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Combined Dynamic Alterations in Urinary VEGF Levels and Tissue ADAM9 Expression as Markers for Lethal Phenotypic Progression of Prostate Cancer

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Abstract

Recent evidence has demonstrated that detection of changes in the levels of urinary vascular endothelial growth factor (VEGF) and tissue a disintegrin and metalloproteinase 9 (ADAM9) is effective in determining prostate cancer progression. To evaluate the combined application of VEGF and ADAM9 as early progression markers of lethal phenotypic cancer, quantification of urinary VEGF and tissue ADAM9 expression was studied in patients with late stage prostate cancer. Tissue biopsies were collected during palliative transurethral resection of prostate (TURP) surgery, and urine samples were collected before hormone therapy and 3, 6 and 12 months post-TURP. We observed a nearly 100% correlation between increasing urinary VEGF levels over time and prostate cancer progression, but no correlation was observed when comparing urinary VEGF concentrations at a single time point and cancer progression. In addition, we also observed correlation of increasing ADAM9 nuclear positive staining and lethal phenotypic transition. Statistical analysis revealed that both the increase in urinary VEGF level and the presence of the tissue ADAM9 nuclear staining were significantly correlated with the

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risk of patients with relapse prostate cancer ($P < 0.05$). Thus, we suggest that combination of detection of changes in urinary VEGF and tissue staining of ADAM9 may be accurate for predicting the mortality of patients with prostate cancer during hormone therapy.

Key Words: ADAM9, prostate cancer, urine VEGF

Introduction

The ability to correctly predict patient outcome earlier than disease progression during therapy represents the most challenging assessment in clinical oncology. Although most patients with prostate cancer remain stable during hormone therapy, the unpredictability of poor prognosis in some patients is the greatest challenge in determining the strategy and timing for advance therapy. Correctly determining cancer progression before any detectable serum prostate specific antigen (PSA) relapse during hormone therapy enables better control and selection of advance therapies. Recent studies of prostate cancer have demonstrated a correlation between increased levels of vascular endothelial growth factor (VEGF) (1) and a disintegrin and metalloproteinase 9 (ADAM9) (7, 24) relative to cancer progression. VEGF is one of the most critical angiogenic factors involved in vascular permeability, endothelial cell proliferation and motility (19). Increased microvascular density in the cancer milieu, therefore, correlates with the metastasis potential of cancers (14). The level of VEGF can be easily detected in the serum (11) and urine (1) of cancer patients. In addition, urinary VEGF levels are a useful predictive marker for progression-free survival in patients with prostate cancer following radiotherapy (3).

ADAM9 expression increases during prostate cancer progression (7, 24), and clinical analyses have demonstrated a correlation between increased ADAM9 level and shortened PSA relapse-free survival rate in patients with prostate cancer (7). The expression of ADAM9 has been shown to be upregulated by stress and reactive oxygen species (ROS) in prostate cancer cells (24). It is also likely that the induction of ADAM9 is controlled by androgen in androgen-dependent prostate cancer cells but is constitutively overexpressed in androgen-independent cancer cells (21). In addition, inhibition of ADAM9 expression has been shown to sensitize prostate cancer cells to the therapeutic effects of both radiotherapy and chemotherapies (13). Peduto further hypothesized that targeting ADAM9 could be a potential strategy for treating patients with cancer (20). Hence, it is conceivable that the level of ADAM9 in prostate cancer cells might be an indicator of malignant development.

Although serum and urine VEGF and tissue ADAM9 levels can be used as markers to predict mortality after radiotherapy (3, 13), evidence is still lacking as to whether VEGF levels can be used as a marker to predict prostate cancer progression during hormone therapy, especially for patients suffering late-stage cancer with lower urinary tract symptoms (LUTS). This study sought to determine the prognostic value of urinary VEGF levels in combination with immunohistochemical analysis of ADAM9 for prostate cancer patients undergoing hormone therapy and transurethral resection of prostate (TURP) surgery. Our results indicated that dynamic VEGF alterations (before and after hormone therapy and TURP) in addition to ADAM9 nuclear staining together served as accurate early markers of lethal phenotypic progression.

Materials and Methods

Patient Cohort

Patients with prostate cancer and normal control were collected for this study (Tainan Hospital, IRB2007-007). Informed consent was obtained from each patient. A total of 8 prostate cancer patients and 9 normal healthy controls were collected during the same period. The patient cohort was carefully selected and included those exhibiting late-stage prostate cancer and severe LUTS who were ready for treatment with a combination of hormone therapy and TURP. Tissue samples were evaluated by a pathologist (C.C.L.), and demonstrated the Gleason Grade range between 5 and 8. In addition, the concentration of serum PSA was evaluated each time during the therapies (Table 1). The age-matched normal controls were evaluated to have no urological disease. Urine was collected during early and mid-day of the test. Histories of smoking, hypertension, hair staining and diabetes were indicated (Table 2).

Sample Preparation

Urine was collected prior to the initiation of hormone and TURP therapies and at 1, 3, 6, and 12 months after TURP surgery. Specimens were collected and stored at -70°C until analysis. Urine and tissue samples were anonymized before being released from the Tainan Hospital Pathology Department tissue bank for this study. Tissue biopsies were collected at the time of TURP surgery and were evaluated before being released.

Table 1. Combination of conditions of prostate cancer patients to predict cancer outcome during the first year of TURP and hormone therapy

Patient	1	2	3	4	5	6	7	8
Urine VEGF velocity slop	Down	Up	Down	Even	Down	Up	Up	Up
ADAM9 Nuclear staining	–	+	–	–	+/–	+	+	+
Gleason Score	2+3/10	3+4/10	2+3/10	2+3/10	3+2/10	3+4/10	3+2/10	4+4/10
Serum PSA before therapy (ng/ml)	15.1	>500	27.4	10.0	>500	>500	>500	>500
Serum PSA during first year of therapy (ng/ml)	≤ 0.2	2.5-5.8	2.5-7.0	0.5-10.0	0.5-28	25-98	0.2-6.0	≥ 100
3-year survival	Stable	Relapse	Stable	Stable	Stable	Relapse	Relapse	Relapse

+: 100% nuclear positive staining; –: 100% nuclear negative staining; +/–: mixture of nuclear positive and negative staining; Spearman's correlation coefficient, $P = 0.012$.

Table 2. Urinary VEGF levels in the control group with multi-year history of smoking, alcohol drinking, hair staining, diabetes and hypertension

Patient	Urine Collection[a]	Age	Smoke (Pack/Day)	VEGF (pg/ml)	Smoking Period/ Years	Alcohol/ Year	Hair-Staining/ Year	Diabetes	Hypertension
1		54	1.2		30	20	3		
	First			11.34 ± 0.61					
	Mid-day			13.10 ± 0.32					
2		42	1		20	15			
	First			12.64 ± 0.24					
	Mid-day			10.14 ± 0.12					
3		44			25	20		Yes	
	First			31.25 ± 5.12					
	Mid-day			15.45 ± 0.24					
4		46	1		30	20			Yes
	First			21.77 ± 0.21					
	Mid-day			57.97 ± 0.28					
5		45	2		25	N/A	2		
	First			41.51 ± 0.16					
	Mid-day			44.85 ± 0.99					
6		46	1		30	20			
	First			10.20 ± 0.12					
	Mid-day			36.02 ± 0.73					
7		48	1		20	N/A			
	First			11.00 ± 0.37					
	Mid-day			8.56 ± 0.00					
8		55	1		20	35			
	First			10.57 ± 0.00					
	Mid-day			10.86 ± 0.16					
9		57	1		35	N/A			
	First			45.33 ± 0.50					
	Mid-day			14.48 ± 0.24					
Summary	Median = 46	Median = 1	Average = 21.42 ± 16.19	Median = 25	Median = 20				

Cell Culture

The human prostate cancer epithelial cell line PC3 was purchased from the American Type Culture Collection (ATCC). All cells were cultured in T-medium (Invitrogen, Carlsbad, CA, USA) with 5% FBS (Invitrogen) and Pen/Strep at 37°C.

Animal Study

Six week-old Balb/c nude mice were used. To produce metastatic tumors, mice were injected at the

left ventricle with 5×10^5 luciferase expressing PC3 cells (PC3-luc). The cells were resuspended in PBS and injected with a 29-gauge needle in a volume of 50 µl. Mice were monitored twice weekly. The metastatic PC3 tumors were determined by examination using bioluminescence imaging and collection of tumors.

Enzyme-Linked Immunosorbent Assay (ELISA)

Urinary VEGF was quantified by ELISA (R&D Systems, Minneapolis, MN, USA) following standard procedures supplied by the manufacturer. The color development was read at 450 nm using an ELISA reader, and the results were expressed as the mean absorbance of triplicate samples ± standard error (SE) in pg/ml.

Immunohistochemical Staining

Five-micron thick paraffin-embedded tissue sections were deparaffinized and rehydrated. The tissue sections were incubated for 2 h with primary antibodies: mouse monoclonal anti-human VEGF (1:50; Millipore, Billerica, MA, USA) and mouse monoclonal anti-ADAM9 (1:100; R&D Systems). Specificity was verified by replacing the primary antibody with an isotype IgG control. Three different views per patient slide were analyzed. The "+" indicates positive staining observed in all the area that analyzed, "+/–" indicates at least one of three areas revealed negative staining, "–" indicates no positive staining in all three views.

Western Blot Analysis

Cells cultured to 80-95% confluency were lysed in a lysis buffer as previously described (22). Protein lysates were then subjected to SDS-PAGE, and the blot was incubated with an anti-human ADAM9 monoclonal antibody (1 µg/ml; R&D Systems), anti-human Lamin B (a nuclear marker; GeneTex, Irvine, CA, USA), α-tubulin (a cytosol marker; GeneTex), and then visualized using ECL (GE Healthcare Life Science, Piscataway, NJ, USA).

Nuclear Extraction

To separate the nuclear extraction and cytosol fractions, tumors were collected and washed with ice-cold PBS. Cell lysis buffer (20 mM HEPES, pH 7.0, 10 mM potassium chloride, 2 mM magnesium chloride, 0.5% Nonidet P-40, 1 mM sodium vanadate [Na_3VO_4], 2 µg/ml aprotinin, 1 mM PMSF) were added to the cell pellet and lysed in a Dounce homogenizer with 30 strokes. Nuclei were collected by centrifugation for 5 min at 1,500 *g* and then resuspended in NETN buffer (0.5% NP-40, 20 mM Tris, pH 8.0, 50 mM NaCl, 50 mM NaF, 100 µM Na_3VO_4, 1 mM DTT, 1 mM PMSF). The nuclei mixture was sonicated and centrifuge at 12,000 *g* for 20 min and the nuclear lysate (supernatant) was collected.

Statistical Analysis

All data were subjected to One Way ANOVA analysis of variance and expressed as the means ± SD, otherwise mentioned specifically in the text. A *P*-value of < 0.05 was considered statistically significant.

Results

Evaluation of VEGF as an Early Prediction Marker for Lethal Phenotypic Prostate Cancer

The patients in this study were divided into stable and relapse groups according to the outcome of three-year survival following hormone therapy. No PSA elevation was observed during the first year of hormone therapy for all patients (Fig. 1, A and B) indicating the limitation of using serum PSA to predict outcome in the early period of therapy. In addition, one patient remained stable with an initial PSA of ≥500 ng/ml (Fig. 1C) and a Gleason score of 3+2 (Table 1) indicating that better markers are required for patients during hormone therapy. To determine if there was any correlation between VEGF levels and cancer progression, urine VEGF levels were compared individually prior to the initiation of therapy (pre-OP) and at 3 months after initiation of hormone therapy and TURP (3-month post-OP). Although no significant correlation between urinary VEGF concentrations during the first year of hormone therapy was observed between the two groups (Fig. 1, A and B), dynamic studies of urine VEGF concentrations for each patient demonstrated a high correlation with cancer progression (Fig. 1D). Increasing VEGF concentrations indicated that relapse was likely to occur. By comparison, decreasing levels of VEGF over time were observed in the stable group. Hence, the dynamic alteration of urine VEGF concentrations (VEGF slope) between pre-OP and 3-month post-OP was a clear indication of lethal phenotypic prostate cancer transition before PSA relapse could be detected.

ADAM9 Staining as a Marker for Predicting the Development of Lethal Phenotypic Prostate Cancer

Previous reports have shown that ADAM9 expression increases in patients with malignant prostate cancer (7, 24). This raises the possibility of using ADAM9 as an independent prognostic marker for

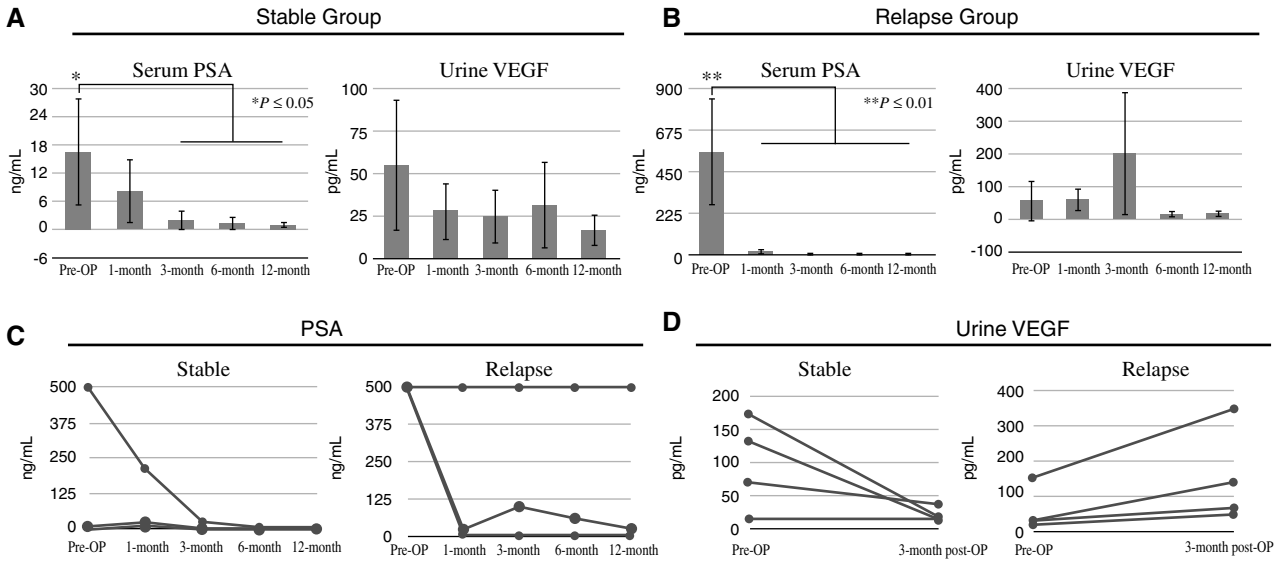

Fig. 1. Summary of changes in the concentrations of serum PSA and urine VEGF changes in patients with prostate cancer before and after TURP and hormone therapy. Patient groups were divided into (A) stable and (B) relapse groups according to the outcome obtained 3 years after hormone therapy. Kinetic analysis of (C) serum PSA and (D) urine VEGF in individual patients with prostate cancer that were either stable (left) or relapse (right) after therapy. Samples were collected during the first year after hormone therapy. (*$P \leq 0.05$, **$P \leq 0.01$).

PSA relapse-free survival following hormone therapy. To determine the potential of using urine VEGF expression and tissue ADAM9 levels as combined markers for lethal phenotypic prediction in patients treated with hormone therapy and TURP and to determine if addition of VEGF tissue staining could further enhance the prediction, tissue staining was performed in the same patient cohort and compared between the two patient groups. Tissues collected before TURP and stained for VEGF or ADAM9 in the refractory prostate cancer group are shown in Fig. 2. VEGF demonstrated an overall equal intensity of staining between low-grade region (black square) and high-grade tumor (red square) of the same tissue. In contrast, the majority of ADAM9-positive staining was observed in the high-grade cancer region of hormone-refractory prostate cancer patients (Fig. 2, A and B, red square of both 10× and 40×). Furthermore, comparison of the tissues of hormone-responsive (stable) and hormone-refractory (relapse) prostate cancer patients tissues demonstrated no difference in VEGF staining intensities (Fig. 2C). However, strong immunostaining of ADAM9 could only be observed in the samples of relapse patients. In addition, the majority of ADAM9 staining was noted to be located in the nuclear region of relapsed prostate cancer (Fig. 2D). To further elucidate ADAM9 localization in metastatic prostate cancer samples, mice underwent intracardiac injection of PC3 cells, and tumor was determined at the metastatic loci (Fig. 2E). Nuclear extraction from the metastatic tumors showed positive staining of ADAM9 expression in the nuclear fraction of xenograft tumor indicating that ADAM9 nuclear localization correlated with the metastatic prostate cancer cells.

Combination of Urinary VEGF Changes and ADAM9 Nuclear Positive Staining Enhances the Prediction of Lethal Phenotypic Prostate Cancer

The combination of urinary VEGF alterations and ADAM9 nuclear staining enable accurate prediction of lethal phenotypic prostate cancer progression during early hormone therapy (Table 1). Statistical analysis by Spearman's correlation coefficient demonstrated that both the increase in urinary VEGF level and the presence of the tissue ADAM9 nuclear staining were significantly correlated with the risk of the patient with relapse prostate cancer ($P = 0.012$). Logistically, a patient could have a biopsy before hormone therapy to evaluate possible risk based on ADAM9 tissue staining. This would be followed by the examination of kinetic variations in urinary VEGF before and during hormone therapy. This combination of VEGF and ADAM9 measurements could serve as a new predictor of early progression-free survival after hormone therapy and before any serum PSA rebound could be detected. In addition, combination of measurement of urinary VEGF concentration and ADAM9 positive nuclear staining was highly correlated with histopathological grading of prostate cancer stages of the patients (Table 1). Among them,

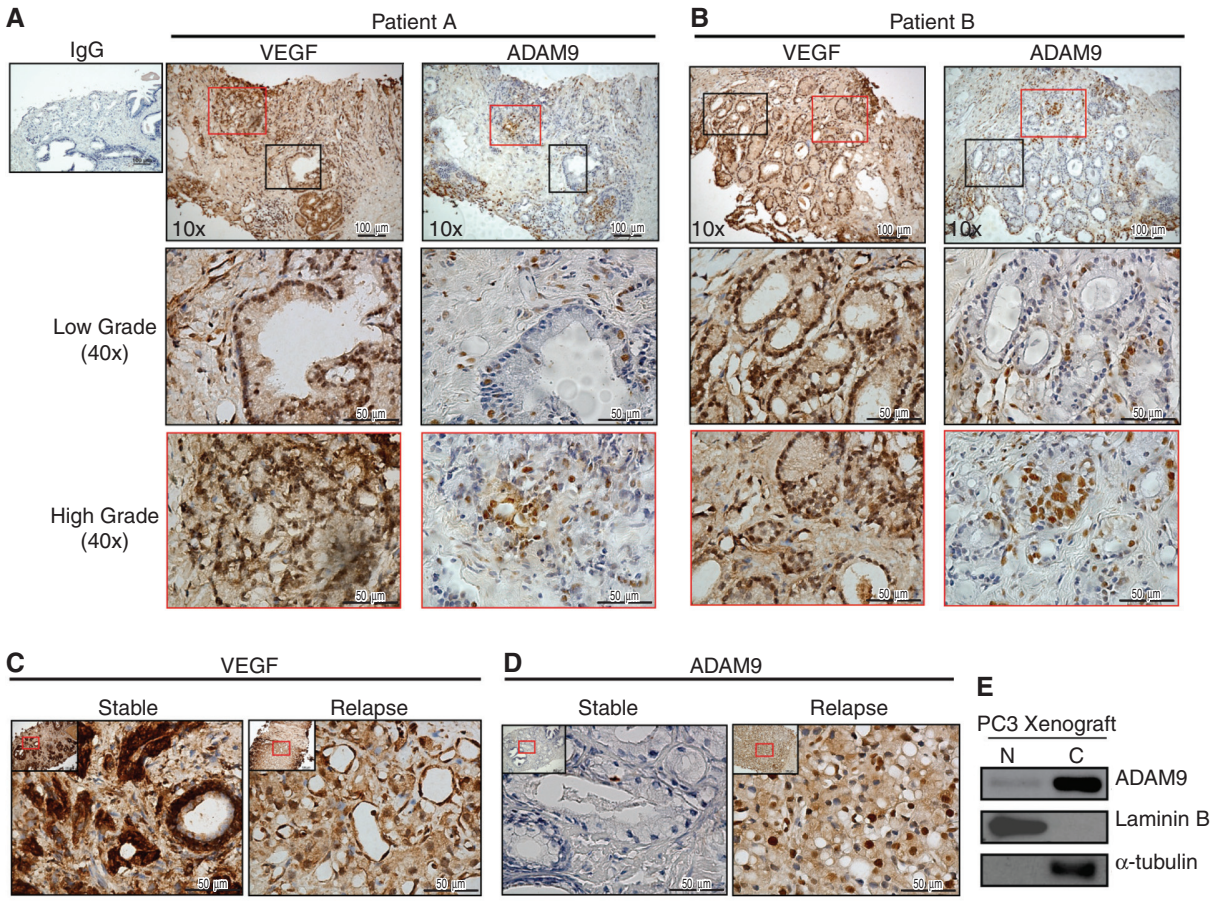

Fig. 2. Comparison of VEGF and ADAM9 tissue staining as lethal phenotypic markers of prostate cancer. (A, B) Two samples of hormone-refractory cancer patients revealed equal expression of VEGF staining in low-grade (black square and enlarged image at the bottom) and high-grade (red square and enlarged image at the bottom) tumor regions (insert, IgG control). ADAM9 staining, by comparison, revealed strong nuclear staining only in the high-grade regions. (C) Enlarged (40×) image of VEGF staining showed no difference in the samples of stable and relapse patients. (D) Increased ADAM9 nuclear staining in relapsed prostate cancer tissues compared to stable tissues (Bar, 50 µm; insert: low-power field). (E) Western blot of ADAM9 expression on nuclear protein extracted from freshly isolated tumors taken after intracardiac injection of PC3 prostate cancer cells and metastasis to adrenal gland. Top, Western blot for ADAM9; middle, lamin B, a nuclear envelope protein, to show the integrity of the nuclear extracts; bottom, α-tubulin, a cytoplasmic marker (N: nuclear extract; C: cytosol extract).

one relapse patient with Greason score of 3+2 but positive in ADAM9 nuclear staining and increased of urine VEGF concentration during therapy. This indicates addition of ADAM9 staining and urine VEGF evaluation could have a better prediction of therapeutic effect before and during hormone therapy. Nevertheless, our data indicated that ADAM9 staining and urine VEGF velocity before and during hormone therapy could enhance the prediction accuracy of serum PSA values.

Urinary proteins are highly correlated with personal health conditions and/or behaviors, such as uremia (2) or cigarette smoking (5, 9). To determine if smoking interfered with urine VEGF concentrations, we examined a group of healthy controls in regards to smoking, alcohol consumption and the use of hair dye. The results showed that urine VEGF expression in control group was lower compared to the patients with prostate cancer suggesting that smoking, alcohol consumption and the use of hair dye did not affect our data (Table 2).

Discussion

We report in this study initial quantification of urinary VEGF levels and tissue ADAM9 expression in patients with either stable or recurrent prostate cancer. One-year analysis of urine VEGF variations after palliative TURP and hormone therapy demonstrated that kinetic elevation of VEGF highly correlated with prostate cancer progression. Recent studies have indicated that overexpression of VEGF in prostate cancer cells and the cancer microenvironment enhances malignant progression (10, 12). In

addition, Gustavsson *et al*. showed strong correlation of increasing VEGF secretion and hormone-resistant prostate cancer progression (8). Mazzucchelli *et al*. demonstrated that down-regulation of VEGF expression was associated with a better outcome of anti-androgen treatment (17). They also indicated that increasing VEGF staining in vascular endothelial cells of hormone-resistant prostate tumor was correlated with increasing of Gleason score. Furthermore, Wade and Kozlowski, using magnetic resonance imaging (MRI), showed increased size in the blood vessel in the tumor of hormone-refractory patients compared to hormone-responsive patients (26). These results indicate that elevation of VEGF secretion and angiogenesis activities after hormone therapies has a higher risk in developing hormone-refractory prostate cancer (HRPC) progression that may correlated with age induced cancer progression (15). Therefore, VEGF secretion in tumor and urine after hormone therapy could be a good candidate to predict HRPC progression (1, 18). However, evidence is still lacking in showing urine VEGF as an accurate marker for predicting lethal phenotypic transition of patients undergoing hormone therapy.

The study by Chan *et al*. has indicated that the kinetics of VEGF expression during and after radiotherapy highly correlates with disease progression (3). Lin *et al*. further demonstrated the expression of LGR8 and related biomarkers, such as VEGF, could crosstalk to relaxin receptors and induced hepatocellular carcinoma progression (16). Our study indicated that the dynamic alteration of urine VEGF was a good indication of lethal phenotypic prostate cancer transition before PSA relapse could be detected. These observations suggested that the levels of VEGF at a single time point in prostate cancer may not necessarily be related to malignancy; more exactly, the dynamic alterations of VEGF in a patient's urine could be a more favorable candidate marker. Although studies have indicated increasing VEGF concentrations in the urine and tissue of hormone-refractory prostate cancer patients (3, 18), detail mechanisms of the role of VEGF in hormone therapy are still unclear. It is possible that alternations in tumor microenvironment induced by therapeutic agents may play an important role. It has been implicated that blood vessels in hormone-refractory tumors are more resistant to hormone-withdraw therapies.

In addition to the correlation between increasing VEGF levels and cancer progression, increases in ADAM9 staining have been shown to correlate with cancer progression and ROS production in cancer cells (21, 24). Furthermore, knockdown of ADAM9 was shown to enhance the therapeutic effects of radiation and chemotherapy (13). Our data demonstrated that combined kinetic analysis of urinary VEGF concentrations and ADAM9 expression in tissue biopsies could serve as markers for lethal phenotypic transition after hormone therapy and TURP. We also noticed that increased nuclear staining of ADAM9 expression correlated with lethal phenotypic transition.

Our study further demonstrated that the combination of changes in VEGF and ADAM9 nuclear staining could improve prognosis prediction during the early phase of hormone therapy. During cancer therapies, intracellular ROS in prostate cancer cells is induced in response to a variety of exogenous stressors such as radiation therapy, chemotherapeutic agents, androgen stimulation, overcrowding and serum deprivation (13, 21, 24). However, ROS may also initiate a downstream signaling cascade that aids in the survival and progression of tumor cells (4, 23). Previously, we demonstrated an increased expression of ADAM9 in response to lethal concentrations of hydrogen peroxide, overcrowding and serum deprivation (21, 24). In addition, studies of biopsy specimens from relapsed patients have revealed elevated levels of ADAM9 expression in the cancerous portions of the gland; on the other hand, ADAM9 expression decreased in the adjacent healthy prostate tissue (7, 24). We also demonstrated nuclear positivity of ADAM9 protein expression in the metastatic xenograft mouse model indicating possible ADAM9 nuclear translocation during malignant progression. Although we demonstrated that the increase in ADAM9 nuclear localization correlated with lethal phenotypic transition, the role of ADAM9 in the nucleus is still unclear. Current studies indicated that ADAM9 may be involved in stress response (6, 13, 21, 24) and possibly enhances stress response by ADAM9 after nuclear translocation. However, further studies are necessary to clarify the role of ADAM9 in this pathway. Tsai *et al*., indicating increasing of calcium influx during stress stimulation through phospholipase-C independent manner that enhances PC3 stress responses (25). It is arguable that ADAM9 might activate the stress regulatory pathways, such as calcium concentration in cancer cells. Nevertheless, increased ADAM9 nuclear staining could serve as an indicator of lethal phenotypic transition.

In summary, we demonstrated that assessment of changes in VEGF urinary levels in combination with ADAM9 nuclear expression resulted in effective lethal phenotypic prediction for patients with prostate cancer undergoing hormone therapy. Furthermore, we suggest that the combined use of both markers substantially increased the accuracy of this prediction. As such, urinary VEGF changes and ADAM9 nuclear expression might serve as vital tools for establishing disease recurrence and selection of patients that would benefit most from earlier and more advanced therapies.

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