.001

Table 2 Summary of IMD and DTCMV scores

	Mean ± SD	Median	Range
IMD (frequency)	11 ± 6	10	2–32
DTCMV (μM)	82 ± 29	79	37–258

sis-free survival were determined using univariate and bivariate (stratified) log-rank analysis, with the continuous variables grouped into two (above and below median values), three (for disease stage), or four (quartiles; for a single analysis of IMD *versus* survival) bands. A stepwise multivariate Cox regression was also performed to further test for the independence of vascularity measurements from clinical parameters. A significance level of 0.05 was used throughout.

RESULTS

Scoring Reproducibility and Variability. Both tumor vascularity scoring methods were validated for intra- and interobserver reproducibility. Ten randomly selected specimens were scored twice by the same scorer (R. A. C.), and 20 specimens were scored by two independent observers (R. A. C. and C. M. L. W.). For both methods, there were significant correlations between the repeat measurements obtained by the same scorer and by two different scorers (Table 1). The CVs for interobserver reproducibility were 38 and 14% for IMD and DTCMV, respectively. Inter- and intratumor variability were compared using ANOVA. For all 111 patients, the CVs for differences between patients were 55 and 35% for IMD and DTCMV, respectively. These values were higher than those for intratumor variability, which yielded CVs of 9 and 11% for DTCMV and IMD, respectively. There was significantly more variability between tumors than within tumors ($P < 0.001$ for both methods).

As expected, high microvessel density was associated with a shorter DTCMV. There was therefore a weak but significant inverse correlation between the measurements of IMD and DTCMV ($r = -0.44$; $P < 0.001$).

Vascularity Measurements. The data obtained using the two methods are summarized in Table 2. There were 44, 43, and 24 stage I, II and III tumors, respectively. Eight tumors were adenocarcinoma, and 17, 60, and 26 squamous cell carcinomas were well, moderately, and poorly differentiated, respectively. The mean age of the 111 women was 50 years, with a range of 29–81 years. Tumor size information was available for 68 tumors, and the mean tumor diameter was 4 cm, with a range of 2–8 cm. There were no correlations between the IMD and DTCMV values and tumor stage, grade of differentiation, patient age, and maximum tumor diameter. Patients were stratified
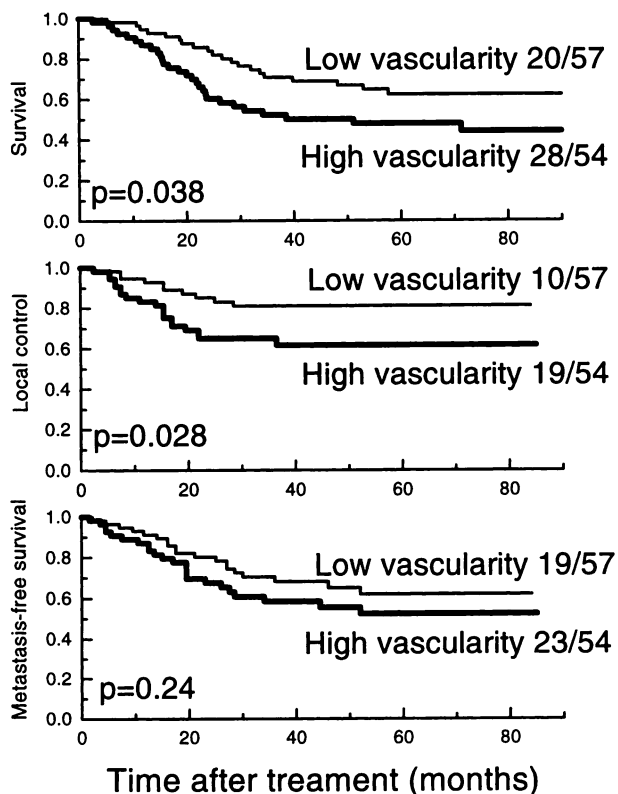
Fig. 1 Treatment outcome in relation to the IMD for 111 cervix carcinoma patients treated with radiotherapy alone with a median of 80 months of follow-up. Patients were stratified according to the median value. The number of patients and events in each arm are indicated.
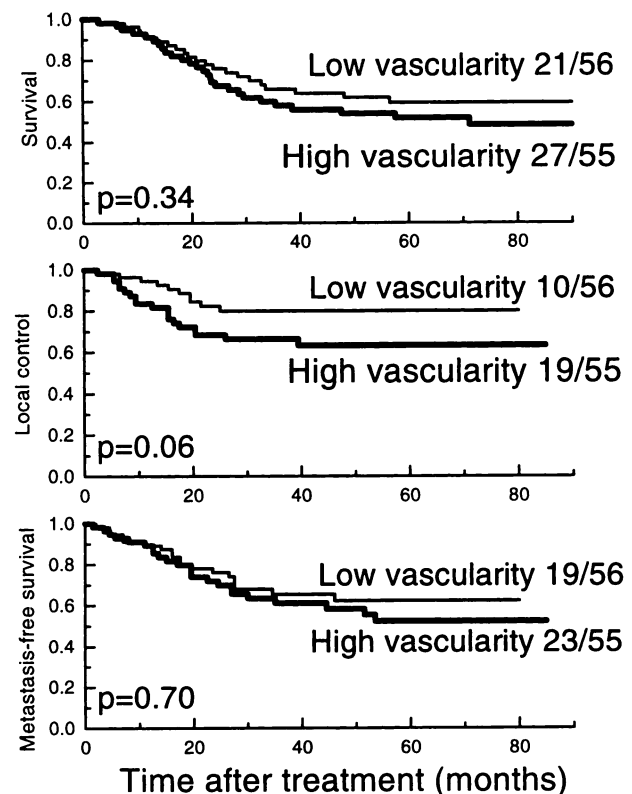

Fig. 2 Treatment outcome in relation to the DTCMV for 111 cervix carcinoma patients treated with radiotherapy alone with a median of 80 months of follow-up. Patients were stratified according to the median value.

according to the median IMD and DTCMV values for all 111 tumors to obtain low (IMD $\leq$ 10; DTCMV > 79 μm) and high (IMD > 10; DTCMV $\leq$ 79) vascularity groups (Table 2).

Correlations with Outcome. Patients were stratified into two groups, high vascularity and low vascularity, according to the median values (Table 2). There were no significant differences in the distributions of ages, stages, differentiation grades, and maximum tumor diameter between the two groups. On univariate analysis, high vascularity measured as high IMD was a significant prognostic factor for overall survival and local recurrence-free survival but not for metastasis-free survival (Fig. 1). Patients with poorly and well-vascularized tumors had 5-year survival levels of 65 and 50%, respectively. High vascularity measured as short DTCMV was associated with a poorer prognosis, but the differences were not significant (Fig. 2).

An additional analysis of IMD *versus* survival was carried out, stratifying patients according to quartiles. Although the numbers of patients in each arm were small, there was an indication of a possible threshold effect. The quartile of patients with the lowest vascularity tumors had a good prognosis (67%), but the other three quartiles had a similar survival rate (39–48%).

For this group of 111 patients, stage was not significantly associated with survival (P = 0.090), local control (P = 0.63),

or metastasis-free survival (P = 0.32). This lack of correlation can be explained by the patients having a limited range of advanced stage disease (stage Ib–III). For a larger group of 264 patients that included the smaller consecutive cohort of 111 patients, stage was a significant prognostic factor for survival (P < 0.01), local control (P = 0.007), and metastasis-free survival (P = 0.01).

The patients were stratified according to disease stage in a bivariate analysis examining vascularity in relation to treatment outcome (Figs. 3 and 4). Because the median IMD and DTCMV values for all 111 patients were used to stratify the data, there were unequal numbers in the different arms. In general, there was a trend for tumor vascularity to be more important with advancing disease stage. On bivariate analysis, after allowing for the effects of stage, tumor vascularity was a significant prognostic factor for local control by both methods (IMD, P = 0.030; DTCMV, P = 0.033) but not for overall survival or metastasis-free survival.

Multivariate Analysis. To further evaluate the independence of IMD and DTCMV as prognostic factors, a multivariate Cox regression analysis was carried out. In an analysis of 111 patients including IMD, DTCMV, stage, and age, IMD emerged as the most important prognostic variable. After allowing for IMD, no other prognostic variable was associated with treatment outcome (Table 3).
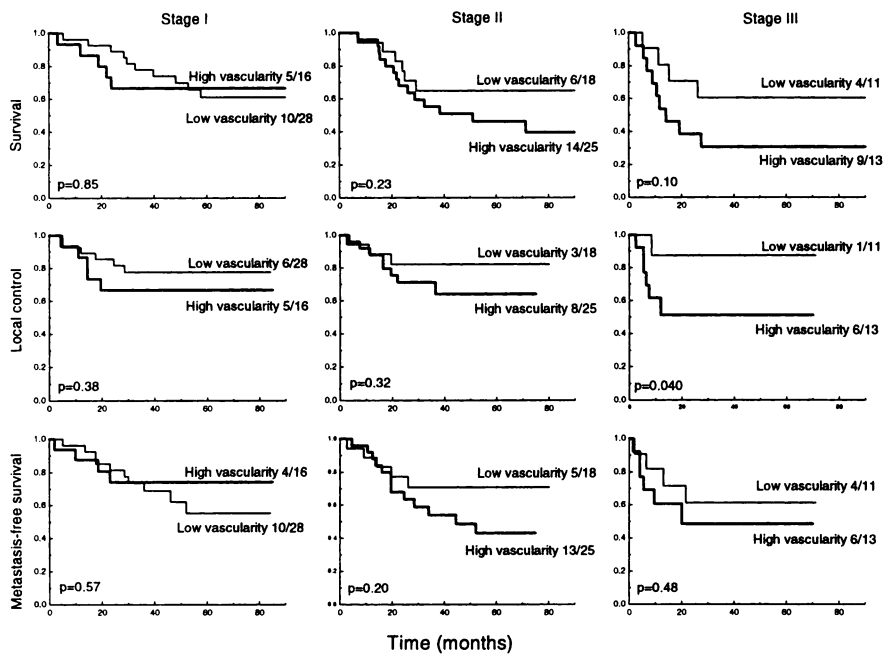
Fig. 3 Survival, local control, and metastasis-free survival *versus* IMD stratified according to the median value. Patients were divided into groups according to stage (stage I, II, and III disease). The overall significance levels allowing for stage for survival, local recurrence-free survival, and metastasis-free survival were $P = 0.080$, $P = 0.030$, and $P = 0.32$, respectively.

Fig. 4 Survival, local control, and metastasis-free survival *versus* the DTCMV stratified according to the median value. Patients were divided into groups according to stage (stage I, II, and III disease). The overall significance levels allowing for stage for survival, local recurrence-free survival, and metastasis-free survival were $P = 0.16$, $P = 0.033$, and $P = 0.32$, respectively.
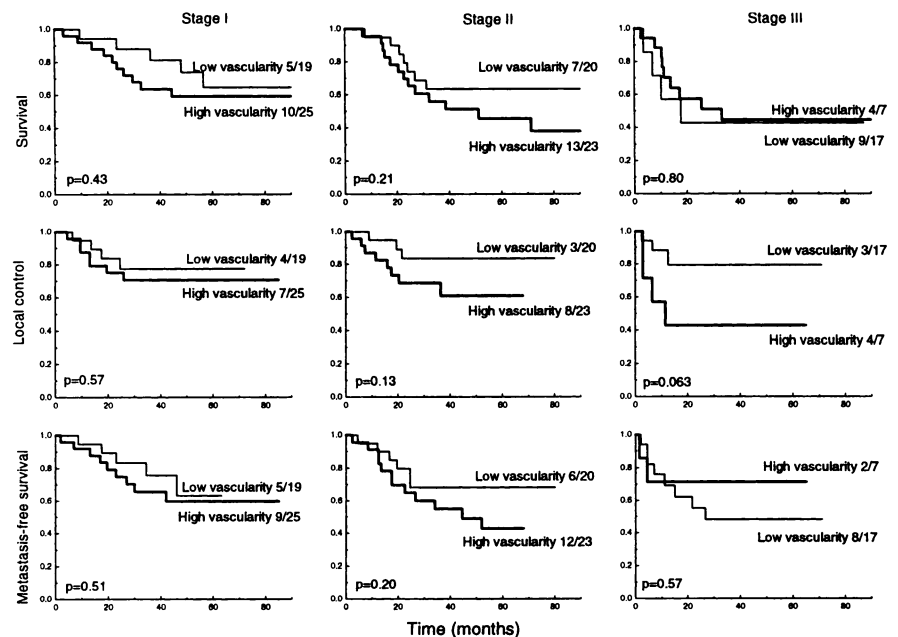
DISCUSSION

Our findings of the prognostic significance of IMD measurements are in agreement with a number of recent studies in carcinoma of the cervix. Using the IMD vascular hot spot technique as recommended by an International Consensus statement (3), three studies found an association between poor outcome and high vascularity (13, 14, 19). However, in all three studies, patients had mainly stage Ia, Ib, or II disease treated with a variety of modalities. We have confirmed and extended these findings in a large number of patients with locally ad-

vanced carcinoma of the cervix (stage: bulky Ib, II, and III) treated with radiotherapy alone. In addition, this study has shown that measurement of tumor vascularity using the hot spot technique is an independent prognostic parameter within multivariate analysis. Although two recent studies using the same method have concluded that IMD measurements have no prognostic value (15) or that high vascularity was associated with improved survival (16), the majority of reports using the hot spot method have shown that high angiogenesis is associated with poor prognosis. It is also of note that high tumor vascularity

Table 3 Cox multivariate analysis showing the relative risk for outcome in 111 cervix tumors

Variable	Survival		Local control		Metastasis-free survival	
	RR (95% CI)[a]	P	RR (95% CI)	P	RR (95% CI)	P
IMD (frequency)	1.8 (1.0–3.2)	0.041	NS[b]	0.24	NS	0.44
DTCMV (μM)	NS	0.82	2.3 (1.1–4.9)	0.034	NS	0.25
Stage	NS	0.12	NS	0.57	NS	0.32
Age	NS	0.97	NS	0.96	NS	0.45

[a] RR, relative risk; CI, confidence interval.
[b] NS, not significant.

is associated with poor outcome irrespective of whether treatment is predominately by surgery (12, 13) or radiotherapy alone (the present study). The association between increased vascularity and poor outcome is likely to be related to the known role of angiogenesis in promoting tumor growth and progression (1).

Over the past 30 years, a wide variety of methods have been used to measure tumor vascularity in carcinoma of the cervix. A similar finding (well-vascularized tumors had a significantly poorer overall and disease-free survival) has been reported in a study of 39 patients with carcinoma of the cervix with mixed treatment and a short follow-up (median, 18 months; Ref. 12). The latter study measured tumor vascularity as the DTCMV. Using a similar technique to that group, we found a similar but nonsignificant trend toward a poorer survival for patients with highly vascular tumors.

However, our results contradict some older studies that showed that well-vascularized tumors were associated with improved survival (9, 10, 11, 18). The latter was interpreted as high vascularity being associated with well-oxygenated tumors and therefore a good prognosis after radiotherapy. The disparity in the findings may be due to the methods used to quantify vascularity in the earlier work. The latter were either intercapillary distance (9, 10) or morphometry (11, 20, 21) and did not consider the areas of neovascularization. We have previously used these older methods in the same series of patients studied as part of this work (18, 22). Measurements were made of the intercapillary distance on factor VIII-stained sections and the microvessel density (morphometric analysis) on Masson's trichrome-stained sections. Using both end points, high vascularity was associated with increased survival, but the differences were not significant. It may be that tumor hypoxia, which has been shown to stimulate angiogenesis (23), leads to areas of neovascularization (and therefore to increased IMD). The latter may be associated with an increased heterogeneity of vessel distribution and thus lower overall vascularity as measured by the "older" methods. These findings emphasize the importance of the method chosen to evaluate vascularity and the need to standardize the technique so that meaningful comparisons can be made between groups and support the International Consensus that the hot spot technique is the method of choice (3).

An additional observation from our study is the significant association between tumor vascularity and local recurrence rather than metastatic disease, as might be predicted from the known role of angiogenesis in the metastatic process. This may be related to neovascularization stimulating local tumor growth (24) before the development of metastasis, and the natural history of cervix cancer in which patients may die of local recurrence before any metastases are detected (25).

In conclusion, the results of this study indicate that patients with highly vascularized cervix tumors have a poorer 5-year survival and increased local recurrence. We have also shown that the hot spot technique is the method of choice in carcinoma of the cervix. The latter is relatively simple to perform and shows good correlation between observers. In the future, the identification of highly vascular tumors may allow the targeting of new antiangiogenic drugs toward patients who are likely to benefit.

ACKNOWLEDGMENTS

REFERENCES

1. Folkman, J. What is the evidence that tumors are angiogenesis dependent? N. Engl. J. Med., 285: 82–86, 1990.

2. Weidner, N., Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N. Engl. J. Med., 324: 1–8, 1991.

3. Vermeulen, P. B., Gasparini, G., Fox, S. B., Toi, M., Martin, L., McCulloch, P., Pezzella, F., Viale, G., Weidner, N., Harris, A. L., and Dirix, L. Y. Quantification of angiogenesis in solid human tumours: an international consensus on the methodology and criteria of evaluation. Eur. J. Cancer, 32A: 2474–2484, 1996.

4. Horak, E. R., Leek, R., Klenk, N., Lejeune, S., Smith, K., Stuart, N., Greenall, M., Stepniewska, K., and Harris, A. L. Angiogenesis assessed by platelet/endothelial cell adhesion molecule antibodies as an indicator of node metastases and survival in breast cancer. Lancet, 340: 1120–1124, 1992.

5. Weidner, N., Carroll, P. R., Flax, J., Blumenfeld, W., and Folkman, J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am. J. Pathol., 143: 401–409, 1993.

6. Bochner, B. H., Cote, R. J., Weidner, N., Groshen, S., Chen, S., Skinner, D. G., and Nichols, P. W. Tumor angiogenesis is an independent prognostic indicator of invasive transitional cell carcinoma of the bladder. J. Natl. Cancer Inst., 87: 1603–1612, 1995.

7. Srivastava, A., Laidler, P., Davies, R. P., Horgan, K., and Hughes, L. E. The prognostic significance of tumor vascularity in intermediate-thickness (0.76–4.0-mm thick) skin melanoma. Am. J. Pathol., 133: 419–423, 1988.

8. Lauk, S., Skates, S., Goodman, M., and Suit, H. D. A morphometric study of the vascularity of oral squamous cell carcinomas and its relation to outcome of radiation therapy. Eur. J. Cancer Clin. Oncol., 25: 1431–1440, 1989.

9. Kolstad, P. Intercapillary distance, oxygen tension and local recurrence in cervix cancer. Scand. J. Clin. Lab. Investig., 22: 145–157, 1968.

10. Awwad, H. K., Nagger, E. L., Mocktar, N., and Barsoum, M. Intercapillary distance measurement as an indicator of hypoxia in carcinoma of the cervix uteri. Int. J. Radiat. Oncol. Biol. Phys., *12:* 1329–1333, 1986.

11. Siracka, E., Siracky, J., Pappova, N., and Revesz, L. Vascularisation and radiocurability in cancer of the uterine cervix: a retrospective study. Neoplasma (Bratisl.), *29:* 183–188, 1982.

12. Schlenger, K., Hockel, M., Mitze, M., Schaffer, U., Weikel, W., Knapstein, P. G., and Lambert, A. Tumor vascularity: a novel prognostic factor in advanced cervical carcinoma. Gynecol. Oncol., *59:* 57–66, 1995.

13. Bremer, G. L., Tiebosch, A. T. M. G., van der Putten, W. H. M., Schouten, H. J. A., de Haan, J., and Areds, J. Tumor angiogenesis: an independent prognostic parameter in cervical cancer. Am. J. Obstet. Gynecol., *174:* 126–131, 1996.

14. Wiggins, D. L., Granai, C. O., Steinhoff, M. M., and Calabresi, P. Tumor angiogenesis as a prognostic factor in cervical carcinoma. Gynecol. Oncol., *56:* 353–356, 1995.

15. Rutgers, J. L., Fleming, T., and Vargas, P. M. Angiogenesis in uterine cervical squamous cell carcinoma. Int. J. Gynecol. Pathol., *14:* 114–118, 1995.

16. Kainz, C., Speiser, P., Wanner, C., Obermair, A., Tempfer, C., Sliutz, G., Reinthaller, A., and Breitenecker, G. Prognostic value of tumour microvessel density in cancer of the uterine cervix stage Ib to IIb. Anticancer Res., *15:* 1549–1552, 1995.

17. West, C. M. L., Davidson, S. E., Roberts, S. A., and Hunter, R. D. Intrinsic radiosensitivity and prediction of patient response to radiotherapy for carcinoma of the cervix. Br. J. Cancer, *68:* 819–823, 1993.

18. Davidson, S. E., Ngan, R., Wilks, D. P., Moore, J. V., and West, C. M. L. A comparison of four methods for assessing tumour vascularity in carcinoma of the cervix. Int. J. Oncol., *5:* 639–645, 1994.

19. Dinh, T. V., Hannigan, E. V., Smith, E. R., Hove, M. J., Chopra, V., and To, T. Tumor angiogenesis as a predictor of recurrence in stage Ib squamous cell carcinoma of the cervix. Obstet. Gynecol., *87:* 751–754, 1996.

20. Revesz, L., Siracka, E., Siracky, J., Delides, G., and Pavlaki, K. Variation of vascular density within and between tumors of the uterine cervix and its predictive value for radiotherapy. Int. J. Radiat. Oncol. Biol. Phys., *16:* 1161–1163, 1988.

21. Siracka, E., Revesz, L., Kovac, R., and Siracky, J. Vascular density in carcinoma of the uterine cervix and its predictive value for radiotherapy. Int. J. Cancer, *41:* 819–822, 1988.

22. Davidson, S. E., Moore, J. V., West, C. M. L., and Hunter, R. D. Comparison of two methods to assess tumour vasculature in human cervical carcinoma. Int. J. Radiat. Biol., *60:* 169–173, 1991.

23. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature (Lond.), *359:* 843–845, 1992.

24. Weidner, N., and Folkman, J. Tumoral vascularity as a prognostic factor in cancer. *In:* V. T. DeVita, S. Hellman, and S. A. Rosenberg (eds.), Important Advances in Oncology, pp. 167–190. Philadelphia: Lippincott-Raven Publishers, 1996.

25. Perez, C. A., Kuske, R. R., Camel, H. M., Galakatos, A. E., Hederman, M. A., Kao, M. S., and Walz, B. J. Analysis of pelvic tumor control and impact on survival in carcinoma of the uterine cervix treated with radiation therapy alone. Int. J. Radiat. Oncol. Biol. Phys., *14:* 613–621, 1988.

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