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Intratumoral Vascularity as a Prognostic Factor in Cancers of the Urogenital Tract

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INTRODUCTION

A POSITIVE correlation of increasing intratumoral microvessel density (IMD) with various measures of tumour aggressiveness has been reported for a long list of solid tumours arising at multiple body sites. This list includes not only cancers of the breast, head and neck, lung, skin, stomach, colon, rectum, bone marrow, and central nervous system, but also those arising in the prostate, bladder, ovary, cervix, endometrium and testis. In most of these studies increasing IMD was found to have independent prognostic significance when compared to traditional prognostic markers by multivariate analysis. These reports are derived from numerous independent medical centres located around the world and these, as well as other evidence supporting the importance of tumour vascularity in the growth and spread of cancer, have been recently extensively reviewed [1]. Also, recent work, especially for prostate carcinoma, suggests that the assessment of IMD may first achieve widespread practical application in urogenital tract tumours. This review will focus on those studies of urogenital tract tumours wherein measuring tumour angiogenesis has been useful as a prognostic factor.

REPORTS OF A POSITIVE ASSOCIATION OF INTRATUMORAL VASCULARITY WITH TUMOUR AGGRESSIVENESS

Male urogenital tract

Prostate carcinoma. Focusing on prostate carcinoma, Wakui and associates [2] highlighted endothelial cells with antivimentin and quantitated intratumoral angiogenesis with a computerised image analysis system. They determined the blood capillary density ratio (BCDR) in the entire tumour area of the section. The BCDR was the ratio of the blood capillary area (x) to the tumour area (y) minus the luminal area of the tumour glands (z) [$BCDR = x/(y - z)$]. They found that in Gleason's low and intermediate grade tumours, the BCDR was significantly higher in prostate carcinomas that developed bone marrow metastasis than those than did not ($P < 0.001$). Similarly, to determine how IMD correlated with metastasis in prostate carcinoma, my colleagues and I used our own previously developed technique [3, 4] and counted microvessels within the initial invasive carcinomas of 74 patients (29 with metastasis, 45 without) [5]. The mean number of microvessels in tumours from patients with metastases was 76.8 microvessels per 200x field (median, 66; s.d., 44.6). The counts within carcinomas from patients without metastases were significantly lower, 39.2 (median, 36; s.d., 18.6) ($P < 0.0001$), and the incidence of metastasis increased with increasing IMD (Figure 1). IMD increased with increasing Gleason's score ($P < 0.0001$), but this increase was present predominantly in the poorly differentiated tumours (Figure 2). 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 IMD alone. Thus, the assay of IMD within invasive tumours may prove valuable in selecting patients for aggressive adjuvant therapies in early prostate carcinoma. Fregene and associates [6] quantitated microvessels in 23 nonmalignant and 34 malignant prostatectomy specimens. The findings were correlated with Whitmore–Jewitt stage and, based on the number of microvessels, the authors were able to distinguish stage D from all other pathological stages ($P = 0.004$). They concluded that tumour angiogenesis in prostate cancer may have both clinical and pathological significance. Brawer and coworkers [7] studied radical prostatectomy specimens from 32 patients with prostate cancer using a computer-aided image analysis system to measure IMD, after immunostaining with anti-F8RA/vWF. Their field size measured 1.71 mm^2, and the validity of the image analysis method was verified by comparing manual counts obtained by two of the authors with the computer counts ($P < 0.001$, $r = 0.98$, $n = 20$ fields compared). They found that increasing IMD was an independent predictor of pathological stage and, presumably, malignant potential. In fact, increasing IMD was superior to histological grade and preoperative prostate specific antigen (PSA) levels in distinguishing organ-confined tumours from those having extracapsular extension or pelvic lymph node metastasis. Also, they found that glands containing prostate intra-epithelial neoplasia (PIN) had a rim of neovascularity that was greater than that found around benign glands in 18 out of 25 specimens. Veslainen and associates [8], while using a somewhat heterogeneous group of diagnostic specimens

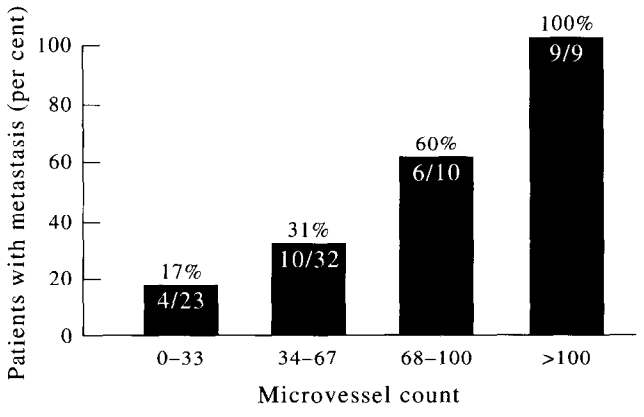

Figure 1. Metastatic disease among 74 patients with invasive prostate carcinoma in relation to microvessel count in progressive 33 microvessel increments. This plot shows how the incidence of metastatic disease increased as vessel counts increase, becoming 100% for patients having invasive carcinomas containing microvessel counts of >100 per 200 × field. Reprinted by permission of the *American Journal of Pathology*, from Weidner *et al., Am J Pathol* 1993, Vol. 143, pp. 401–409.

from 88 patients, collagen type IV immunostaining, and a relatively small field size of approximately 0.196 mm^2, found that increasing IMD correlated with decreased overall patient survival by univariate analysis ($p = 0.0268$). However, with multivariate analysis, Gleason's score included all the available independent prognostic information. Hall and associates [9] immunostained 25 transurethral resection specimens for F8RA and determined IMD, and found that increasing IMD correlated with serum PSA levels, tumour grade, and flow ploidy status. Moreover, IMD was higher in patients who failed radiotherapy and was associated with a significantly worse actuarial outcome at 4 years ($P = 0.0003$). Very recently, Bostwick and colleagues [10] evaluated 186 randomly selected needle biopsy samples and matched radical prostatectomy specimens collected from multiple independent institutions. The needle biopsy specimens were immunostained for F8RA, and an optimised IMD was determined with a digital image analysis system. The Gleason score was also determined on the needle biopsies, and pathological stage was determined from the radical prostatectomy specimens and independently verified by independent review. Prediction of extraprostatic extension was significantly increased when the optimised IMD was added to the Gleason's score and serum PSA level ($P = 0.003$). Detailed probability tables were developed from the values of Gleason's score, serum PSA level and optimised IMD. For example, a patient with a Gleason's score of 7, a serum PSA of 12 ng/ml, and an optimised IMD of 25 had a 49% chance of extraprostatic extension, whereas, a patient with the same Gleason's score and PSA level, but an optimised MVD of 500, had a 91% chance of extraprostatic extension. This is a very important and practical study showing that IMD can be added to other prognostic factors to assist in therapeutic decision making. Finally, Silberman and associates [11] immunostained 109 radical prostatectomy specimens for CD31 that had invasive prostate carcinoma of Gleason sum 6 or 7. They measured IMD in an area of 3.14 mm^2 in the hot spot, and found no correlation with pathological stage. However, these investigators also immunostained an additional 87 radical prostatectomy specimens, which contained Gleason's score 5 to 7 prostate carcinomas, and measured IMD in the same manner. They found that the IMD was significantly higher in those patients that had progressive carcinomas versus those that did not ($P < 0.0001$). Moreover, IMD and Gleason's score were independent predictors of progression. The authors concluded the IMD was an independent significant predictor of progression, after radical prostatectomy, for Gleason's score 5 to 7 tumours, and because these comprise the majority of prostate carcinomas, it is this group for which discrimination of biological potential is most needed.

To my knowledge, no papers have been reported describing a totally negative association of IMD with various measures of tumour aggressiveness for prostate carcinoma. Measuring IMD may find widespread application in clinical practice as a prognostic marker in patients with invasive prostate carcinoma.

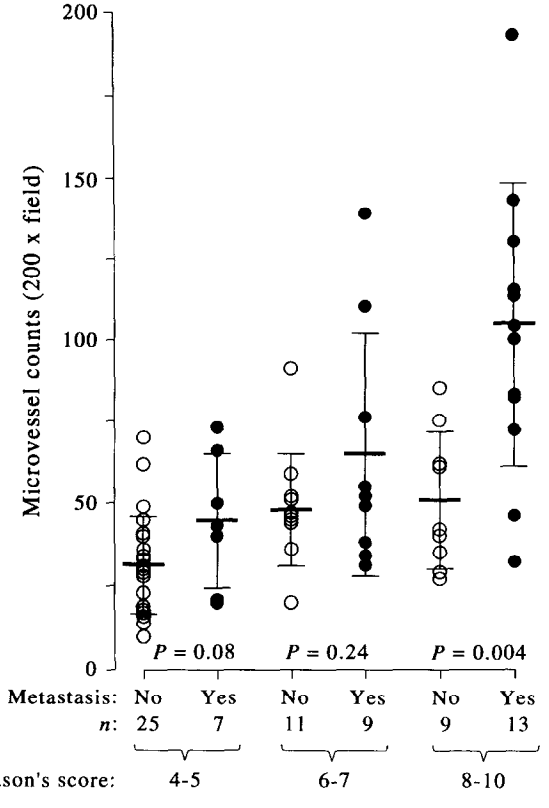

Figure 2. The variation of microvessel counts with the presence or absence of metastasis within each Gleason's score grouping. Shown is the plot of microvessel counts from the tumours of patients with or without metastatic disease according to the three Gleason's score groupings, 4 and 5 (well-differentiated carcinoma), 6 and 7 (moderately differentiated carcinoma), and 8–10 (poorly differentiated carcinoma). Also provided are the numbers (*n*) within each category and the *p* values as determined by the Wilcoxon rank sum test. Reprinted by permission of the *American Journal of Pathology*, from Weidner *et al., Am J Pathol* 1993, Vol. 143, pp. 401–409.

Bladder carcinoma. To evaluate IMD as a prognostic factor in transition cell carcinoma (TCC) of the bladder and to determine its relationship with other prognosticators, Dickinson and associates [12] immunostained 45 TCCs for CD31 and quantitated IMD using Chalkley point eyepiece graticle. Univariate analysis showed that overall survival correlated with stage, histological grade, and IMD, but not relationship was observed between stage and grade and IMD. Multivariate analysis showed that IMD was an independent prognostic factor and as good as stage in predicting overall survival ($P = 0.026$). Jaeger and associates [13] immunostained 41 primary invasive TCCs for F8RA and determined IMD within hot spots in a 0.74 mm^2 area. IMD correlated with the presence of lymph nodal metastases ($P = 0.001$) (Figure 3), whereas T stage, histological grade, and lymphatic-vascular invasion did not. Most recently, Bochner and colleagues [14] immunostained 164 bladder TCCs for CD34 and measured IMD in a 200x field (approximately 0.74 mm^2) within the hot spot or immediately adjacent to each tumour. IMD was significantly associated with both disease-free (Figure 4), overall survival (Figure 5) and disease progression not only in organ-confined TCCs, but also in carcinomas invasive beyond the bladder wall and/or those with lymph nodal metastases. Moreover, IMD was found to be an independent prognostic factor when evaluated in the presence of histological grade, pathology stage, and regional lymph nodal status. Importantly, Grossfeld and associates [15] have studied thrombospondin-1 expression in invasive bladder carcinomas and documented its association with over-accumulation of p53 tumour angiogenesis, and tumour progression. Thrombospondin-1 is a known inhibitor of angiogenesis, and the loss of the wild-type (wt) allele of the *TP53* gene, which is usually associated with overaccumulation of immunostainable p53, may cause a decline in thromobospondin-1 expression. In this recent study, TCCs from 163 patients were immunostained for thrombospondin-1 (monoclonal antibody MA-II), p53 (monoclonal antibody Pab 1801), and CD34 (endothelial

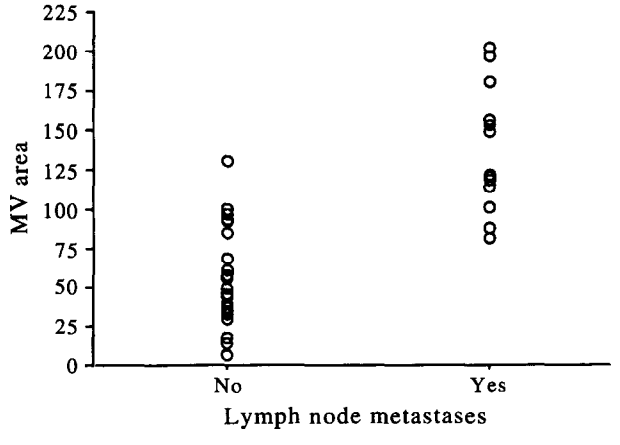

Figure 3. The correlation of microvessel density and lymph node metastases determined in a 200x microscopic field (approximately 0.74 mm^2) for invasive transitional cell carcinomas of the bladder. Reprinted by permission of the *Journal of Urology*, from Jaeger, *et al., J Urol* 1995, Vol. 154, pp. 69–71.

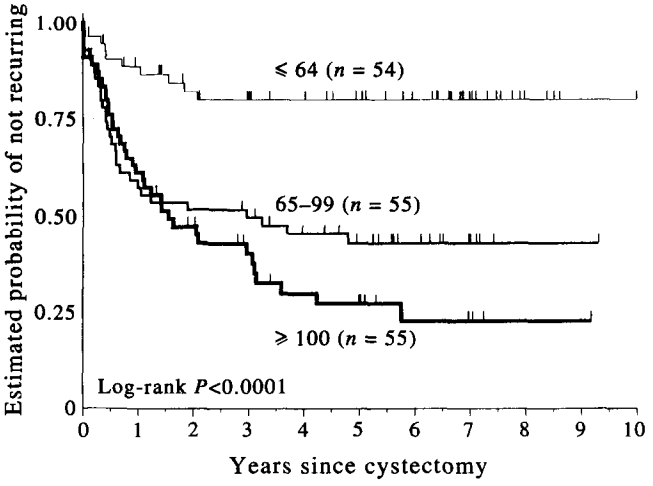

Figure 4. Kaplan–Meier recurrence-free survival plots for patients with invasive bladder carcinoma. Probability of remaining recurrence free in 164 patients with invasive transitional cell carcinoma of the bladder with low (≤64), intermediate (65–99), and high (≥100) microvessels per 200 × microscopic field. Each tick mark represents a patient who had no evidence of disease recurrence at the time of last follow-up. Reprinted by permission from Bochner *et al., J Natl Cancer Inst* 1995, Vol. 87, pp. 1603–1612)..

marker for assessment of IMD). Patients having TCCs with low thrombospondin-1 expression exhibited decreased overall survival when compared to patients with moderate or high tissue levels of expression. Moreover, thrombospondin-1 expression was an independent predictor of disease recurrence and overall survival compared with tumour stage, lymph node status, and histological grade.

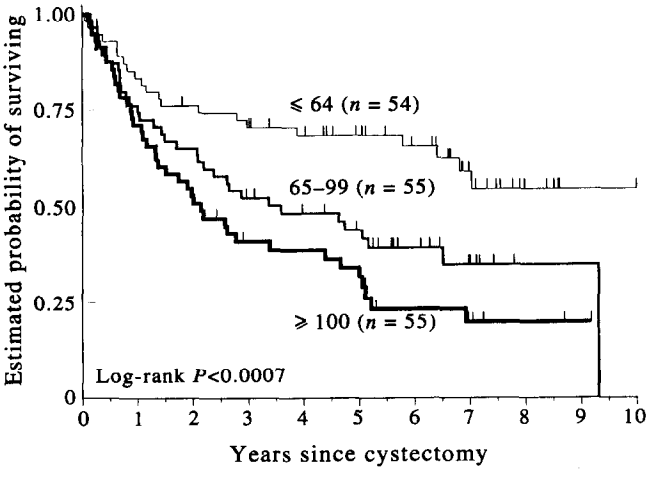

Figure 5. Kaplan–Meier overall survival plots for patients with invasive bladder carcinoma. Probability of survival in 164 patients with invasive transitional cell carcinoma of the bladder with low (≤64), intermediate (65–99), and high (≥100) microvessels per 200 × microscopic field. Each tick mark represents a patient who had no evidence of disease recurrence at the time of last follow-up. Reprinted by permission from Bochner *et al., J Natl Cancer Inst* 1995, Vol. 87, pp. 1603–1612).

However, it was not independent of overaccumulation of p53. In fact, relatively low thrombospondin-1 expression was significantly associated with nuclear p53 immunostaining and high IMD. The inverse relationship between thrombospondin-1 expression with overaccumulation of p53 and increased IMD is compatible with the hypothesis that p53 affects tumour angiogenesis by regulating the level of thrombospondin-1 expression. Therefore, thrombospondin-1 appears to be a turmour suppressor, possibly acting in part through down regulation of tumour angiogenesis and this inhibitory function is lost when *TP53* gene mutations occur.

To my knowledge, no papers have been reported describing a totally negative association of IMD with various measures of tumour aggressiveness for bladder carcinoma. As with prostate carcinoma, assessment of IMD TCC of the bladder may become a widely used prognostic factor.

Renal cell carcinoma. Yoshino and associates [16] immunostained 84 renal cell carcinomas (RCCs) for F8RA and assessed IMD in a 200 × field (approximately 0.74 mm^2) within the neovascular hot spot. The mean number of microvessels in patients with metastases was significantly higher than microvessel counts in patients who were disease free for more than three years ($P = 0.004$). The survival of patients with less than 30 microvessels per 200 × field was significantly higher than that of patients with more than 30 microvessels ($P = 0.007$). Multivariate analysis showed that IMD was the only significant predictor of prognosis in 45 patients with T1-2 and M0 tumours ($P = 0.028$). The authors concluded that IMD is probably one of the most important prognostic predictors in RCC. Basic fibroblast growth factor (bFGF) is a potent angiogenic factor, which has been detected in body fluids of patients with various malignancies including RCC, and Nunus and associates [17] showed that cytoplasmic expression of bFGF in primary RCC correlates with shortened patients survival. Duensing and colleagues [18] analyzed serum bFGF levels in 23 patients with advanced RCC and progressive disease in various organs. Increased bFGF correlated with progressive pulmonary metastases compared to those without progressive pulmonary disease ($P = 0.0006$). The findings suggest that certain angiogenic factors elaborated by types of specific tumour may influence exactly where metastases develop. Of additional importance, Nguyen and associates [19] measured urine bFGF levels in 950 patients with a wide variety of cancers of the bladder and other organs and from a control group of health patients and patients with non-cancer-related diseases. They found that 31% of normal bladder patients and 47% of patients with active metastatic cancers had elevated urine bFGF. Survival among cancer patients was approximately 85% for those with normal urine bFGF and approximately 71% for those with elevated urine bFGF. Brown and colleagues [20] have presented data suggesting that vascular endothelial growth factor (VEGF) is a very important angiogenic factor in both RCC and TCC.

However, some investigators have not found a positive correlation of IMD with outcome or stage. MacLennan and coworkers [21] immunostained 97 consecutive RCCs for F8RA and counted the hot spot MVD in a 0.1855 mm^2 area (400 × field). The mean IMD was very high (i.e. 741 microvessels per mm^2); a not unexpected finding given the highly vascular nature of the average or usual RCC. There was no correlation between IMD and stage, tumour grade, or cancer-specific survival.

Testicular carcinoma. Olivarez and associates [22] studied Neovascularisation in 65 clinical stage A testicular germ-cell tumours; 43 were subsequently found to have pathological stage B disease as shown after finding occult lymph nodal metastases. The primary tumours were immunostained for F8RA and IMD determined in a 400 × field. Univariate analysis showed a significant correlation between higher IMD and higher stage ($P = 0.02$), but multivariate analysis, which included venous invasion, lymphatic invasion, presence of embryonal carcinoma, angiogenesis, and absence of yolk sac tumour, showed that only the absence of yolk sac tumour was significantly predictive of occult lymph nodal metastases. The authors concluded that prospective use of angiogenesis quantitation needs to be defined, and further studies are needed on germ cell tumours before definitive conclusions can be made.

Female urogenital tract

Cervix. Smith-McCune and colleagues [23] were among the first to suggest a relationship between cervical carcinoma and tumour angiogenesis. These investigators immunostained 23 cervical specimens for F8RA which contained either normal or various grades of cervical intraepithelial neoplasia (CIN). A region of increased neovascularisation was found along the basal lamina subtending the dysplastic epithelium when compared to normal adjacent squamous epithelium. The intensity of the neovascular response increased with increasing grade of CIN, and it did not correlate with either inflammatory response or human papilloma virus subtype. Subsequent work by Guidi and associates [24] have confirmed these findings and further suggested that EGF is an important angiogenic factor in cervical neoplasia. They observed increased mRNA for VEGF, epithelial–stromal vascular cuffing, and IMD in invasive cervical carcinoma and high-grade CIN as compared to low-grade CIN and benign squamous epithelium. However, Wiggins and coworkers [25] were the first to show prognostic utility of IMD in invasive cervical carcinoma. They immunostained invasive squamous carcinomas from 29 patients for F8RA and determined IMD in a 200 × microscopic field. They found increased IMD in invasive squamous carcinoma relative to controls and noted that IMD correlated with vascular space invasion ($P = 0.017$). Four patients with negative lymph nodes and vascular invasion, but with high IMDs developed recurrent carcinoma within 1 year of surgery. They reported no correlation of IMD with lymph nodal status, parametrial involvement, depth of invasion or gross disease. They concluded that IMD could be of prognostic value in patients who do not have other risk factors for recurrence. Almost simultaneously, Kainz and associates [26] reported their findings on 43 patients with invasive squamous carcinoma of the cervix of FIGO stages IB, IIA or IIB. Using F8RA to highlight microvessels and to assess IMD in a 200 × microscopic field (approximately 0.74 mm^2) in the hot spot, they found that patients having tumours with low IMD had a poorer survival ($P = 0.01$). They found no correlation of IMD with lymph nodal status, vascular space

involvement or stromal reaction. This paradoxical result might be explained by Kohno and colleagues [27] who found that relatively high IMD predicted greater tumour response to intra-arterial chemotherapy or invasive cervical cancers. In contrast, Bremer and associates [28] studied invasive squamous carcinomas of the cervix from 114 patients (FIGO stages IB and IIA), after the specimens had been immunostained for *Ulex europaeus-1* lectin to highlight vessels. Microvessels were counted in 8 to 10 microscopic fields and expressed as a mean per mm^2 stroma. Relatively high IMD correlated with pelvic lymph nodal metastases and shortened disease-free survival. Multivariate analysis showed that IMD was independent of the other prognostic factors studied, including lymph nodal status. Using a computerised image analysis system (i.e. determination of the closest individual microvessel to a random point), needle core biopsies and F8RA immunostaining, Schlenger and associates [29] studied 42 specimens from patients with advanced stage cervical squamous carcinomas (i.e. FIGO stages Ib–IVa). Relatively high IMD significantly predicted shorter disease-free survival ($P = 0.025$) and overall survival ($P = 0.032$); and multivariate analysis revealed IMD to be the strongest independent prognostic factor in this group of patients. The other prognostic factors studied included age, FIGO stage, tumour size, histological grade, lymph node status and lymphatic–vascular invasion.

However, some investigators have not found a positive correlation of IMD with outcome or stage in patients with invasive squamous carcinoma of the cervix. Rutgers and associates [30] studied 70 patients and measured IMD in biopsy specimens in a single 400 × field on slides immunostained for F8RA. Patients were followed for 21 months and no correlation between disease status and IMD was found. The short follow-up, limited numbers of patients and biopsy-only counting (which could result in missing the neovascular hot spot) could have biased the findings.

Endometrium. Very few studies assessing tumour angiogenesis in endometrial carcinoma have been published, yet the tumour types occurring in the endometrium should lend themselves very well to these studies, and finding a positive correlation between the intensity of tumour angiogenesis and various measures of tumour aggressiveness would come as no surprise. In fact, Abulafia and associates [31] have done some very important seminal work in this area, and their results are quite promising. Using F8RA to highlight vessels, these investigators determined microvessel density in 19 patients with benign endometrial conditions, 24 patients with endometrial hyperplasias and 34 patients with FIGO stage 1 endometrial carcinomas. Patients with complex hyperplasia had significantly higher microvessel densities than either benign controls or those with simple hyperplasia, but at the same time significantly lower than patients with carcinoma. Moreover, myometrial invasive carcinomas had significantly higher IMD than those without invasion. Increasing histological grade also correlated with increasing IMD. The authors concluded that complex hyperplasia and endometrial carcinomas are angiogenic, and that for FIGO stage 1 carcinomas, greater depth of invasion and tumour grade are directly correlated with angiogenic intensity. Thus, there appears to be a progressive increase in tumour angiogenesis with more aggressive preneoplastic hyperplasias and overt endometrial neoplasias. I hope that many more studies in this area of endometrial neoplasia are in progress and will soon be published.

Ovary. Hollingsworth and associates [32] were the first to report a correlation of IMD in ovarian carcinoma and patient survival. They immunostained 43 advanced stage (FIGO III and IV) ovarian carcinomas for CD34 and measured IMD in both 200× and 400× microscopic fields. Both higher FIGO stage and IMD correlated with shortened disease-free survival by univariate analysis. With multivariate analysis, FIGO stage was the best predictor of overall survival; however, the average IMD at 400× was found to be the best predictor of disease-free survival. These findings were recently confirmed by Gasparini and associates [33] who immunostained 60 cases of FIGO stage III or IV ovarian carcinoma for CD31 and counted microvessels in the neovascular hot spots. Age, performance status, histotype, extent of residual disease, response to chemotherapy and IMD all significantly correlated with overall survival. In multivariate analysis, only histotype, extent of residual disease and performance status retained significance. Also, IMD, age and performance status were able to predict significantly responsiveness to platinum-based chemotherapy by univariate analysis, but only, IMD and performance status were able to predict responsiveness by multivariate analysis. The authors concluded that the determination of IMD seemed to be useful for a more accurate selection of those patients (i.e. those with low vascularised tumours) who have a higher probability of gaining benefit from platinum-based chemotherapy.

However, some investigators have not found a positive correlation between IMD and outcome or response to platinum-based chemotherapy in patients with ovarian carcinoma. van Diest and colleagues [34] immunostained 49 ovarian carcinomas for *Ulex europaeus-1* lectin to highlight vessels. All patients were FIGO stage III or IV, and, following survival analysis, a tendency for worse prognosis with high IMD was found, although statistical significance was not reached. Before meaningful conclusions can be reached regarding ImRD in ovarian carcinoma, additional studies need to be performed, especially after a standardised scheme is developed to determine IMD reproducibility. IMD levels tend to be very heterogeneous in ovarian cancers.

CONCLUSION: EXPLANATIONS FOR THE ASSOCIATION OF IMD WITH TUMOUR AGGRESSIVENESS

The association between IMD and various measures of tumour aggressiveness can be explained in a number of ways. First, a highly angiogenic primary tumour with a high IMD is more likely to seed distant sites with highly angiogenic clones. Second, solid tumours are composed of two discrete yet interdependent components (i.e. the malignant cells and the stroma they induce), and measuring IMD could be a valid measure of the success that a particular tumour has in forming this very important stromal compartment. Also, the endothelial cells of this stromal component may be stimulating the growth of the tumour cells in a reverse paracrine manner. If true, the more microvessels and, thus, more endothelial cells, the greater this paracrine growth stimulation. Third, the density of the microvessel

bed within a tumour is probably a direct measure of the size of the vascular window through which tumour cells pass to spread to distant body sites. The larger that window, the greater the number of circulating tumour cells from which a metastasis could develop. Finally, if it is true that endothelial cells play a very active role in the metastatic process and that tumour cells are actually more passive than previously thought, then IMD could be a measure of those endothelial-derived forces promoting metastases. I believe that all of these factors are acting together to encourage tumour growth and metastasis. Indeed, it is no surprise that IMD correlates with various measures of tumour aggressiveness.

Finally, it should be emphasised that tumour angiogenesis alone is not sufficient to cause metastases. Tumour cells must also proliferate, penetrate host tissues and vessels, survive within the vasculature, escape the host's immune system and then begin growing at a new body site. Although I am highly optimistic that measuring IMD in invasive prostate and/or transitional cell carcinoma of the bladder will become a practical clinical prognostic factor, it remains to be seen whether IMD will be universally reproducible and continue to be a predictor of metastasis or patient outcome when utilised in a prospective manner by pathologists in many different centres. As tumour therapies become more effective in preventing tumour recurrence, the ability of a prognostic test to stratify patients into various prognostic categories becomes diminished. With a 100% cure rate or death rate, all prognostic tests for predicting patient survival become meaningless. In any event, the well-documented association of increasing IMD with various measures of tumour aggressiveness have increased our understanding of the critical role of angiogenesis in human tumour growth and metastasis.

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