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An observational study of plasma vascular endothelial growth factors (VEGF) A and D expression in non-localized prostate cancer

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Abstract

Background—The aim of the study was to measure plasma levels of the vascular endothelial growth factors (VEGF) A and D in serially collected blood specimens from non-localized prostate cancer (PCa) subjects.

Methods—Plasma VEGF A and D levels were measured in two serial specimens 3–6 months apart in two groups of non-localized stage PCa patients. Group 1 was comprised of patients with biochemical relapse after localized PCa treatments and/or patients with clinically metastatic hormone-sensitive stage PCa prior to receiving hormonal therapy. Group 2 included patients failing hormonal therapy for non-localized hormone-sensitive stage PCa. VEGF A and D levels were compared within each cancer group between the two time-points using the Wilcoxon Rank Sum test.

Results—At the first time-point in Group 1 ($n = 46$), median VEGF-A and D levels were measured at 5.2 (pg/ml) (range = 0–97) and 319 (range = 172–780) (pg/ml). For Group 2 ($n = 34$) VEGF-A level was 9.6 pg/ml (range = 0–78) and VEGF-D level was 377 pg/ml (range = 243–989)

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for the first measurement. Median time-period for the serial second specimen was 189 days in Group 1 and 84 days in Group 2. At the second time-point, in Group 1, VEGF-A levels were 0.0 pg/ml ($P = 0.0002$) while VEGF-D increased to 349 pg/ml ($P = 0.002$). For Group 2 patients at the second time-point, median VEGF-A was 0.0 pg/ml ($P = 1.0$) and VEGF-D was measured at 442 pg/ml ($P = 0.008$).

Conclusions—Higher plasma VEGF-D than VEGF-A expression in advanced PCa stages suggests a greater role for VEGF-D dependent lymph angiogenesis in advanced stage PCa, which needs further evaluation.

Keywords

Prostate cancer; Angiogenesis; VEGF; Chemo-hormonal treatments

Introduction

Morbidity and mortality from prostate cancer (PCa) in the United States (U.S.) was estimated at 240,890 new cases and 33,720 deaths in 2011 [1], which fortunately reflects an on-going decreasing annual PCa-specific mortality rate in the previous decade. This healthy trend has coincided with an era of extensive prostate-specific antigen (PSA) screening for cancer diagnosis and the use of novel therapeutic strategies in targeting specific biological pathways implicated in PCa progression. Some of these pathways include mutations in BCL-2, epidermal growth factor receptor (EGFR), clusterin, heat shock protein 27 (HSP27), mitogen activated protein kinase (MAPK), phosphoinositide 3'-kinase/AKT kinase (PI3K/Akt) and vascular endothelial growth factor (VEGF) along with mutations in the androgen receptor [2]. Chemotherapy drugs, such as docetaxel which targets the BCL-2 axis, have already been approved as standard treatment options in the castration-resistant stage. In addition, clinical trials targeting aberrant angiogenesis and the lymph angiogenesis pathways, which result in the development of new blood and lymphatic vessels from pre-existing structures, are on-going. Aberrant angiogenesis and lymph angiogenesis pathways contribute to tumorigenesis and metastasis in a variety of solid tumors [3] and appear to be specifically active in contributing to an “angiogenesis switch” in PCa [4] with the development of new bone and/or lymph node metastasis during tumor progression.

The VEGF family of proteins, including VEGF-A, VEGF-C, and VEGF-D, have been implicated in PCa angiogenesis and lymph angiogenesis pathophysiology. VEGF expression in PCa is increased in tumor cells more than in benign prostatic tissue [5], and in metastatic PCa cells a greater enhancement of VEGF production is observed in cells with high versus cells with low metastatic potential [6]. VEGF peptides are secreted into circulation and, consequently, circulatory VEGF levels have been measured in the peripheral blood of PCa patients at different stages of disease progression and following treatments for evaluating circulatory peptide levels as potential diagnostic, prognostic or predictive markers [7–10]. The treatment effects of androgen deprivation therapy (ADT) in hormone-sensitive PCa and chemo-hormonal therapies in the castration-resistant PCa stage on circulatory VEGF peptide levels is unknown. In this study, two serial levels of plasma VEGF-A and D were measured in a cohort of advanced PCa patients that included patients being followed after the initiation of ADT for hormone-sensitive non-localized stage PCa and those undergoing systemic treatments for the castrate-recurrent stage. Levels were measured 3–6 months apart in order to determine any effects of systemic treatments on circulatory VEGF-A and D levels.

Materials and methods

Patient and plasma processing methods

Research blood specimens for the measurement of VEGF peptides in this study were obtained from an Institutional Review Board approved PCa patient biospecimen databank collected and stored at a tertiary level hospital (University of Rochester, NY). This databank had enrolled PCa patients in all stages of cancer progression between April 2005 and January 2008. Specimen collection was performed on all enrolled subjects after obtaining an informed consent for serial blood and urine specimen collection with access to medical records restricted to authorized research personnel while subjects were receiving cancer stage-specific treatments. Clinical annotation of all enrolled subjects included research subject demographics, pathology, cancer treatments, laboratory and imaging results, PSA measurements and clinical outcomes on follow up.

For the present study, subjects with non-localized stage disease enrolled in the registry were identified. Group 1 included either patients who had completed primary PCa treatments and had experienced biochemical relapse or newly diagnosed subjects with nonlocalized stage PCa. All subjects in that group also had to be ADT-naïve at the time of their first specimen collection. Additionally, subjects in that group with biochemical progression alone, and without clinical progression after primary PCa therapy, had confirmation of a serially rising serum PSA level on two separate occasions measured 4 weeks apart prior to enrollment in the registry. Measurements of plasma VEGF-A and VEGF-D peptides were performed in two research specimens for all Group 1 patients.

The Group 2 cohort comprised subjects identified from the PCa databank who were experiencing disease progression while receiving ADT for the non-localized, metastatic hormone-sensitive PCa stage. The selection criteria for these subjects to have castration-resistant PCa (CRPC) progression in the databank was based on either a confirmed clinical progression during ADT, detected on imaging scans, or a biochemical progression with serially increasing PSA while receiving ADT on two occasions at least 4 weeks apart with the first of the two dates deemed to be the date of progression to castration resistance. Clinical variables collected on patients in this group included all treatment interventions for CRPC stage, including secondary hormonal treatments or systemic chemotherapy. All CRPC patients had two serial research specimen collections after enrollment to the registry, with the first research specimen collection being performed at the time of ADT failure and the second 3–4 months later. These specimens were used for the measurement of plasma VEGF-A and VEGF-D peptides in Group 2. Serum PSA measurements, using enzyme-linked immunosorbent assay (ELISA) for both groups, were performed as part of routine clinical practice and subjects were enrolled in the databank based on their PSA criteria, as mentioned above.

Specimen methods

Blood samples (5 ml) were collected from all patients in tubes containing 7.2 mg of ethylenediaminetetraacetic acid (K2-EDTA) anti-coagulant. All specimens were transported for processing within 45 mins after phlebotomy. An initial centrifugation was performed at 180 g for 10 mins at 20 °C to separate platelet-rich plasma. After removal of the supernatant, the sample was centrifuged again at 3000 g for 10 mins at 20 °C to obtain platelet-poor plasma, which was stored in quantities of 500 µl at –70 °C and was accessed for assays without undergoing freeze–thaw cycles prior to measurements of VEGF proteins. Platelet-poor plasma was used for performing enzyme immunoassays for VEGF-A and VEGF-D on two serial specimens on all patients included in the research cohort. Specimen processing

remained uniform during the course of patient enrollment and serial collection to the repository.

Enzyme immunoassay

Platelet-poor plasma specimens with K2-EDTA anticoagulant were thawed for a quantitative sandwich ELISA to determine VEGF-A and VEGF-D concentrations using commercially available kits from R&D Systems (Minneapolis, MN, USA) following manufacturer's instructions. Briefly, 200 µl of plasma or standards were added to each ELISA plate well and specimens were allowed to incubate for 2 h with horseradish peroxidase (HRP)-conjugate before being washed and prior to adding VEGF conjugates. After incubating with conjugate, wells were rinsed again and a stop solution was added to each well. Using a microplate reader (Syngery HT, Biotek, Winooski, VT), wells were measured at 450 nm with correction at 562 nm, if the coefficient of variation was higher than 10 within a sample. The standard curve had an $r^2 = 0.99$. Corrected optical density (OD) values were used to calculate VEGF concentrations. All samples were evaluated in duplicate and an internal control was included in each plate. All results were reported for analysis in pg/ml for each specimen.

Statistical analysis

Results of PSA, VEGF-A and VEGF-D assays are reported as median levels with inter-quartile ranges. The Wilcoxon signed rank test was used to compare serial levels of measured PSA, VEGF-A and VEGF-D within the two groups. For each specimen, the Wilcoxon rank-sum test was used to compare the values between Group 1 and Group 2 subjects with a significance level (P) set at 0.05 for all comparisons. The analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC).

Results

From the PCa databank, 46 subjects were identified as meeting the eligibility criteria for Group 1, of which 20 subjects had radiographic evidence of metastatic hormone-sensitive PCa, while 26 subjects had biochemical progression alone (serially rising serum PSA levels) following previous treatments for primary PCa. All 20 subjects with clinically metastatic disease initiated ADT after collection of the first research specimen. The second specimen collection was performed 4 months after initiating ADT (Table 1). Subjects in Group 1 with biochemical progression alone ($n = 26$) were followed without initiating ADT treatments during the serial collection of two research specimens. The reasons for not initiating cancer interventions in this population were at the discretion of the treating physician. The most common reason recorded was asymptomatic disease with indolent biochemical relapse in this group. Nineteen of these 26 subjects underwent a radiological diagnostic work-up for their biochemical relapse, which included a bone scan and computed tomography (CT) imaging of their abdomen/pelvis, which was negative for clinical metastasis. At the discretion of the treating physician, no imaging work-up was initiated in the remaining seven of these 26 patients, due to low PSA levels. The mean PSA level of these seven patients during biochemical progression was 0.38 ng/ml (range = 0.03–0.89 ng/ml) at the time of the first specimen collection after enrolment to the registry and 0.45 ng/ml (range = 0.01–1.22 ng/ml) at the time of the second collection (4 months after the initial research specimen). The clinical characteristics of the Group 1 cohort are further elaborated in Table 1.

In Group 2, 34 subjects were identified as having progression to castration resistance while receiving ADT for non-localized stage hormone-sensitive disease. Of these 34 subjects, 24 were started on additional treatments after progression to the castration-resistant stage which

included chemotherapy or additional hormonal agents. The chemo-hormonal therapeutic agents added to ADT in these 24 subjects included: docetaxel (14/24); mitoxantrone (3/24); ketoconazole (2/24); bicalutamide (2/24); cisplatin/etoposide (1/24) and paclitaxel (2/24). The remaining 10 subjects remained on ADT alone. The reasons for not offering additional treatments in these 10 patients after progression on ADT were increased age with multiple co-morbid conditions (9/10) and an indolent biochemical relapse post ADT (1/10). The clinical and demographic features of this patient group are shown in Table 2.

Plasma VEGF-D level was measured in the two research specimens in all 46 subjects in Group 1, while VEGF-A levels were successfully measured in both research specimens in only 43 patients as three patient specimens at both time points were found to have insufficient plasma volume for the VEGF-A assay. In Group 2, all subjects (34/34) had VEGF-D assays performed on the first of the two research specimens, which was collected at the time of ADT failure, along with 29/34 second specimens. VEGF-D could not be assayed in five second specimens due to inadequate plasma volume. Similarly VEGF-A was measured in 30/34 first specimens and 17/34 second plasma specimens in this group based on the availability of sufficient specimen volume from the repository.

Results for VEGF-A and VEGF-D peptide levels for Group 1 and Group 2 subjects with corresponding PSA measurements for the two time points are shown in Tables 3A and 3B, respectively. The median plasma VEGF-D levels in Group 1 were 319.5 pg/ml (inter-quartile range (IQR) = 281.4–377.6) and 349.9 pg/ml (IQR = 281.4–506.8) for the first and second specimens, respectively, while median plasma VEGF-A levels were 5.2 pg/ml (IQR = 0–17.1) for the first specimen and 0.0 pg/ml (IQR = 0.0–0.0) for the second specimen. The PSA values, VEGF-A and VEGF-D levels in Group 1 subjects receiving ADT ($n = 20$ for clinically metastatic disease) and Group 1 subjects followed without ADT ($n = 26$ subjects experiencing biochemical progression alone) is also presented in Table 3A for the two time points.

For the first specimen collected at the time of the ADT failure in Group 2, median levels of plasma VEGF-D were 377.0 pg/ml (IQR = 323.1–509.8 pg/ml) and 442.1 pg/ml (IQR = 361.9–582.8 pg/ml) for the second specimen. Median plasma VEGF-A levels in this group of subjects at the time of ADT failure were 9.6 pg/ml (IQR = 0.0–38.4 pg/ml) and 0.0 (IQR = 0.0–41.9 pg/ml) for the second specimen. Median VEGF-D and VEGF-A for patients who received additional treatments ($n = 24$) and subjects without additional treatments ($n = 10$) are provided in Table 3B.

Discussion

The role of the VEGF family of growth factors in the different stages of PCa progression has been extensively reported for VEGF-A [7–11]. Secreted isoforms of VEGF-A are elevated in patients with localized disease compared to healthy controls [12,13] while elevated levels of VEGF-A in the blood of castration-resistant stage patients correlate with poor overall survival [9]. In localized PCa, VEGF-A expression patterns at the time of radical prostatectomy have been reported to correlate with risk of developing subsequent metastatic disease [14], suggestive of a prognostic role for VEGF-A expression. Furthermore, in advanced cancer, VEGF-A-dependent tumor-associated angiogenesis and progression has been proposed, based on the altered expression patterns for VEGF-A during the transition from the hormone-sensitive to castration-resistant state in cell lines [15], *in vivo* models and in the resected tissue of patients treated with hormonal therapy [16,17].

However, expression patterns and the pathophysiological role VEGF-D might play in PCa is less well known. VEGF-D expression in PCa lymph angiogenesis has been proposed on the

basis of its tissue expression patterns and its correlation with sentinel lymph node metastasis [18,19]. Expression of VEGF family members in PCa tissue may also be cancer stage-dependent, with higher VEGF-A expression in localized PCa relative to VEGF-D and a higher VEGF-D expression relative to VEGF-A in more advanced cancer stages [7]. Secreted VEGF peptide levels in blood also appear to parallel this tissue expression pattern in a stage-specific manner for both VEGF-A and VEGF-D.

To our knowledge no prior studies have quantified the relative fractions of VEGF-A and VEGF-D in non-localized stages of PCa or investigated the effects of advanced stage-specific treatments on circulatory VEGF peptide expression. In this study, we have reported the levels of secreted VEGF-A and VEGF-D in uniformly processed platelet-poor plasma collected specifically for biomarker development in two subsets of patients with non-localized PCa. Plasma VEGF-A and VEGF-D levels were measured serially, in patients both before and after initiating hormonal treatments for hormone-sensitive stage PCa and in patient both before and after systemic chemotherapy for castration-resistant stage PCa. VEGF levels were measured in platelet-poor plasma in an attempt to avoid any confounding effect from physiological, non-cancer related VEGF protein pooled in the alpha granules of platelets, which can spuriously elevate circulatory protein levels [11]. Higher levels of secreted VEGF-D, compared to VEGF-A, were detected in all ADT-naïve subjects with non-localized cancer (Group 1) in both serial research specimens. While elevated VEGF-D to VEGF-A expression has been previously reported in non-localized PCa patients after initiating ADT treatments [7], in this study this was also observed in ADT-naïve subjects. VEGF-D levels were elevated in ADT-naïve subjects regardless of whether clinical metastases were present or whether the patient had suffered biochemical failure alone following primary PCa treatments. Interestingly, no decrease in the VEGF-D levels in the 20 patients who received ADT was found, despite observing a biochemical response to ADT (Table 3A: median PSA level decreased from 2.6 ng/ml to 0.2 ng/ml; $P = 0.0003$), while ADT treatment was associated with decreased plasma VEGF-A levels (Table 3A). These observations suggest that VEGF-D lymph angiogenesis may not be hormonal-pathway dependent in this stage of PCa. However, the small patient cohort in group 1 precludes the making of firm conclusions.

A similar trend of higher plasma VEGF-D than VEGF-A was also detected for the group of patients failing ADT (Group 2) and serial measurements detected a further rise in VEGF-D at the second time-point (442.1 pg/ml versus 377.0 pg/ml; $P = 0.008$) with or without additional treatments for castration resistance. This suggests that pathophysiological pathways increasing circulatory VEGF-D levels may be inadequately suppressed with currently used therapeutic approaches. This clinical observation will need larger patient cohorts and better molecular characterization to be more definitive if it is to have any therapeutic implications.

Finally, while there was some heterogeneity noted in the cohorts, it was also observed that plasma VEGF-A levels, unlike VEGF-D levels, decreased over the period of the two specimen collections when stage-specific interventions were performed for patients in either the castration-resistant stage or those undergoing ADT in the hormone-sensitive stages, suggesting that there may be, at the very least, a time-limited therapeutic interaction with VEGF-A. However, in addition to limited patient numbers, the specimen capture and observations were not performed over an extended time period and were also associated with the clinical outcomes in these patient cohorts, all of these factors being limitations of this pilot project.

These clinical observations, however, support previous findings of a higher VEGF-D expression, compared with VEGF-A, being detected in the prostatic tissue of patients with

advanced stage disease [7]. Furthermore, the increase in plasma VEGF-D levels observed in two serially collected specimens, despite an adequate PSA-based response to hormonal and/or chemotherapy treatments in the initial months of these interventions, suggests inadequate VEGF-D targeting with the current standard therapies. Larger prospective studies are therefore needed to confirm these preliminary findings, along with a better understanding of the molecular role VEGF-D may play in advanced PCa. The limitations of this pilot study include a relatively small specimen set, which will need to be confirmed in larger clinical cohorts of advanced PCa patients as well as at the molecular level prior to making definitive conclusions on the role of lymph angiogenesis-associated VEGF growth factors in advanced stage PCa.

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Table 1

Clinical and demographic characteristics of patients in Group 1

Clinical characteristics	Patients experiencing biochemical failure post primary prostate cancer treatment (<i>n</i> = 26/46)	Patients with clinical metastatic hormone sensitive prostate cancer (<i>n</i> = 20/46)
Age (years): median (Q1,Q3)	71.5 (64,78)	65 (60.5,70)
PSA at the time of biochemical failure/clinical metastatic disease (ng/ml): median (range)	0.41 (0.03–9.12)	2.63 (0.10–152.00)
Time between pre-ADT and post-ADT initiation specimen (days): mean (range)	194 (68–358)	186 (61–336)
Clinical T stage at first diagnosis: <i>n</i> (%)		
T1	2 (7.7)	2 (10)
T2	7 (26.9)	4 (20)
T3+4	9 (34.6)	6 (30)
T4	0 (0.0)	1 (5)
TX (unverifiable)	8 (30.8)	7 (35)
TNM staging of lymph nodes: <i>n</i> (%)		
N0	12 (46.2)	11 (55)
N1	0 (0.0)	4 (20)
NX	14 (53.8)	5 (25)
Time from diagnosis to ADT initiation: years (Q1,Q3)	2.61(0.41, 4.20)	4.01 (0.57, 8.22)
Biopsy Gleason score at initial diagnosis: <i>n</i> (%)		
6	8 (32)	4 (20)
≥7	13 (52)	12 (60)
8	4 (16)	4 (20)
Unknown	1 (3.8)	0 (0)
Definitive local therapy: <i>n</i> (%)		
None	0 (0)	3 (15)
Radical prostatectomy	20 (76.9)	13 (65)
Radiation therapy	9 (34.6)	7 (35)

Q1,Q3, inter-quartile ranges; ADT, androgen-deprivation therapy; TNM, tumor, nodes, metastasis.

Table 2

Clinical and demographic characteristics of patients in Group 2

Clinical characteristics	Patients experiencing biochemical failure post primary prostate cancer treatment (<i>n</i> = 34)
Age (years): median (Q1,Q3)	75.5 (67.5,84)
PSA at the time of progression to castration resistance (ng/ml): median (range)	10.2 (0.45–1352.09)
Time between 1 st and 2 nd specimen (days) (<i>n</i> = 30): mean (range)	92 (53–170)
Clinical T stage at first diagnosis: <i>n</i> (%)	
T1	2 (5.9)
T2	4 (11.8)
T3+4	5 (14.7)
T4	1 (2.9)
TX (unverifiable)	22 (64.7)
TNM staging of lymph nodes: <i>n</i> (%)	
N0	4 (11.8)
N1	4 (11.8)
NX	26 (76.5)
Time from diagnosis to ADT failure: years (Q1,Q3)	2.77 (0.08, 5.00)
Biopsy Gleason score at initial diagnosis: <i>n</i> (%)	
6	4 (11.8)
=7	10 (29.4)
8	16 (47.1)
Unknown	4 (11.8)
Definitive local therapy: <i>n</i> (%)	
None	13 (38.2)
Radical prostatectomy	10 (29.4)
Radiation therapy	15 (44.1)
Type of treatment after ADT failure	
No further treatment	10
Chemotherapy	24

Q1,Q3, interquartile ranges; ADT, androgen-deprivation therapy; TNM, tumor, nodes, metastasis.

Table 3A

Plasma VEGF-A and VEGF-D results for Group 1 cohort

Subjects	Variable	First specimen			Second specimen			P-value *
		N	Median	IQR	N	Median	IQR	
All	PSA (ng/ml)	46	1.0	0.3–4.0	45	0.3	0.0–1.2	.0002
	VEGF-A (pg/ml)	43	5.2	0.0–17.1	43	0.0	0.0–0.0	<.0001
	VEGF-D (pg/ml)	46	319.5	281.4–377.6	46	349.9	281.4–506.8	0.002
No ADT (<i>n</i> = 26)	PSA(ng/ml)	26	0.4	0.2–2.3	25	0.3	0.1–1.2	0.47
	VEGF-A (pg/ml)	26	3.4	0.0–16.4	26	0.0	0.0–1.0	0.04
	VEGF-D (pg/ml)	26	302.2	269.7–382.2	26	319.8	271.2–457.5	0.07
ADT (<i>n</i> = 20)	PSA (ng/ml)	20	2.6	1.0–10.9	20	0.2	0.0–1.1	0.0003
	VEGF-A (pg/ml)	17	9.4	3.5–17.1	17	0.0	0.0–0.0	0.0002
	VEGF-D (pg/ml)	20	322	282.9–362.5	20	395.3	327.0–561.0	0.009

IQR, inter-quartile range; PSA, prostate-specific antigen; ADT, androgen deprivation therapy.

* Wilcoxon signed rank test.

Table 3B

Plasma VEGF-A and VEGF-D results for Group 2 cohort

Subjects	Variable	First specimen			Second specimen			P-value *
		N	Median	IQR	n	Median	IQR	
All	PSA (ng/ml)	34	10.0	2.9–53.1	30	9.0	1.2–58.4	0.51
	VEGF-A (pg/ml)	30	9.6	0.0–38.4	17	0.0	0.0–41.9	1.00
	VEGF-D (pg/ml)	34	377.0	323.1–509.8	29	442.1	361.9–582.8	0.008
No chemo or hormonal therapy at the time of ADT failure	PSA (ng/ml)	10	2.4	0.8–6.9	7	1.1	0.7–8.0	0.94
	VEGF-A (pg/ml)	10	13.8	0.0–40.7	1	–	–	–
	VEGF-D (pg/ml)	10	374.7	326.4–418.5	6	494.7	335.6–569.7	0.03
Chemotherapy at the time of ADT failure for CRPC	PSA (ng/ml)	24	14.6	6.3–64.8	23	27.5	4.2–117.3	0.28
	VEGF-A (pg/ml)	20	9.6	0.5–23.9	16	1.6	0.0–56.0	1.00
	VEGF-D (pg/ml)	24	377.0	317.9–556.9	23	435.3	361.9–689.1	0.05

IQR, inter-quartile range; PSA, prostate-specific antigen; ADT, androgen deprivation therapy; CRPC, castration-resistant prostate cancer.

* Wilcoxon signed rank test.