Prognostic and Predictive Significance of Haemostatic and Angiogenic Parameters in Cancer Bladder Patients

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Abstract: Recent studies demonstrated a key role of angiogenesis, thrombosis and fibrinolysis in tumour invasion and metastasis. We aimed to clarify the potential link between angiogenic factor {vascular endothelial growth factor (VEGF)}, prothrombotic factor {von Willebrand factor (vWF)} and fibrinolytic markers {tissue plasminogen activator (t-PA), plasminogen activator inhibitor-1 (PAI-1) and D-dimer} with disease progression and metastatic dissemination in bladder cancer patients. The study enrolled forty five bladder cancer patients classified into three groups: 20 patients with locally invasive tumours, 15 patients with regional lymph nodes involvement and 10 patients with distant metastasis. In addition to 15 subjects served as a control group. Enzyme linked immunoassay method was used for measurement of VEGF, vWF, t-PA, PAI-1 and D-dimer. Enhanced angiogenesis was evident by high level of VEGF with subsequent high release of endothelial vWF. Also activation of fibrinolytic system was pronounced by elevated t-PA, PAI-1 and D-dimer. In addition, highest values of these factors were associated with relatively advanced tumour stage, as they showed a significant direct correlation with the stage of bladder cancer. Regression analysis proved that VEGF, vWF, t-PA, PAI-1 and D-dimer are potential prognostic markers in bladder cancer patients. These findings may have future implications for the treatment of patients with metastastatic disease.

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1. Introduction:

Some haemostasis and angiogenesis-related factors such as platelets, vWF, fibrinogen, PAI-1, D-dimer and VEGF have been highlighted as new potential response and survival predictors in cancer patients ⁽¹⁾.

Metastasis is a multi-step process involved in the alterations of cell-cell adhesion, angiogenesis, degradation of extracellular matrix, escape of immune surveillance and cell-matrix adhesion ⁽²⁾. Degradation of extracellular matrix is important for tumour growth and invasion, which in part is regulated by the plasminogen activation system. Cell matrix adhesive interaction plays an important role in the normal organization and stabilization of the cell layer in epithelial tissue. However in tumour cells the adhesive interaction of these cells and the subendothelial matrices is essential for their invasive and metastatic capabilities, and the molecules that mediate this adhesive process may facilitate tumour cells to metastasize ^(3,4).

Vascular endothelial growth factor (VEGF) is one of the most important angiogenic mediator both in physiological and pathological states ⁽⁵⁾. Angiogenesis and coagulation system activation are

associated with tumour growth and metastasis⁽⁶⁾. Their aberrancies are integral parts of the pathobiology of cancer growth and dissemination ⁽⁷⁾. We reported previously that enhanced platelet activation in cancer bladder patients associated with release of platelet derived angiogenic factor (VEGF), plays an important role in tumour growth and dissemination ⁽⁸⁾.

Von Willebrand factor (vWF) is a glycoprotein, synthesized mainly in endothelial cells and in megakaryocytes ⁽⁹⁾. It mediates the adherence of platelets to subendothelial matrices during vascular-endothelial damage and acts as a carrier protein for coagulation factor VIII ⁽¹⁰⁾. Increased plasma vWF have been reported in patients with various types of cancer such as prostate cancer ⁽¹¹⁾, cervical and ovarian carcinoma ⁽¹²⁾, head and neck cancer ^(13, 14) and colorectal cancer ^(1, 15, 16). Moreover, high plasma vWF concentrations often correlates with advanced tumour staging and may have prognostic significance in these patients ^(11, 12, 13, 14, 15, 16).

It has been demonstrated that the fibrinolytic system, in particular the urokinase-type plasminogen activator system (uPA), is involved in the process of tumour cell invasion and metastasis. uPA binds to the urokinase-type plasminogen activator receptors (uPAR), which is present on tumour cells and monocytes, thus facilitating the conversion of plasminogen to plasmin. Plasmin is a protease not only able to cleave the fibrin network of a clot but also degrades the extracellular matrix, thereby allowing tumour cells and monocytes to invade the extracellular matrix and surrounding tissues^(17, 18).

Elevated tumour levels of uPA, uPAR and PAI-1 are associated with poor prognosis in various malignancies, including colorectal cancer ^(3, 4, 19, 20), bladder and renal cell cancer ⁽²¹⁾, squamous cell cancer ⁽²²⁾ and breast cancer ^(23, 24, 25).

D-dimer is a degradation product of crosslinked fibrin clots and reflects fibrin concentration. D-dimer levels are increased in patients with enhanced fibrin formation and have been reported as sensitive indicators for deep venous thrombosis and pulmonary embolism ^(26, 27). In addition, preoperative plasma D-dimer level correlates with tumour stage and prognosis for patients with lung cancer ^(28, 29, 30, 31) and colorectal cancer ^(32, 33).

Von Willebrand factor and other coagulation/fibrinolysis factors should be seriously considered as potential future prognostic and predictive indicators in bladder cancer patients. We aimed to evaluate the level of angiogenic factor (VEGF), coagulation factor (vWF), fibrinolytic parameters (t-PA, PAI-1 and D-dimer) and their possible relation to tumour progression and prognosis in cancer bladder patients. It is hoped that a better understanding of these factors will ultimately lead to the development of more targeted strategies that may have a positive effect on the process of tumour growth and dissemination.

2. Patients and Methods:

This study was conducted on forty five patients (32 males and 13 females having mean age of 63.57 ± 10.52) with urinary bladder cancer admitted to Urology Department, Theodor Bilharz Research Institute, Giza, Egypt. Results were compared to those of fifteen age and sex matched healthy subjects (10 males and 5 females having a mean age of 59.71 ± 10.45) that served as a control group.

The study protocol was approved by the international committee for the protection of human participants and confirmed by the guidelines of the 1975 Declaration of Helsinki.

All studied patients' groups were subjected to detailed history taking, thorough

clinical examination, abdominal and pelvic ultrasonography, chest X-ray, Computed Tomography (CT), urine cytology and histopathological diagnosis of urinary bladder biopsies obtained by cystoscopy.

Accordingly, patients were classified clinically into three groups:

- 20 patients (13 males and 7 females having a mean age of 62.23 ± 10.41) with primary locally invasive urinary bladder cancer (de novo urinary bladder carcinoma) with no regional lymph nodes involvement and distant metastasis.

- 15 patients (12 males and 3 females having a mean age of 63.26±10.34) with urinary bladder cancer accompanied by regional lymph nodes involvement.

- 10 patients (7 males and 3 females having a mean age of 65.23 ± 8.41) with urinary bladder cancer accompanied with regional lymph nodes involvement as well as distant metastasis.

According to the results of the above mentioned investigations for each case, determination of the stage using Tumour-Node-Metastasis (TNM) staging system were done.

Depending on histopathological examination of cystoscopic bladder biopsies, patients were classified into three types:

-31 out of 45 patients (68.9%) with transitional cell carcinoma.

-13 out of 45 patients (28.9%) with squamous cell carcinoma.

-1 out of 45 patients (2.2%) with adenocarcinoma.

For all studied subjects, five ml blood samples were collected under complete aseptic conditions by clean venipuncture without venous stress, samples were distributed into the following tubes:

- One ml blood was collected on EDTA containing tube for complete blood picture.

- Two hundred μ l Na citrate (3.8%) containing vacutainer was completed to 2 ml with blood in a ratio of 1:9. Centrifugation at 3000 rpm for 20 minutes was done and plasma samples were separated and preserved in small aliquots and stored at -20°C for assay of haemostatic parameters (vWF, t-PA, PAI-1 and D-dimer). Two ml of blood was left to stand for clot formation. Serum was separated and divided into small aliquots for assay of liver and kidney function tests and serum level of VEGF.

All individuals were subjected to general investigations including an automated haemogram (using ACT differential, Beckman, France), liver and kidney function tests using autoanalyzer (Hitachi 736, Hitachi, Japan).

Haemostatic parameters were assayed for all studied groups by enzyme immunoassay, including assay of plasma level of vWF (Helena, Laboratories), tPA (Hyphen Biomed, France), PAI-1 (Stago Diagnostica, USA), Plasma D-dimer (Hyphen Biomed, France). In addition, determination of serum level of VEGF was also done by enzyme immunoassay technique (using R&D System, VEGF, USA). All specific assays were carried out according to manufacture's guidelines.

Analysis of data

SPSS for Windows version 9.0 computer program (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. Means of different groups were compared using one-way ANOVA. Pearson correlation coefficient 'r' was used to measure the relationship between two variables. Stepwise multiple regression analysis was employed to evaluate any association between both angiogenic factor (VEGF) and haemostatic parameters (vWF, tPA, PAI-1 and D-dimer) with tumour progression and prognosis (tumour stage and distant spread) in bladder cancer patients. For all tests, a P value of less than 0.05 was considered statistically significant.

3. Results:

The results of studied parameters in patients' and control groups are shown in Table (I). Table (II) shows the result of the studied parameters in control, locally invasive tumours, tumours with LN involvement and tumours with distant metastasis.

Cancer Bladder patients showed increased serum angiogenic factor (VEGF) level compared to controls (P<0.05). A significant high VEGF level was detected in patients with regional LN involvement compared to those with localized tumours (P<0.05). Also a significant high VEGF level was noted in patients with distant metastasis compared to both locally invasive tumours (P<0.05) and those with regional LN involvement (P<0.05).

Our study showed a significant increase in plasma vWF in patients' groups compared to control group (P<0.05). A significant high level was detected in locally invasive tumours compared to control group (P<0.05). Also vWF was significantly higher in patients with regional LN involvement compared to both control group (P<0.05) and patients with locally invasive tumours (P<0.05). In addition, vWF was significantly higher in patients with distant metastasis compared to control (P<0.05), locally invasive tumours (P<0.05) and tumours with regional LN involvement (P<0.05) and tumours with regional LN involvement (P<0.05).

Regarding the fibrinolytic system, our data showed a statistical significant difference in patients'

group compared to control group. A significant high plasma level of both t-PA and PAI-1 was observed in locally invasive tumours compared to control group (P<0.05). In addition, both t-PA and PAI-1 were

(P<0.05). In addition, both t-PA and PAI-1 were significantly higher in tumours with regional LN involvement compared to control (P<0.05) and locally invasive tumours (P<0.05). Moreover, the highest levels of t-PA and PAI-1 were observed in patients with distant metastasis and their levels were significantly higher compared to control (P<0.05), locally invasive tumours (P<0.05) and tumours with regional LN involvement (P<0.05).

D-dimer level was significantly raised in patients' group compared to control group (P<0.05). A statistical significant difference was observed in locally invasive tumours compared to control group (P<0.05). Also, a statistical significant rise was detected in tumours with regional LN involvement in comparison to both control (P<0.05) and locally invasive tumours (P<0.05). In addition, a statistical significant difference was noticed on comparing tumours with distant metastasis with control (P<0.05), locally invasive tumours (P<0.05) and tumours with LN involvement (P<0.05).

Correlation analysis revealed a significant direct correlation between VEGF and vWF (r=0.734, P<0.05), suggesting the influence of angiogenic factors in increasing the production of endothelial vWF by the newly formed blood vessels. On the other hand, the angiogenic factor (VEGF), procoagulant factor (vWF) and fibrinolytic factors (t-PA and D-dimer) showed a significant direct correlation with the stage of bladder cancer (r=0.875 and r=0.579 respectively). These results give an evidence for the role of angiogenic and haemostatic factors in tumour growth and distant dissemination of malignant tumours (table: III).

Stepwise multiple linear regression analysis revealed that the angiogenic factor (VWGF), haemostatic factor (vWF) and fibrinolytic factors (t-PA and D-dimer) are independent determinant for the stage of bladder cancer tumour {(f=11.982, p=0.000), (f=2.456, p=0.024), (f=4.059, p=0.001) and (f=12.857,p=0.000) respectively. These data give strong evidence for the importance of these parameters as prognostic markers for bladder cancer tumours.

		Control Group (n=15)	Patients' Group (n=45)
VEGF	Mean±S.D.	58.93±29.14	257.77±172.0*
(pg/ml)	Range	10-115	52-762
vWF	Mean±S.D.	112.46±16.80	143.51±34.08*
(%)	Range	82-130	89-195
tPA	Mean±S.D.	5.7±2.7	8.77±3.4.2*
(ng/ml)	Range	3.0-9.0	4.5-19.5
PAI-1	Mean±S.D.	55.30±21.20	81.56±16.29*
(IU/ml)	Range	34.0-78.0	38.0-180.0
D-dimer	Mean±S.D.	224.40±99.7	484.44±119.26*
(ng/ml)	Range	93-342	210-907

Table I: Results of studied parameters in control and patients' groups

*: Statistically significant from control (P<0.05)

Table	II:	Results	of	the	studied	parameters	in	control,	locally	invasive	tumours,	tumours	with	LN
	in	volvemer	nt ar	nd tu	mours w	ith distant m	ieta	stasis.						

		Control Group	Locally Invasive	Tumour with LN	Tumour with distant
		(n=15)	Tumour (n=20)	Involvement	Metastasis (n=10)
				(n=15)	
VEGF	Mean±S.D.	58.93±29.14	114.40±45.55 ^a	277.60±76.72 ^{ab}	514.80±106.60 ^{abc}
(pg/ml)	Range	10-115	52-205	152-390	384-762
vWF	Mean±S.D.	112.46±16.80	126.10±33.53 ^a	160.00±26.03 ^{ab}	174.26±14.24 ^{abc}
(%)	Range	82-130	89-190	100-190	160-195
tPA	Mean±S.D.	5.7±2.7	7.6±3.9 ^a	9.4±3.9 ^{ab}	15.9±4.3 ^{abc}
(ng/ml)	Range	3.0-9.0	4.5-11.5	4.3-13.5	10.5-19.5
PAI-1	Mean±S.D.	55.30±21.20	63.5±32.9 ^a	98.0±26.4 ^{ab}	129.0±36.2 ^{abc}
(IU/ml)	Range	34.0-78.0	38.0-100.0	71.0-128.0	95.0-180.0
D-dimer	Mean±S.D.	224.40±99.7	341.1±98.8 ^a	631.7±112.6 ^{ab}	881.3±36.8 ^{abc}
(ng/ml)	Range	93-342	210.0-443.0	354-741	819-907

a: Statistically significant from control group (P<0.05)

b: Statistically significant from locally invasive tumours (P<0.05)

c: Statistically significant from tumours with LN involvement (P<0.05)

Table	III:	Correla	ation	analy	ysis b	etwee	n studi	ed
	para	meters	and	both	stage	e and	grade	of
	bladder cancer tumour							

	Stage of tumour (TNM system)
VEGF	r=0.875*
vWF	r=0.427*
tPA	r=0.414*
PAI-1	r=0.113
D-dimer	r=0.579*

*: Correlation is significant at (P<0.01)

4. Discussion:

The development of metastasis is a stepwise process that starts when cancer cells separate from a primary tumour, migrate across blood vessel walls into blood stream and disperse throughout the body to generate new colonies. During the transit into the circulating system, tumour cells are exposed to fluid mechanical forces, plasma proteins and the vascular cells such as platelets. All of which may affect their survival and extravasations from the vasculature ⁽¹⁶⁾.

As we previously reported ⁽⁸⁾, our recent study demonstrated a significantly raised serum levels of VEGF in both transitional cell carcinoma and squamous cell carcinoma in comparison to control subjects. Moreover, its level showed a direct correlation with the tumour stage. VEGF has been associated with angiogenesis, lymphangiogenesis and regional lymph node metastasis and was reported to have anti-apoptotic and proliferative role ⁽³⁴⁾. Also Suzuki et al., (2005) ⁽³⁵⁾, reported that VEGF expression is an important predictive factor of pelvic lymph node metastasis in bladder cancer patients.

vWF plays a very important role in the pathogenesis of metastasis, by promoting the binding of tumour cells to platelets, and subsequently, to vascular endothelium ^(36, 37, 38). The binding of vWF to several types of collagen may contribute to the attachment of platelets to the extracellular matrices of subendothelium, furthermore, a direct interaction between vWF and neoplastic cells has been demonstrated ⁽³⁹⁾. This interaction forms heterotypic cellular emboli, which are not easily recognized by the immune system and have more chance of

attaching to the endothelial surfaces than single tumour cells $^{(36, 37)}$.

Elevated plasma vWF had been reported in different types of cancer. Our study demonstrated a significantly higher plasma vWF levels in bladder cancer patients compared to healthy controls, and the highest levels were observed in patients with metastatic disease. Elevations in vWF plasma levels in disseminated disease reflect the enhancement of angiogenic activity (as evident by high serum VEGF levels and its direct correlation with plasma vWF) to sustain a larger tumour cell burden and the metastatic progression ⁽¹⁵⁾. In addition, the release of thrombin by tumour cells may induce vWF production in endothelial cells and enhance the adhesion of tumour cells ⁽¹⁶⁾. Furthermore, the metastatic status of these patients may represent an effect of the adhesive property of vWF, which seems to play a crucial role during the course of haematological spread ⁽¹⁶⁾. Accordingly, vWF may serve as a potential biological marker of disease progression in these patients.

It is well known that the fibrinolytic system is of importance in inflammation, wound healing and fibrosis development. However, it is also important in the process of tumour invasion and metastasis ⁽³⁾. The present study demonstrated high plasma level of both t-PA and PAI-1 compared to control subjects and the highest levels were reported in patients with distant metastasis.

Numerous independent studies have demonstrated that patients with low levels of u-PA and PAI-1 in their primary tumour tissue have significantly better survival than patients with high levels of either factor ^(23, 24). Tumour cells can express everything required for regulation of the fibrinolytic pathway on their cell surface. They possess both the (u-PA) and urokinase-type the tissue-type plasminogen activator (t-PA) and can also produce plasminogen activator inhibitor-1 (PAI-1)^(40, 41). Indeed, tumour cells are known to carry the specific PA receptors (u-PAR) (CD 87) on their membranes, which can facilitate the activation of the fibrinolytic system ⁽⁴²⁾. Extravasation and intravasation of solid malignant tumours is controlled by attachment of tumour cells to components of the basement membrane and the extracellular matrix, by local proteolysis and tumour cell migration ⁽¹⁷⁾. Recent data strongly suggest that the delicate balance between plasminogen activators and their inhibitors plays a role in tumour invasion, tumour cell progression and metastasis and associated with shortened disease free and or overall survival in patients affected with malignant solid tumours. Levels of one or more of these markers have been recognized as predictors of disease-free interval and long-term survival in some patients with malignant disease (17, 22, 24, 41). Moreover,

intravesical administration of PAI-1 significantly inhibits tumour progression in an in vivo model of bladder cancer ⁽⁴³⁾. The above mentioned data clarify the clinical impact of fibrinolytic system in tumour invasion and metastasis, and the future relevance in anti cancer therapy.

Plasma D-dimer levels have been shown to be increased in patients with various solid tumours including lung, prostate, cervical, ovarian, breast and colon cancer ⁽³³⁾. An elevated plasma D-dimer level indicates activation of coagulation and fibrinolysis ⁽³²⁾. A close interaction exists between venous thromboembolic disease and cancer. In the present study, we demonstrated a high plasma D-dimer level in cancer bladder patients compared to controls. Moreover, its level showed a significant correlation with the stage of the tumour. These data suggest that tumour progression is associated with activation of coagulation and fibrin formation, which is both implicated in cancer proliferation and metastatic dissemination (44). Accordingly, elevated plasma Ddimer might be a sign of poor prognosis and preoperative plasma D-dimer level can be used to predict postoperative survival.

In summary, our data indicates that serum VEGF and plasma vWF, t-PA, PAI-1 and D-dimer levels are elevated in a stage-dependent manner, and higher levels correlate with metastatic diseases. Accordingly, they can serve as potential biological markers of disease progression in bladder cancer patients.

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