Tissue-Based Biomarkers of Tumor-Vascular Interactions

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Abstract

The vascular systems are key components of the tumor microenvironment and angiogenesis is recognized as a hallmark of cancer. Although studies have indicated that the prognosis of certain cancer patients might be improved by targeting tumor-associated blood vessels, there is a lack of markers that can predict the clinical response to such anti-tumor therapy and thereby stratify patients for optimal management. Microvessel density (MVD) and other angiogenesis markers are known to be effective prognostic factors, but information on response prediction is virtually lacking. In addition to the use of novel endothelial proteins and markers for improved tumor imaging and targeting strategies, the potential practical value of selected histologic indicators for better stratification and predictive purposes needs to be more deeply explored and validated in future studies.

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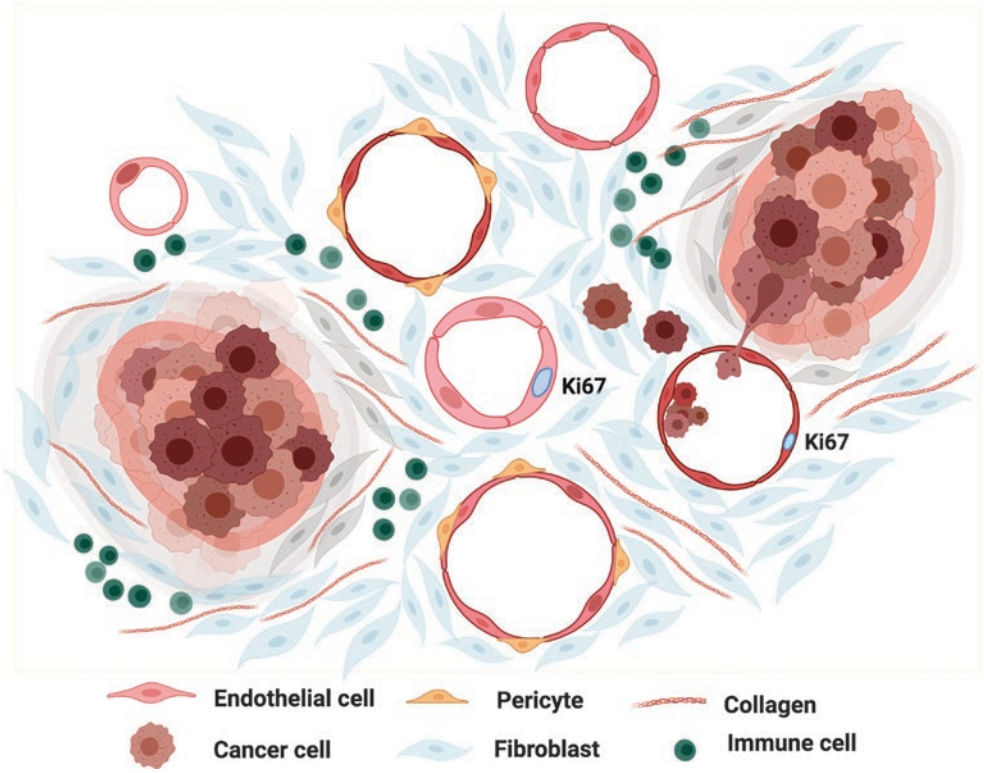

Tumor-associated vessels are unevenly distributed with variation in diameter and shape. They show increased endothelial proliferation (e.g., by Ki67 expression) and are more immature with decreased pericyte coverage. These atypical vessels are more prone to invasion by tumor cells as an early marker of vascular dissemination

Take-Home Lessons

- Tumor-associated blood vessels are different from normal vessels
- Angiogenesis in malignant tumors is most often associated with increased endothelial proliferation and less pericyte coverage
- Vascular proliferation is frequently a stronger prognostic factor than standard microvessel density
- There is no clear association between vascular markers and response to neoadjuvant or adjuvant treatment
- Glomeruloid microvascular proliferation (GMP) is a form of aberrant vascular phenotype with increased occurrence in malignant tumors, being associated with decreased survival in several cancer types

Introduction

In 1971, Folkman suggested that the growth of malignant tumors is dependent on the process of angiogenesis and that tumors can be treated by attacking their blood supply [1]. Since then, mechanisms of angiogenesis have been explored [2–5], and multiple cell types and regulatory pathways have been shown to interact in this complex process, e.g., tumor cells, endothelial cells, perivascular cells, tumor fibroblasts, inflammatory cells, and circulating endothelial progenitor cells from the bone marrow [2, 6, 7]. Studies have indicated an effect of anti-angiogenesis treatment on certain human cancers, such as metastatic colorectal carcinoma, breast cancer, and other tumors [8–10]. A few attempts have been made to identify predictors of response to anti-angiogenesis treatment or traditional chemotherapy [11–15]. Although identification of predictive factors would be important for individual patients and for cost-effective clinical practice,

this search has not been convincing in the angiogenesis field [16], in contrast to the reported value of various angiogenesis markers as significant prognostic factors.

Notably, is it possible to classify or grade the vascular response in malignant tumors on a routine basis, so that this information can be used for improved prognostication as well as for response prediction? Histologic grading of tumor-associated angiogenesis was suggested by Brem et al. in 1972 [17] and was later modified by Weidner and Folkman with the introduction of microvessel density (MVD) as a prognostic indicator for breast cancer [18]. Although MVD has later been shown to predict patient prognosis in multiple clinical studies, this marker has some limitations [19]. Hlatky et al. stated that microvessel density is not a simple measure of the angiogenic dependence of tumors, but is rather a reflection of the metabolic burden of the supported tumor cells. The authors proposed that there would be no direct relationship between microvessel density and the tumor response to anti-angiogenesis therapy.

More recently, other prognostic features of angiogenesis have been reported such as vascular proliferation [20–24] and vascular maturation status [24–26]. Also, architectural patterns like vascular nesting or glomeruloid microvascular proliferation (GMP) have been focused and studied in relation to the diversity of tumor-associated angiogenesis and aggressive tumor features including reduced survival in human cancers [27–29].

In addition to markers of tumor-associated angiogenesis, studies have also reported the frequency and impact of vascular invasion, i.e., the ability of tumor cells to enter blood vessels or lymphatic vasculature, and the different influence of these characteristics on tumor progress in various organs [30–33].

Since there is limited data on the prediction of response to anti-angiogenic treatment or standard chemotherapy using histology-based markers of tumor angiogenesis, this needs to be further explored and validated in translational studies of clinical trials, with respect to response prediction in the era of precision treatment and cost-effective medical practice.

It should be mentioned, although not reviewed here, that the process of angiogenesis in solid tumors is not only a local process, but systemic aspects have gained increasing attention [3]. Thus, it has been shown that populations of circulating bone marrow-derived endothelial progenitor cells can differentiate into mature endothelial cells and contribute to pathological neovascularization. These cells can be detected in tissue sections by immunohistochemistry. However, the relative contribution and role of circulating endothelial progenitor cells to tumor neovascularization in humans is not well understood.

Further, the premetastatic niche concept represents an important part of the systemic interactions and regulatory cross-talk between primary tumors, bone marrow and distant tissues that can be influenced to receive or resist metastatic cells. From a diagnostic point of view, circulating cells, e.g., tumor cells, endothelial precursor cells, or other classes of cells, have also received much attention lately as representing a key part of the "liquid biopsy" concept [34]. These diagnostic modalities will likely supplement the tissue-based assessment of primary and metastatic lesions in the future.

Markers of Angiogenesis

Microvessel Density

In 1972, Brem, Cotran, and Folkman suggested criteria for histologic grading of tumor-associated angiogenesis [17], based on the combined assessment of vasoproliferation (number of microvessels within a microscopic field), endothelial cell hyperplasia (number of endothelial cells lining the cross section of a capillary), and endothelial cytology (nuclear changes in proliferating endothelium). In 1988, Srivastava et al. showed in a small study that histologic quantification of microvessels provided significant prognostic information in melanoma [35]. In 1991, Weidner and Folkman reported criteria for microvessel density (MVD) and demonstrated prognostic value in breast cancer [18, 36]. After highlighting the vessels or individual endothelial cells by pan-endothelial markers like Factor VIII (von Willebrand's factor) or CD31, microvessels were counted in the most active area of the tumors, i.e., within hot-spots (Fig. 2.1). Subsequently, after these important papers, MVD has been widely studied for prognostication in several types of malignant tumors, like breast cancer [18, 36], endometrial cancer

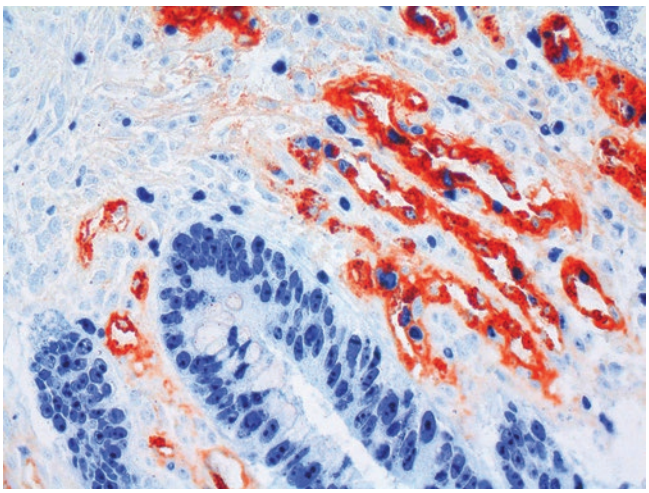

Fig. 2.1 Microvascular proliferation: Microvessels in red (Factor VIII) with some dividing endothelial cells in blue (Ki67). Tumor cells (to the left) show a high degree of proliferation (Ki67 positive nuclei)

[37], lung cancer [23], malignant melanoma [35, 38], and prostate cancer [39, 40]. MVD has been a significant prognostic factor in a majority of studies reported, although some have been negative [41]. In a large meta-analysis of breast cancer [42], including 43 studies and almost 9000 patients, MVD was a significant but rather weak prognostic factor. The conclusions implied that other angiogenic markers might potentially add prognostic information and should be studied.

Modifications of this method have been reported, by using Chalkley counts or image analysis and morphometric measurements based on random area selection [43–45]. The Chalkley counts, giving a relative area estimate of immunostained vessels, may increase the reproducibility of counts within a given hot spot [42]. Tissue sampling is important since there is considerable heterogeneity within individual tumors [46]. However, these methods have not increased the practical value of microvessel counts.

Whereas most studies suggest that microvessel density is a significant prognostic factor, data on response prediction are very limited. Paulsen et al. reported in 1997 that clinical response to neoadjuvant doxorubicin monotherapy for locally advanced breast cancer could not be predicted by MVD [11]. Similar conclusions were reached by others [12]. Further, Jubb et al. [13] concluded that MVD, in addition to VEGF and TSP-1 expression, did not correlate with treatment response or patient outcome in the series of metastatic colorectal carcinoma for which the effect of bevacizumab was first shown [8].

In a study by Tolaney et al. in 2015 [47], a trial of preoperative bevacizumab treatment followed by a combination of bevacizumab and chemotherapy in HER2-negative breast cancer patients was performed to determine how vessel morphology and function was influenced by bevacizumab. The clinical response appeared to reflect the process of vascular normalization primarily in patients with high baseline tumor microvessel density, especially among triple negative breast cancers. In a recent clinical trial study from 2021 of locally advanced or large breast cancer, Krüger et al. examined tissue-based angiogenesis markers for their potential predictive value and found that high baseline MVD significantly predicted response to neoadjuvant bevacizumab treatment [48]. In contrast, microvessel proliferation and the GMP vascular phenotype did not predict response but were instead associated with aggressive tumor features, including basal-like and triple negative tumor phenotypes. Taken together, more data on the predictive value of different tissue-based and other angiogenesis markers is clearly needed. Recently, the introduction of more refined analysis algorithms have been presented [26, 49]. In the latter study, Mezheyeuski et al. reported that the use of novel digitally scored vessel-density-related metrics might identify stroma-normalized microvessel density in the invasive margin as a candidate marker for benefit of adjuvant 5-FU-based chemotherapy in colon cancer. Also, in a study by Corvigno et al., vessel distribution and high "vessel distance" were found to be significantly associated with poor survival in both renal cell and colorectal cancers [50].

Vascular Proliferation

There is limited knowledge of endothelial cell proliferation in human cancers (Fig. 2.1), and its prognostic or predictive importance is not well described in most tumor types. A few studies of breast, lung, prostate, and colorectal tumors have reported a vascular proliferation rate ranging from 0.15% to 17% [20–23, 25, 51–53]. Eberhard et al. studied endothelial cell proliferation in six types of human tumors and found a range from 2.0% (prostate) to 9.6% (glioblastomas) within vascular hot spots [25]. Fox et al. showed a mean labeling index for endothelial cell proliferation in breast cancer of 2.2%, being highest in the tumor periphery [51]. Notably, there was no correlation between endothelial cell proliferation and microvessel density in any of these studies, similar to what others have reported [53]. In a study of 21 colorectal carcinomas, Vermeulen et al. found an average endothelial proliferation labeling index of 9.9%, compared to 21% in vascular hot spots [21]. In a recent study of lung cancer, Ramnefjell et al. found a value of 2.9% in lung cancer [23].

In the early studies, there was no information on the importance of vascular proliferation for patient prognosis. In 2006, Stefansson et al. showed for the first time that vascular proliferation (i.e., proliferating microvessel density, pMVD; microvessel proliferation, MVP) was an independent prognostic factor, shown in endometrial cancer, and pMVD was superior to microvessel density by multivariate analysis [24]. In this study, the median vascular proliferation index (VPI), i.e., the percentage of microvessels, within hot spot areas, with evidence of proliferating endothelial cells by Ki67 staining, was 3.9%, with a range of 0–21% within the tumor tissue. Microvessel proliferation (MVP) was found to be increased in cases with presence of tumor necrosis, and with high tumor stage (by FIGO categories). In the same study, vascular proliferation was an independent prognostic factor by multivariate analysis in addition to histologic grade, vascular invasion by tumor cells, and tumor stage.

In subsequent studies of breast cancer, using three independent cohorts including 499 patients, Arnes et al. found that median vascular proliferation ranged from 0.95% to 1.95% and was associated with estrogen receptor negative tumors and reduced patient survival, whereas microvessel density was not significant [54]. It was further shown by Nalwoga et al., in two breast cancer cohorts including 431 cases, that vascular proliferation was significantly increased in estrogen receptor negative cases and in tumors with a

basal-like or triple negative phenotype [55]. In 2021, Krüger et al. found a median vascular proliferation of 5.2% among 128 patients with locally advanced breast cancer, being associated with basal-like and triple negative phenotypes [48]. Increased vascular proliferation in basal-like compared to luminal breast cancer was recently shown by Kraby et al. [56]. The mechanism for such a relationship in breast cancer is not known. It was found that basal-like and triple negative cancers were associated with VEGF expression [57], a key regulator of breast cancer angiogenesis [58], and VEGF-driven angiogenesis might contribute to the increased vascular proliferation that we found among basal-like tumors. Notably, in a study of locally advanced breast cancer, response to anti-VEGF therapy by bevacizumab was predicted by overall MVD although not by microvessel proliferation [48].

It was reported in 2009 by Gravdal et al. that when combining Ki-67 for endothelial proliferation with a marker of immature endothelium, Nestin, the prognostic sensitivity was increased [59]. By studying prostate cancer, Nestin/Ki67 co-expression, as a marker of vascular proliferation, was four to fivefold higher in castration-resistant cancers and metastases compared with localized tumors and prostatic hyperplasias. Still, even among localized cancers, high vascular proliferation was a strong and independent predictor of biochemical failure, clinical recurrence, and time to skeletal metastasis by multivariate analysis. In castration-resistant cancers, vascular proliferation was associated with reduced patient survival. In a more recent study of prostate cancer, vascular proliferation was found to be associated with EMT factors Twist and Snail [60]. In breast cancer, by Nestin/Ki67 co-expression, a median vascular proliferation of 2.7% was found by Krüger et al. [61]. There were significant associations with estrogen receptor negative tumors as well as basal-like and triple negative phenotypes. In this study, vascular proliferation was an independent predictor of death from breast cancer. In lung cancer, the median vascular proliferation (by Nestin/Ki67) was 2.9% [23].

Interestingly, in a study by Haldorsen et al., microvascular proliferation in endometrial cancers was compared with imaging parameters obtained from preoperative dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and diffusion-weighted imaging (DWI) to explore the relationship between these markers and their potential ability to identify patients with poor outcome [62]. Notably, microvessel proliferation was found to be negatively correlated to tumor blood flow by MRI, possibly reflecting an abnormal and reduced functionality in newly formed tumor-associated vasculature. In this study, vascular proliferation was significantly associated with reduced patient survival, similar to what was previously found [24].

In a study by Stefansson et al. in 2015, a 32-gene expression signature was found to separate tumors with high versus low microvascular proliferation [63]. This 32-gene signature associated with high-grade tumor features and reduced survival by independent cohorts. Interestingly, copy number studies revealed a strong association between microvessel proliferation and 6p21 amplification. VEGF-A is known to be located in the 6p21 chromosomal region [64], and integrated analyses demonstrated significant associations between increased vascular proliferation and VEGF-A mRNA expression, pointing to a possible angiogenesis driver mechanism in endometrial cancer. In a previous study of endometrial cancer, VEGF-A was significantly associated with vascular proliferation and reduced patient survival [24]. In locally advanced breast cancer, this 32-gene angiogenesis signature was associated with vascular proliferation and a basal-like tumor phenotype, although not with response to anti-VEGF therapy by bevacizumab [48].

Vascular Maturation

The structural integrity and maturation status of blood vessels, i.e., the degree of coverage by cells like pericytes, has been reported [3, 65], and several factors are known to contribute to pericyte recruitment [66, 67]. Reduced maturation appears to accompany the atypical structure of vessels in malignant tumors [27, 68]. Also, tumor-associated pericytes are often abnormal when present [69]. Vascular maturation, as estimated by pericyte coverage, appears to be a dynamic process. In prostate cancer, androgen ablation therapy may induce a downregulation of intra-tumoral VEGF followed by selective regression of immature tumor microvessels by apoptosis of endothelial cells not covered by pericytes [70]. The authors suggested that vessel maturation status of individual tumors might predict the efficacy of anti-VEGF tumor treatment. In 2001, Jain proposed that anti-angiogenic therapy might lead to improved maturation and normalization of the tumor vasculature thereby increasing the efficacy of combined treatment including chemotherapy or radiation [71, 72]. In a clinical study, injection of anti-VEGF was followed by increased maturation of tumor-associated vessels [73], as has also been reported in experimental studies [74, 75]. It was shown that anti-VEGFR2 treatment creates a "normalization window" of the vasculature for increased efficacy of additional radiation treatment by upregulation of Ang1 and degradation of the basement membrane by MMP activation [76]. In a trial of preoperative bevacizumab followed by a combination of bevacizumab and chemotherapy in HER2-negative breast cancer, Tolaney et al. reported that the tumor response appeared to reflect vascular normalization, primarily in patients with high tumor microvessel density [47].

Data on human tumors are limited with respect to clinical correlates and outcomes. In early clinical studies of this

marker, Eberhard et al. reported vascular maturation in six human tumor types and found a wide range in pericyte coverage index from 13% (glioblastoma) to 67% (breast cancer) [25], although no clinical or prognostic evaluation was presented. In a study of lung cancer [77], a better outcome was found for tumors with high vascular maturation. The mean vascular maturation index (VMI) was 46%, and high VMI was associated with low microvessel density and absence of nodal metastases. In contrast, a report on breast cancer showed no prognostic impact of VMI [78]. In both studies, the basement membrane antibody LH39 was used as a maturation marker. The authors concluded that differences between various tissues in vascular proliferation and maturation might be relevant for the suitability of anti-angiogenic treatment. In a study of endometrial cancer in 2006, Stefansson et al. showed that median pericyte coverage, as estimated by the α-SMA coverage index (SMAI), was 35%, and lower SMAI was significantly associated with increased vascular invasion by tumor cells and impaired patient prognosis [24].

In a study of colorectal cancer from 2016, semi-quantitative and digital image analyses-based scoring identified significant associations between low expression of perivascular PDGFR and shorter overall survival. Notably, perivascular PDGFR-α and PDGFR-β remained independent factors for survival by multivariate analyses [26].

Glomeruloid Microvascular Proliferation

Although tumor vessels frequently have abnormal structure, architectural and cytologic atypia might be difficult to assess, and there is no consensus on how to report vascular morphology in a reproducible way. Some studies have suggested pattern-based angiogenesis markers, such as glomeruloid microvascular proliferations (GMP) (Fig. 2.2). GMP, also called "microvascular nests" or "glomeruloid bodies," are focal proliferative buddings of a mixture of vascular cells (primarily multilayered endothelial cells in addition to pericytes and macrophages) that superficially resemble renal glomeruli [79–82]. In standard tissue sections, GMPs generally consist of 15–100 cells; one or more vascular lumens are usually present, especially in more mature GMPs.

GMPs represent a defining histologic feature of glioblastoma multiforme [79, 80] and have been associated with increased aggressiveness in brain tumors [83, 84]. GMP-like patterns have also been sporadically reported in other tumors, including gastrointestinal carcinomas, thymomas, and different vascular tumors [81, 85–89]. However, until quite recently, human tumors have not been studied systematically.

In animal studies, Dvorak and coworkers induced the formation of "glomeruloid bodies" from preexisting microvessels in mouse skin, through the injection of an adenoviral vector expressing VEGF-A_{164}, indicating that the formation of the GMP phenotype might represent a VEGF-A dependent and dysregulated angiogenic response [90]. The formation of new blood vessels through several steps, each with a distinctive morphology, was described in detail; these include *mother vessels* (MOV), *glomeruloid microvascular proliferation* (GMP), and *arterio-venous malformations* (AVM) [27, 81, 82, 91]. The GMP phenotype was dependent on the continued presence of VEGF-A_{164}, and as VEGF-A_{164} expression declined, GMPs underwent apoptosis and progressively devolved into smaller, more normal-appearing microvessels [82]. Thus, the GMP generated in this model also required exogenous VEGF-A_{164} for their maintenance, and this finding is likely relevant to GMP in human tumors. All of the tumor types known to form GMP also express VEGF-A. Another human parallel appears to be the POEMS syndrome, where increased VEGF-A levels are associated with glomeruloid vascular proliferations in the skin, i.e., glomeruloid hemangioma [85].

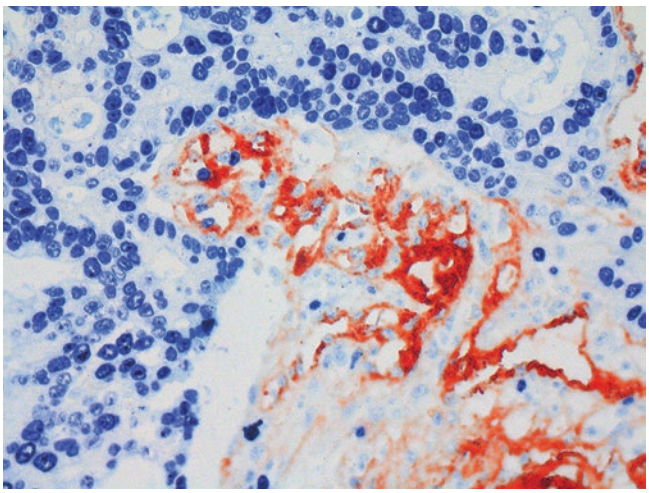

Fig. 2.2 Glomeruloid microvascular proliferation (GMP) (red vessels, Factor VIII), with a few dividing endothelial cells in blue (Ki67), and marked proliferation in tumor cells (Ki67)

In a study by Straume et al. in 2002 of more than 700 human cancers (breast, endometrial, prostate, melanoma), approximately 20% of the cases were considered GMP positive (range 13–23%). Presence of GMP was significantly related to poor prognosis [29], and this has been confirmed in studies of non-small cell lung cancer [92] and pancreatic cancer [93]. This angiogenic phenotype was found to be a better predictor of outcome than microvessel density [16].

In the series of nodular melanomas [29], 23% were GMP positive, and the presence of GMP was significantly associated with aggressive tumor features like increasing lesion thickness (a.m. Breslow) and ulceration. In survival analysis, GMP was an independent prognostic factor along with Clark's level of tumor invasion and ulceration, and GMP was

of greater value in this regard than standard microvessel density. To extend these studies, the presence of GMP in relation to the expression of several different angiogenic factors and their receptors in melanoma was evaluated [94]. GMP was associated with increased endothelial cell expression of VEGF receptor-1 (FLT-1), VEGF receptor-2 (KDR), and Neuropilin-1. The expression of VEGF-A protein in tumor or endothelial cells was not associated with the presence of GMP, whereas VEGF-A expression was significantly stronger in GMP endothelium compared with non-GMP endothelium within the tumors. There was a significant association between lack of Tie-2 expression in tumor-associated endothelial cells and the presence of GMP, whereas there was no association with the expression of angiopoietin-1 (Ang-1) [94]. Taken together, our findings indicate that increased expression of VEGF receptors on the endothelium in melanomas was associated with presence of GMP, whereas the opposite was found for Tie-2, a receptor that has been linked to vessel maturation [10]. Expression of bFGF was decreased in GMP endothelium, and this has been associated with a less mature vasculature [29].

In our initial study [29], 17% of breast carcinomas were GMP positive, and presence of GMP was related to the ductal histotype, high grade, estrogen receptor negativity, and HER2 expression. Regarding prognosis, GMP was found to be an independent prognostic indicator by multivariate analysis, providing additional information beyond basic variables such as tumor size, histologic grade, and lymph node metastases. Notably, GMP was not correlated with microvessel density (MVD) which was not prognostic in this patient cohort. These findings indicate that GMP may provide a novel prognostic marker, indicative of a more aggressive vascular phenotype.

Further studies on breast cancer indicated that GMP is associated with multiple markers of aggressive tumors like estrogen receptor negativity and a basal-like phenotype [95], and the GMP vascular phenotype has been associated with presence of *BRCA1* germline mutations and p53 alterations [96]. *BRCA1*-related breast cancers have a distinct profile on microarray analysis [97] and also a characteristic spectrum of *TP53* mutations [98]. Our data suggest that *BRCA1* mutations might induce a genetic profile of which GMP is an important manifestation and part of the tumor phenotype. Of relevance, BRCA1 protein has been associated with inhibition of VEGF transcription and secretion in breast cancer cells [99].

We previously found a significant association between GMP and pathologic expression of p53 protein [96], whereas p53 overexpression was not associated with increased microvessel density. The relationship between p53 and angiogenesis could involve several different mechanisms: 1. p53 is known to suppress the expression of VEGF [100] and interacts with the transcription factor Sp1 [101]; 2. p53 degrades hypoxia inducible factor 1 [102]; 3. p53 downregulates the expression of bFGF binding protein [103]; and 4. p53 upregulates thrombospondin-1 expression [104].

In a study of locally advanced breast cancer, treated with standard chemotherapy, Akslen et al. found that the presence of GMP, occurring in 21% of the cases, was significantly associated with high-grade tumors and *TP53* mutations in addition to basal-like and HER2 positive subtypes of breast cancer as defined by gene expression data [15]. The GMP phenotype was significantly associated with a lack of treatment response and progressive disease, indicating a potential predictive value. In these tumors, GMP was also correlated to a gene expression signature for tumor hypoxia response, pointing to a possible mechanistic relationship. In a randomized clinical trial of neoadjuvant bevacizumab treatment of locally advanced breast cancer, GMP was associated with aggressive tumor features, although not with treatment response, which was predicted by baseline microvessel density [48].

In a study of metastatic melanoma, GMP in primary tumors (25%) or metastatic tissue (12%) did not predict the response to bevacizumab monotherapy, although limited tissue from metastatic lesions could decrease sensitivity [105].

In endometrial cancer, GMPs were found to be significantly associated with increasing histologic grade, diffusely invasive growth pattern, presence of necrosis, vascular invasion, deep myometrial invasion, and high clinical stage [24]. This study also indicated an association between GMP formation and increased vascular proliferation, by Factor VIII/Ki67 co-expression. The findings provide further evidence that GMP is an angiogenic marker of high-grade and aggressive tumors.

In prostate cancer, GMP was present in 13% of cases [29] and was associated with high preoperative levels of serum PSA. The GMP phenotype was an independent predictor of time to biochemical failure as determined by multivariate analysis.

In other tumor types, GMP was a significant prognostic factor in a study of non-small cell lung cancer [92]. A total of 25% of these tumors were GMP positive, and the frequency of GMP was not associated with basic factors such as histologic grade or clinical stage. Similar to our findings [29], there was no association between GMP status and microvessel density in these lung cancers. There was no correlation between VEGF-A expression and the frequency of GMP, although this phenotype was more often seen in Ang-1 positive tumors. Multivariate analysis indicated that GMP was a significant and independent prognostic factor, whereas microvessel density was not. Taken together, these data support our initial observation that GMP might be a novel and significant tissue-based angiogenesis marker for potential clinical use.

Other Vascular Patterns

There has been some additional focus on architectural patterns of angiogenesis in malignant tumors [106]. It seem that qualitative features, rather than quantitative metrics of microvessel density and other markers, may provide some prognostic relevance in certain tumor types, like glioblastomas of the brain, and ocular melanomas. Some studies have focused on the distribution pattern of microvessels within tumors. The EDVIN concept ("edge versus inner") suggests that comparing vessel counts at the edge of the tumor with the inner area might give a better picture of the angiogenic activity and patient survival. The prognostic value of EDVIN was shown in studies of breast and colorectal cancers [107].

Quantification of vascular pattern by image analysis has shown increased prognostic impact by use of syntactic structure analysis [108]. Studies of pheochromocytomas, which are highly vascular tumors of the adrenal medulla, have shown that complex and irregular vascular patterns are associated with malignant behavior [109].

Vascular Molecular Phenotypes

Can certain vascular immunomarkers discriminate between endothelial cells in benign tissues and "activated" tumor-associated endothelium? If so, these markers could be applied in tumor imaging and therapeutic targeting, in addition to response prediction and prognostication. This field is very promising but not well developed, and it is not the primary topic of this chapter. Chi et al. reported expression differences between endothelial cells from various sites of the vascular system [110]. Also, proteins are differentially expressed in tumor-associated endothelium [111, 112], and such endothelial markers might provide "zip codes" or "maps" for homing of anti-tumor peptides like LyP1 [113]. St. Croix et al. showed multiple novel antigens being expressed selectively in tumor endothelium from colorectal cancers, some of them associated with the cell membrane (TEM1, TEM7, TEM8), or extracellular matrix [114]. In the same setting, studies from our team indicate that when using the marker Nestin for immature endothelium, in addition to Ki67 as a proliferation marker, enhanced and significant prognostic information can be obtained from tissue sections [59, 61].

Pan-endothelial markers, such as Von Willebrand's Factor (Factor VIII), CD31, and CD34, are frequently used to visualize endothelial cells by immunohistochemistry when estimating microvessel density. Some reports suggest that CD105/endoglin, a TGF-β receptor involved in vascular development and remodeling, might be suitable as a marker of active angiogenesis in malignant tumors, as well as a therapeutic target on tumor-associated vessels [115]. Microvessel density by CD105 was superior and independent as a prognostic factor in breast cancer [116]. Similar results were presented for lung cancer [117] and prostate cancer [118], whereas no advantage of CD105 was found in studies of endometrial cancer [119] and malignant melanoma [120].

VEGF and its receptors may be present on tumor cells and vessels and might represent targets for imaging and treatment [121]. It was shown that activated microvessel density (aMVD), as estimated by VEGF/KDR staining on endothelial cells, was highest in the tumor periphery and superior to standard microvessel density (sMVD) as a prognostic factor evaluated by multivariate survival analysis of non-small cell lung cancer [122].

Expression of bFGF on tumor-associated endothelial cells was inversely associated with lymph node metastases and pathological stage of non-small cell lung cancer [123]. Similar findings, together with a prognostic role, have been found for prostate cancer [124] and malignant melanoma [125]. These findings further support the diversity of tumor-associated vessels.

Other angiogenesis markers have been explored, like the expression of tumor-specific endothelial (TEM) antigens [126–128]. Expression of certain integrins, like αvβ3, has been associated with tumor vasculature [129], and this marker might also be applied for imaging [130] and treatment strategies [131]. The main challenge will be to validate such proteins in further studies. It is not clear whether simple histology-based tissue markers will prove effective in comparison with other classes of angiogenic markers, like circulating endothelial cells. Taken together, studies of vascular markers are important for our understanding of tumor-associated angiogenesis, vascular imaging techniques, and the development of therapeutic modalities. Whether gene expression signatures might capture the complexity of malignant tumors and better reflect their angiogenesis capacity should be studied in more detail.

Markers of Vascular Invasion

One important hallmark of cancer progression is the ability of tumor cells to migrate into vascular channels, i.e., blood vessels or lymphatic vasculature, as an early step of metastatic spread [132]. In breast tumors, vascular invasion is usually considered to be lymphatic vessel involvement (LVI) more often than blood vascular invasion (BVI) [31], but there are few studies in this field. Vascular invasion, as observed on standard tissue sections, is associated with an increased risk of tumor recurrence, metastasis, and death from disease [31, 133]. Lymphatic invasion is particularly important as a prognostic factor in early stage breast cancer [134, 135]. Gujam et al. highlighted that immunohistochemistry discriminates better between BVI and LVI, and this

distinction improves the prognostic value of vascular invasion compared to standard sections [32, 33, 136–138].

A potentially different impact of blood vessel invasion as compared with lymphatic involvement has not been well established, for example, in relation to the molecular subtypes of breast cancer. This might be due to the lack of firm criteria to separate blood vessel and lymphatic invasion. Usually, CD31 staining for blood vessel endothelium and D2-40 for lymphatic vessels are applied, although overlapping staining patterns exist. Still, D2-40 expression is considered to be specific for lymphatic endothelium. In a breast cancer study by Klingen et al., blood vessel invasion, present in 15% of the cases, showed strong associations with non-luminal tumors such as the basal-like, triple negative, and HER2 positive subgroups [32]. In survival analysis, BVI was significantly associated with recurrence-free and breast cancer-specific survival, whereas LVI was not. When adjusting for basic factors, BVI was an independent prognostic marker, indicating that this feature might be recorded in breast cancer diagnostics, although more studies need to confirm these findings. Development of even more specific markers for blood vessels would be desirable in a routine setting to identify patients at a higher risk for early systemic spread. The potential use of such diagnostic approaches for improved therapy among cases with blood vessel invasion should be considered.

We previously reported that basal-like breast cancers appear to have increased angiogenesis with more microvessel proliferation and higher frequency of the glomeruloid microvascular pattern (GMP) when compared with other breast cancer subtypes [54, 55]. These findings suggest a possible relationship between increased angiogenesis and blood vessel invasion among basal-like breast cancers. The relationships between vascular proliferation, immature vessels, and vascular invasion have also been shown in endometrial cancer [24].

Notably, studies of disseminated tumor cells from the bone marrow, as well as expression profiles of primary tumor cells, suggest that hematogenous spread is often an early event in tumor progression [139]. Early systemic dissemination of breast cancer cells is associated with a specific expression signature, and the molecular pathways associated with primary hematogenous spread and lymphatic dissemination appear to be different [140]. The present data suggest that blood vessel invasion by tumor cells is strongly associated with aggressive tumor subtypes (basal-like, triple negative, HER2 positive). Blood vessel invasion has also been related to interval breast cancer presentation compared with screen-detected tumors [32]. Based on such findings, it might be of practical importance to examine the presence of blood vascular invasion in breast cancers.

It has been suggested that the basal-like phenotype of breast cancer may be related to non-lymphatic spread [141], and findings indicate a reduced risk of axillary lymphatic spread in triple negative breast cancer [142]. Although the presence of metastases in axillary lymph nodes predicts the development of distant metastases, 20–30% of patients with node-negative breast cancer develop metastatic spread at distant sites [143]. Early systemic dissemination of breast cancer cells is associated with a specific gene expression signature [140].

In a large study of endometrial cancer, 18% of the tumors showed blood vessel invasion, whereas 31% of the tumors revealed lymphatic involvement [30]. Both BVI and LVI were associated with features such as high histologic grade and diffuse tumor growth. Patients without vascular invasion had the best prognosis and those with BVI (with or without LVI) had the worst outcome, whereas patients with LVI had an intermediate survival by univariate analysis. Both BVI and LVI had independent prognostic importance. Such findings support the biological importance of vascular spread through the haematogenic and lymphatic routes in endometrial cancer. The significant correlation found with clinical phenotype indicates that these markers may be relevant for patient management.

In further studies of endometrial cancer, certain gene expression patterns were associated with vascular invasion by tumor cells as examined in standard sections [144]. Thus, a vascular invasion signature of 18 genes was significantly associated with patient survival and clinicopathologic phenotype. Vascular involvement was related to gene sets for epithelial-mesenchymal transition, wound response, endothelial cells, and vascular endothelial growth factor (VEGF) activity. Further, expression of Collagen 8 and MMP3 were associated with vascular invasion, and ANGPTL4 and IL-8 showed a relationship to patient survival. These findings indicate that vascular involvement within primary tumors is associated with gene expression profiles related to angiogenesis and epithelial-mesenchymal transition. This 18-gene expression signature was furthermore studied in multiple cohorts of breast cancer and found to associate with aggressive features like high tumor grade, hormone receptor negativity, HER2 positivity, a basal-like phenotype, reduced patient survival, and response to neoadjuvant chemotherapy [145]. The 18-gene vascular invasion signature was associated with several other gene expression profiles related to vascular biology and tumor progression, including the Oncotype DX breast cancer recurrence signature. Taken together, the findings indicate that markers for vascular invasion by tumor cells in the primary tumor, including gene expression patterns, might provide information that indicates an increased risk of metastatic spread.

Concluding Remarks/Summary

It has become increasingly evident that some malignant tumors can be treated by attacking their blood supply. At the same time, both experimental and clinical data have demonstrated that tumor-associated angiogenesis is more complex than reflected simply by the number of microvessels on tissue sections. In the era of targeted therapy, companion biomarkers are becoming crucial to increase treatment efficacy by defining subgroups of patients with high probability of response to the treatment [13, 16], similar to the role of HER2 in breast cancer management. Whereas this is a "hallmark of tailored treatment," such markers have not yet been successfully established in the field of anti-angiogenesis therapy. In the case of anti-VEGF regimens, there is no simple relationship between presence of the target (VEGF) and treatment response [13], and no reliable association with the "end-point" of angiogenic stimulation, i.e., microvessel density, has been found. At the same time, there is a relative lack of translational studies of human tumors, and tissue-based angiogenesis markers should therefore be further studied and validated. Markers reflecting the angiogenic response in primary tumors, such as vascular proliferation and vascular maturation status, need to be examined across different tumor types to increase the evidence of their potential utility, especially as predictive factors. The presence of glomeruloid microvascular proliferation (GMP), reflecting some of the increased irregularity and complexity of tumor-associated angiogenesis, and a marker of VEGF-driven angiogenesis, should be considered. Furthermore, a refined immunophenotypic profiling of the tumor vasculature might improve the basis and indications for novel imaging techniques and treatment targets. Complementary systemic biomarkers, such as circulating endothelial progenitor cells, are likely to gain increased importance. Different markers might be combined into profiles to obtain a balance between high-technology methods and simpler cost-effective techniques.

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