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Computerized Whole Slide Quantification Shows Increased Microvascular Density in pT2 Prostate Cancer as Compared to Normal Prostate Tissue

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BACKGROUND. Contrast enhanced imaging enables powerful, non-invasive diagnostics, important for detection and staging of early prostate cancer. The uptake of contrast agent is increased in prostate cancer as compared to normal prostate tissue. To reveal the underlying physiological mechanisms, quantification of tissue components in pathology specimens may yield important information. Aim of this study was to investigate whether microvascularity is increased in prostate confined cancer (pT2).

METHODS. Radical prostatectomy specimens of 26 patients were selected for organ confined peripheral zone tumors which were restricted to one side of the prostate. Microvessels were visualized by immunohistochemistry against CD31. Specimens were scanned using a computer controlled microscope and scanning stage and vessels were recognized automatically. Pseudocolor mappings were produced showing number of vascular profiles (MVD), vascular area (MVA) and perimeter (MVP) in an overview of the entire prostate transection. MVD is a common measure for vascularity, whereas MVA represents the 3D vascular volume and MVP the perfused surface area. Mean, coefficient of variation and 75th percentile of these parameters were calculated automatically in manually indicated areas, consisting of the entire tumor area and the corresponding normal area in the contra lateral side of the prostate.

RESULTS. The mappings clearly indicate areas of increased vascularity in prostate transections. In tumor tissue an increase was found compared to normal tissue of 81%, 49%, and 62% for mean MVD, mean MVA and mean MVP, respectively (P<0.001 for all comparisons). In contrast, the heterogeneity in tumor vasculature was significantly decreased as compared to normal prostate (P<0.001).


KEY WORDS: prostate cancer; microvasculature; digital image analysis; quantification; whole slide scanning

INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer among men and the second highest cause of cancer related death [1]. In the last decades, introduction of PSA screening has resulted in earlier detection of prostate cancer, leading to a continued slight decline in mortality [1]. The in vivo visualization of prostate cancer is continuously improving as a result of new

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developments in imaging techniques (MRI, ultrasound) and the use of contrast agents. The contrast enhancement in these imaging modalities reflects hemodynamic changes in the prostate, providing functional rather than anatomical data. Close comparison with detailed histopathology results has contributed to the understanding of MRI data. Unfortunately, the detection of small tumors remains an important challenge to the urologist and radiologist. Insight in the characteristics of the microvascularity of normal prostate and prostate cancer will increase the understanding of contrast-enhanced radiologic imaging.

Microvascularity may be assessed quantitatively, for instance by counting the number of vascular profiles (microvascular density, MVD) in an immunohistochemically stained tissue section. MVD is considered an important marker for neo-angiogenesis which in turn is responsible for local growth and metastasis in tumors [2]. Compared to normal tissue, increased MVD has been shown in primary tumor and metastases of melanoma [3], breast cancer [4], cervical cancer [5], stomach cancer [6], lung cancer [7] as well as prostate cancer [8,9]. In prostate cancer MVD has been reported to be associated with Gleason score, stage and tumor progression, although somewhat contradictory results were found [10–14]. In general, contradictory results may be attributed to differences in either the composition of the patient group or because of differences in the quantification procedure. Parameters that influence the outcome of the counting procedure include the choice of antibody used to highlight vascular profiles, the method of selection of the area to be measured and the actual counting method.

In prostate specimens, the widely used CD34 monoclonal antibody (MAb) is less optimal for microvessel staining, because of abundant stromal staining. CD31 MAb is preferred over anti-von Willebrand factor, because of stronger staining of the vascular endothelium [15]. In many published studies, the actual counting of vascular profiles is performed manually, applying the so called Weidner procedure [16]. Using this procedure, microscopic profiles are counted in visually identified “vascular hot spots.” It was shown that especially localizing these hot spots is prone to observer bias, limiting the applicability of such procedures [17,18]. Use of a Chalkey counting grid was recommended to improve reproducibility [18]. Because the grid is applied to visually identified hot spots as well, the main source of variability is not reduced with this method. Application of digital image analysis has been shown to enhance reproducibility of both selection of the measurement area and of the actual counting of vascular profiles [17,19]. Moreover, digital image analysis may yield additional information describing the geometry of vascular profiles (e.g., vascular area and perimeter).

The aim of the present study was to investigate differences in microvascular characteristics between pT2 prostate tumors and normal prostate tissue. An optimized quantification technique was applied, which tries to avoid the deficiencies that lead to ambiguities, by selecting an optimal staining technique combined with computerized quantification. Characteristics of the microvasculature in unilateral peripheral zone pT2 tumors were compared with the corresponding contra lateral normal peripheral zone. Digital image analysis was used to construct spatial mappings for three parameters, microvessel density (MVD), vascular area (MVA) and vascular perimeter (MVP), in a whole transverse section of the prostate. These mappings were used to construct pseudocolor overviews of vascular parameters. Also, vascular parameters could be assessed objectively in tumor and normal tissue, avoiding subjective hot spot selection.

**METHODS**

**Patient Selection and Pathology**

To decrease inter-tumor variability, this study was restricted to peripheral zone tumors, which constitute 80% of prostate cancer and are known to be more proliferative and have higher invasive potential as compared to transition zone tumors [20]. Twenty-six radical prostatectomy specimens with unilateral peripheral zone pT2 tumors were included in this study. All patients showed negative resection margins and did not receive neo-adjuvant therapy. The serum PSA level and patient age at time of radical prostatectomy were obtained from case history.

All Prostatectomy specimens had been completely embedded, as described previously [21]. In summary, specimens were cut into serial 4 mm transverse slices perpendicular to the dorsal rectal surface. The slices were photographed with a CCD-camera. Apical and basal slices were then sectioned parasagittally. Depending on the size of the prostate, the remaining slices were divided in two or four parts in order to fit routine cassettes. The tumor margins were marked on haematoxylin and eosin (H&E) stained sections by an experienced uropathologist (CAHK). Tumor was then outlined on the macroscopic photographs in order to reconstruct tumor extension and multifocality. For this study, also, a mirror image of equal size compared to the tumor was marked in the contra lateral normal peripheral zone tissue. Mild to moderate chronic inflammation and various subtypes of atrophy were accepted as “normal” variations because of their high frequency of occurrence in prostates at the age at which
adenocarcinoma occurs. Severe and extensive inflammation and multifocal high grade PIN, however, were regarded as abnormal. The Gleason grade and score were determined using the 2005 ISUP Modified Gleason System [22]. Tumor volume was determined by measuring the largest tumor diameter and the diameter perpendicular to the largest diameter in all tissue sections containing tumor. Section tumor area was calculated from these diameters by assuming elliptical tumor shape, was integrated over all sections was regarded as abnormal. The Gleason grade and score

The estimated tumor volume.

and multiplied by the section thickness to arrive at the

tissue sections containing tumor. Section tumor area

diameter perpendicular to the largest diameter in all

by measuring the largest tumor diameter and the

were determined using the 2005 ISUP Modified

Gleason System [22]. Tumor volume was determined

was shown on the computer monitor. Subsequent

interactive correction was limited to removal of falsely

recognized non-vascular objects and drawing of

vascular profiles not recognized by the automated

procedure. Mapping of MVD, microvascular area (MVA) and perimeter (MVP) was performed by calculating these parameters (cumulative) in a small circular neighborhood around each pixel of the mapping and assigning a pseudocolor to the pixel expressing these parameter values. Properly assessed in 2D sections, MVD may be regarded as a measure of length density per volume unit, MVA as a volume per volume measure and MVP as a surface area per volume measure [24]. Especially MVP may be of interest, as this measure represents the (relative) available vascular diffusion surface. Mappings were produced for a local neighborhood with radius 235 \( \mu \)m (area = 0.173 mm\(^2\), corresponding to one single microscopic fields using a 20× objective). A transverse section through the entire prostate consisted of two to four standard histological sections. Images of the sections comprising a complete transverse section of the prostate were included in a single mapping (see e.g., Fig. 1A,B).

In addition to the production of these pseudocolor mappings, quantitative data were extracted from scanned specimens in manually delineated regions comprising the tumor and contra lateral normal tissue. To reduce the effect of region size, the region in normal prostate tissue was drawn with size and shape comparable to the tumor area. For MVD, MVA, and MVP the mean value, coefficient of variation (CV) and 75th percentile were calculated for each prostate over these two regions. The 75th percentile gives information on the higher vascularized areas and is therefore better comparable to the hot spot MVD than the mean MVD. The CV, defined as 100% × standard deviation/mean value, is a useful tool to compare variability between populations independent of their respective means [25].

**Statistical Analysis**

Because a number of vascular parameters showed a distribution which deviated significantly from normal (\( P < 0.05 \)), non-parametric statistics were used throughout this study. To compare data from tumor and normal tissue pair-wise, Wilcoxon signed ranks test was used. Spearman correlation analysis was

**Immunohistochemistry**

CD31 immunohistochemistry was performed on sections serial to the H&E stained sections containing marked regions. Tissue sections of 4 \( \mu \)m were deparaffinised, rehydrated and pre-treated using an EDTA antigen retrieval system. Endogenous peroxidase activity was blocked by using 3% \( \text{H}_2\text{O}_2 \) in methanol for 10 min. Next, specimens were rinsed with water and phosphate buffered saline (PBS). A mouse anti-human CD31 monoclonal antibody (Clone JC70A, DakoCytomation, Copenhagen, Denmark) was used as the primary antibody at a dilution of 1:10. The JC70A, DakoCytomation, Copenhagen, Denmark) was used as the primary antibody at a dilution of 1:10. The

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mouse anti-human CD31 monoclonal antibody (Clone

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used as the primary antibody at a dilution of 1:10. The

were incubated overnight at 4°C. Specimens

were washed in PBS and incubated with PowerVision

(Poly-Hrp-Anti Ms/Rb/Rt IgG Biotin-free, Immunologic, The Netherlands) for 30 min. Specimens were washed in PBS for 10 min and subsequently incubated with Power-DAB (Immunologic, The Netherlands) for 5 min resulting in brown staining of vascular endothelium. Finally, specimens were washed in water, dehydrated and mounted with Quick-D (Klinipath, The Netherlands). Sections used for quantitative analysis were not counterstained.

**Automated Analysis of Microvasculature**

To allow analysis of the spatial distribution of vessel characteristics, a mapping technique was developed based on a previously described scanning system [23]. Sections were scanned exhaustively using an AxioCam MRc (Carl Zeiss, Germany) connected to an AxioPlan 2 Imaging microscope (Carl Zeiss, Germany). The microscope was equipped with a computer controlled scanning stage (8 specimen stage, Márzhäuser GmbH, Wetzlar, Germany controlled by a Ludl MAC5000 controller, Ludl Electronic Products Ltd., Hawthorne, NY). Images were acquired using a 10× objective (Plan Apochromat, NA = 0.32), resulting in a specimen level pixel size of 1.06 × 1.06 \( \mu \text{m}^2 \). All image acquisition and processing were performed using custom written macros in KS400 image analysis software (version 3.0, Carl Zeiss, Germany). Each microscopic field of vision was focused automatically before acquisition. Images of consecutive individual microscopic fields were automatically acquired and stitched together into large 24 bit RGB TIFF images. Recognition of vascular profiles was performed based on the RGB color image data. Resulting binary images were sampled down by a factor 2 and the result of automated vessel recognition was shown on the computer monitor. Subsequent individual correction was limited to removal of falsely recognized non-vascular objects and drawing of vascular profiles not recognized by the automated procedure. Mapping of MVD, microvascular area (MVA) and perimeter (MVP) was performed by calculating these parameters (cumulative) in a small circular neighborhood around each pixel of the mapping and assigning a pseudocolor to the pixel expressing these parameter values. Properly assessed in 2D sections, MVD may be regarded as a measure of length density per volume unit, MVA as a volume per volume measure and MVP as a surface area per volume measure [24]. Especially MVP may be of interest, as this measure represents the (relative) available vascular diffusion surface. Mappings were produced for a local neighborhood with radius 235 \( \mu \)m (area = 0.173 mm\(^2\), corresponding to one single microscopic fields using a 20× objective). A transverse section through the entire prostate consisted of two to four standard histological sections. Images of the sections comprising a complete transverse section of the prostate were included in a single mapping (see e.g., Fig. 1A,B).

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applied to study correlation between vascular parameters and patient age and tumor characteristics (Gleason grade and score, serum PSA level, tumor volume). All probabilities from statistical tests are based on two-sided testing, significance being defined as $P < 0.05$. All statistics were performed using SPSS software (SPSS, Inc., Chicago, IL).

**RESULTS**

Characteristics of the microvasculature could be quantified successfully in all 26 radical prostatectomy specimens. Mean patient age was 60.4 years (range 47–70 years) with mean preoperative PSA of 6.43 ng/L (range: 2.33–12.1 ng/L). Tumors were generally small with a volume ranging from 0.3 to 4.8 cm$^3$ (mean 1.56 cm$^3$). The Gleason grade ranged from 2 to 5 (mean 3.33) with mean Gleason score 6.5 (range 5–9). Immunohistochemical staining of microvessels using the CD31 MAb resulted in specific, easily identifiable vascular profiles (Fig. 1 C,D,G,H). Automated recognition of vascular profiles was generally of high quality, requiring only a minimum of interactive correction.

In general, pseudocolor mappings provided a distinct overview of the spatial distribution of microvascular characteristics. Figure 1 shows examples of mappings for two cases. The first example case clearly shows enhanced MVD (Fig. 1A) and MVP (Fig. 1B) in tumor compared to normal tissue. Inspection of the CD31 stained histological sections confirms this observation, showing an increased number of vascular profiles in the tumor (Fig. 1C,D). In the second example case (Fig. 1E–H), a similar increase was seen, be it less pronounced (especially regarding MVP).

Tables I–III show results for the quantitative data derived from mappings, averaged over all patients. Figure 2 shows the 10-based logarithm of the ratio between tumor and normal tissue for these parameters. A value of 0 indicates no difference and values $> 0$ indicate increased values in tumor tissue. In the majority of cases, mean and 75th percentile of MVD and MVP and to a lesser extent MVA were increased in

![Fig. 1. Pseudocolor mappings for two prostate transections (A,B and E,F) containing peripheral zone pT2 tumor, as well as corresponding CD31-stained histological images (100× magnification; C,D and G,H). Different regions are indicated by: T, tumor region; N, normal region; U, Urethra. Shown are microvessel density (MVD) and microvessel perimeter (MVP).](image)

<table>
<thead>
<tr>
<th>TABLE I. Microvessel Density (MVD), Averaged Over 26 Patients</th>
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<td>Mean ($1/mm^2$)</td>
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Coefficient of variation defined as $100\% \times$ standard deviation/mean. Ratio is the ratio between data from tumor and normal tissue. Statistical results ($P$-values) from Wilcoxon signed ranks test.
tumor as compared to normal tissue. Mean MVD, MVA, and MVP showed a mean increase of 81%, 49%, and 62%, respectively. Increase in the 75th percentile was less pronounced (70%, 51%, and 55% for MVD, MVA, and MVP, respectively). All differences were statistically significant ($P < 0.001$). In contrast, the heterogeneity of the vasculature, expressed in the CV, was found decreased in tumor as compared to normal tissue for all parameters ($P < 0.001$).

In the area used as reference normal peripheral zone tissue, mild to moderate inflammation was present in 16 out of the 26 cases. Simple atrophy and focal atrophy with cysts (according to the recent Working Group Classification) [26] were focally present in 18 and 7 cases, respectively. Postatrophic hyperplasia and an incidental gland with HGPIN were present in only one case each. In none of the cases extensive HGPIN or severe inflammation was present in the area used as normal reference control tissue. No significant differences were observed in vascular parameters (MVD, MVA, MVP) between cases with inflammation and/or atrophy compared to cases without inflammation and/or atrophy in the normal tissue.

No correlation was found between patient age or tumor characteristics (PSA, volume, Gleason grade and score) and vascular parameters (MVD, MVA, MVP). A significant age—volume correlation was found ($r = 0.49, P = 0.012$).

### DISCUSSION

In this study, a consistent significant increase in vascular parameters MVD, MVA, and MVP was found in tumor versus normal prostate. This is in concordance with previous studies [8,9,27,28]. Bigler et al. found on average a twofold increase in MVD in 15 cases of prostate cancer compared to normal prostate tissue. MVD was averaged over five randomly selected regions. Kaygusuz et al also found a two-fold increase in MVD, measured as the mean of three hot spots. This figure is slightly higher than the 1.8-fold increase (1.7-fold for 75th percentile) found in the present study. Bigler et al studied in general larger tumors with a higher stage (50% /pT3) compared to the present study. Kaygusuz compared between tumor and benign hyperplasia, which may exhibit microvascular properties differing from those in normal peripheral zone. The present study is focused on small (pT2) tumors, as these are most difficult to visualize in vivo. In contrast, only a 1.47-fold increase was found for MVD assessed in hot spots in the study of Eberhard et al. [28], which is markedly smaller than the 1.7-fold increase observed in the present study. Eberhard does not give information about tumor volume, stage or zonal origin of normal tissue.

Chalkey point counting showed a 2.4-fold increase in vascularity for pT2 tumors compared to normal
prostate [8]. The Chalkey procedure yields information regarding the area of vascular profiles [24], comparable to the MVA in the present study. Results of the present study show only a 1.5-fold increase in tumor for the 75th percentile of MVA. This difference may be explained by the way the Chalkey procedure is carried out. The Chalkey procedure is most often applied as described by Fox et al. [18], in which the graticule is rotated to obtain a maximum number of hits. However, this is not in agreement with the original publication by Chalkey, in which the grid is placed at random over the structures of interest [29,30]. Both MVD quantification according to Weidner and the Chalkey procedure as described by Fox do not fully satisfy the basic concepts of stereology [30] and therefore may lead to biased results [31,32].

In a study comparing MVD assessed according to Weidner et al. [16] and Chalkey point counting it was concluded that in general the two methods show only moderate correlation [30]. It was also shown that for a number of tumor types (e.g., prostate) only MVD correlated with prognosis, whereas for other tumors the Chalkey count exhibited more clinically relevant information. This underlines our vision that different parameters may yield complementary information, and should ideally be assessed simultaneously [33]. The use of digital image analysis is therefore recommended [34]. MVD might not be the most important vascular parameter [18]. In the present study, especially the increase in MVP, which may be regarded as a relative measure for the 3D vascular diffusion surface, may contribute to increased uptake of radiological contrast agents in prostate cancer as compared to normal prostate tissue.

Both the method according to Weidner and the Chalkey technique as proposed by Fox assess vascular parameters in so-called vascular hot spots, which are considered of highest biological relevance [18]. It has been shown that identification of the hot spot is subject to observer bias and is poorly reproducible [17,35]. It has also been shown that hot spot selection may be performed objectively by exhaustive sampling of a specimen using digital image analysis [17,19]. However, as the variation between sections from a single tissue block and between tissue blocks is considerable [36], the value of locating a single hot spot in a section remains doubtful. Reproducibility may be increased by averaging over a number of highly vascularized areas [37]. In the present study, both the mean value and the 75th percentile over the entire tumor area and contra lateral normal tissue were calculated, to achieve a compromise between reproducibility and assessment of the most relevant information. As discussed above, the 75th percentile provides information on the higher vascularized areas and is therefore better comparable to the hot spot than the mean value.

The heterogeneity, expressed in the CV of vascular parameters MVD, MVA, and MVP was decreased in tumor as compared to normal tissue in the present study. The same observation may be derived from data published by Bigler et al. [9]. An explanation for this phenomenon may be that the strongly increased levels of angiogenic factors (e.g., VEGF) [8] result in a "saturation" of the vascular bed. It was argued that vessel density in tumors may exceed the metabolic requirements [38]. Probably, this process may lead to a more uniform, strongly overvascularized tissue.

In this study no correlation was found between microvascular parameters and Gleason score. Conflicting results concerning this issue have been published in the literature. Arakawa et al. [10] and Bettencourt et al. [11] found a significantly increased MVD with increasing Gleason score. Erbersdobler et al. [39] found a 1.5-fold difference between transition zone tumors, with a mean Gleason score of 5, and peripheral zone tumors with mean Gleason score of 7. Probably, the fact that only peripheral zone tumors were used exhibiting a small range of Gleason scores (score 5 or higher) accounts for this.

CONCLUSION

In the present study application of an automated procedure to quantify vascular parameters showed significant differences between the vascular bed in tumor tissue as compared to normal prostate tissue.
Mean and 75th percentile of MVD, MVA, and MVP were increased in tumor, showing enhanced vascularity. In contrast, the heterogeneity in MVD, MVA, and MVP was decreased in tumor as compared to normal tissue.

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