Original Article

Practical Application of Angiogenesis and Vasculogenic Mimicry in Prostatic Adenocarcinoma

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Abstract

Background: Tumor growth depends on vascular blood supply. The novel discovery of non-endothelialized vessel-like channels in malignant tumors called vasculogenic mimicry has provided new insights about tumor behavior and also serves as a potential target for drug therapy. Although the association between vasculogenic mimicry and poor prognosis has been established in some tumors, there are only a few studies concerning prostatic carcinoma.

Methods: Using a histochemical and immunohistochemical dual staining method for PAS-CD34 and special immunohistochemical staining for laminin, we studied the presence and pattern of non-endothelialized channels known as vasculogenic mimicry as well as the quantity of endothelialized vessels designated as microvessel density in usual paraffin sections of 20 low-grade and 20 high-grade prostatic adenocarcinomas by routine light microscopy.

Results: We found a direct positive relationship between higher microvessel density and tumor grade (P<0.001), presence of vascular invasion (P<0.001) and percent of involved tissue (P<0.001); however, no such relationship was found with vasculogenic mimicry and only a weak correlation was noted between vasculogenic mimicry and perineurial invasion (P=0.03).

Conclusion: Unlike other cancers and despite the results of *in vitro* studies on prostatic adenocarcinoma, we were not able to demonstrate a significant relationship between vasculogenic mimicry channels and histologic grading as one of the most important prognostic factors; however, this may be due to an inherent limitation of prostatic tissue imposed by abundant smooth muscle fibers stained by this method. On the other hand, microvessel density scoring appears to be an important, simple, and applicable histologic tool for prostatic cancer evaluation in daily practice.

Keywords: angiogenesis modulating agents, angiogenic proteins, prostatic neoplasms

Introduction

It is well known that induction of blood vessel formation is necessary for neoplasms to grow larger than 1 mm^3 in volume.[1] Tumors stimulate angiogenesis either by directly secreting vessel-forming substances or by activating angiogenic compounds in the extracellular matrix.[2] The term "angiogenesis" means formation of new vessels by sprouting from pre-existing vessels. These new vessels are lined by endothelial cells as their parent vessel. Aggressive tumors, however, may gain blood and nutrients from *de novo* vessels produced by themselves. These new vessels have no endothelial lining and are mainly composed of basement membrane-like material. These channels are called vasculogenic mimicry (VM) and the process is called "vasculogenesis".[3]

Several authors have stated that angiogenesis, measured as microvessel density (MVD) correlates well with pathologic stage,[1] tumor progression,[4] and prostate specific antigen (PSA) recurrence[2] in prostatic carcinoma. Maniotis et al. first described the importance of VM channels in uveal malignant melanoma.[5]

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They showed the presence of these channels when stained by the periodic acid schiff (PAS) method and noted that their presence was highly indicative of more aggressive tumor behavior and poor prognosis.[5] Both the presence and patterns of VM channels have been of much interest in recent years. Using PAS staining, seven morphologic patterns of VM including straight lines, arcs, loops, and networks have been described in uveal melanoma[6] and the presence of more complicated patterns e.g., back to back loops and networks have been very strongly associated with death from metastatic melanoma.[3] In a comprehensive multidisciplinary study, Sharma et al. observed the ability of dunning rat and human prostatic carcinoma cells to form VM channels in 3-dimensional cell culture.[7] They concluded that the potential of forming VM networks is seen in aggressive prostate cancer *in vitro*. They also demonstrated that Metastat®, an inhibitor of matrix metalloproteinase (MMPs) decreased the formation of VM networks in aggressive prostate cancer,[7] indicating possible therapeutic implications. In recent years, the prognostic and therapeutic importance of VM has been studied in a broad range of cancers including prostate,[8] breast,[9] ovary,[10] astrocytoma,[11] soft tissue sarcomas,[12] osteosarcoma,[13] and hepatocellular carcinoma.[14] Most of these studies indicate some association between angiogenesis or VM proliferation and aggressiveness of tumor behavior. Targeting these nutritional sources has provided new therapeutic strategies for advanced cancer. In fact, some anti-angiogenic drugs such as Endostatin[15] and Bevacizumab (Avastin®)[16] have already been used successfully to treat advanced cancers such as non-small cell lung cancer and metastatic colon cancer. *In vitro* targeting of angiogenesis by Endostatin may fail in blocking or disrupting VM[17] indicating that angiogenesis and VM are different processes and require different drugs. Recent reports indicate *in vitro* selective targeting of VM with anti-laminin antibodies.[18] All these studies open a new horizon in cancer chemotherapy, particularly in advanced cases. In this study we attempt to find a practical approach relating angiogenesis or vasculogenesis with histologic grade and other pathologic findings in prostatic adenocarcinoma. Finding such a relationship, particularly in advanced prostate cancer, may assist in better patient stratification for newer therapeutic strategies and better monitoring.

Subjects and Methods

In this cross-sectional study, 65 pathology specimens of prostatic adenocarcinoma diagnosed in our center during a 12-year period (1996 – 2007) were reviewed. In order to examine an adequate volume of tumor, needle biopsy specimens were initially excluded and only prostatectomy (simple and radical) specimens were selected. Twelve cases were further excluded due to poor fixation or processing. The remaining 53 cases were reviewed and classified according to the 2005 ISUP modified Gleason grading system.[19] The initial five grades or patterns and final combination scores of the two most common patterns in this system are similar to the traditional Gleason system.

Finally 20 low grade (Gleason grades or patterns 2 or 3) and 20 high grade (grades or patterns 4 or 5) prostatectomy samples were selected for special staining and further studies. Additional histopathological findings including tumor volume and vascular or perineurial invasion were also recorded in each case. Paraffin sections of all samples were submitted for PAS-CD34 dual staining to characterize endothelial cells and glycosylated basement membranes of vessels as well as vessel-like (VM) channels. The technique was adopted from Yue and Chen with some modifications.[11] Laminin staining was also performed to visualize basement membrane using the monoclonal antibody and IHC method according to the manufacturer's instructions. The applied monoclonal antibodies (mouse anti-human; DakoCytomation Inc., Carpenteria, CA) as well as other IHC reagents (DAKO Corporation, Carpenteria, CA) and cytochemical reagents were provided by a local distributor. Microvessel density (MVD) was determined by the mean number of small CD34-positive vessels counted in ten microscopic high power fields (HPF) selected from two hot spots on each slide. Hot spots were determined while scanning the entire slide under low microscopic magnification (100×). The surface area of each HPF (400×) of the microscope (Olympus model CX-21; Olympus Optical Co., Ltd.) had been previously calibrated with an ocular micrometer that was 0.16 square millimeters. Small vessel-like structures in the tumor that were PAS-positive but CD34-negative were presumed to be VM channels particularly if they contained red blood cells. The presence as well as pattern (linear, tubu-

Table 1. Main histologic findings in two study groups

	Low grade	High grade	*P*-value
Number of cases	20	20	—
Age mean (range)-years	71.7 (61 – 84)	69.9 (52 – 87)	—
MVD* mean (range)	17.56 (12.3 – 22)	27 (21 – 35.6)	<0.001
VM** positive	12 (60%)	14 (70%)	0.51
VM pattern:			0.65
Linear	5 (41.67%)	5 (35.71%)	
Tubular	6 (50%)	6 (42.86%)	
Network	1 (8.33%)	3 (21.43%)	
Vascular invasion	1 (5%)	10 (50%)	<0.001
Perineurial invasion	13 (65%)	13 (65%)	—
Tumor volume (range)	41.5% (20 – 80%)	52.5% (20 – 80%)	0.12
Margin involvement***	9 (90%)	13 (76.5%)	0.40

*Microvessel density as mean number of CD34-positive vessels per microscopic high power field (400×); **Vasculogenic mimicry; only the number of apparently positive cases are stated; ***Surgical margin involvement studied only in radical prostatectomy specimens (10 low-grade; 17 high-grade)

lar, and network) of VM channels were recorded in each case. Laminin-stained slides were also used to confirm the topography of the basement membrane of the vessels and vessel-like structures (both stain positively). In each run of PAS-CD34 staining, sections of established high-grade uveal melanoma were used as positive controls for VM. Sections of normal kidney were also prepared as positive controls for laminin staining. The recorded data were analyzed by statistical methods using SPSS V.14 software (SPSS Inc., Chicago, Illinois). Significance testing was applied using an unpaired *t*-test for parametric variables and the Chi-square method for non-parametric data. *P* values equal or less than 5% were presumed to be statistically significant.

Results

The specimens included 27 radical and 13 simple prostatectomies composed of 20 low grade (grades 2 or 3) and 20 high grade (grades 4 or 5) carcinomas. Patients' mean age was 70.8 year (range 52 – 87). The PAS-CD34 stained slides revealed endothelial cells of small vessels (Figure 1) as well as non-endothelialized PAS-positive channels presumed as vasculogenic mimicry (Figure 2). A summary of the histologic findings in the two groups is presented in Table 1.

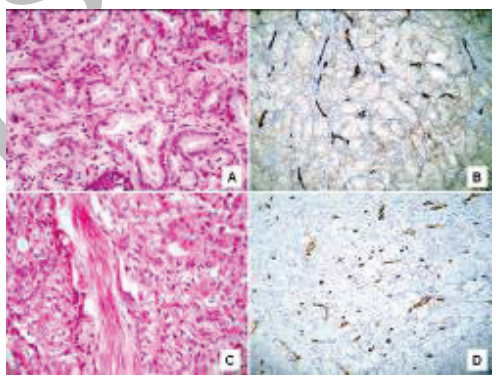

Figure 1. A) Low grade prostate adenocarcinoma as seen in routine H&E staining (250×). **B)** The same specimen stained by CD34 marker for endothelial cells (CD34-PAS dual stain. (Vessels appear brown-black). Note low density of small vessels (250×). **C)** High grade prostate adenocarcinoma (H&E 250×). **D)** High grade tumor stained by CD34 marker. Compare with B (250×).

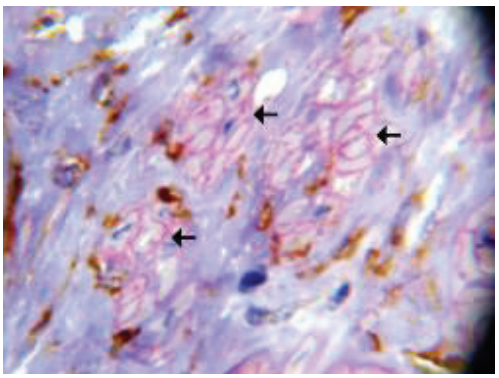

Figure 2. PAS-positive VM channels in tumor (arrows). Note lack of staining for CD34 endothelial marker (CD34-PAS dual stain; 400×).

The MVD score in the high grade group was significantly higher than the low grade tumors (P<0.001) whereas no significant relationship was found between grade and VM presence (P=0.51) or pattern (P=0.65). Tumors with higher MVD were more frequently associated with vascular invasion (P<0.001) but not with perineurial invasion (P=0.13).There was also a direct relationship between MVD score and percent of involved tissue (P<0.001). The relationship between VM and neurovascular invasion was quite different with MVD. It appeared that tumors with more detectable VM channels were more frequently associated with perineurial invasion (P=0.03), whereas no such association could be shown with vascular invasion (P=0.53). Similarly, no relationship was detected between the pattern of VM and either perineurial (P=0.76) or vascular (P=0.53) invasion in tumors.

Discussion

Establishing better prognostic factors and newer treatment modalities for prostate cancer has long been a matter of much debate. Pathologic grading as presented by Donald Gleason in 1966 is still routinely used as a powerful guide although with some modifications. The latest modification of this grading system presented by a consensus of uropathologists worldwide in 2005 is the most reliable method[19] and we applied this method to all of our 65 cases. In this 5-tiered system, the tumors are initially classified into five grades or patterns and the final grading score for each patient is calculated by adding the numbers of two most common patterns seen in each tumor (e.g., Gleason score: 3+4=7). Grades 1, 2, and 3 and combined scores less than 7 are considered low grade whereas grades 4 and 5 and scores more than 7 are regarded as high grade. Histologic grading is easily accessible, correlates well with stage and is one of the most important prognostic factors considered in selecting treatment modalities. For these reasons we preferred grading as the cornerstone of our study.

Various cell markers have been used for identification of endothelial cells. The most common markers detected by IHC methods are factor VIII, CD31, and CD34 but it has been shown that CD34 is superior to CD31 for characterization of vascular proliferation.[2,11] In fact CD31 may fail to identify the association between MVD and pathologic stage; even though CD31 is known as the most specific antibody for endothelial cells.[2] Brawer[1] using factor VIII for MVD quantification found an important correlation with stage; however, Silberman[4] using CD31 was unable to find such a relationship, but at the same time he found MVD as an independent significant predictor of prostate cancer progression after radical prostatectomy.[4]

de la Taille[2] found a close association between high MVD score and stage pT3 in prostate cancer. He also reported MVD obtained by the use of CD34 (but not CD31) as an independent predictor of PSA recurrence.[2] Using histologic grade as a reliable prognostic factor and CD34 as the most reliable endothelial marker we found a significant correlation between MVD score and Gleason grading (P<0.001). We also were able to show a close association between increasing MVD and vascular invasion (P<0.001) as well as tumor volume estimated as percent involved tissue (P<0.001), however, no significant relation was found between the number of small vessels and perineurial invasion (P=0.13). Perineurial invasion is best recognized at the posterior prostatic zones studied in radical prostatectomy specimens. We were not able to examine this area in 13 out of our 40 cases because we had only 27 radical excisions and this may be a limiting factor. Our findings support the reports of Brawer,[1] Silberman,[4] and de la Taille[2] indicating that MVD evaluation, easily feasible on paraffin sections, may be used as an auxiliary predictor of the degree of tumor progression.

Our study is based on the principle that the walls of VM channels are similar to blood vessels in having an extracellular matrix and glycosaminoglycans that offer them PAS stainability; however, by definition the VM channels have no endothelial lining and do not stain with endothelial markers such as CD31 or CD34.[9,11,14] In 2001 Sharma and coworkers[7] confirmed the finding of VM channels as a poor prognostic sign in prostate cancer and suggested further investigations. Their work was principally based on the *in vitro* study of cancer cells in culture media rather than on virtual patient samples.[7] Liu et al.[8] who studied experimental models of prostate carcinoma found VM-like channels and in a therapeutic approach, they were able to induce VM channel thrombosis and tumor infarction following PSA targeting drugs.[8]

The importance of more complicated patterns of VM channels was first reported in aggressive uveal

melanomas.[20] Consequently, other workers found a relationship between more complex patterns and more progressive tumors.[3,7,9] We used PAS-CD34 dual staining because it seemed more applicable in routine pathology laboratories. Laminin staining was also applied to confirm the nature of the basement membrane. We found that prostatic smooth muscle cells that are present in large numbers produce confounding effects in visualizing VM channels. In fact these cells are normally covered by a delicate basement membrane-like material making the diagnosis of VM difficult. Therefore, the best way of confirming the presence of VM appears to be their visualization within the tumor or demonstration of RBC within these channels. By this way we were not able to show any relationship between Gleason grading and the presence (P=0.51) or pattern (P=0.65) of VM channels.

Lack of strong relationship between VM and tumor grade has also been reported in cerebral astrocytoma.[11] These authors observed VM phenomenon in some malignant astrocytomas, however, the CD34 expression in some of the anaplastic tumor cells made VM detection impossible.[11] There was also no obvious correlation between the presence or pattern of VM with vascular invasion (P=0.53) or percent of tissue involved by tumor (P=0.16) but there appears to be some direct relation between the presence of VM and perineurial invasion (P=0.03). Although this may only be a spurious finding, it may also be possible that the tumor cells extend via the perineurial vessel-like or VM channels rather than perineurial lymphatic vessels as previously believed. The most practical limiting factor in this study was the presence of many smooth muscle fibers embedded in basement membrane-like material that interfere with vasculogenic mimicry. This limitation is not present in uveal melanoma, cerebral astrocytoma or even breast carcinoma.

In conclusion we found a meaningful direct correlation between the MVD score and prostatic tumor grade, presence of vascular invasion and percent of involved tissue but no such associations were found with VM. Better detection methods and using more specific markers for VM may be promising in recognition of the pathophysiology of prostatic cancer growth, presenting a prognostic tool and finding a potential therapeutic target in advanced cases. Further studies should also consider clinical staging as well as long-term patient follow up to find a closer relationship with patient outcome and survival.

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