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ORIGINAL ARTICLE

Clinical significance of microvessel density and proliferation in prostate cancer core biopsy

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Summary

Purpose: *To investigate the microvessel density (MVD) and proliferation in prostate cancer (PC) core biopsies.*

Methods: *Core biopsy samples of PC tissue from 45 patients were routinely processed and embedded in paraffin. The samples of PC formed the investigated group (n=25), while samples of benign prostatic hyperplasia (BPH) served as controls (n=20). From paraffin blocks, 3-5 μm-thick sections were made and routine hematoxylin-eosin method and immunohistochemical ABC method with Ki67 and CD34 antibodies were applied. Immunohistochemical expression of Ki67 and CD34 was stereometrically quantified.*

Results: *The median number of Ki67 and CD34 positive cells per mm2 in PC were significantly higher in comparison to the median of these cells in BHP. The average age and Gleason score in patients with high proliferation index (proIDX) and MVD index (mvdIDX) was significantly greater in comparison to those with low proIDX and low mvdIDX. The absolute values of Ki67 expression were in highly positive and significant correlation with the absolute values of CD34 expression. Highly significant correlation was found between Gleason score and proIDX and mvdIDX.*

Conclusion: *This study showed that PC expressed significantly higher values of Ki67 and CD34 in comparison to BPH. The values of proIDX and mvdIDX obtained by core biopsy could clearly show the level of cancer progression expressed through highly correlated Gleason score. In this way it is possible to identify the patients at high risk for disease progression.*

Key words: *immunohistochemistry, microvessel density, proliferation, prostate cancer*

Introduction

Prostate cancer is the second most often diagnosed tumor in males. About 1.1 million people with PC were diagnosed in 2012, of whom 70% of cases (795,000) were from developed countries. The highest incidence of PC was noted in Australia/New Zealand, North America, North and West Europe, while the lowest was in Asia. The disease most often affects male population over the age of 50, and often results in significant mortality. It was noticed that the increasing incidence of the tumor and the resulting mortality followed the extension of lifespan [1-3].

The identified risk factors for the incidence of PC are old age, positive family history, race and ethnicity, geographic location, androgen hormones, economic and social factors, while in recent decades obesity is especially pronounced [4-6].

PC is characterized by diverse and often unpredictable biological behavior. On the one hand,

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tumors with high malignant potential form metastases prior to any signs or symptoms, while on the other, some cancers remain localized for a long time, often without pronounced symptoms. Literature data suggest that various growth factors and numerous molecules can be connected with the progression and prognosis of PC [7,8].

For early diagnosis of PC, digital rectal examination (DRE), prostate specific antigen level (PSA), transrectal ultrasound (TRUS) and prostate biopsy are used [9-11]. Histopathological confirmation of the diagnosis of PC is necessary not only for consideration of the biological behavior of the tumor but also for the therapeutic approach and selection of treatment. Among the most significant prognostic factors associated with the PC is disease extension. Since locoregional control of disease depends on the proliferation activity of the tumor and the degree of angiogenesis [12], the aim of this study was to investigate MVD and proliferation index in prostate adenocarcinoma in the tissue samples obtained by core biopsy.

Methods

Patients and tissue samples

The research included 45 patients who had core biopsy performed at the polyclinic Dr Vezmar in Kragujevac from February 2014-December 2015. Suspicious DRE or TRUS findings and/or serum PSA>4.0 ng/mL were indications for biopsy of prostate tissue. According to the guidelines for histopathological examination, core biopsy of prostate tissue was taken from both left and right prostate lobes, 10-12 samples per patient. The obtained samples were fixed in 4% neutral buffered formalin, routinely processed, embedded in paraffin blocks and archived at the Centre for Pathological Anatomical Diagnostics, Clinical Centre Kragujevac. PC was histopathologically diagnosed on routine microscope preparations, while Gleason score was concomitantly defined by using standard procedures. The tissue samples of PC obtained by core biopsy formed the examined group (n=25), while the samples with BPH served as controls (n=20). The study protocol was approved by the local Ethics Committee.

Histopathology and immunohistochemical examination

Serial sections, 3-5 μm thick, were made on paraffin blocks of all tissue samples obtained by core biopsy, and routine hematoxylin-eosin method for histopathological verification of the lesions and immunohistochemical ABC method with Ki67 and CD34 antibodies were applied.

Representative tissue samples, 3 μm thick, were heated at 55°C to melt the paraffin, deparaffinized in xylene (3-5 min) and then rehydrated through graded ethanols. Antigen retrieval was enhanced by autoclaving slides in sodium citrate buffer (pH 6.0) for 30 min. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide-methanol buffer for 25 min. To reduce nonspecific background, staining of the section was incubated with 10% normal bovine serum albumin for 30 min at room temperature. Rabbit monoclonal Ki67 antibody (Abcam, Cambridge, UK; 1:100) and monoclonal mouse CD34 (clone QBEmd 10) antibody (Dako, Glostrup, Denmark, Ready to use) were incubated at 4^0 C overnight. Immunostaining was performed by the avidin-biotin-peroxidase complex (ABC) method (Vectastain ABC-Elite kit, Vector Laboratories, Burlingame,-CA). Staining was visualized with 3,3diaminobenzidine tetrachloride (DAB). The slides were counterstained with Mayer hematoxylin and mounted on Canada balsam. Negative controls were done by replacing the primary antibody with phosphate buffered saline (PBS).

Quantification of immunohistochemical staining

In evaluating the expression of Ki67, only stained nuclei were taken into account, while for the evaluation of Ki67-positive cells per mm^2 the multipurpose test system M42 by Weibel [13] was used. Objective micrometer (Reichert Wien 2mm/200) was used to determine the measuring area of 0.016 mm^2.

For testing Ki67, positive cells/mm^2 were counted successively by 5 "hot spots". The absolute value of the density of positive cells in the "hot spot" was determined stereometrically [13]. The arithmetic mean of the obtained values of the "hot spots" represented the final number of Ki67-positive cells per mm^2 per case. The median was subsequently determined and the absolute values of the density of positive cells were divided into two groups: those with low expression level (values ≤ the median value) and those with high level of expression (values >the median value). These values represented the proIDX.

MVD was calculated by counting microvascular CD34 positive structures, under 400x magnification, whereas first were selected areas with highest MVD ("hot spots"). Every single cell or field marker was counted as microvascular structure. For the determination of MVD the multipurpose test system M42 was also used and measured a field of 0.016 mm^2 with Olympus BH-2 microscope. For the investigation of MVD per mm^2, 5 "hot spots" were counted successively, and the absolute value of positive vascular structures density in "hot spot" was determined stereometrically [13]. The final result was from the study of 5 consecutive fields on average. After having obtained data regarding the number of microvascular structures for each patient, the median was determined, according to which the patients were divided into two groups: those with low grade of angiogenesis (MVD in tumor ≤ than median value), and those with high grade of angiogenesis (MVD > than median value). From absolute determined values of MVD regarding deviation from median, the MVD index was obtained.

Table 1. Relationships between prostate cancer/benign prostatic hyperplasia and other clinicopathological variables

	n	*Minimum*	*Maximum*	*Mean*	*SD*	*p value**
Age						0.308
PC	25	58	84	71.3	6.8	
BPH	20	57	83	69.1	7.7	
Gleason score						-
PC	25	6	10	7.7	1.2	
PSA						0.006*
PC	25	4.40	111.30	25.63	23.63	
BPH	20	0.49	31.48	10.89	6.69	
Prostate volume (mm)						0.994
PC	25	22.3	115.0	50.2	23.8	
BPH	20	22.0	99.5	50.2	21.8	

*Student's t-test
PC: prostate cancer, BPH: benign prostatic hyperplasia, PSA: prostate specific antigen, SD: standard deviation

The expression of the aforementioned markers was evaluated independently by two pathologists.

Statistics

Median levels of Ki67 and hematopoietic progenitor cell antigen CD34 were compared between PC and BPH groups by means of the non-parametric Mann-Whitney U test. Other variables (mean value of age, Gleason score, PSA, prostate volume) between the groups of low and high index of Ki67and MVD, as well between cancer and non-cancer groups were compared using Student's t-test. Kolmogorov-Smirnov test for normality distribution was performed. Correlation analysis (Spearman's rank correlation coefficient and Pearson's correlation coefficient for parametric features) were used. P values <0.05 were considered as statistically significant.

Results

Clinical features

The average patient age with PC was 71.3 years (range 58-84), while in the group with BPH it was 69.1 (range 57-83). No statistically significant difference in age was found between the patients of the 2 groups (p=0.308; Table 1).

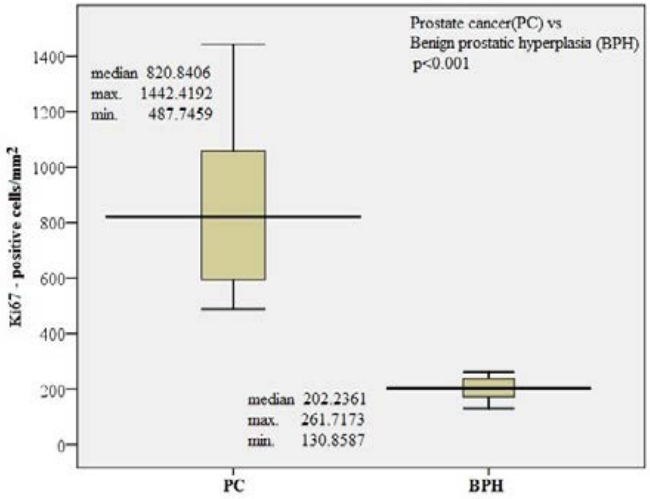

Figure 1. Expression of Ki67 in PC and BPH.

Serum PSA values were significantly higher in the patients with PC compared to the patients with BPH. The average PSA levels were about 2.5-fold higher in cancer patients compared to those with BPH (p=0.006; Table 1).

In Table 1, Gleason score is presented by using descriptive parameters. This score is a characteristic of cancer, i.e. it is a representative of differentiation of the tumor. The average Gleason score in our patients with PC was 7.7

By using the same statistical procedure of testing, no differences in the volume of prostate were found (Table 1), where the average volume in PC and BPH groups was the same (50.02).

Immunohistochemical expression of Ki67 in PC and BPH

Immunohistochemical examination of Ki67 expression absolute values of positive cell density per mm^2 was carried out. Basic characteristics of these values (median, minimum, maximum and discrepancy values) are presented in box-plot diagram (Figure 1). The median number of Ki67 positive cells per mm^2 in PC was significantly higher in comparison to the median Ki67 positive cells per mm^2 in BPH (820.8 vs 202.2, p<0.001, Mann-Whitney U test).

Proliferation index in comparison to other clinicopathological variables

The mean PC patient age in relation to proliferation index Ki67 revealed significantly greater mean age with high proliferation index (Figure 2) in comparison to those with low proliferation index (74.5 vs 67.8 years; p-0.012 ; Table 2; Figure 2).

Table 2. Relationships between expression levels of Ki67 (low/high) and other clinicopathological variables

	n	*Minimum*	*Maximum*	*Mean*	*SD*	*p value**
Age						0.012
LproIDX	12	58	77	67.8	6.0	
HproIDX	13	64	84	74.5	6.1	
Gleason score						<0.001
LproIDX	12	6	8	6.7	0.7	
HproIDX	13	8	10	8.7	0.8	
PSA						0.753
LproIDX	12	4.40	111.30	24.03	29.67	
HproIDX	13	7.42	65.80	27.10	17.46	
Prostate volume (mm)						0.873
LproIDX	12	22.3	98.8	51.0	24.3	
HproIDX	13	32.0	115.0	49.4	24.3	

*Student's t-test
LproIDX: Low levels of Ki67 expression (values ≤ the median value), HproIDX: High levels of Ki67 expression (values > the median value), SD: standard deviation

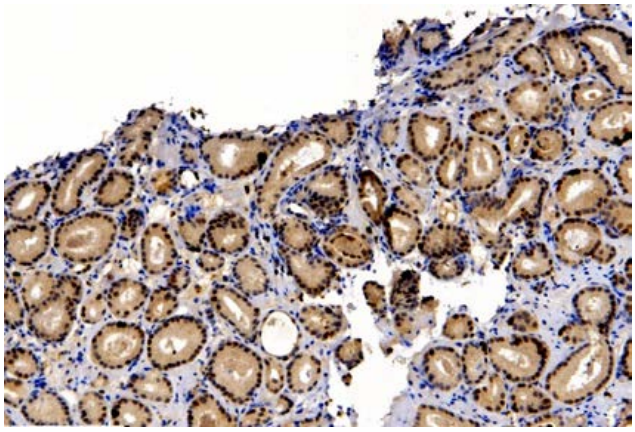

Figure 2. High levels of proliferation index in the elderly patients with prostate cancer (ABC, antiKi67 antibody, x200).

In addition, comparison of Gleason score between these two groups proved that it was significantly greater in the group with high proliferation index (approximately 9 on average) in comparison to low proliferation index (approximately 7 on average) (Table 2).

Mean values of PSA and prostatic volume did not differ significantly between the groups with low and high proliferation indices (p=0.753 vs p=0.873) (Table 2).

Immunohistochemical expression of CD34 in PC and BPH

In the cases of cancer, the median number of CD34 positive cells per mm^2 was significantly higher in comparison to the median CD34 positive cells per mm^2 in the cases of BPH (856.5 vs 339.0, p<0.001, Mann-Whitney U test; Figure 3).

Microvessel density index in comparison to other clinicopathological variables

The mean patient age with high MVD index (Figure 4) was significantly higher in comparison to the patients with lower MVD index (74.5 vs 68.2 years; p=0.024;Table 3).

In addition, comparison of Gleason score between these groups showed that it was signifi-

Table 3. Relationship between expression levels of CD34 (low/high) and other clinicopathological variables

	n	*Minimum*	*Maximum*	*Mean*	*SD*	*p value**
Age						
LmvdIDX	12	58	77	68.2	6.0	0.024
HmvdID	13	64	84	74.2	6.4	
Gleason score						
LmvdIDX	12	6	8	6.7	0.7	<0.001
HmvdID	13	8	10	8.7	0.8	
PSA						
LmvdIDX	12	4.40	36.40	17.36	12.02	0.093
HmvdID	13	7.42	111.30	33.26	29.21	
Prostate volume (mm)						
LmvdIDX	12	22.3	98.8	47.3	21.2	0.577
HmvdID	13	32.0	115.0	52.8	26.6	

*Student's t- test
LmvdIDX: Low levels of CD34 expression (values ≤ the median value), HmvdIDX: High levels of CD34 expression (values > the median value), SD: standard deviation

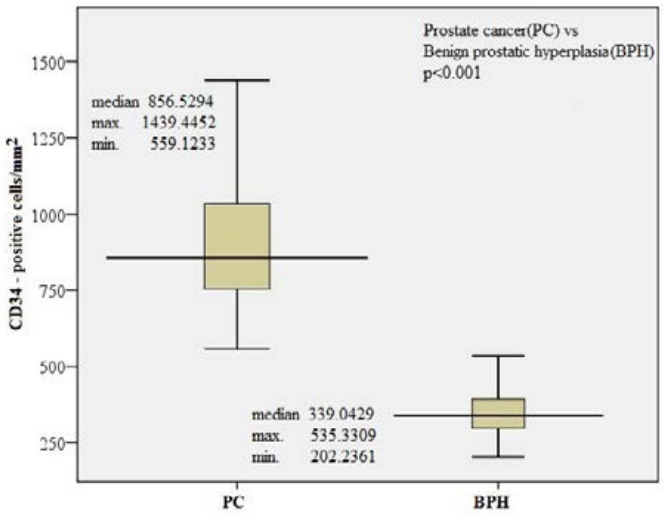

Figure 3. Expression of CD34 in PC and BPH.

Table 4. Correlation matrix–significance of correlations between proliferative and neoangiogenesis index

Correlations			*proIDX*	*mvdIDX*
Spearman's correlation	proIDX	Correlation coefficient	1.000	0.840*
		Sig.		<0.001
	mvdDX	Correlation coefficient	0.840*	1.000
		Sig.	<0.001	

* Correlation significant at the 0.01 level. proIDX: proliferative index, mvdIDX: angiogenesis index

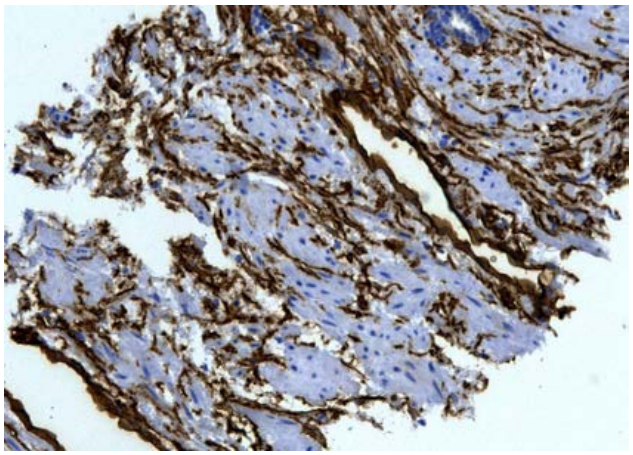

Figure 4. High levels of microvessel density index in the elderly patients with prostate cancer (ABC, antiCD34 antibody, x300).

cantly greater in the group with high MVD index (approximately 9 on average) in comparison to low MVD index (approximately 7 on average; p<0.001;Table 3).

Mean serum PSA levels, even though higher in cases of high MVD index in comparison to low (about 33 vs 17), were not statistically significantly different at the adopted level of reliability (p=0.093; Table 3).

The volume of prostate was not significantly different between groups with low and high MVD indices (Table 3).

Correlation analyses of Ki67 and CD34 expression

The similarity of results and determined significance of other variables in the previous analysis between the level of proliferation and MVD index initiated the need to examine the level of mutual correlation between these two indices.

Spearman's rank correlation revealed high correlation (correlation coefficient=0.840) between the proliferation index and MVD index in the presence of PC, which was significant (p<0.001;Table 4).

Correlation analyses of the examined variables

In order to identify the level and direction of mutual correlation of the variables considered in this study, the results of correlation are presented in Table 5. The expression Ki67 and CD34, considered in absolute value of measurements, as well as the previously measured indices (proIDX and mvdIDX) were in highly positive and significant correlation (p<0.001, which was also similar in correlation coefficient: 0.834).

Highly significant correlation of Gleason score with Ki67 and MVD was also obvious (Table 5). Significant increase of the mean Gleason score in high in comparison to low indices of the analysed antigens proved in the previous analysis, was confirmed by high positive correlation coefficient (0.831) between Gleason score and absolute values of both antigens (Ki67 and CD34); p<0.001).

No good correlation coefficient of 0.343 between PSA and CD34 (absolute value) was confirmed as significant at the adopted level of reliability (p=0.093).

Comparison of the groups with PC and BPH at the beginning of statistical analysis proved that significantly higher PSA values were found in the PC group. Correlation analysis of this diagnostic factor showed moderate positive correlation (correlation coefficient=0.565) between PSA and prostate volume. It was the only significant interdependence of these variables in relation to all compared variables (p=0.003).

Discussion

PC mainly affects elderly male population and its incidence is increasing with age, especially after the age of 60 [14]. In our study, the aver-

Table 5. Correlation matrix - correlation parameters - significance and degree of dependence

Correlations		*PSA*	*Gleason score*	*Prostate vol*	*Ki67*	*CD34*
PSA	Pearson correlation	1	0.321	0.565**	0.066	0.343 0.093
	Sig.		0.118	0.003	0.753	
Gleason score	Pearson correlation	0.321	1	0.122	0.831*	0.831*
	Sig.	0.118		0.561	<0.001	<0.001
Prostate vol	Pearson correlation	0.565*	0.122	1	-0.034	0.117
	Sig.	0.003	0.561		0.873	0.577
Ki67	Pearson correlation	0.066	0.831*	-0.034	1	0.834
	Sig.	0.753	<0.001	0.873		<0.001
CD34	Pearson correlation	0.343	0.831*	0.117	0.834*	1
	Sig.	0.093	<0.001	0.577	<0.001	

* Correlation significant at the 0.01 level. Ki67: positive cells/mm^2, CD34: positive cells/mm^2

age age of patients with PC was 71.3 years, which is in accordance with numerous reports from the literature [15,16]. However, it is known that males aged 30 or 40 also suffer from PC [14,17]. In some studies the rate of cancer identification in biopsy was evaluated as 0.08% for the males aged 30-39 and 1.9% for those aged 40-49 years. Contrary to this, PC is identified by biopsy at the ages 50-59, 60-69 and 70-79 in 13.5%, 34% and 39% respectively [17].

The final diagnosis of PC is identified by biopsy. The indications for biopsy are increased PSA values and suspicious DRE findings [9,10]. Biopsy provides reliable information of disease differentiation, its extension and biological behavior. Histological confirmation of PC diagnosis is primarily necessary for proper therapeutic approach, and besides, biopsy may identify the parameters necessary for tumor prognosis.

By examining Ki67 positive cell expression per surface area, the absolute values of Ki67 positive cell expression was significantly higher in PC in comparison to BPH, hence the proliferation index (proIDX) was significantly higher in PC cases. This was recently confirmed by Adisa et al., who found positive expression of Ki67 reactive cells in only 13.3% of BPH samples [18]. Concerning the proliferation index, our patients with PC were divided in groups with low and high proliferation index of tumor cells. Considering the proliferation index in relation to other clinical parameters, the mean patient age (74.5 years) with verified high proliferation index in PC was significantly higher in comparison to the mean age of patients (67.8 years) with low level of proliferation of tumor cells. This study also showed that the mean Gleason score was significantly higher (approximately 9) in patients with high proliferation index in comparison to the patients with low proliferation index of tumor cells. Another study showed that increased proliferation index provided additional information about clinical stage, size of tumor and Gleason score, whereby the correlation between high proliferation index and bad prognosis of PC was emphasized [19].

Cell proliferation is proportional to the growth and progression of the tumor, and Ki67 is traditionally used as proliferation marker. It is present in cell nuclei and marks not only the cells in division but also all cells in synthetic phases of the cell cycle (in G_1, S, G_2 and M phases). The antigen cannot be detected in G_0 phase [20]. In the last decade a great number of prospective studies appeared which examined the expression of Ki67 and clinical outcomes of various diseases, including tumors. In breast cancer most studies showed strong, statistically significant correlation with clinical outcomes in both univariate and multivariate analyses [20,21]. In gastric cancer, the correlation between Ki67 expression and clinicopathological prognostic parameters was highly significant, whereby a group of Greek surgeons suggest that, in addition to providing important information on cancer prognosis, Ki67 expression can be a useful means for the identification of patients with aggressive course of disease, thus contributing to better therapeutic approach [22].

The hematopoietic progenitor cell antigen CD34 as a marker of vascular endothelial progenitor cells was used in the investigation of angiogenesis, i.e. MVD, in PC tissue. CD34 is a glycoside transmembrane protein which is expressed in endothelial cells [23]. This study proved that absolute values of CD34 positive microvessel structures per surface area were significantly greater in PC in comparison to BPH, hence the MVD index was significantly higher in PC. Significantly increased MVD index in PC was noticed by other authors as well, who, by using univariate analysis, showed that high level of MVD in PC

was correlated with poor disease outcome [19,24].

In the 1970s Folkman introduced the concept of angiogenesis, which received its full affirmation thereafter due to the evidence that tumors support the proliferation of endothelial cells, but the process is two-way since endothelial cells also support the growth of tumor through paracrine mechanisms [25]. The tumor grows until the demand for oxygen surpasses the oxygen depot. The resulting hypoxia induces tumor cells to form a series of proangiogenetic factors which function as highly specific factors of survival for endothelial cells through increased transcription which is regulated by hypoxia-HIF1a and increased stabilization of mRNA. Among these factors vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are the most significant mediators in the process of tumor angiogenesis [26].

Our study proved that the mean age patient was significantly higher in patients with high MVD index. In addition, patients with high MVD index had significantly greater Gleason score in comparison to those with low MVD index in tumor tissue. Reports on the correlation between MVD and Gleason score are rare and contradictory. Erbersdobler et al. proved significant correlation between MVD and the stage of cancer and Gleason score [27], but rare studies did not confirm the correlation between these two parameters [28].

By using Spearman correlation coefficient, significantly high correlation (r=0.840) between the proliferation index and MVD index was proved in the present study. This shows high positive interdependency between proliferation and neovascularization in prostatic carcinogenesis. As proved, advanced age corresponded to high proliferation and MVD indices, which shows that the time factor had decisive influence on the intensity of effect of these processes during prostatic carcinogenesis.

Gleason score is an index of tumor differentiation and is considered a predictor of stage and prognosis of PC. It is known that Gleason score is also correlated with the degree of disease extension [29,30]. Higher Gleason score corresponds to worse stage of disease. The average Gleason score of 7.7 in our patients showed that PC in the examined samples was mostly in progressive stage. Significant increase of the average Gleason score at high in comparison to low proliferation and MVD indices was confirmed by high positive correlation coefficient (r=0.831) of absolute values of both examined antigens (Ki67 and CD34). By examining over 1000 patients Narain et al. found that bioptic Gleason score correlated with the stage of disease and survival. They showed that when Gleason score was < 7, cancer was localized in 59% of the cases; if Gleason score was 7, cancer was localized in 44% of the cases; when Gleason score was > 7, cancer was localized in only 26% of the cases [30].

A great deal of studies show that the incidence of PC has significantly increased lately, which may be related with the widely used PSA as diagnostic test. The determination of serum PSA level of male population was introduced in medical practice in the 1980s [31]. The introduction of this test allowed the diagnosis of PC at early stages, regardless of the patient age [32,33].

PSA is a glycoprotein secreted by epithelial prostatic cells. The increase of PSA level in circulation is a result of the damage of basal membranes of prostatic glands and increased vascularization of the prostatic tissue. By analysing PSA level in our patients with PC and BPH, significantly higher PSA values were measured in those with PC. Correlation analysis showed moderately positive correlation (r=0.565) between PSA and the volume of prostate. At the same time, it is the only significant interdependence of these variables in relation to all the compared variables. The absence of significant correlation between PSA and other considered parameters, primarily Gleason score, indicates the diagnostic limitations of this parameter. In support of this are findings that increased serum PSA values are a biochemical marker not only for prostate cancer but also for traumas, prostatitis and BPH. Also, increased serum PSA levels were detected in the urine retention, after ejaculation, after instrumental manipulations in the urinary tract and after exaggerated physical activity and stress [33]. PSA values were noticed to vary depending on the androgen level, age, race and prostate volume [34,35].

Widely applied in diagnostic practice, PSA is a relevant marker for the presence of cancer in comparison to BPH. However, our analysis showed significant correlation only between serum levels of PSA and prostate volume. Somewhat better correlation between this marker and MVD in PC may be implied, which should be tested on greater number of samples, but this is not the subject of this study. Further research is necessary which would determine its diagnostic sensitivity and specificity of PSA.

Finally, this study showed that PC is followed by significantly higher values of Ki67 and CD34

antigen expression in comparison to BPH. The values of proliferation and MVD indices in the tissue samples obtained by core biopsy of prostate can quite accurately show the degree of cancer progression expressed through highly correlated Gleason score. In this way it is possible to identify patients at high risk for PC progression, which is of great importance in the treatment of patients.

Conflict of interests

The authors declare no confict of interests.

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