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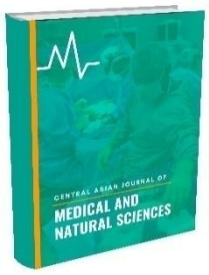

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The Importance of Vascular Density in Progression of Prostate Cancer Undergoing Radical Prostatectomy

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Abstract: Development of vascularization in tumor by supporting the nutrition and dissemination of tumor cells. However, the reduced number of vascular density (VVlow) may bring to hypoxia, which accounts for the selection of resistant clone(s) of tumor cells. This research focused on to evaluate the prognostic importance of blood vessel (VV) and lymphatic vessel (LV) in disease progression in prostate cancer (PCa). Tumor samples from 85 prostate cancer patients undergoing radical prostatectomy (RP) were prepared in duplex as tissue microarrays. All VV and LV were estimated using immunohistochemistry detecting CD34 and podoplanin, respectively, and correlated with clinical data, biochemical recurrence (BR), and proteins analyzed in tumor cells.

VVlow and LV were found in 13% and 24% of patients with informative prostate cancer samples, respectively. VVlow correlated with a shorter time to BR 1, 3, and 5 years after RP in hormone-native patients ($p = 0.021$, $p = 0.024$ and $p = 0.040$, respectively). Also these are shown to be an independent prognostic factor 3 years after surgery (multivariate analysis, $p = 0.041$). Tumors characterized by VVlow expressed the epithelial cell adhesion molecule, EpCAM, less frequently ($p = 0.014$) and revealed a borderline correlation to increased levels of tumor cell invasion marker Loxl-2 ($p = 0.043$). No correlations were found for LV. In summary, VVlow in hormone-native patients undergoing RP has prognostic potential and seems to be related to an aggressive phenotype of tumor cells.

Key words: prostate cancer, tumor progression, vascular density, vascular vessel, lymphatic vessels, hormone-native patients.

Introduction

In fact that, prostate cancer is one of the most actual issues in oncology although in oncourology, and it has become one of the most prevalent diseases among men in most countries around the world at the beginning of the 20th century. In the United States, more than 174,650 new cases were reported in 2019, and the number of deaths exceeded 31,620 [20]. Prostate cancer increased 3.0 times during 2001-2016 years (from 19.1 to 56.5 cases per 100 thousand population) [1] in the structure of the incidence of malignant neoplasms in men in Russia. Over the past 10 years, the mortality rate from prostate cancer ("rough" indicator) among men has increased by 39.0% from 12.9 in 2006. 18.42 cases per 100 thousand population in 2016, the average annual growth rate is -3.21% [1]. In the last 5 years in Uzbekistan there is an increase in the number of patients with prostate cancer, in particular, in 2015 – 372, in 2016 – 443 and by 2019 the number of patients reached 483 (an increase of 23%). There is also an increase in the incidence rate, which in 2015 was 1.2 patients per 100 thousand population, and by 2019 this figure was 1.5 (Tillyashaykhov M.N. et al. 2020). The mortality rate from prostate cancer is 8.09 per 100,000 population. Over the last decade, this figure has grown by 23.54%. (Al Shukri S.X. 2019). The sharp increase in the number of new diagnoses of prostate cancer and mortality from disease over the past decade, it has necessitated improvements in the screening and treatment of these patients. At present, early detection of oncological diseases serves toincrease the effectiveness of patients' treatment. Early diagnosis of prostate cancer allows patients to undergo radical treatment and increase the life expectancy. Over the past 5 years, the rate of prostate cancer in Uzbekistan (stages I and II) has improved, in particular, 30.1% of patients were diagnosed at an early stage in 2015, and this figure was 40.6% in 2019 (Tillyashaykhov M.N. 2020) Biochemical recurrence (BR) is still assumed to be the earliest indicator of patient relapse. It has been estimated that approximately 28% of patients manifest BR within 5 years after surgery and overt metastases approximately 3 years after BR. Further research is needed to identify markers relevant for individualized prognostication and tailoring of therapy.

(Neo) vascularization is considered as one of the imaginary factors impacting tumor development and in significant impacting patients' outcome in different solid tumors including prostate cancer [4,6,9,11]. Rich vascularity might guarantee appropriate nutrition of tumor cells and putatively facilitate their dissemination. However, a low number of vascular vessels may result in poor oxygenation, i.e., hypoxia. In prostate cancer, hypoxia occurs even at early stages of disease [11] and is associated with poor prognosis due to the selection of the most resistant clone(s) of tumor cells [7]. To date, the relationship between vascular vessel and/or lymphatic vessels and patient outcome in prostate cancer has not been fully clarified. The data of researchers reported variable outcomes (i.e., correlations to clinico-pathological parameters and patient survival or lack of) depending on the study design and applied methodology (including origin and type of prostate cancer specimens, type of targeted proteins to detect endothelial cells, area of analysis, type of treatment, etc.)

Thus, in the current study the association between vasculature and the aggressive prostate cancer phenotype and disease progression was investigated in unselected prostate cancer patients, hormone-native patients, and those treated with non-adjuvant ADT. CD34 and podoplanin, two proteins usually used for detection of VV and LV, respectively, were assessed in order to examine the number of vessels, as well as to examine their heterogeneity and elucidate putative clinical relevance. In addition that we also focused on minimal vessel numbers and their possible role in tumor progression or the relationship to clinical parameters.

2. Result

2.1. High changeable of Numbers of Vascular and Lymphatic Vessels in Prostate Carcinoma

In total, 109 tumor samples from 27 patients and tumor samples in 9 patients were informative for CD34 and podoplanin staining (Figure 1A,B), respectively. All examined tumor samples had a diameter of 10 mm (i.e., an area of 0.5 mm^2) and contained between 10 and 1000 tumor cells [15]. Of note, none of the detected VV and LV were invaded by tumor cells.

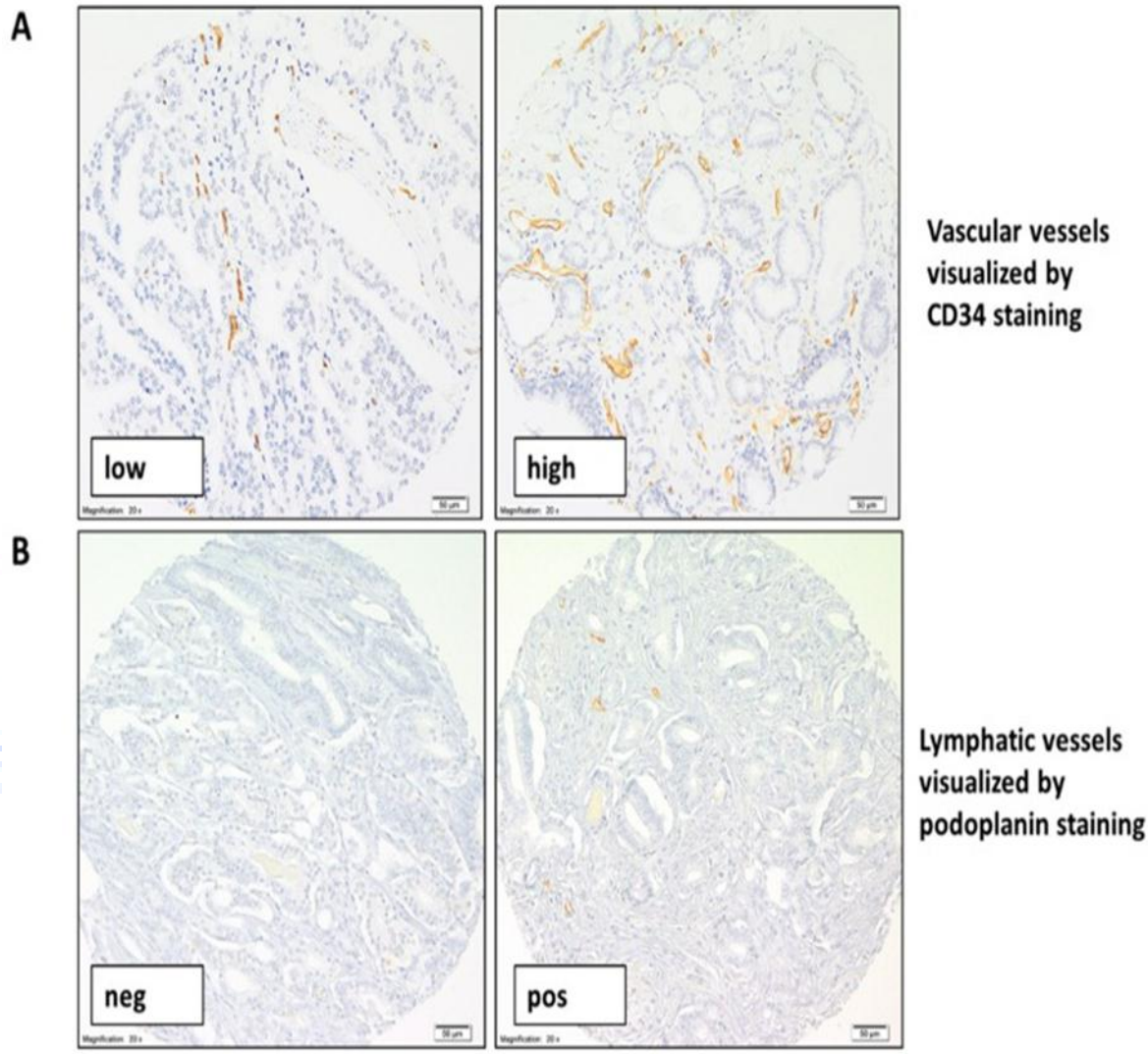


Figure 1

Representative pictures of CD34 and podoplanin staining in prostate cancer (PCa). Representative pictures of CD34 (**A**) and podoplanin (**B**) immunohistochemical staining (brown) in PCa with different number of identified vascular and lymphatic vessels, respectively (magnification 200×).

minVV and maxVV (i.e. minimal or maximal VV) were tested to categorize the patients for further statistical analysis . However, only minVV dichotomized according to the lower quartile (equal 3 VV) into $minVV^{low}$ and $minVV^{high}$ correlated to clinical outcome, and therefore this classification is described in the current study in detail. According to this classification system, 13% (n = 27) patients were characterized by $minVV^{low}$.

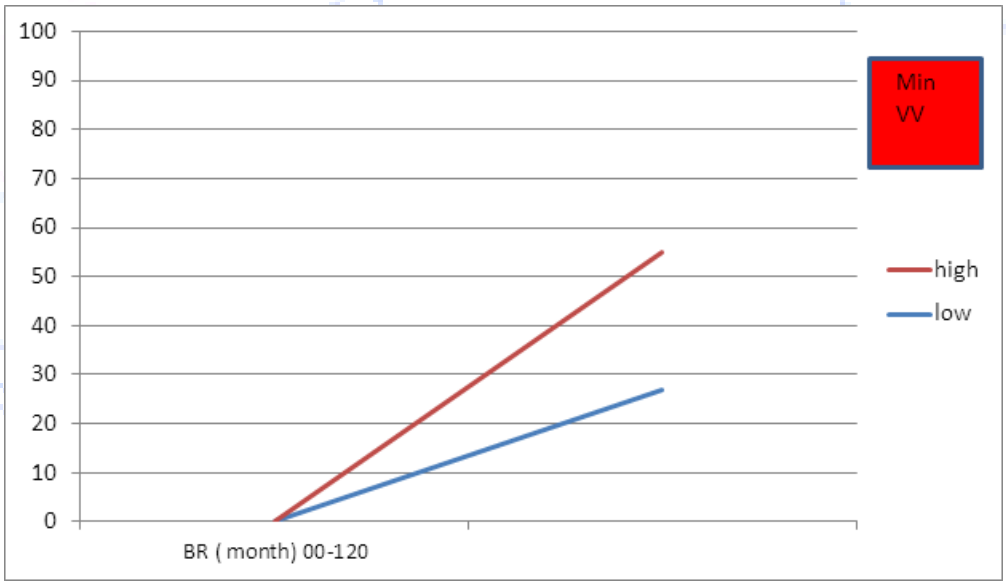
Podoplanin-positive LV (Figure 1B) were detected in 15 (13.7%) of 109 tumor samples with the mean and median total number of LV determined as 1 and ranging from 1 to 16 LV per tumor fragment.

minLV and maxLV were also tested to categorize the patients for further statistical analysis . However, as there were no correlations to clinical data for LV, any positive maxLV was categorized as LV^{pos}, whereas no LV was termed LV^{neg}. In total, 26 (24%) patients were defined as LV^{pos} according to this classification system.

2.2. Clinical Relevance of Low Number of Vascular Vessels

Different cut-offs (i.e., mean, median, quartiles) to categorize the outcomes and clinical subgroups of the study cohort (i.e., unselected cohort, hormone-naïve patients, and patients treated with neoadjuvant ADT) were tested in order to determine putative relevance of vascular and lymphatic vascularity in prostate cancer. No correlations were found between vasculature and clinico-pathological parameters (presented outcomes for minVV and maxLV are described in $minVV^{low}$ correlated with the shorter time to BR (Kaplan-Meier plot, n =85) at timepoints of 1, 3, and 5 years after surgery (p = 0.031, p = 0.029, and p = 0.056, respectively; Figure 2) in the hormone-naïve patients. It appeared to be an independent prognostic factor in this cohort of patients in the multivariate analysis including T status, Gleason score, and minVV status 5 years after surgery (n = 85, p = 0.046, HR—hazard ratio 0.607, 95% CI 0.3810–0.990

Figure 2



Time point (months)	Number of events (min VV low)	(min VV high)	p value
≤36	27	28	0.028
≤60	28	30	0.027
≤120	30	31	0.056

Figure 2

Survival analysis. Association of minVV to shorter time to biochemical recurrence in hormone-nativeve prostate cancer patients. BR: biochemical recurrence. VV indicates vascular vessels.

Table 1. Multivariate analysis. Statistically significant results are bolded. PSA: prostate-specific antigen, minVV^{low}—low number of vascular vessels, minVV^{high}—high number of vascular vessels.

5 Years	Univariate Analysis			Multivariate Analysis		
	p-Value	HR	95% CI	*p*-Value	HR	95% Cl
T3–4 vs. T1–2	<0.001	3.734	2.068–6.742	<0.001	3.337	1.815–6.136
N1–2 vs. N0	0.260	1.691	0.678–4.251	-	-	-
Preoperative PSA ≥ 4 ng/mL vs. < 4 ng/mL	0.076	5.971	0.829–43.030	-	-	-
Age (≥ 64 vs. < 64)	0.515	1.174	0.724–1.903	-	-	-
Gleason scale ≥ 7 vs. < 7	0.010	2.796	1.277–6.120	0.190	1.716	0.765–3.849
minVV^{low} vs. minVV^{high}	0.031	0.584	0.358–0.951	0.046	0.607	0.372–0.991

2.3. Signatures of Aggressive Molecular Phenotype in Tumors Characterized by Low Number of Vascular Vessels

The panel of different prostate cancer aggressiveness- and progression-related proteins was assessed before in tumor cells of individual tumor samples of the examined patients: Ki-67, apoptosis marker (ApopTag), cytokeratins (CK5/6, CK14, CK8/18, CK19), vimentin, E- and N-cadherin, aldehyde dehydrogenase 1 (ALDH1), epidermal growth factor receptor (EGFR), epithelial cell adhesion molecule (EpCAM), B-cell lymphoma-2 apoptosis regulator (Bcl-2). The numbers of VV were compared to those proteins in the individual tumor samples

Tumors with VV^{low} not subjected to any preoperative ADT (i.e., tumors of hormone-naïve subcohort of patients) less frequently expressed the epithelial cell marker EpCAM in tumor cells (n = 109, Chi-squared = 10.314, p = 0.016, Figure 3A) and were usually characterized by the higher Gleason score (n = 109, Chi^2 = 25.116, p < 0.001, Figure 3B). In addition, VV^{low} revealed a borderline correlation to the increased presence of a hypoxia-related marker of tumor invasion on the extracellular matrix, Loxl-2, in tumor cells (n = 178, Chi-squared = 5.669, p = 0.059, Figure 3C).

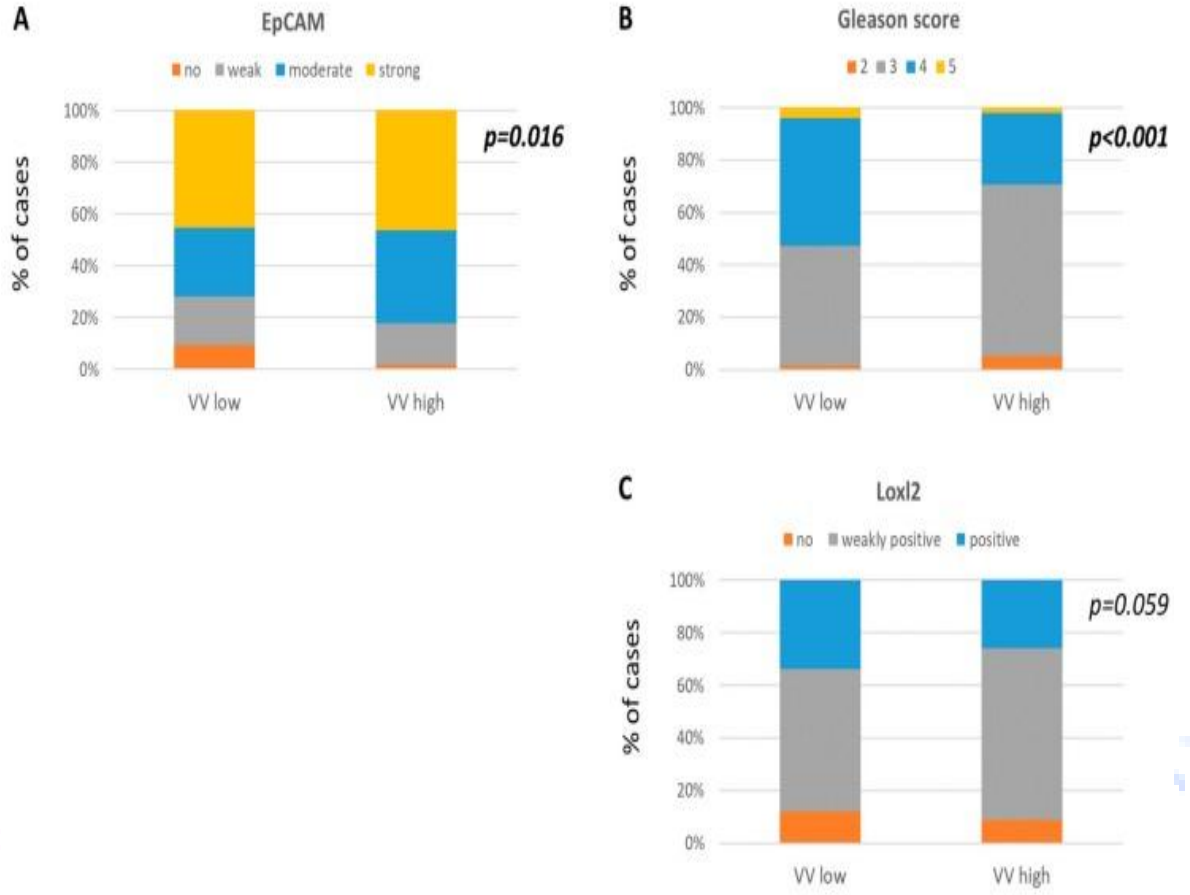


Figure 3

Molecular characteristics of PCa in comparison to VV status. VV^{low} correlations to less frequent (**A**) EpCAM expression, (**B**) higher Gleason score, and borderline correlation to more frequent (**C**) Loxl-2 expression.

Interestingly, when only the lower and upper quartiles of VV were compared (i.e., groups of tumors with VV < 5 and VV > 15, respectively), the tumors with low vasculature were characterized significantly more frequently by Loxl-2 (p = 0.013), proliferation marker Ki-67 (p = 0.034), and the apoptosis marker (p = 0.022; data not shown [18]).

3. Discussion

Tumor (neo)vasculature might play a crucial role in tumor development and progression. Here, we show for the first time that low vasculature correlates to worse clinical outcome in hormone-native prostate cancer patients after radical prostatectomy and is associated with aggressive phenotype of tumor cells. The number of vascular and lymphatic vessels is considered as a significant microenvironmental factor impacting tumor fate in different solid tumors [4,5,10,12]. However, inconsistent study designs and applied methodologies (i.e., different origin, type and area of the analyzed PCa specimens, type of targeted proteins to detect endothelial cells, etc.) result in different outcomes showing both correlations and their lack between number of vessels and clinico-pathological parameters in PCa [11]. In the current study, two commonly used proteins, CD34 and podoplanin, were examined to define the numbers of VV and LV, respectively. Our evaluation method was similar to commonly used so called Weidner method [20]. However, the applied study approach allowed for an examination of the clinical significance of both minimal and maximal numbers of vessels detected within the defined fragments of tumors (area equal 0.28 mm^2) without an a priori assumption that only

high vasculature might support tumor progression. Of note, tissue microarrays (TMAs) used in this study represented a collection of potentially different tumor samples of individual patients prepared in order to study prostate cancer heterogeneity. In addition, to exclude bias caused potentially by ADT [14], clinical relevance of vasculature was compared in the unselected cohort, as well as hormone-native prostate cancer patients and those treated with ADT preoperatively.

VVs were detected in almost all samples, whereas LVs were found in only 27% of tumor samples. Intratumoral heterogeneity of VV and LV numbers was observed in approximately one-third of the patients, which substantiates the high heterogeneity described in PCa for many factors. VV^{low} and LV were found in 32% and 43% of patients, respectively. Only $minVV^{low}$ indicating the lowest numbers of vascular vessels (i.e., the lower quartile of minimal values assigned for a patient) correlated to worse clinical outcome in hormone-naïve PCa patients in the timeframe of 3–5 years after surgery. Of note, in the current study $minVV^{low}$ was an independent prognostic factor in the multivariate analysis, substantiating its prognostic potential in patients without ADT. We did not observe any correlation to clinical outcome in the cohort of unselected PCa patients or for maxVV and LV, nor for minVV in the PCa patients undergoing ADT before the prostatectomy. However, this cohort was relatively small (n = 25) which might have biased the outcome. Those results seem to be counterintuitive as the other groups showed that the higher number of VV [12,19,22,24] and also the presence of LV may increase the risk of BR in the patients undergoing radical prostatectomy after neoadjuvant treatment with androgen blockage [24], correlating with high Gleason score and other clinico-pathological parameters indicating advanced disease . Of note, a high number of VV evaluated using CD105, another protein detecting endothelial cells, was also identified as a significant and independent predictor of biochemical recurrence in prostate cancer patients who underwent radical prostatectomy with ADT [13]. Our results and those of the literature could suggest that vascularization develops differentially under different androgen conditions, regulating tumor development and patient outcome in a different way. In addition, the majority of the studies focused on the so-called “hot spot” with the highest number of VV. This approach might be also biased [28] and result in exclusion of fragments of tumor with low number of vessels, potentially still crucial for progression but driven by different biological mechanisms.

Indeed, in our study tumors characterized by VV^{low} exerted some features of more a aggressive phenotype of tumor cells. They were characterized more frequently by the absence of the epithelial cell marker EpCAM [29], which might suggest that those tumors undergo EMT known to facilitate migration, and even induce stemness [30]. They also more frequently expressed higher levels of Loxl-2, a protein related to invasion of tumor cells on extracellular matrix, and in comparison to highly-vascularized tumors this was also the case for proliferation marker Ki-67 and the apoptosis marker. All those proteins are known to correlate to poorer clinical outcome both in PCa and/or other tumor entities [31,32,33]. Of note, they might be expressed under hypoxic conditions expected to occur in less vascularized samples of tumors [32]. Hypoxia-related markers (such as hypoxia-inducible factor 1-alpha, HIF-1α) were not investigated in this study. However, it might be still speculated that hypoxia occurred in the examined tumors with low numbers of VV, potentially promoting a proliferation/apoptosis imbalance as well as induction of EMT [34] and acquisition of more aggressive phenotype by tumor cells [30]. In concordance, Loxl-2 was shown to be upregulated by HIF-1α in a hypoxic tumor microenvironment in hepatocellular cancer [35], and to induce EMT in colorectal and breast carcinomas [36,37].

4. Material and Methods

4.1. Patient Cohort

Tumor samples from 85 prostate cancer patients were collected following radical prostatectomy at the Department of oncourology at the Cancer Centre of Uzbekistan after patients gave informed consent,

and were prepared in duplex as 2 TMAs as described previously [15,16]. Briefly, two tumor samples were selected for each patient from different areas of the tumor (if the tumor was monofocal) or different tumor foci (if the tumor was multifocal). Patients were characterized by different clinico-pathological parameters and different molecular phenotypes of tumor cells (Supplementary Table S4). Measurement of serum PSA concentration was performed by two methods: (1) up to 2009 using Tandem-E (Hybritech, San Diego, CA, USA) and (2) from 2010 using Access 2 (Hybritech-calibrated, Beckman Coulter, Brea, CA, USA). BR was defined as two consecutive concentrations of PSA above 0.1 ng/mL. The time point of BR was defined as the first PSA concentration above 0.1 ng/mL.

Table 2

Distribution of clinical parameters in the study cohort. neg: negative, pos: positive. Note that not all numbers sum up to 109 due to the missing data. ADT: androgen deprivation therapy.

Distribution of patients with local advanced prostate cancer by TNM system, n=109

№	Group research	T2a-stage	T2b-stage	T2c- stage	**total**
1.	1-control	4 *(3,7%)*	18 *(16,5%)*	5 *(4,6%)*	**27 *(24,8%)***
2.	2-contorl	3 *(2,7%)*	8 *(7,3%)*	6*(5,5%)*	**17 *(15,5%)***
3.	1-main group	11 *(10,1%)*	13 *(11,9%)*	13 *(11,9%)*	**37*(33,9%)***
4.	2-main group	5 *(4,6%)*	15 *(13,8%)*	8 *(7,4%)*	**28 *(25,8%)***
	total	**23*(21,1%)***	**54 *(49,5%)***	**32*(29,4%)***	**109*(100%)***

Distribution of patients according to morphological structure, n=109

	Gleason score 6 (G2)	Gleason score 7 (G3-4)	Gleason score 8 (G3-4)	Gleason score 9 (G3-4)	Total
T2aN0M0	9	4	6	4	**23 *(21,1%)***
T2bN0M0	21	9	8	6	**54 *(49,5%)***
T2cN0M0	11	8	6	7	**32 *(29,4%)***
Total	**41**	**21**	**20**	**17**	**109*(100%)***

Distribution of patients according to age n=109

Age / Sex		40 – 49	50 -59	60 - 69	70 - 79	Older 80 age	total
T2aN0M0	number	1	11	7	3	1	23
	в %	*0,9*	*10,1*	*6,5*	*2,7*	*0,9*	*21,1*
T2bN0M0	number	2	33	19	6	1	54
	в %	*1,8*	*30,3*	*17,6*	*5,5*	*0,9*	*56,1*
T2cN0M0	number	1	9	11	3	1	32
	в %	*0,9*	*8,2*	*10,1*	*2,7*	*0,9*	*22,8*
total	number	4	53	37	12	3	109
	в %	*3,6*	*48,6*	*34,2*	*10,9*	*2,7*	*100*

Preoperative PSA	<4 ng/mL	15	13.7
	4–10 ng/mL	25	22.9
	10–20 ng/mL	28	25.6
	>20 ng/mL	41	37.6

	total	109	
Biochemical recurrence	no	66	60.5
	yes	43	39.5
	total	109	
Tumor focality	monofocal	25	23
	multifocal	84	77
	total	109	

4.2. Immunohistochemistry for CD34 and Podoplanin

Immunohistochemical staining was performed on TMA sections (4–5 µm thick) using commercially available ready-to-use mouse monoclonal anti-CD34 antibody (clone QBEnd10, Agilent Dako, Santa Clara, CA, USA) for VV and anti-podoplanin antibody (clone REF 760–4395, Roche, Switzerland) for LV visualized by EnVision FLEX+ system (Dako) and UltraView DAB Benchmark XT (Roche) system, respectively.

4.3. The Evaluation of VV and LV Density

The immunohistochemical staining was evaluated at the 200× magnification using light microscope (Olympus BX 43, Olympus, Japan). The total number of CD34- or podoplanin-positive VV or LV, respectively, with visible light of lumen was documented in each informative tumor sample (0.6 mm diameter, area 0.28 mm^2).

In order to examine the possible variability of numbers of vessels, vessels were counted separately in two tumor samples of each patient. The tumor sample with the lower number of vessels was assigned minVV or minLV, the sample with the higher number of vessels was considered as maxVV or maxLV (Figure S1). If only one tumor sample was informative, the vessel count of that tumor sample was assigned to the patient. The assigned discrete values for minVV, maxVV, minLV, and maxLV were dichotomized based on different mathematical cut-offs (i.e., mean, median, quartiles) and compared to clinical data and patient survival (Figure S1).

4.4. Immunohistochemistry for Tumor Cell Markers

Immunohistochemical staining for Ki-67, apoptosis marker (ApopTag), cytokeratins (CK5/6, CK14, CK8/18, CK19), vimentin, E- and N-cadherin, ALDH1, EGFR, EpCAM, and Loxl-2 protein was performed, evaluated, and categorized as negative or positive (and for Loxl-2 as negative, weakly positive, or positive)

4.5. Statistical Analysis

Statistical analysis was performed using SPSS software (IBM) version 25 licensed . The comparison of number of vessels and clinical data or proteins detected in tumor cells was performed using Chi-squared or Fisher's exact tests. The association between number of vessels and time to BR was calculated using Mantel Cox test and presented using Kaplan–Meier plots. The uni- and multivariate analysis was performed using the Cox-Hazard-Potential regression model (95% CI). The obtained results were considered statistically significant at $p < 0.05$. Cases with missing data were excluded from the statistical analysis.

5. Conclusions

In summary, tumors with VV^{low} seem to have a more aggressive phenotype and shorter time to BR in hormone-nativeve prostate cancer, suggesting the pivotal role of vascular vessels in regulation of tumor progression. However, further studies are needed to dissect this process in detail.

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