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Analysis of Serum Markers with Regard to Treatment Procedures in Advanced Stage Prostate Cancer Patients

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Background: Biomarkers predicting the efficacy of treatment for locally limited prostate cancer are greatly needed. This knowledge could improve the classification of patients for different methods of treatment and enable better recognition of groups with higher risk of biological recurrence. We prospectively assessed serial blood levels of apoptotic biomarkers and correlated them with response to treatment and clinical factors.

Material/Methods: Blood was collected from 25 patients with prostate cancer before and after surgery, 16 healthy volunteers with benign prostatic hyperplasia (BPH), and 14 patients with metastasized disease. Immunoenzymatic methods were used to determine circulating apoptotic and inflammatory mediators, including tumor necrosis factor α (TNF- α), type I receptor (TNFRI), and type II receptor (TNFRII); FAS ligand (FasL); TNF-related apoptosis-inducing ligand (TRAIL); caspase 8 (Cas8); caspase 9 (Cas9); DNA methylation (metDNA); P-selectin; and high-sensitivity C-reactive protein. The total circulating fragments of cell-free DNA (cfDNA) were measured directly in serum.

Results: Peripheral serum prostate-specific antigen increased rapidly together with cfDNA. A negative correlation was noted between tumor volume and TNFRI and TNFRII. Postsurgery P-selectin level was decreased, and metDNA and TNFRII levels were increased. Three comparisons were made between patient groups: surgical vs. BPH; surgical vs. palliative; and palliative vs. BPH. TNFRI, TNFRII, metDNA, P-selectin, Cas8, and FasL were shown to have significant roles.

Conclusions: The study indicated significant roles for cfDNA, both TNF receptors, metDNA, and P-selectin as serum biomarkers in patients with prostate cancer.

MeSH Keywords: **Apoptosis Inducing Factor • Caspase 8 • Caspase 9 • Prostate-Specific Antigen • Prostatic Neoplasms**

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Background

Prostate carcinoma (PCa) is the main cause of mortality among men and the second most common cause of cancer-related mortality [1]. The treatment strategy for PCa depends on the clinical stage of disease, peripheral serum prostate-specific antigen (PSA), Gleason score, and the patient's age. PSA is an organ-specific marker and can vary with several diseases of the prostate gland other than cancer, such as benign prostatic hyperplasia (BPH) and prostatitis. Consequently, it is difficult to differentiate benign and malignant pathology based on increases in PSA. Furthermore, an estimated 25% of men with PCa do not display elevated levels of serum PSA [2,3]. Researchers have used various PSA figures to improve its clinical utility (age-adjusted PSA range, PSA-velocity, PSA-density, doubling of PSA) [4,5]. PSA testing remains a controversial tool for the early detection of PCa [2,6]. Currently no single serum or urine biomarker is sufficient for highly sensitive and specific detection of PCa and for the differentiation between indolent and aggressive PCa.

Numerous commercially available biomarker tests that use a liquid biopsy for the detection of PCa provide results that qualify patients for prostate biopsy, but PSA cutoff values differ between studies [7]. Presently only 5 of these tests are commercially used (Oncotype Dx Prostate, Prolaris, Decipher, Decipher PORTOS, ProMark) [7]. Therefore, more studies on proper individual use are needed to improve clinical treatment at different stages of disease. Prostate adenocarcinomas are heterogeneous. Based on the architectural pattern associated with the Gleason score, stromogenic, cribriform, and intraductal carcinoma may have more adverse prognostic implications than other grade 4 patterns [8,9]. During metastasis from the primary tumor site, circulating tumor cells (CTCs) enter the circulatory system [10,11] and can serve as biomarkers for liquid biopsy. In the bloodstream CTCs manifest by at least 2 mechanisms: (1) epithelial-mesenchymal transition, in which tumor cells lose their epithelial phenotype and acquire mesenchymal traits [12], and (2) elimination of cells by external forces, such as surgery [12]. CTCs are found as apoptotic populations and as specific subpopulations, depending on the stage of the disease. Analysis via blood (i.e., liquid biopsy) enables assessment of tumor transformation and the biology of the tumor [13]. In the future such analyses could reduce the number of unnecessary invasive diagnostic tests (e.g., prostate biopsy) in patients with false-positive PSA, especially in the subgroup of individuals with "gray zone" PSA values. These methods could complement the processes of staging the cancer, with the biomarkers offering valuable information about an individual patient's disease. In clinical practice, additional tests are necessary and are routinely used to complement imaging tests in choosing the optimal treatment option for patients. Radical treatment methods include surgery, radiotherapy (RT), or a combination of both, sometimes in further combination

with hormonotherapy. Radiotherapy, chemotherapy, and hormonotherapy are also used in palliative treatment. Surgery is a valuable choice of treatment for removing tumors in younger patients with PCa in early clinical stages. RT destroys tumor tissue by killing the tumor cells, and it possibly modulates the patient's immune response.

Here we describe the situation in which cells actively continue apoptosis after surgery and radiotherapy. This prospective study was intended to assess this modulation and to find any correlation between clinical features and mediators of apoptosis, such as blood/serum-based indicators. In the future, such indicators could provide information about response and resistance factors in therapy.

Material and Methods

Patients characteristics

Thirty-nine patients with PCa and 16 healthy volunteers with benign prostate hyperplasia (BPH) were recruited by the Urology Department and Radiotherapy Department at the Multidisciplinary Hospital in Gorzow (Poland) and enrolled in this study. Participants were tested with pretreatment biopsies. All patients were clinically diagnosed with PCa confirmed by histopathological examination and categorized according to clinical stage. Among the patients, 25 had prostatectomy procedures (surgical group) and 14 underwent palliative radiotherapy for bone metastases (palliative group). No participants had any other type of tumor. Surgical patients had not undergone chemo- or endocrinological therapy before the surgery. Palliative patients underwent endocrinological therapy. For each patient the following data were collected: clinical stage, Gleason score, serum PSA level, and transrectal ultrasonography-based prostate volume. Peripheral serum was obtained before and after surgery and before radiotherapy. Written informed consent was obtained from all patients and healthy volunteers (BPH group). The study was approved by the ethical committee of the Medical Chamber in Zielona Góra No 18/129/2019. Clinical characteristics of the patients are presented in Table 1.

Radiotherapy treatment

The radiation procedure was performed using a 6-MV photon beam. Palliative patients received 3-dimensional conformal radiotherapy with a conventional hypofractionalization of 4–8 Gy for a total dose of 6–20 Gy.

Blood collection

Blood samples were taken from the median cubital vein between 7: 00 and 8: 00 AM using S-Monovette tubes (Sarstedt,

Table 1. Clinical characteristics of patients.

	PCa (surgical)	PCa (palliative)	Control – BPH
Patients: Number of patients (%)	25 (64.1)	14 (35.9)	16 (100)
Feature:	Average (min–max)		
Prostate volume mL	47.12 (30–80)	45.21 (20–84)	59.88 (35–90)
Age years	66.28 (53–82)	69.29 (55–88)	69.44 (51–82)
PSA ng/mL	12.62 (6–67)	103.61 (4.7–616)	3.39 (0.2–11)
	Number of patients (%)		
Gleason score:			
1–6	12 (48)	3 (21.43)	
7	10 (40)	2 (14.29)	
8–10	3 (12)	9 (64.28)	
Pathological stage:			
T1	6 (24)	0 (0)	
T2a	5 (20)	0 (0)	
T2b	8 (32)	0 (0)	
T2c	4 (16)	8 (57.14)	
T3a	2 (8)	2 (14.29)	
T3b	0 (0)	1 (7.14)	
T4	0 (0)	3 (21.43)	
Groups of medications use:			
Statin	10 (40)	1 (7.1)	2 (12.5)
Antidiabetic	5 (20)	2 (14.29)	0 (0)
Antihypertensive	15 (60)	4 (28.57)	2 (14.29)
Cardiological	12 (48)	2 (14.29)	0 (0)
ADT	0 (0)	14 (100)	0 (0)

PCa – prostate cancer; BPH – benign prostatic hyperplasia; PSA – prostate-specific antigen; ADT – androgen deprivation therapy;

Austria). Within 60 min, they were centrifuged at 1000 *g* and 4°C for 20 min. Aliquots of serum were stored at -80°C until further analysis.

Apoptotic and inflammatory mediators

Serum tumor necrosis factor α (TNF α) and high-sensitivity C-reactive protein (hsCRP) were determined by R&D Systems kit (USA) and DRG International (USA), and their detection limits were 0.038 pg/mL and 0.001 mg/L, respectively. Tumor necrosis factor type I receptors (TNFRI) and type II receptors (TNFRII) were evaluated by Abcam kits (UK), and their detection limits were 1 pg/mL and 5 pg/mL, respectively. FAS ligand (FasL) and TNF-related apoptosis-inducing ligand (TRIAL) as well as caspase 8 (Cas8) and caspase 9 (Cas9) were evaluated by Thermo Fisher kits (USA). The detection limits for FasL, TRIAL, Cas8, and Cas9 were 0.07 ng/mL, 5 pg/mL, 0.1 ng/mL, and 0.4 ng/mL, respectively. DNA methylation (metDNA) was measured by a Cayman kit (USA) with a detection limit of 1.17 ng/mL. P-selectin was determined by DRG Diagnostics (USA) with a limit detection of 0.6 ng/mL. All samples were analyzed in duplicate or triplicate in a single assay to avoid interassay variability. The average intra-assay coefficient of

variation for the kits was <5–8%. The total circulating fragments of cell-free DNA (cfDNA) were measured directly in serum using a Quant-iT™ DNA high-sensitivity assay kit and a Qubit fluorometer (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The samples were analyzed in duplicate, and the mean of the 2 measurements was used as the final value. The intra-assay CV for the Quant-iT™ DNA high-sensitivity assay was <2%.

Statistical analysis

Since a change of concentrations before and after surgical procedure of the analyzed biomarkers over time was assessed, multilevel (hierarchical) modeling was applied [14]. Because random variation in results was expected from the current longitudinal models, additional random effects were structured. In turn, in the intergroup comparisons, due to the non-normal distribution of morphological factors, the nonparametric Wilcoxon's test for independent samples was carried out. The computation was performed in the R statistical platform [15]. Similar interpretation of the results obtained by the implemented statistical models was also provided in our previous study [16].

Table 2. Comparison between groups: surgical vs. benign prostatic hyperplasia (BPH); surgical vs. palliative; palliative vs. BPH.

Comparison	Mean±SD	Mean±SD	Median	Median	p-Value
Surgical vs. BPH	Surgical	BPH	Surgical	BPH	
PSA ng/ml	12.6±13.6	3.4±2.7	8.0	3	p=0.000
hsCRP mg/l	6.7±3.8	5.8±4.9	6.5	3.4	p=0.005
TNFR1 pg/ml	57±16.1	86.3±114.8	51.3	55.3	p=0.02
TNFR2 pg/ml	1128±150	1368±240	1157	1337	p=0.00
Cas8 ng/ml	0.111±0.004	0.114±0.005	0.109	0.112	p=0.02
Surgical vs. palliative	Surgical	Palliative	Surgical	Palliative	
PSA ng/ml	12.6±13.6	104±160	8	65	p=0.000
hsCRP mg/l	6.7±3.8	9.1±4.3	6.5	11.3	p=0.05
TNFR2 pg/ml	1128±150	1507±248	1157	1485	p=0.00
metDNA ng/ml	69.1±5.8	67.7±6.5	69.2	66.5	p=0.06
P-selectin ng/ml	14.2±4	192±92	13	238	p=0.00
Palliative vs. BPH	Palliative	BPH	Palliative	BPH	
hsCRP mg/l	9.1±4.3	5.8±4.9	11.3	3.4	p=0.002
P-selectin ng/ml	192±92	14.6±4.8	238	14.2	p=0.000
metDNA ng/ml	67.7±6.5	72.1±5.2	66.5	73.5	p=0.05

SD – the standard deviation; BPH – benign prostatic hyperplasia; PSA – prostate-specific antigen; hsCRP – high sensitivity C-reactive protein; TNFR1 – type I receptors, tumour necrosis factor; TNFR2 – type II receptors, tumour necrosis factor; Cas8 – caspase 8; metDNA – DNA methylation.

Results

Surgical group

The modifications as morphological changes in apoptotic cell death were analyzed before and after surgery and were compared between advanced and BPH groups. The results showed that hsCRP and P-selectin decreased after surgery: 0.74 mg/L ($P=0.00$) and 0.90 ng/mL ($P=0.01$), respectively. TNFR2 and metDNA increased significantly after surgery by 34 pg/mL ($P=0.04$) and 1.6 ng/mL ($P=0.02$), respectively. The effect of surgery on TNFR1, FasL, TNFR2, Cas8, Cas9, and cfDNA was insignificant. The inclusion of clinical factors in the surgical group resulted in the following changes.

Tumor

An increase in tumor volume by 1 cm³ was associated with decreases in TNFR1 and TNFR2 by 0.58 pg/mL ($P=0.04$) and 2.28 pg/mL ($P=0.01$), respectively. Each increase of PSA by 1 ng/mL was associated with an increase of cfDNA by 4.25 ng/mL ($P=0.03$).

Age

Cas9 decreased after surgery by 0.35 ng/mL ($P=0.0123$) for each year of patient age (therefore, in the older population this decrease was more rapid than in younger patients). In the oldest subjects, compared with younger, the cfDNA and TNFR2 increased by 7.82 ng/mL ($P=0.0372$) and 12.6 pg/mL ($P=0.0055$) per year, respectively.

Medications

Cardiological medications caused an increase in FasL receptor of 0.01 ng/mL ($P=0.01$) and a decrease in P-selectin by 4 ng/mL ($P=0.0013$). Antidiabetic medications caused a decrease in metDNA by 7.24 ng/mL ($P=0.0047$) and increased TNFR1 by 12.9 pg/mL ($P=0.0368$). Medication-free treatment resulted in increases in Cas8 and metDNA by 0.007 ng/mL ($P=0.01$) and 4.58 ng/mL ($P=0.00$). Other factors, including hsCRP, TNF- α , TNFR1, and Cas9, were not affected by the use of medications.

Intergroup results

The statistically significant differences between groups (surgical vs. BPH group, surgical vs. palliative group, palliative vs. BPH group) are presented in Table 2. Additionally, an increase

in FasL by 0.67 ng/mL ($P=0.008$) was found to correlate with an increase in PSA in the palliative group. The other factors were statistically insignificant.

Discussion

The constant process of apoptosis is an important biological phenomenon. When it malfunctions, it can lead to uncontrolled cell proliferation resulting in cancer [17,18]. Three main types of changes in apoptosis are observed: (1) activation of caspases, (2) DNA and protein breakdown, and (3) membrane changes and recognition by phagocytic cells [19]. Patients with PCa undergo different treatment modalities that possibly influence cellular immunity in different ways. Radical prostatectomy is a very effective method because the whole organ with the tumor is removed. Surgery and RT result in a type of immunosurveillance for cancer cells. The absence of prostate-associated lymphocytes is one of the possible mechanisms by which this immunosurveillance is avoided [20]. The mechanism of cellular cytotoxicity depends on the particular TNF family. They lead to an activation of the effector caspases pathways in the final stage of apoptosis. This process ends when contact with the target cell is lost, which is synonymous with cell death [21]. Caspases are a family of proteases that play a central role in the processes of apoptosis (e.g., caspase-2, -3, -6, -7, -8, -9, and -10). They are defined as either tumor suppressors or oncogenes due to their proteolytic activity. Caspases are the initiators and the executioners of apoptosis. The initiation pathways form both the intrinsic (mitochondrial) and extrinsic (the death receptor) pathways of apoptosis [22]. The intrinsic pathway begins within a cell. This process is triggered by factors such as genetic damage, hypoxia, severe oxidative stress, and extremely high concentrations of cytosolic Ca^{2+} [23]. Cas9 is part of a large quaternary protein structure known as apoptosome (made up of cytochrome c, Apaf-1, and Cas9), which activates caspase 3 via cytoplasmic release of cytochrome c [24]. The extrinsic death receptor pathway binds death ligands to death receptors. The activation of TNF- α results from the binding of 2 receptors (TNFRI and TNFRII) on the cell membrane. The best-known death receptors are TNFRI and a related protein Fas (CD95) whose ligands are TNF and Fas ligand (FasL), respectively [25]. After the binding of the death ligand to the death receptor and then to an adaptor protein, a death-inducing signaling complex is created. With the presence of Cas8, a cascade is initiated, leading to apoptosis [22]. Another receptor belonging to the TNF family, TRIAL, has the ability to induce apoptosis in cancer cells, while being nontoxic for physiological cells [26].

The effects of surgery and medication in apoptosis

Within our study sample, an increased volume of the prostate gland correlated with decreases in TNFRI and TNFRII, and

an increase in PSA was correlated with an increase in cfDNA. After the removal of the prostate gland, an increase in TNFRII and metDNA and a decrease in hsCRP and P-selectin were observed. With each year of age of the patients, TNFRII and cfDNA increased and Cas9 decreased. The medications taken by patients had the following effects on the status of apoptosis mediators. Antidiabetic drugs caused a decrease in metDNA and an increase in TNFRI, while cardiological drugs led to a decrease of P-selectin and an increase of FasL. No use of medication resulted in an increase in metDNA and Cas8.

Intergroup results

This study investigated the correlation of the inflammation biomarker hsCRP with the existing tumor and its stage. An increase in both TNFRI and TNFRII was observed in the BPH group in comparison with the early-stage and the palliative group. The BPH group also showed an increase of immunoreactions to Cas8 compared with the early-stage group. FasL was correlated with an increased PSA concentration in the palliative group. A study by Rodríguez-Berriguete et al. [27] reached similar conclusions. They observed that tumor expression of TNF- α and TNFRI was significantly correlated with a poor prognosis and with clinicopathological features. BPH is characterized by a fast cell transformation; therefore, within this group, immunoreactions to both TNF- α receptors increased compared with normal prostate glands [28]. In another study, Chadha et al. [29] found that TNFRI and TNF- α are supportive factors alongside PSA in distinguishing men with and without PCa.

cfDNA

A large number of studies have indicated that PCa patients present with higher cfDNA concentrations than controls [30–36]. However, other studies have reported nonspecific increases in cfDNA in cancer patients [37,38]. According to the results of our study, surgical patients did not show any significant differences in the concentration of cfDNA, which was similar to findings from van der Pol and Mouliere [39] in a study on early-stage brain tumors. However, higher PSA was correlated with higher cfDNA, which was also found in previous studies [40,41]. Importantly, the current study confirmed that the concentration of cfDNA increases with age. Circulating cfDNA is the fraction of DNA released by tumor cells to the bloodstream, and it is affected by the size of the tumor and its stage and cell type [42,43]. The time from the initiation of cell death to the final cellular fragmentation depends on injurious agents and the apoptotic pathways [44]. In addition, the final concentration of cfDNA in the bloodstream depends on the rate of degradation and clearance [45,46].

MetDNA

Some epigenetic changes are described as conversions in the structure of DNA, with the main change being DNA methylation (hypermethylation and hypomethylation). These disturbances can lead to malignant transformations. In comparisons of the early stages of neoplastic transformation with PCa metastasis, hypomethylation has been observed to prevail in the latter [47]. The methylation pattern and its changes can directly contribute to tissue transformation into cancer [48]. As a consequence of this process, either hypermethylation or hypomethylation takes place [49–51]. The measurements of these DNA alterations (methylation or mutations) in blood have been used in the diagnosis of PCa [36,52]. Our findings indicate statistically significant differences between metDNA levels in patients from different groups: early-stage vs. surgery, early-stage vs. palliative, and palliative vs. BPH.

Medication

The study additionally investigated how the use of medications affected metDNA levels. It was observed that the use of antidiabetic drugs resulted in a decrease in the metDNA level compared with no use of medications.

P-selectin

P-selectin (GMP-140, CD62P) is an adhesion molecule localized in platelet granules and in Weibel-Palade bodies of vascular endothelium. P-selectin is induced by inflammatory mediators, and

its main task is to bind circulating leukocytes to vascular endothelium during the inflammatory response to activate the processes connected with binding leukocytes to platelets [53]. An increase in P-selectin expression on the surface platelets could be a biomarker in some types of cancer, including breast, lung, kidney, and colon cancers [54,55]. Our current study, as in a previous one [56], showed that the P-selectin level decreased after surgery and increased in the palliative group. Cardiological medications reduced the level of this adhesion molecule.

Conclusions

Various medical studies suggest that defects within apoptotic pathways play a crucial role in carcinogenesis. In this small sample study, we observed that cfDNA, both TNF receptors, metDNA, and P-selectin played a significant role in this process. A larger study could elucidate the exact role of these factors. Monitoring them together with PSA level could be a useful further tool in testing the biochemical recurrence or progression of the disease.

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Conflict of interests

None.

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