Chapter 20
Assessing Tumor Angiogenesis in Histological Samples

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20.1 Introduction

Angiogenesis is a hallmark of cancer [1] and occurs in most human tumors. It has been shown that angiogenic tumors are more likely to develop metastasis and exhibit resistance to standard cancer therapies [2], making tumor angiogenesis a prognostic and sometimes predictive biomarker [3, 4]. Although new imaging technologies, such as dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) or positron emission tomography-computed tomography (PET-CT) scans, are clinically available to visualize tumor angiogenesis in vivo [5, 6], histological assessment of tumor angiogenesis remains a technique of interest, as it can provide information on the capillary level of newly developed microvessels in different parts of the tumor [7]. Via histological examination, the relationship between tumor microvessels and other clinicopathological tumor characteristics can be evaluated as well [8].

Although studies examining the grading of tumor angiogenesis date back to the 1980s [9], the introduction of more specific endothelial markers and the quantitative immunohistochemical study by Weinder et al. [10] in the early 1990s have made tumor angiogenesis a topic of active research by many investigators. However, due to a lack of standard methods for the identification and quantification of capillaries and inter- and intra-observer variation, the prognostic role of tumor angiogenesis has not been confirmed [11]. It is important to note that the angiogenic profiles of tumors do not always correspond to the histological grade in breast cancer and some other solid cancers [12, 13]. Additionally, the therapeutic effects of antiangiogenic agents in relation to angiogenesis activity are diverse [14]. These issues complicate understanding the role of tumor angiogenesis in tumor progression.

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Quantification of tumor angiogenesis by counting microvessels in immunostained tissue sections was ranked as category III (meaning: "all factors which are not sufficiently studied to demonstrate their prognostic value") based on the 1999 consensus of the College of American Pathologists [15–17], which has not yet been updated. However, with increasing evidence of the clinical usefulness of anti-angiogenic therapies in various cancers, the assessment of tumor angiogenesis to determine cases in which these new therapies are more likely to produce better results is transitioning from research laboratories to routine diagnostic pathological laboratories.

In this chapter, we briefly discuss the current optimal protocol based on the "Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumors" [18]. We also briefly explain double staining for concurrent detection of mural and endothelial cells and evaluation of proliferating (Ki-67-positive) endothelial cells as markers of maturation [19]. In view of emerging digital pathology and new imaging technologies, we will also discuss the topic of computer-assisted image analysis for evaluating the morphology and characteristics of tumor microvessels.

20.2 Methodology

Immunohistochemistry (IHC) is a common technique for visualizing microvessels in tumor samples. For the details of basic IHC protocols, please refer to Current Protocols in Molecular Biology by Goldstein et al. [20].

In general, the IHC technique consists of antigen retrieval, selection of a specific antibody, a sensitive detection method, and negative and positive controls.

20.3 Materials

1. Silane-coated or charged microscope slides (e.g., Superfrost Plus®)
2. Slide staining tray
3. Coplin jars
4. Dry incubator or oven at 37 °C
5. Xylene or xylene substitute (e.g., HistoChoice®)
6. Ethanol (100 %, 90 %, and 70 %)
7. Normal goat serum
8. Phosphate-buffered saline (PBS) or Tris-buffered saline (TBS)
9. Tween 20
10. Methanol
11. 30 % hydrogen peroxidase
12. Tris-EDTA buffer, pH 9.0
13. Water bath at 95 °C, pressure cooker, or microwave oven

14. Antibodies to CD31 (JC70; Dako M0823) or CD34 (QBEND10; Dako M7165).[1]
15. Detection system: Avidin/biotin detection technology (VECTASTAIN Elite ABC system, Vector Laboratories) or Chain polymer detection systems (Dako EnVision™)
16. Chromogens: diaminobenzidine (Dako), 3-amino-9-ethylcarbazole (AEC, Dako), 5-bromo-4-chloro-3-indoxyl phosphate, and nitroblue tetrazolium chloride (BCIP/NBT, Dako)
17. Hematoxylin for counterstaining, if desired
18. Permanent (for DAB) or aqueous mounting medium (for Vector® Blue, Vector® Red or other alcohol-soluble chromogens)
19. Microscope coverslips
20. Chalkley graticule (25 dot)

20.4 Methods

It is important to note that similar to any other IHC method, optimization and standardization are necessary for obtaining high-quality staining with low nonspecific background. Specifically, when performing quantitative measurements by image analysis, minimum non-specific staining will yield more precise results. If IHC is being conducted in a non-histopathology research laboratory, we highly recommend that the basics of IHC protocols be reviewed before conducting the protocol. Otherwise, histopathology laboratories that have their own in-house IHC protocols can follow those protocols. However, attention must be paid to the antigen retrieval and antibody selection steps.

Basic Protocol

1. Cut 4-μm sections from formalin-fixed, paraffin-embedded (FFPE) tissue blocks and mount them onto silane-coated or charged slides. Using a pencil, label the slides with the specimen and primary antibody to be used.
2. Dry the slides at 37 °C in a dry incubator overnight.
3. Dewax the slides using two changes of xylene (or a xylene substrate) in a Coplin jar for 10 min each. Note: These jars can be stored and used for several cycles until they become cloudy. Xylene-containing jars with tightened lids should be kept in a fume cabinet between cycles. The sections should not dry out from this step onward.
4. Rehydrate the sections by passing them through graded ethanol solutions (100 %, 90 %, and 70 %) in Coplin jars for 2–5 min each. Then, rinse the

[1] CD31 and CD34 are the most commonly used endothelial markers due to their consistent and reliable results in paraffin-embedded tissues; however, depending on the objective of the study, other markers, such as vascular endothelial growth factor receptors (VEGFRs) or CD105, can be used.

sections in water for 5 min and PBS or TBS for 5–10 min. Note: Like xylene, ethanol can be reused before replacement with fresh solutions.

5. Pre-treatment with heat-induced epitope retrieval (HIER) is required and performed by placing sections in Tris EDTA buffer, pH 9.0.
   Note: The antibody specification sheet usually has a recommended method. Optimal results are obtained in our laboratory by using a water bath; pre-heat the water to 65 °C, perform epitope retrieval treatment at 95 °C for 20 min, and then cool to room temperature for 30 min in the same buffer. The same results can be obtained using the same buffer in a pressure cooker for 2 min. We have observed tissue damage resulting from microwave oven heating due to the sudden increase in temperature.
6. Rinse in PBS/TBS 3 times. Place the slide rack in a Coplin jar with PBS/TBS, drain, and refill with fresh solution 3 times for 5 min each.
7. Block endogenous peroxidase activity by incubating the sections in 0.3 % H_2O_2 in methanol for 30 min and 3 % H_2O_2 in methanol for 10 min. Note: This step is not required if an alkaline phosphatase detection system is used.
8. Rinse with PBS/TBS 3 times for 5 min each. Note: PBS with 0.1 % Tween-20 (PBS-T) can be used as a wash buffer from this step onward.
9. Block non-specific antibody binding sites by applying 200–300 μl of 5 % goat serum in PBS to each section after placing the sections in a staining tray. Make sure the tissue sections are thoroughly covered with blocking serum. Incubate for 30 min at room temperature.
10. Place the slides back in the rack and rinse with PBS/TBS 3 times for 5 min each.
11. Carefully dry the slides using KimWipes. Place the slides in a staining tray and carefully apply diluted primary antibody. Make sure the entire tissue section is covered with the antibody. Add IgG1 to the negative control slide. Note: 1:50–1:100 dilutions of primary antibodies can be applied at room temperature for 30–60 min. Lower antibody concentrations (1:200–1:300) can be used when the sections are kept at 4 °C overnight.
12. Rinse slides 3 times with PBS-T for 5 min each.
13. Utilize the appropriate detection system based on the primary antibody and sensitivity. Incubate the slides for 30 min at room temperature.
    Note: For CD31/CD34, chain polymer-conjugated technology, which avoids endogenous tissue biotin, is a fast one-step method with acceptable sensitivity. The Envision™ system can be used to detect any primary antibody of mouse or rabbit origin. However, due to the hydrophobic dextran backbone in this system, multiple washes with PBS-T are required. When the avidin/biotin system is used, endogenous biotin needs to be completely blocked before applying the appropriately diluted biotinylated secondary antibody. Attention should be paid to the species of the primary antibody.
14. Rinse the slides 3 times with PBS-T for 5 min each.
15. Apply the chromogen substrate for 5–10 min at room temperature.
16. Check the intensity of the internal control (i.e., medium-sized vessels) staining under a light microscope. Note: The color intensity needs to be optimized for image analysis.

17. Rinse the slides in tap water.
18. Place the slides in a jar of hematoxylin for approximately 1–2 min.
19. Wash the slides with normal tap water until the water is clear.
20. Dry the slides with four changes of 100 % ethanol, and clear them with three changes of xylene, each for a few seconds. Note: If an alcohol-soluble chromogen is used, skip this step. Use a separate set of ethanol and xylene jars for the dehydration step. Do not mix with the deparaffinization jars.
21. Apply a few drops of an appropriate mounting solution to each section, and place a coverslip on top. Note: If Aquamount medium is used, seal each coverslip with clear nail polish around the edges.

20.4.1 Identification of Tumor Blood Vessels

Examine the negative control slide to ensure the absence of nonspecific staining. Tumor microvessels have different sizes (diameters range from 10 to 200 μm) and morphologies and sometimes show a collapsed lumen (Fig. 20.1). Tumor-associated vessels can be concentrated within discrete areas due to the higher concentration of growth factors in these areas, which are called hot spots. The entire tumor should be scanned at low power (40× or 100×), and 3–5 hot spot areas should be selected. This step is very subjective. The tumor periphery usually exhibits higher microvessel density than the central areas. Avoid necrotic areas. The presence of red blood cells in the lumen is not a requirement. Many of the tumor-associated vessels have a collapsed lumen due to increased solid pressure from cancer cells and components of the microenvironment.

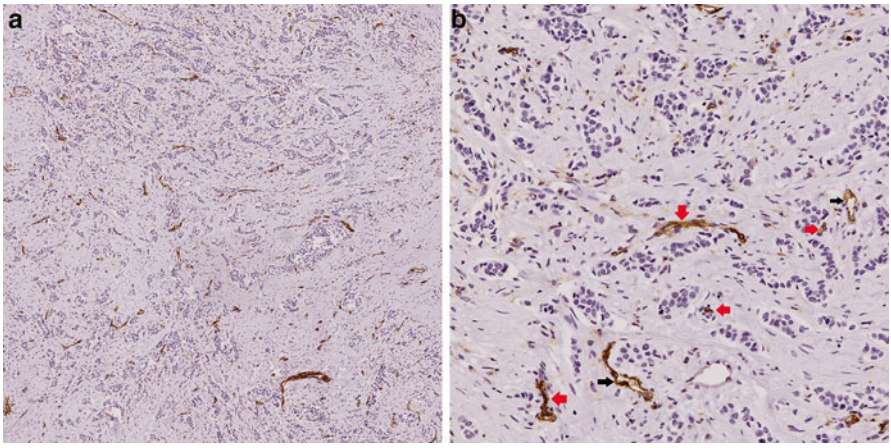

Fig. 20.1 Immunohistochemical staining of blood microvessels in breast cancer tissue. Intratumoral microvessels show strong CD31 staining (**a**) (5× objective). *Black arrows* show capillaries with open lumen, and *red arrows* show microvessels with collapsed lumen (20× objective) (**b**)

20.4.2 Measurement of Mean Microvessel Density (MVD)

Manual Counting Once hot spot areas are selected, use a higher magnification (200× or 400×) to count the stained structures, regardless of the size or presence of the lumen in the selected area, and record this number. Repeat this step for all hot spots. Calculate the mean of these counts. Check the microscope manufacturer's handbook to calculate the field of view in millimeters for each objective. The area of view can be calculated using the following formula: Area (mm^2) = π (Field of view/2) [2].

The MVD can be calculated using the following formula:

$$\mathrm{MVD}\left(\mathrm{mm}^{-2}\right) = \text{Mean vessel count} / \mathrm{Area}\left(\mathrm{mm}^{2}\right).$$

Chalkley Counting A Chalkley eyepiece graticule that fits the microscope is required. It is a 25-point eyepiece graticule and should be rotated such that the maximum number of dots overlaps stained microvessel structures. Record the number of overlapping dots (maximum score 25). Repeat this for all selected hot spot areas. The mean of these scores is the Chalkley count of that tumor section (Fig. 20.2).

20.4.3 Assessment of Microvessel Morphology and Patency

For this purpose, image analysis is very useful. The microscope should be equipped with a digital camera. After selecting hot spot areas, snapshots of these areas can be recorded. These pictures can then be analyzed by image analysis software. The perimeter, diameter, and area of highlighted tumor-associated vessels can be measured by the software tools (Fig. 20.3).

If a whole slide scanner is available, the accompanying software can usually provide morphological characteristics of selected structures after they are highlighted on the virtual slide (Fig. 20.4).

20.4.4 Measurement of Microvessel Proliferation

The ratio of tumor to endothelial cell proliferation (TCP/ECP) has been shown to be a marker of angiogenesis-independent tumor growth [21]. Angiogenesis inhibitors alone or in combination with other therapies are an accepted treatment in some cancers. To determine the tumors for which these therapies are most effective, endothelial cell proliferation has been shown to be a more reliable marker than MVD [22]. Double IHC staining on the same section with

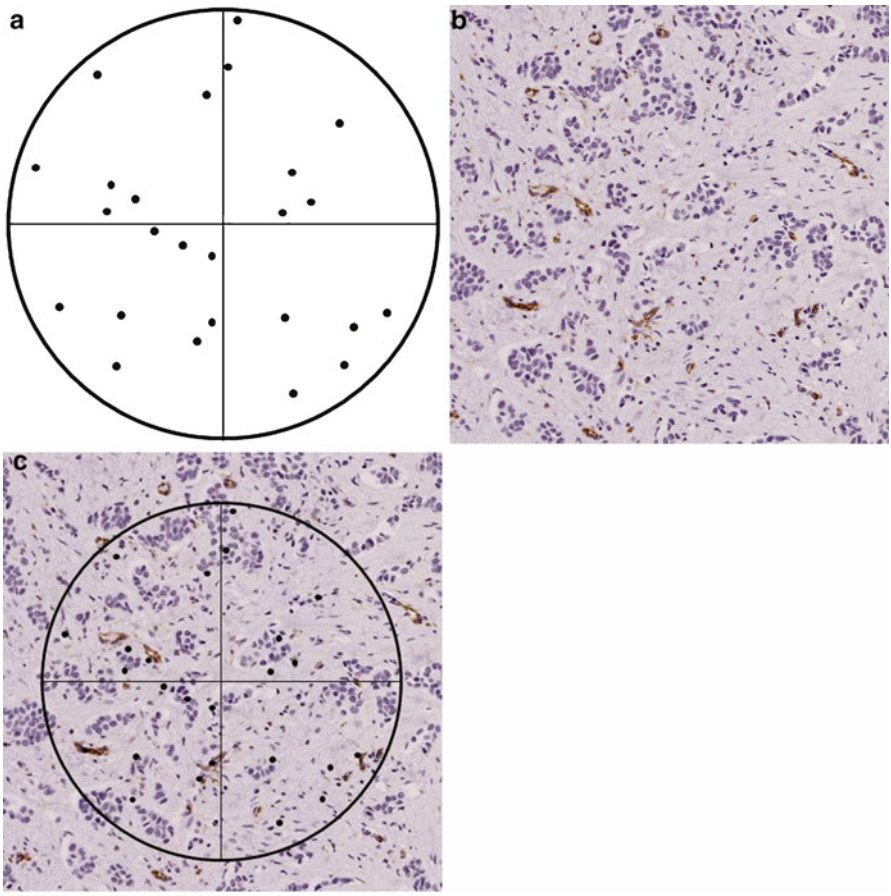

Fig. 20.2 The Chalkley method for estimating microvessel density. A Chalkley graticule (**a**). Intratumoral microvessels of breast cancer tissue stained for CD31 (**b**) are overlaid with a representation of the Chalkley grid (**c**). *Dots* that overlap microvessels are counted

antibodies to endothelial markers in conjunction with antibodies to proliferation markers (e.g., Ki67) can differentiate endothelial cells in proliferative versus quiescent states. The observer should be aware that tumor cells and inflammatory cells in the microenvironment contain abundant proliferating cells in addition to endothelial cells. Additional attention should be paid to detecting proliferating endothelial cells in microvessels with collapsed lumen. However, optimization and standardization of the double-staining IHC technique may not be simple in non-histology laboratories. This method can be easily substituted by staining for each marker on two separate serial sections. Virtual slides created by whole slide scanners allow simultaneous assessment of selected areas on both sections, therefore differentiating pericyte-positive versus -negative microvessels (Fig. 20.5).

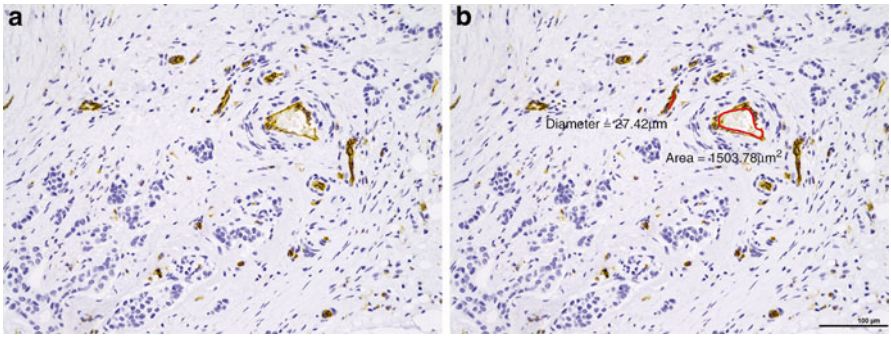

Fig. 20.3 Morphometric image analysis of microvessels. An image from a hot spot was captured by a digital camera attached to a light microscope (**a**). Microvessels stained for the expression of CD31 in breast cancer tissue can be analyzed by software programs to extract morphometric characteristics such as their area and diameter (**b**)

20.4.5 Assessment of Microvessel Maturity

In the early phases of tumor angiogenesis, endothelial cells proliferate and tube formation occurs. These microvessels are immature and sometimes not perfused. After recruitment of pericytes and establishment of the basement membrane, the endothelial cells become quiescent, and microvessel maturation takes place [19]. Evaluation of the presence of pericytes adjacent to endothelial cells is considered a useful tool in the calculation of the vessel maturation index. For this purpose, double staining of tumor sections for endothelial markers and pericyte markers (e.g., SMA) or serial section staining can be useful (Fig. 20.6).

20.4.6 Assessment of Hypoxic Markers

Despite the occurrence of active angiogenesis in most cancers and the increased number of microvessels, most of the newly formed vessels are not functionally normal, which makes hypoxia a common characteristic of tumors. Hypoxia can induce angiogenic pathways and, therefore, enhance angiogenesis [23]. Many hypoxia-related markers have been assessed in clinical settings and used as prognostic markers.

Carbonic anhydrase IX (CAIX), a hypoxia-induced enzyme that is overexpressed in tumor cells and has a pH regulatory function, has been shown to be a surrogate of hypoxia in some types of solid tumors [24] (Fig. 20.7).

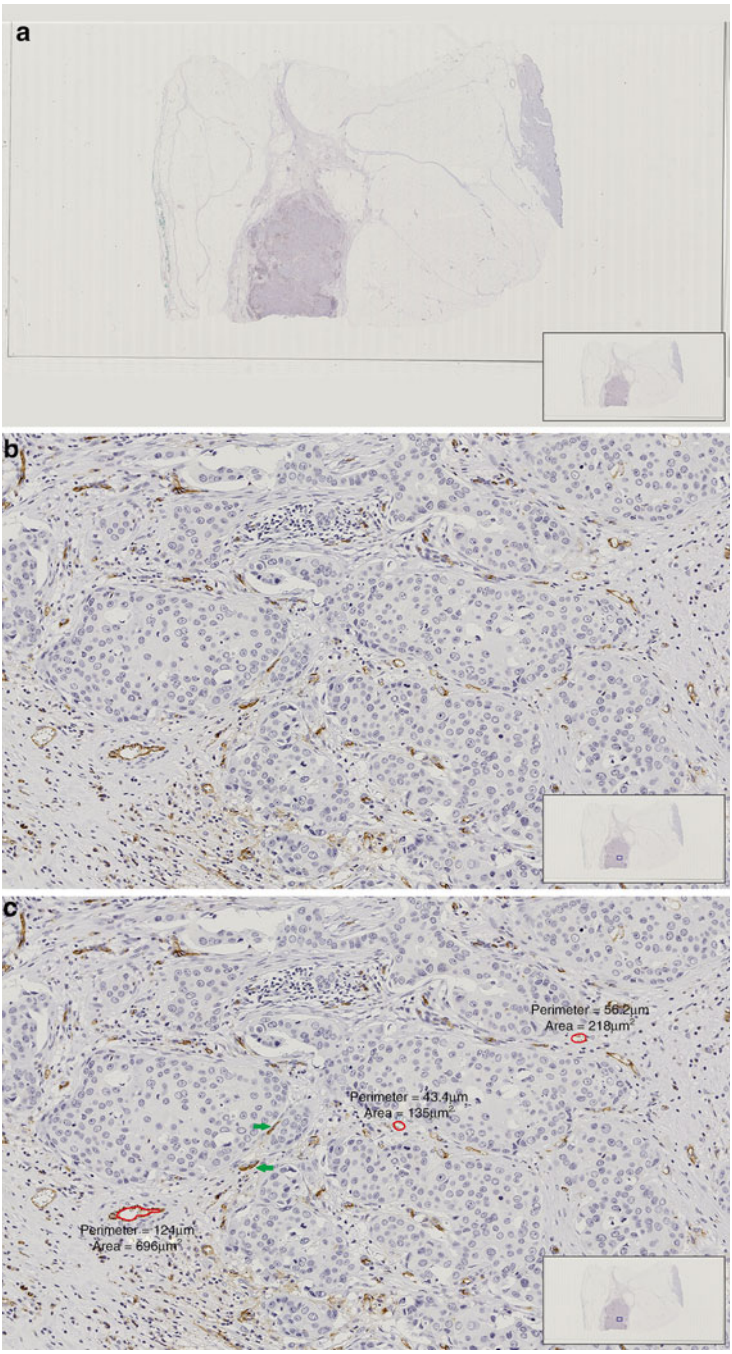

Fig. 20.4 Virtual slide of the entire tissue created by a slide scanner. The image of a breast cancer tissue section stained for CD31 was created using a Hamamatsu NanoZoomer slide scanner (**a**). Microvessels stained for CD31 in a hot spot (magnified digitally, equivalent to a 20× objective) (**b**). Morphometric analysis by Hamamatsu NDP Analyze software. *Green arrows* show microvessels with collapsed lumen (**c**)

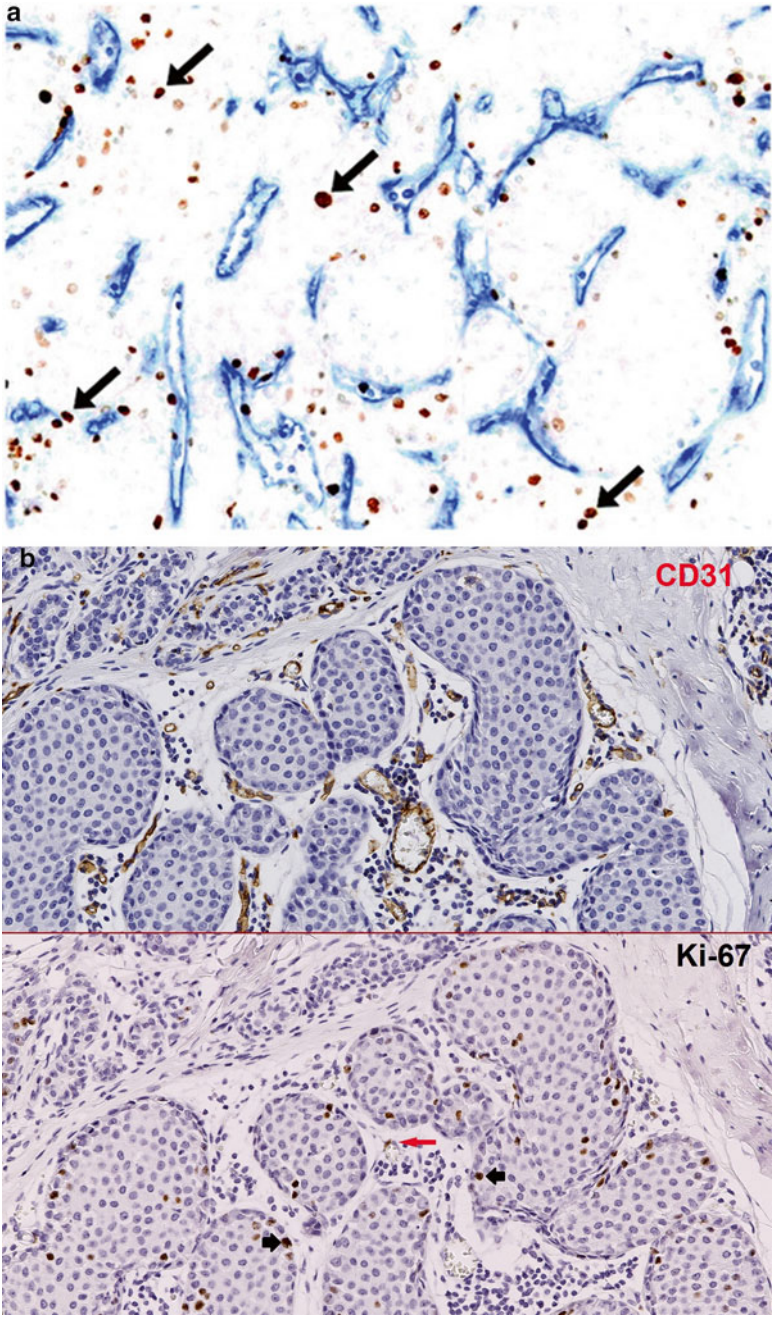

Fig. 20.5 Proliferation assessment of microvessels. Primary tumor tissue sections from patients with renal cell carcinoma were stained with a mixture of CD31/CD34 antibodies to visualize blood vessels (*blue*) and with anti-Ki-67 (*brown*, *arrows*) to monitor the proliferation status of both the endothelial and tumor cell compartments (**a**, adapted from Ref. [22]). CD31 and Ki-67 staining in two serial sections (4 μm) of breast cancer tissue (**b**). The *red arrow* indicates a proliferating endothelial cell. *Black arrows* indicate proliferating tumor cells

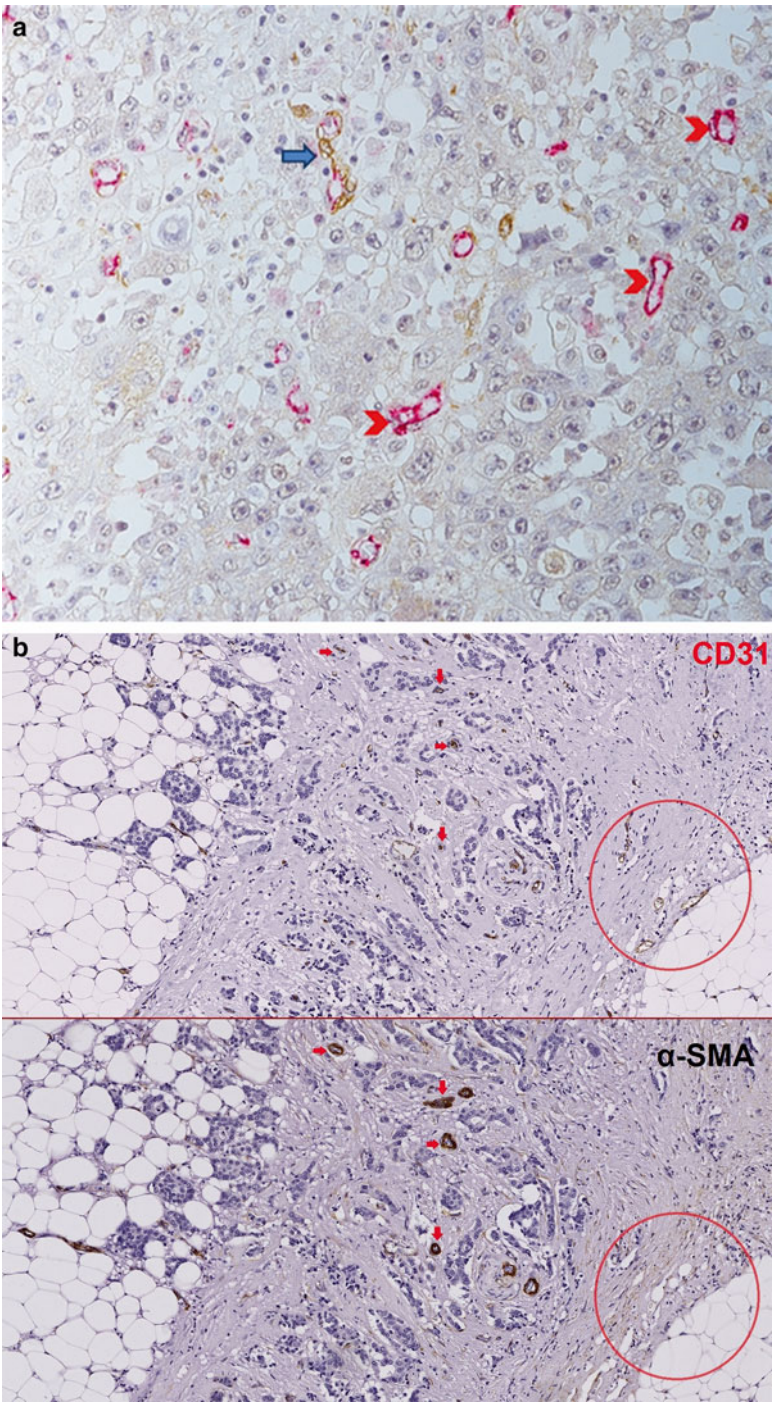

Fig. 20.6 Pericyte coverage of microvessels. Double-immunostaining of pericytes and endothelial cells in breast cancer tissue was performed; CD31 (*red*) and α-SMA (*brown*) staining are shown (**a**). The *blue arrow* indicates a vessel that is CD31+/α-SMA+; the *red arrowheads* indicate vessels that are only CD31+ (Adapted from Ref. [19]). CD31 and α-SMA staining in two serial sections (4 μm) of breast cancer tissue (**b**). *Red arrows* indicate microvessels with strong pericyte coverage. A *red circle* indicates an area where the microvessels are only CD31+

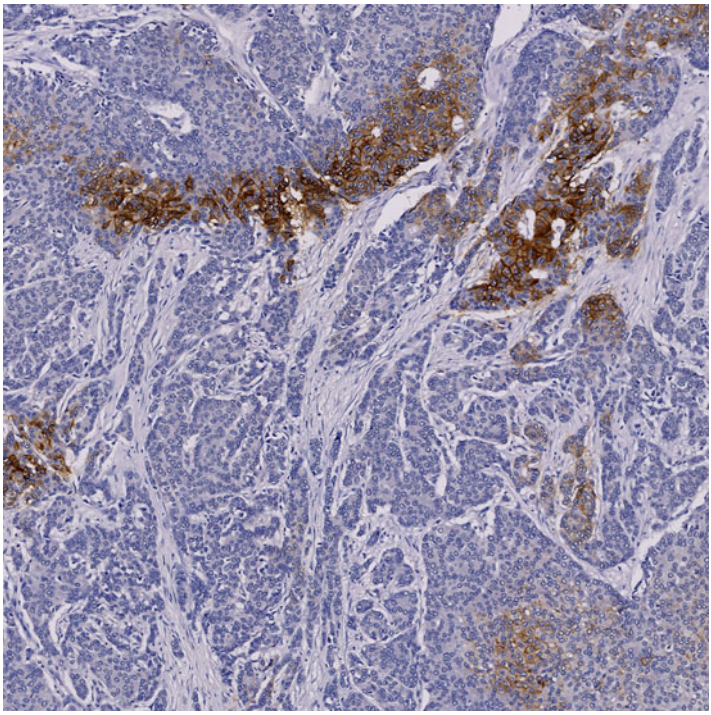

Fig. 20.7 Carbonic anhydrase IX staining of breast cancer tissue. The strong focal membranous and cytoplasmic staining of CAIX indicates hypoxia

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