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## *Review*

# Evaluation of microvascular density in tumors: pro and contra

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**Summary.** Microvascular density (MVD) counting protocols have become the morphological gold standard to assess the neovasculature in human tumors. This method requires the use of specific markers to vascular endothelium and of immunohistochemical procedures to visualize microvessels. MVD determined in primary tumors is significantly associated with metastasis and prognosis in several tumors and is most predictive in those tumors that induce significant angiogenesis, namely carcinomas of breast and prostate, and haematological malignancies.

There is such a wide range of antibodies and suppliers, antigen retrieval methods, designation of high and low vessel count groups, patient study groups and data interpretation, that it is exceedingly difficult to compare results.

**Key words:** Angiogenesis, Endothelial cell, Microvascular density, Tumor growth

### Historical background

As early in 1972, Brem et al. proposed a microscopic angiogenesis grading system to assess the angiogenic status of the tumor vasculature. Based on the analysis of the vascular density, the number of endothelial cell (EC) nuclei and endothelial cytology, an angiogenic score was determined and used to establish an angiogenic rank order of different human brain tumors.

In 1988, Srivastava et al. studied the vascularity of 20 intermediate thickness skin melanomas (0.76 to 4.0 mm level of invasion). Vessels were highlighted with *Ulex europaeus*-1 agglutinin conjugated with peroxidase, and the stained histological sections were analyzed with a semi-automatic image analysis system. The 10 cases that developed metastases had a vascular area at the tumor base that was more than twice that seen in the 10 cases without metastases. Age, sex, Breslow's tumor thickness, and Clark's level of invasion were similar in the two groups.

In 1991, Weidner et al. developed a new method to perform microvascular density (MVD) counting studies within tumors. The first step in Weidner's approach is the identification by light microscopy of the area of highest neovessel density, the so called hot spot, by scanning the whole tumoral section at low power, then, individual microvessels are counted at a higher power (x200 field) in an adequate area (e.g. 0.74 $mm^2$ per field using x20 objective lens and x 10 ocular). The technique for identifying neovascular hot spots is very similar to that for finding mitotic hot spots for assessing mitotic figure content within breast tumors and is subjected to the same kind of inter- and intra-observer variability. Sclerotic, hypocellular areas within tumors and immediately adjacent to benign breast tissues were not considered in MVD determinations. Any stained EC or clusters separate from adjacent vessels are counted as a single microvessel, even in the absence of vessel lumen. Vessels with muscular walls were not counted. Vessel lumen and red cells were not used to define a microvessel. Each single count is expressed as the highest number of microvessels identifed at the hot spot. By using this approach, Weidner et al. (1991) showed that intratumoral MVD in mammary tumors with poor prognosis and metastasis is twice as high in patients with mammary tumors with good prognosis and without metastasis, and confirmed this correlation also in prostate carcinoma (Weidner et al., 1993). Other studies

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performed on different patients databases by different investigators at different medical centers have observed the same association of increasing intratumoral vascularity with various measures of tumor aggressiveness, such as higher stage at presentation, greater incidence of metastases, and/or decreased patient survival.

Some Authors used the Chalkley count which consists of applying a 25-point Chalkley eyepiece graticule (Chalkley, 1943) on several hot spots. Briefly, three or four areas of tumor are chosen. The graticule is applied to each hot spot using a specific magnification (x250) with a corresponding defined Chalkley grid area (0.196 $mm^2$). The graticule is oriented to allow the maximum number of points to hit on or within the areas of stained microvessel profiles. This technique, suggested as a standard in an international consensus (Vermeulen et al., 1996) is considered to be a simple and acceptable procedure for daily clinical use (Fox et al., 1994; Hansen et al., 1998). Hansen et al. (2000) studied a population based group consisting of 836 patients with operated primary, unilateral invasive breast carcinomas and demonstrated that there were significant correlations between high Chalkley counts and axillary lymph node metastasis, large tumor size, high histological malignancy grade, and histological type.

### Counting procedure

Manual counting remains variable and poorly reproducible. The counting procedure relies on the subjective distinction of individual vessels by the observer. This distinction may be particularly difficult in areas where a single tortuous vessel can be sectioned several times and, depending on the observer, counted as one or multiple microvessels (Simpson et al., 1996).

Different methods based on computerized image analysis have been developed to quantify immunohistochemical staining (Lehr et al., 1997) and have therefore been proposed to eliminate the subjective distinction of microvessels. These computer systems have their own general and specific problems, above their capital and running costs. Measurements of endothelial area (EA) corresponding to the surface of immunostained endothelial structure (Simpson et al., 1996; Schoell et al., 1997), microvessel perimeter and microvessel area, consisting of the EA plus the vessel lumen (Barbareschi et al., 1995), has been as a more accurate index of tumor vascularization.

Total microvascular area (TVA) is occupied by microvessels per unit area of the tumor in a limited number of fields (three or four), subjectively selected from the most vascularized areas (hot spots). Branching count is the number of vessel ramifications per 100 vessel sections. There is usually a positive correlation between MVD and branching count (Korkolopoulou et al., 2001). However, these different methods do not eliminate another factor of variability, which is the selection of the tissue area to be analyzed.

### Multiple blocks or single block?

It has been suggested that multiple blocks per tumor should be assessed for MVD (De Jong et al., 1995). De Jong et al. (1995) found a high average coefficient of variation of approximately 24% if more than one tissue block was analyzed as compared to 15% when only counts within one section of one block were made, indicating that careful stanning of all available tumor material might be necessary to identify the relevant hot spots.

### Hot spots or randomly chosen microscopic fields?

Microvessel quantification is usually performed, either on vascular hot spots corresponding to the most vascularized area (Weidner et al., 1991) or on randomly chosen microscopic fields (Oh et al., 2001). The selection of vascular hot spots is subjective and depends on the experience and training of the observer (Vermeulen et al., 1997). There is little agreement as to the optimal number of hot spots to assess, which currently ranges from 1 to 5. In many studies the mean value of the vessel count in four fields was retained as the final value of MVD, others counted at least two fields for each tumor. This number also has an important bearing on the efficacy of the method, since tumors have a limited number of identifiable hot spots.

Vascularization quantification on randomly chosen microscopic fields is dependent on the arbitrary selection of a limited number of fields in a restricted area of a tumor section and does not take into consideration the heterogeneous distribution of microvessels in tumor tissue (Vermeulen et al., 1996). A higher magnification gives an increased resolution, which enables more microvessels to be identified, but to the detriment that all fields at too high a magnification become an angiogenic hot spot. Conversely, low magnification, with its lower resolution, will identify a smaller number of vessels, and will dilute out the hot spot.

### Use of panendothelial cell markers

Interest in grading tumor angiogenesis was stimulated with the advent of non-specific endothelial markers, such as endogenous alkaline phosphatase and lectins, but only since the latter part of 1980s, as more specific endothelial markers have become available, has the quantification been performed.

The use of different panendothelial cell markers may account for some of the variation in the estimation of MVD. When applied properly, anti factor VIII-related antigen (RA) remains the most specific endothelial marker, providing very good contrast between microvessels and other tissue components.

Although apparently more sensitive and superior on paraffin sections, CD31 strongly cross-reacts with plasma cells (De Young et al., 1993). This complication can markedly obscure the microvessels in those tumors

with a prominent plasma cellular inflammatory background. Another disadvantage of CD31 staining is the frequent antigen loss due to fixatives containing acetic acid. Using anti-CD31 antibodies, regions with a prominent inflammatory infiltrate might be erroneously taken for a vascular hot spot at low magnification.

CD34 is an acceptable alternative and the most reproducible EC highlighter in many laboratories, but CD34 will highlight perivascular stromal cells and has been noted to stain a wide variety of stromal neoplasms (van de Rijn and Rouse, 1994; Traweek et al., 1991).

It is noteworthy to emphasize that none of the above markers are able to discriminate between quiescent versus activated/proliferating endothelium. Three antibodies, namely E-9 (Wang et al., 1993, 1994), CD105 (endoglin) (Kumar et al., 1999; Minhajat et al., 2006) and LM-609 to integrin $\alpha v\beta_3$ (Thorpe et al., 1994) seem to be specific for activated/proliferating endothelial cells.

CD105, a proliferation-associated and hypoxia-inducible protein, is preferentially expressed in the activated EC participating in neoangiogenesis, especially tumors, and is undetectable or weakly expressed in vessels of normal tissues (Kumar et al., 1999; Marioni et al., 2005, 2006; Minhhajat et al., 2006; Sandlund et al., 2006).

Tie-2/Tek, an endothelium-specific receptor tyrosine kinase (Dales et al., 2004a) and vascular endothelial growth factor (VEGF) receptors (Dales et al., 2004b) also identify stromal vessels.

## Topography

Topography is important in differentiating tumor vessels into those supplying the invading tumor edge (i.e. the zone of tumor/normal tissue interaction) and those serving the inner tumor area.

The peripheral tumor areas are composed of typical capillaries with EC, derived form pre-existing vessels. In contrast, the central areas of the tumor are made up of tube-like endothelial structures and pseudo-vascular channels lined by tumor cells, not EC. These tumor-lined spaces, commonly known as 'vasculogenic mimicry', are probably generated directly by the tumor cells rather than through the expression of angiogenic factors (non-angiogenesis-dependent pathway). The two vascular channels are inter-communicating (Ribatti et al., 2003; Yue and Chen, 2005; Zhang et al., 2007).

Although areas of hot spots are not infrequently seen within the inner tumor area, they usually predominate at the edge of tumor. The vascular density was high in the tumor areas adjacent to normal tissues, but decreased gradually towards the inner tumor areas, although to a variable degree between different types of tumors.

As was pointed out by Thomlinson and Gray (1955) one can think of the supported tumor cells as forming a viable cuff around a vessel, with cuff size being roughly indicative of the metabolic burden of the cancer cells. Cuff size tends to vary inversely with tumor metabolic demand. Tumors that have high rates of oxygen or nutrient consumption, such as glioblastomas, have small cuffs only two or three cells wide and have a high vascular density. In contrast, tumors of low metabolic demand, such as chondrosarcomas, have a relatively large cuff size, with many cell layers supported and a relatively low vascular density.

## Certain human tumor types can exhibit lower MVD than the corresponding normal tissues

The measurement of MVD is not sufficient to reveal the functional or angiogenesis status of tumor neovasculature. MVD for human lung, mammary, renal cell and colon carcinomas are lower than those of their normal tissue counterparts (Eberhard et al., 2000). In lung carcinoma, for example, MVD was found to be only 29% that of normal lung tissue. MVD for glioblastomas was found to be 78% that of normal brain tissue and pituitary adenomas are less vascular than the normal pituitary gland (Turner et al., 2000). The apparent paradox can be partially explained by the lower oxygen consumption rate of tumor cells (Steinberg et al., 1997). All tumor vessels are not equal in their ability to provide oxygen and nutrients to the tumor cells they support. Tumor vessels can themselves be hypoxic and carry little oxygen, or they can have oscillating rather than directed blood flows and thus be ineffective at transporting oxygen and nutrients (Hickley and Simon, 2006; Brahimi-Horn and Pouyssegur, 2006; Gruber and Simon, 2006). Inhibiting hypoxic vasculature would shrink a tumor mass less than would inhibiting oxygen-rich vessels (Boyle and Travers, 2006).

In addition, tumor cells are known to tolerate oxygen deprivation and to be resistant to apoptosis under hypoxic conditions (Graeber et al., 1996). Both the lowered oxygen consuption of tumor cells and their tolerance of hypoxic conditions promote increased intercapillary distance in tumors relative to their normal counterparts.

## Prognostic value of MVD

MVD would be a good indicator of therapeutic efficacy, but it has not been as useful for efficacy as it has for prognosis. Since the early studies, hundreds of reports have examined the prognostic value of MVD in several forms of cancers. Most of these studies report positive correlation between MVD and tumor recurrence. Several studies on MVD and prognosis gave positive results in patients with solid tumors, such as head and neck, lung, gastric, colorectal, liver, pancreatic, renal, bladder, ovarian, endometrial and breast cancers and neuroblastoma (Lindmark et al., 1996; Chandrachud et al., 1997; Shimizu et al., 2000; Cantu De Leon et al., 2003; Kusamura et al., 2003; Cernea et al., 2004; Lackner et al., 2004; Coulvelard et al., 2005; Ribatti and Ponzoni, 2005; Zhao et al., 2005; Fernandez-Aguilar et al., 2006; Ribatti et al., 2006; Yao et al., 2007). In

gliomas, MVD appears correlate with outcome in high-grade, but not low-grade tumors, and does not correlates with tumor cellularity in the infiltrating portions of the tumors (Korkolopoulou et al., 2002; Sharma et al., 2006). More recently, a positive correlation between MVD and tumor recurrence has been established also in hematological tumors (Korkolopoulou et al., 2001, 2003, 2005; Ridell and Norrby, 2001; Alexandrakis et al., 2004; Lundberg et al., 2006; Vacca and Ribatti, 2006). Nevertheless, despite the initial confirmatory publications, numerous reports appeared in the literature that fail to show a positive association between increasing tumor vascularity and reduced patient outcome, and caution as to the clinical utility of tumor angiogenesis is being urged (Bossi et al., 1995; Busam et al., 1995; Marrogi et al., 2000; Hillen et al., 2006). However, many of these negative studies may result from significant differences in methodologies.

### MVD has not been shown to be a valid measurement to guide or evaluate antiangiogenic treatment

It is widely assumed that tumors with high MVD are good candidates for clinical trials of antiangiogenic therapies, whereas tumors that typically have low MVD are thought to be poor candidates for such clinical trials (Lenz, 2005; Rhee and Hoff, 2005; Cooney et al., 2006; Zhong and Bowen, 2006; Laquente et al., 2007). However, experimental evidence shows that both poorly vascularized and highly vascularized tumors can respond to antiangiogenic therapy. MVD, accordingly offers no indication as to which patients might be most responsive to antiangiogenic therapy (Lenz, 2005; Rhee and Hoff, 2005; Cooney et al., 2006; Zong and Bowen, 2006; Laquente et al., 2007). In addition, although a decrease in MVD following antiangiogenic therapy can give an indication of the antivascular activity of a particular agent, MVD as a single end point fails to provide an adequate measurement for resolving the vascular response to antiangiogenic agents.

Measuring a slight decrease, no change, or even an increase in MVD is still consistent with a vessel inhibition, because MVD is a dynamically complex quantity that is influenced by the initial vascular suppression plus the consequent interaction between the vascular and tumor-cell compartments (Hlatky et al., 2002)

During tumor regression induced by an angiogenesis inhibitor, MVD may decrease if capillary dropout exceeds tumor cell dropout, increase if tumor cell dropout exceeds capillary dropout, or remain the same if disappearance of capillaries and tumor cells parallel each other. Therefore, the detection of a decrease in MVD during treatment with an angiogenesis inhibitor, suggests that the agent is active. However, the absence of a decrease in MVD does not indicate that the agent is ineffective.

Singhal et al. (1999) showed that multiple myeloma that was resistant to high-dose chemotherapy regressed in response to treatment with a single antiangiogenic agent thalidomide, even though not all tumor regressions were accompanied by a decrease in MVD. The observation of tumor regression without a corresponding decrease in MVD does not indicate that mechanisms other than antiangiogenesis play a causal role in treatment response. The tumor cell population may simply decrease in direct proportion to, and as a direct consequence of, the loss of its supporting vasculature. Likewise, tumors undergoing antiangiogenic intervention may also follow a "shrink to fit" adaptation, which as a result may not lead to reduced MVD counts. It has consequently been argued that MVD reduction may not be the appropriate and expected readout of the success of antiangiogenic interventions in human tumors (Hlatky et al., 2002).

Changes in MVD do not independently measure vascular inhibition, but rather, reflect the changing ratio of the vascular component of the tumor to its tumor-cell component. Under antiangiogenic therapy, capillary inhibition or elimination occur first, followed by tumor-cell elimination, and both influence MVD.

Moreover, experimental antiangiogenic experiments in murine tumor models usually have a significant reduction of the MVD as a primary experimental readout of an antiangiogenic intervention. Human tumors have different growth kinetics compared with experimental tumors.

### The relationship between MVD and intercapillary distance

Intercapillary distance is determined at the local level by the net balance between angiogenic factors that stimulate and those that inhibit vessel growth, as well as by nonangiogenic factors, such as the oxygen and nutrient consumption rates of tumor cells (Yoshii and Sugiyama, 1988; Mikhail et al., 2004). In turn, MVD is determined by intercapillary distance, which in a tumor is dictated by the thickness of the perivascular cuff of tumor cells. Tumors that have high rates of oxygen and nutrient consumption have small cuffs only two to three cells wide and have high vascular density (Gordan and Simon, 2007; Mabjeesh and Amir, 2007). On the contrary, tumors with low rates of oxygen consumption have relatively large cuff sizes and a relatively low vascular density (Gordan and Simon, 2007; Mabjeesh and Amir, 2007). This is an important parameter as it is the goal of an antiangiogenic tumor therapy to reduce the intercapillary distance to a such a degree that it becomes rate-limiting for the growth of the tumor.

### Drawbacks

The lack of standardized protocols and variations in the manual and automated counting of immunoreactive vascular spots are the primary variables to account for the variation of published data. For example, MVD counts in breast tumors have been reported to range from

less than 20 $mm^2$ to more than 200 $mm^2$ (Fox, 1997). The mean MVD of the published mammary tumor reports is between 80 $mm^2$ and 90 $mm^2$. The use of different panendothelial cell markers may account for some of the variation.

Other drawbacks concern the: 1) Cut off size in different experimental tumors; 2) Controversy about prognostic value; 3) Relationship between MVD and tumor necrosis in different experimental tumors.

## Conclusions

It is likely that neoangiogenesis may become an integral part of a more a consistent tumor staging system and routine prognostic evaluation. It has also come to light that careful estimation of neoangiogenesis using markers such as CD 105, as opposed to MVD estimation using panendothelial markers such as CD 31, is crucial to an accurate determination of prognosis and identification of a subset of high-risk patients who would be likely to benefit from a careful selection of optimal molecular targeted antiangiogenic therapies in common malignancies.

For a reliable and reproducible assessment of angiogenesis for all of the assays, validation procedures and quality control protocols are mandatory.

Finally, methodologies to assess tumor angiogenesis may be further enhanced due to the continuous discovery of new antibodies for proliferating and for neoplastic endothelium and to the improvement of assay techniques.

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