Osteopontin and Angiogenic Factors as New Biomarkers of Prostate Cancer

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Purpose: The novel biomarkers that would identify patients at risk for relapse and metastatic spread are needed. The aim of this study was the evaluation of serum levels of osteopontin (OPN) and tumor endogenous angiogenic factors such as vascular–endothelial growth factor (VEGF), vascular-endothelial growth factor receptor 2 (VEGF R2), endostatin, angiostatin and thrombospondin 1, in prostate cancer (PC) patients.

Material and Methods: Blood concentrations of the analyzed parameters were determined in 40 prostate cancer patients eligible for radiotherapy as well as in a control group consisting of 25 volunteers. Commercial ELISA kits were used for the analysis.

Results: Significantly higher levels of OPN (101.49 ng/mL vs. 59.88 ng/mL; \( P = .04 \)), endostatin (252.60 ng/mL vs. 223.55 ng/mL; \( P = .043 \)), angiostatin (47 ng/mL vs. 13 ng/mL; \( P = .047 \)), VEGF (262.1 pg/mL vs. 138.0 pg/mL; \( P = .056 \)) and VEGF R2 (11188.81 pg/mL vs. 9377.50 pg/mL; \( P = .047 \)) were detected in PC patients compared with the control group. In PC patients we showed a positive correlation between OPN level and TNM clinical stage (\( R = 0.36; P = .02 \)) and negative correlation between OPN level and hemoglobin concentration (\( R = -0.33; P = .04 \)).

Conclusion: The study showed higher levels of the angiogenic factors in PC patients compared with the control group and identified OPN as an indicator of the PC clinical stage as well as a decreased hemoglobin level.

Keywords: osteopontin; angiogenesis factors; prostate cancer.

INTRODUCTION

Prostate cancer is the second most common malignancy in men. Despite an overall good prognosis for prostate cancer patients it is estimated that among radically treated patients as many as 25% will experience recurrence of the disease during the first 3 years after treatment. There is now an ongoing intense search for factors responsible for the increased risk of relapse in individual patients, including osteopontin (OPN) or angiogenic factors as potential cancer aggressiveness predictors. In the cancer progression a process of angiogenesis plays an important role being critical in the phenomena of an invasion and metastasising. PC is recognizable by a low vessel density and a slow cell proliferation. Production of numerous anti-angiogenic factors such as: angiostatin, endostatin, prostate specific antigen (PSA), thrombospondin 1, interleukin 10 (IL-10), interferons and retinoids may be responsible for such a PC characteristic.4 Presently these factors are a subject of both pre-clinical and clinical studies.

The prominent PC propensity to bone metastasis indicates that bone metabolism markers may be potentially utilized as prognostic factors. There is a special interest in osteopontin (OPN) which is a representative of a scavenger protein family. In healthy individuals it is involved in such processes as: an early immune response, inhibition of a cellular apoptosis as well as a stress or pressure induced bone modelling.5 OPN increased concentration has been observed in numerous pathological conditions. It is responsible for an initiation and progression of atherosclerotic lesions as it facilitates a deposition of calcium in vascular walls. Presumably, the protein plays a role in a recurrent coronary stenosis. It also affects the growth and proliferation of tumour cells, and metastasising easier by promoting binding tumour cells with integrins. On the other hand suppressed expression of OPN significantly inhibited cell invasiveness and anchorage-independent growth.6,7 OPN involvement in the formation of new vessels is still under investigation.8,9 OPN can stimulate angiogenesis because it promotes the endothelial cell survival due to interaction with \( \alpha \beta 3 \) integrin.10 Other investigators found that OPN produced by nontumor cells plays a host protective role in prostate tumor development.11 There are also pre-clinical evidence suggesting that OPN is involved in inducing chemo-resistance.12

The aim of this study was the analysis of serum levels of OPN and tumour angiogenic factors including: angiostatin, endostatin, thrombospondin, vascular-endothelial growth factor (VEGF) and its receptor 2 (VEGF R2).
PATIENTS AND METHODS

Study population
The study included 40 prostate cancer patients eligible for radical radiotherapy. Inclusion criteria were adult male with pathologically confirmed prostate cancer and written informed consent. Patients with distant metastases, previous oncological treatment due to another cancer or previous radiotherapy to the pelvis area were excluded from the study. Before the treatment, all patients underwent blood tests, prostate biopsy, magnetic resonance imaging (MRI) of the pelvis as well as per rectum examination in order to determine classic prognostic factors including a maximum PSA level, clinical stage according to the TNM classification and a tumour grade according to the Gleason scoring system. Additionally, in each patient the prostate volume was determined based on computed tomography (CT) performed for radiotherapy planning.

The control group was recruited from healthy men who responded to an invitation letter for prophylactic tests in our Oncology Center. After excluding prostate cancer (based on PSA level and per rectum examination) a randomly chosen 25 men were proposed to participate in our study as a control group. Patients with previous oncological treatment due to another cancer were excluded from a study. The study protocol was approved by the Bioethical Committee of Ludwik Rydygier Collegium Medicum in Bydgoszcz of Nicolaus Copernicus University in Torun. All the individuals participating in the project were given an informed written consent. The European Union “Program of the Development of Collegium Medicum of Nicolaus Copernicus University” and a grant for young researchers (MN-5/WL/SD) were the sources of the study funding.

The concentration of hemoglobin was tested on the first day of radiotherapy. The peripheral blood was taken between 7.30 and 8.30 a.m. from the basilic vein of the forearm to sterile Vacutainers (Becton Dickinson, Franklin Lakes, New Jersey, USA) with 3.2% citrate solution as well as to clot. Test-tubes were centrifuged for 15 minutes in 4°C at the speed of 1500 g. Prior to the analysis the blood samples were divided into Eppendorf sterile tubes and stored in 80°C. Concentrations of the analysed biomarkers were measured with the enzyme-linked immunosorbent assays. Detailed data of the test are given in Table 1.

Table 1. Types of the tests used in the study.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Material</th>
<th>Name of the test</th>
<th>Company</th>
<th>City, state</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF R2</td>
<td>Serum</td>
<td>Human VEGF Immunoassay R&amp;D Systems</td>
<td>Minneapolis, Minnesota</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>VEGF R2</td>
<td>Serum</td>
<td>Human Soluble VEGF R2 Immunoassay R&amp;D Systems</td>
<td>Minneapolis, Minnesota</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Plasma</td>
<td>Human Osteopontin Immunoassay R&amp;D Systems</td>
<td>Minneapolis, Minnesota</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>Endostatin</td>
<td>Plasma</td>
<td>Human Endostatin Immunoassay Quantikine (DNSTO) R&amp;D Systems</td>
<td>Minneapolis, Minnesota</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>Angiostatin</td>
<td>Plasma</td>
<td>Human Angiostatin ELISA Kit RayBiotech</td>
<td>Notorois, Georgia</td>
<td>USA</td>
<td></td>
</tr>
<tr>
<td>Trombospordin 1</td>
<td>Plasma</td>
<td>Human Trombospordin-1 Immunoassay R&amp;D Systems</td>
<td>Minneapolis, Minnesota</td>
<td>USA</td>
<td></td>
</tr>
</tbody>
</table>

Results
A clinical characteristics of the patients is presented in Table 2. An average age was 67 years (range: 56 – 81) in the tested group and 64 years (range: 51 – 77) in the control group (P = .4). The most significant difference considered OPN measurements. A median level of OPN in the PC patients was 101.49 ng/mL compared with 59.88 ng/mL in the healthy men (P < .001). These results are presented in Figure 1. In the PC group a median level of thrombospondin 1 was lower in the tested group than in the controls, however the difference was not significant (25.88 pg/mL vs. 262.1 pg/mL; P = .615) (Figure 2) and a negative correlation between OPN level and hemoglobin concentration (R = –0.33; P = .04) (Figure 3). Only the median level of thrombospondin 1 was lower in the tested group than in the controls, however the difference was not significant (27111.1 ng/mL vs. 31246.4 ng/mL; P = .047) (Figure 4). A significantly higher median value of VEGF R2 was noted in the patients compared with the control group (11188.81 pg/mL vs. 9377.50 pg/mL; P = .047). Also the median level of VEGF was about twice as high in the tested group than in the controls, however the difference was not significant (27111.1 ng/mL vs. 31246.4 ng/mL; P = .615) (Figure 1). A significantly higher median value of VEGF R2 was noted in the patients compared with the control group (252.60 ng/mL vs. 223.55 ng/mL; P = .043). Similarly, the median level of angiostatin was more than three times higher (47 ng/mL vs. 13 ng/mL; P = .047) in the treatment group (Figure 5).

We did not find any correlation between the baseline levels of the angiogenic factors and such clinical factors as: Gleason score, PSA level, patient’s age and prostate...
There was also no correlation between the angiogenic factors and osteopontin.

**DISCUSSION**

The aim of this study was to evaluate the levels of endogenous factors regulating tumour angiogenesis in prostate cancer patients. Due to contradictory reports on the matter we decided to take into consideration a wide range of tested parameters in order to evaluate their relationship with clinical prognostic factors and to obtain a starting point for further tests in a larger group of patients.

Biomarkers that distinguish highly aggressive from moderately aggressive tumours and complement PSA measurements are still required. Our study proved almost twice as high level of osteopontin in the prostate cancer patients compared to the healthy men (Me=101.49 ng/mL vs. 59.88 ng/mL; \( P < .001 \)) which is compliant with a number of published reports on an increased osteopontin level in the course of different tumours. The results indicate that OPN is a cancer biomarker and is related to a diseases clinical stage, histologic grade and early tumour progression in multiple cancer types. It is also a predictor of disease-free and overall survival in various malignancies.

It is noteworthy that there were significant discrepancies in OPN levels in individual researchers which may result from a diverse biology of particular types of cancers. On the other hand, Vordermark et al. conducted an experiment in which available ELISA tests for OPN determination generated diverse outcomes in the same blood sample which makes comparing results between researchers using different diagnostic kits practically impossible. The overexpression of OPN in prostate cancer cell lines induced their proliferation, invasion and, most notably, enhanced ability to intravasate blood vessels.

In prostate cancer patients an increased expression of VEGF and OPN are each associated with an increased frequency of biochemical failure and they also correlate with each other.

In our study there was no correlation between the levels of VEGF and OPN, probably due to the small amount of patients. OPN levels are also higher in patients with bone metastases and it was suggested that OPN could be a predictor of treatment response in metastatic castrate-resistant prostate carcinoma after chemotherapy.

In the presented study we showed a correlation between a baseline osteopontin concentration in PC patients and TNM stage (\( R=0.36; \ P = .02 \)). In the literature there is no data which could be compared to our results. Clinical observations on OPN levels in other types of cancers are close to our results. In the study including head and neck cancer patients Snitcovsky et al. showed higher levels of OPN in higher clinical stages (\( P = .009 \)).

By contrast, Hui et al. did not show correlation between OPN and clinical stage in nasopharyngeal cancer patients. However, the authors observed higher levels of the protein in individuals with distant metastases compared to controls (Me=894 ng/mL vs. 513 ng/mL; \( P = .005 \)). The correlation between OPN levels and a tumour grade was found in bladder cancer patients.

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**Table 2. Clinical characteristics of the tested group.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean (range)</td>
<td>67 (56 - 81)</td>
</tr>
<tr>
<td>Maximum PSA level before radiotherapy (ng/mL), mean (range)</td>
<td>23 (4.4 - 100)</td>
</tr>
<tr>
<td>Histological grade - Gleason score</td>
<td></td>
</tr>
<tr>
<td>- median (range)</td>
<td>6 (3 - 9)</td>
</tr>
<tr>
<td>- divided by groups, n (%)</td>
<td></td>
</tr>
<tr>
<td>2-6</td>
<td>31 (77.5)</td>
</tr>
<tr>
<td>7</td>
<td>4 (10)</td>
</tr>
<tr>
<td>8-10</td>
<td>5 (12.5)</td>
</tr>
<tr>
<td>Clinical stage according to TNM classification, n (%)</td>
<td></td>
</tr>
<tr>
<td>T2aN0M0</td>
<td>7 (17.5)</td>
</tr>
<tr>
<td>T2bN0M0</td>
<td>10 (25)</td>
</tr>
<tr>
<td>T2cN0M0</td>
<td>6 (15)</td>
</tr>
<tr>
<td>T3aN0M0</td>
<td>14 (35)</td>
</tr>
<tr>
<td>T3bN0M0</td>
<td>3 (7.5)</td>
</tr>
<tr>
<td>Prostate volume (cm3), mean (range)</td>
<td>69 (26.9 – 143.3)</td>
</tr>
<tr>
<td>Haemoglobin level (ng/dL), mean (range)</td>
<td>13.8 (11.7 – 16.8)</td>
</tr>
</tbody>
</table>

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**Figure 1.** Osteopontin and trombospondin-1 levels in prostate cancer patients and control group patients.
An increase of osteopontin expression could possibly be connected with an oncogenic transformation of the prostate epithelial cells.\(^9\) In a recently published experimental trial it has been proven that an overexpression of OPN isoform b and c may lead to the prostate cancer cells resistance to the docetaxel based chemotherapy.\(^{16}\) In the presented study the increased concentration of OPN was correlated with lower hemoglobin levels (R = -0.33; \(P = .04\)). In PC patients there is no available research to which our results might be related. Consistently to our findings Snitcovsky et al. found a negative correlation between OPN and hemoglobin concentrations (R = -0.39; \(P = .04\)) in head and neck cancer patients.\(^{13}\) The authors suggested that OPN may be an indicator of tumor hypoxia. Le et al. confirmed this conclusion proving a negative correlation between OPN and tumor oxygen partial pressure (pO\(_2\)) (R = -0.42; \(P = .003\)) determined with the Eppendorf microelectrode in head and neck cancer patients.\(^{17}\) Such patients are characterized by the mutated expression of VHL gene which is involved in a cell reaction to hypoxia. Two DAHANCA (Danish Head and Neck Cancer Group) studies, both conducted in head and neck cancer patients, offered more proof to support the thesis that osteopontin may be a marker of tumour hypoxia. In the study by Nordsmak et al. OPN levels correlated negatively with tumour pO\(_2\) measured with an electrode before treatment.\(^{18}\) In the randomized DAHANCA 5 study by Overgaard et al. OPN expression correlated negatively with the prognosis of irradiated patients. The prognosis improved after nimorazole treatment, an agent which sensitizes hypoxic cells to ionizing radiation. The researchers concluded that OPN level may help to select potential beneficiaries of the nimorazole treatment.\(^{19}\) Moreover, an experimental study showed that OPN expression increased under the influence of hypoxia in a culture of human glioblastoma multiforme cells.\(^{20}\) In 34 head and neck carcinoma patients the OPN concentration at normal hemoglobin values was almost 3 times higher compared with decreased hemoglobin levels (\(P = .02\)).\(^{21}\) An experimental study showed that hypoxia does not influence the OPN expression. Four nasopharyngeal cancer cell lines were incubated in hypoxic conditions and with Western blot technologies the intracellular HIF-1 \(\alpha\) protein concentration as well as OPN level were evaluated. A significant increase of HIF-1 \(\alpha\) was noted, whereas the OPN level remained the same. It was also shown that reoxygenation of previously hypoxic cancer cells did not influence the OPN concentration.\(^{14}\) In a prostate cancer cell line study Riemann et al. evaluated an influence of hypoxia and extracellular acidosis on genes expression. They demonstrated that the expression of mRNA for OPN had decreased in hypoxic conditions and increased in an acid environment.\(^{22}\) The above results prove that the influence of hypoxia on the OPN level is not unequivocally confirmed and more studies are necessary in this field.

Among the five angiogenesis regulators we tested a

Figure 2. Correlation between clinical stage according to TNM classification and osteopontin level (ng/ml). (TNM groups correspond to: 1 – T2aN0M0, 2 – T2bN0M0, 3 – T2cN0M0, 4 – T3aN0M0, 5 – T3bN0M0).

Figure 3. Correlation between haemoglobin level (mg/dl) and osteopontin level (ng/ml) in the tested group of patients.

Figure 4. VEGF and VEGF R2 levels in prostate cancer patients and control group patients.
published research showed that endostatin may influence prostate cancer and other tumours. Levels, are characterized by a decreased incidence of tumours, including prostate cancer. It may result from the fact that VEGF R2 has also an affinity to VEGF C and D which both play an important role in lymphangiogenesis while prostate cancer disseminates rather via blood than lymphatic vessels. The immunohistochemistry of prostate cancer shows a 100% VEGF R1 expression, whereas VEGF R2 expression is changeable and its intensity depends on a tumour grade. In our cancer patients group no correlation was observed between VEGF R2 level and the classic prognostic factors. It may result from the fact that VEGF R2 has also an affinity to VEGF C and D which both play an important role in lymphangiogenesis while prostate cancer disseminates rather via blood than lymphatic vessels. In the tested group we observed significantly increased endostatin levels compared with the controls. So far, increased levels of circulating blood endostatin were detected in patients with carcinomas of the breast, kidney, liver, ovarian, prostate, head and neck as well as in non-Hodgkin lymphomas and soft tissue sarcomas. Tests on rats and mice demonstrated that a high endostatin concentration resulted in a regression of a number of tumours, including prostate cancer. The TSP-1 down-regulation correlates with a progression in proliferative diseases. Rofstad et al. consider thrombospondin 1 a positive factor in irradiated patients since it increases the efficiency of radiotherapy by lowering the fraction of tumour hypoxic cells and sensitizing tumour endothelial cells to ionizing radiation.

CONCLUSIONS
On the basis of the obtained results we assume that the process of tumor angiogenesis plays an important role in the prostate cancer pathogenesis. Among the analysed parameters the greatest difference between the PC patients and healthy individuals was shown for osteopontin levels. The protein expression correlated positively with prostate cancer clinical stage and negatively with hemoglobin concentration. OPN should be considered a novel biomarker which may complement PSA measurements and improve a diagnostic and prognostic accuracy.

ACKNOWLEDGEMENT
Funds necessary to perform the study were obtained from a scholarship financed by the European Union as a part of the European Social Fund – the “Program of Development of Collegium Medicum of Nicolaus Copernicus University” as well as a grant for research facilitating the development of young researchers (MN-5/WL/SD) ; granted to TW.
CONFLICT OF INTEREST
The authors report no conflict of interest.

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