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Correlation between Tissue and Released VEGF Levels in Urine of Bladder Cancer Patients

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Abstract: Purpose. Angiogenesis is a complex multistep process essential for tumor growth. Vascular endothelial growth factor (VEGF) is a potent endothelial cell mitogen and vascular permeability-inducing agent. The aim of this study was to analyze whether tissue and urinary VEGF levels of bladder cancer patients are correlated and study their correlation with classical clinicopathological parameters and bilharizial bladder cancer patients. The study group was consisted of consecutive series of 120 bladder cancer patients. VEGF levels were measured by an enzyme immunoassay method (EIA). We evaluated EIA method for quantifying tissue and urinary VEGF levels with intra-assay CVs of 7.3-9.2% and 6.7-9.5%; and inter-assay CVs of 8.3-9.1% and 6.7-9% respectively. The recovery of tissue VEGF level was 96-99% and urinary VEGF was 97-101%. A high significant association was observed between tissue and urinary VEGF levels ($p<0.001$). The present analysis reveals significant positive association between VEGF levels and late stage, high grad, SCC type and bilharzial bladder cancer patients. Moreover, 71.2 % of SCC patients infected with bilhariziasis showed significant high VEGF levels ($p<0.001$) compared to their counterparts with non-bilharzial bladder cancer or TCC type infected with bilhariziasis. Association between increased VEGF levels in tumor tissue and urine of bladder cancer patients may be useful as urinary VEGF may act as a surrogate for VEGF levels within the tumor and be a valuable non-invasive tumor marker. Also, the observations of increased VEGF were more frequent in SCC patients infected with bilhariziasis may suggest a potential role of VEGF in bilharzial bladder cancer vasculogenesis, these results if confirmed, could suggest possible differences in the angiogenic process to evaluate for appropriate antiangiogenic therapy.

Key words: Tissue and urinary VEGF levels, bladder cancer, SCC, bilhariziasis, bilharzial bladder cancer

INTRODUCTION

Carcinoma of the bladder is a significant cause of morbidity and mortality[1]. It represents the most common human cancer in Egypt[2]. There is a universal correlation between the endemicity of schistosoma haematobium, genitourinary bilaharziasis and the frequency of bladder cancer. Because of the geographic coincidence of bladder cancer and endemic bilhariziasis, a causal relationship has been hypothesized and established between tumor and schistosoma haematobium infestation[3]. Although numerous explanations have been proposed for this association, the nature of this relationship still needs to be clarified.

Bladder cancer, like all solid malignancies[4], is dependent on angiogenesis to grow progressively and metastasize efficiently[5]. One of the most studied angiogenic factors is vascular endothelial growth factor (VEGF). VEGF is a secreted, heparin-binding homodymeric glycoprotein of ~ 46 kD, with several protein variants resulting from alternative splicing of VEGF mRNA. It is a potent endothelial cell mitogen, morphogen and vascular permeability-inducing agent[6]. VEGF binds to either one of two tyrosine kinase receptors, the fms-like tyrosine kinase (flt) and the kinase domain receptor (KDR). These receptors are found predominately on endothelial cells[7] and activation leads not only to proliferation and increased vascular permeability but also to the expression of a number of proteolytic enzymes involved in the process of angiogenesis[8].

Studies of VEGF in bladder cancer have been conducted primarily by molecular techniques[9], immunohistochemical methods[10] and microvessel density[11]. Although these methods are important in investigating angiogenic characteristic of VEGF; they are subjective, cumbersome for clinical applications

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and their reproducibility is controversial. In contrast, enzyme immunoassay (EIA) method allows rapid quantitation and objective assessment of VEGF for clinical purposes. In the present study, we attempted to determine whether increased level of VEGF in tumor tissues are correlated with urinary levels of VEGF in bladder cancer patients and to study their association with classical clinicopathological parameters.

MATERIALS AND METHODS

Patients: We studied bladder cancer tissue specimens from 120 patients who had undergone transuretheral biopsy or resection and cystectomy. After written consent was obtained from the patients for measuring VEGF in tissue and urine samples. Each tissue specimen was snap frozen in liquid nitrogen. Fresh midstream urine samples were also obtained before the surgery as a routine part of the patient's work-up. Patient demographic data and medical history were obtained at the study entry and patients who had undergone previous treatment was excluded from the study. All patients underwent cystoscopy as a reference standard for detection of bladder cancer and all tumors or suspicious lesions were either resected or biopsed. A portion of the biopsy was stained with hematoxylin and eosin to evaluate the histopathological diagnosis; only lesions containing more than 90% cancer cells were included in this study. Another portion of the biopsy was placed in liquid nitrogen and stored at -80C for performing estimation of VEGF content.

Among the 120 bladder cancer patients, 73 cases were SCC and 47 cases were TCC type. Bilhariziasis was diagnosed in 67 samples. Clinical staging has been done according to the TNM classification (UICC, 1992)[12] as follows: 59 early stage and 61 late stages. Histological grading was diagnosed according to criteria recommended by the World Health Organization (WHO, 1973)[13]. Fifty-five patients were low grade (I-II) and 65 were high grade (III) tumors. Lymph node involvement was diagnosed in Fifty-four ones.

Preparation of samples: All steps of sample preparation were devised in our laboratory at Biochemistry Department, National Research Center (Gizza, Egypt) and carried out at 4°C. Tissue pieces weighing 50 mg were homogenized with Dounce homogenizer in 1 ml ice-cold 50mM Tris extraction buffer, pH 7.4 (containing: 5m EDTA, 10ml L^{-1} Triton-X100 and the following protease inhibitors: 1 μg mL^{-1} pepstatin, 0.5 μg mL^{-1} leupeptin and 0.2 mM phenylmethylsulfonylfluoride). All reagents were from Sigma (Saint-Louis, MI). The resulting homogenate was centrifuged for 5 min at 20,000x gravity. Supernatant (cell lysate) was frozen at -80°C. Fresh midstream urine samples (20ml.) obtained before cystoscopy were centrifuged at 1000x gravity for 10 min. The supernatant was separated and stored at-80°C.

Schistosomiasis antibodies: Schistosomiasis antibodies were detected serologically by using Cellogent® Schistosomiasis H kit supplied by Dade Behringwerke AG (Marburg, Germany).

VEGF enzyme-immunoassay (EIA): Human VEGF level was measured using an enzyme-immunoassay Quantikine kit purchased from R and D Systems (Abingdon, UK). This assay uses a quantitative sandwich technique. This system uses a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody raised against recombinant human VEGF. In brief, both VEGF standards and samples (cell lysate and urine supernatant) were placed in the wells pre-coated with murine monoclonal antibody against VEGF; the wells were incubated for 2 hours and allowed to react at room temperature. The resulting immune complexes were bound onto the plate and any unbound reactants were removed by a washing step, followed by addition of horse-radish peroxidase conjugated murine antibody directed to another epitope of VEGF molecule to "sandwich" the immunobilized reaction complex, the wells were incubated for 2 hours on a rotator. Substrate-chromogen solution was used to develop the color after removal of unbounded components. The optical density was read using spectrophotometer set at 490nm. The concentrations of VEGF were determined by comparing the optical density of test samples to the standard curve. Each sample was analyzed in triplicate and the mean values were used as the final concentration. To control for differences; cell lysate concentration VEGF expression was standardized to the total protein content (pg mg^{-1} protein) using Bradford method [14] with bovine serum albumin as the calibrator, while urine samples were normalized by creatinine content (pg mg^{-1} creatinine), the urinary creatinine levels were determined based on Jaffe reaction (Sigma Diagnostics, Saint-Louis, MO).

Statistical analysis: The relation between tissue and urinary VEGF levels were assessed using Linear regression analyses. Kruskal-Wallis and Mann-Whitney U tests were used to evaluate the differences of VEGF levels in relation to clinicopathological parameters and bilhariziasis. All analyses were performed with Statistical Package for the Social Science (SPSS version 10.0 for Windows)). All

reported p values are two-sided. Statistical significance in this study was set as p <0.05.

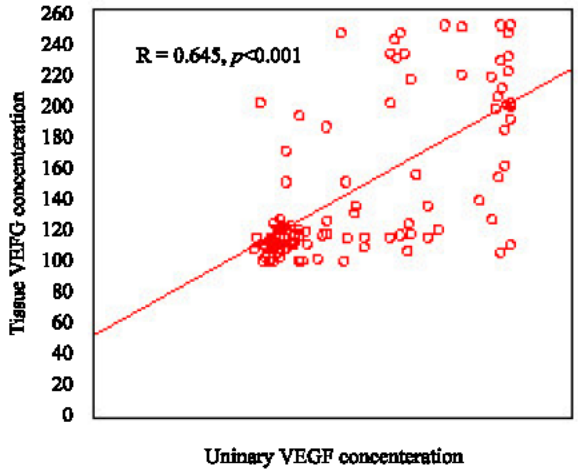


Fig. 1: Correlation of tissue and urine VEGF values in individual subjects. R describes the status of correlation

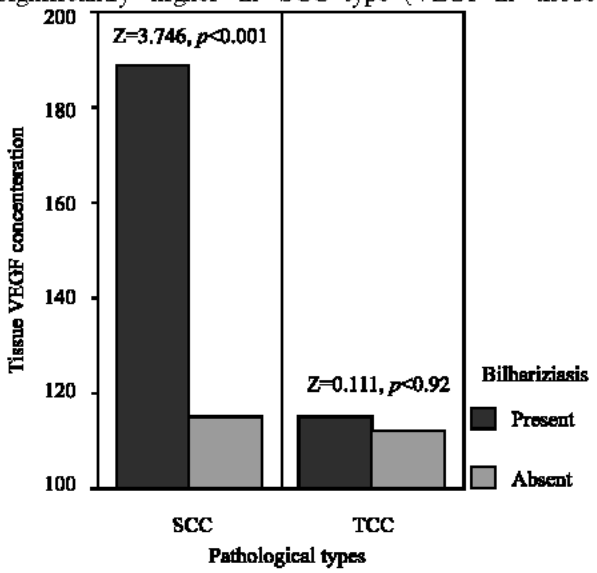
RESULTS

VEGF EIA performance characteristics: We tested the precision of the assay by measuring three tissue lysate pools and urine samples five times in one assay i.e.
inter-assay (within an assay) and in six consecutive assays i.e. intra-assay (between assays). Results are shown in (Table 1).

For VEGF analytical recovery experiments we used three pools for tissue and urine samples (i.e. tissue lysate: 117.5, 199 and 250.2 pg mg $^{-1}$ protein and supernatant of urine samples: 115, 190.2 and 258 pg mg $^{-1}$ creatinine). We assayed each sample in duplicate after addition of three different amounts of VEGF manufactured-supplied kit calibrators (31.2, 125 and 500pg mL $^{-1}$). The calculated recovery range for tissue and urine was 96% to 99% and 97% to 101% respectively, as shown in (Table 2).

Correlation of VEGF level between tissue specimens and urine in individual bladder cancer patient: Using linear regression analysis, we investigated whether there is a correlation between tissue and urinary VEGF levels from the same individuals. As shown in (Fig. 1), tissue and urinary VEGF levels were significantly correlated (R= 0.645 at p<0.001).

Correlation between VEGF levels and classical clinicopathological parameters: We evaluated whether there were differences in VEGF levels in bladder cancer patients regarding to the classical clinicopathological factors (Table 3). Concerning pathological types, mean rank of VEGF was significantly higher in SCC type (VEGF in tissue



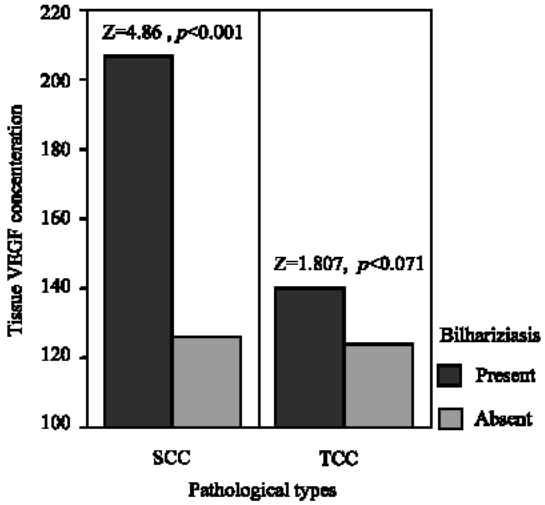


Fig. 2: Comparison of VEGF mean levels in different pathological types based on bilhariziasis (significant difference at p<0.001, nonparametric Mann-Whitney U test)

59.48 pg mg $^{-1}$ protein and urinary VEGF was 60.17 pg mg $^{-1}$ creatinine) than TCC type (VEGF in tissue 35.22 pg mg $^{-1}$ protein and urinary VEGF was 34.03 pg mg $^{-1}$ creatinine). Similarly, mean rank of tissue and urinary VEGF levels were greater in bilharzial bladder cancer patients (tissue and urinary VEGF levels were 58.2 pg mg $^{-1}$ protein and 61.9 pg mg $^{-1}$ creatinine, respectively) than in those with non-bilharzial ones (tissue and urinary VEGF levels were 34.9 pg mg $^{-1}$ protein and 27.4 pg mg $^{-1}$ creatinine, respectively)
(p <0.001).

Table 1: Assay precision of VEGF in studied bladder samples

	Tissue VEGF (pg mg $^{-1}$ protein)		Urinary VEGF (pg mg $^{-1}$ creatinine	
	Mean	CV%	Mean	CV%
Intra-assay(with-in run) (n=5)	117	8.8	115	8.7
	198	7.3	190	6.7
	250	9.2	258	9.5
Inter-assay (between run) (n=6)	117.5	9.1	115.3	9
	199	8.5	190.2	7.5
	250.2	8.3	258.3	6.7

Table 2: Analytical recovery of VEGF in tissue and urine of bladder cancer patients

	Tissue VEGF (pg mg $^{-1}$ protein)			Urinary VEGF pg mg $^{-1}$ creatinine		
Samples	Added	Apparent (measured)	Recovered %	Added	Apparent (measured)	Recovered %
Pool 1	117.5	32.5	96	115	31	101
Pool 2	199	32.2	97	190.2	31.1	97
Pool 3	250.2	31.5	99	258	32.2	97

Table 3: Tissue and urinary VEGF Levels in relation to clinicopathological parameters

Clinicopathological	VEGF level in tissue (pg mg $^{-1}$ protein)			VEGF level in urine (pg mg $^{-1}$ creatinine)		
Parameters (No.)	Mean rank	Median	Z	Mean rank	Median	Z
Pathological type						
SCC (73)	59.48	135	4.04[a]	60.17	192	4.3[a]
TCC (47)	35.22	112		34.03	120	
Bilharzial status						
Present (77)	58.2	135	3.77[a]	61.9	190	5.58 [a]
Absent (43)	34.9	113		27.4	116	
Clinical Stages						
Early (59)	34.8	116	5.3[a]	30.4	125	6.8[a]
Late (61)	65.6	135		69.9	197	
Histological grades						
Low grade (55)	42.1	125	2.6[a]	39.8	186	3.3[a]
High grade (65)	57.3	117		59.2	127	
Lymph node status						
Positive (54)	55.92	135	1.6	58.47	192	2.4[a]
Negative (66)	46.24	112		44.24	120	

[a]Significant difference in VEGF levels at $p< 0.001$ (Z value is non-parametric analysis)

Table 4: Correlation between tissue, urinary VEGF levels and pathological types based upon bilhariziasis

Bladder cancer	VEGF level in tissue[a] (pg mg $^{-1}$ protein)		VEGF level in urine[a] (pg mg $^{-1}$ creatinine)	
patients	Mean rank	Median	Mean rank	Median
SCC with bilhariziasis(n=52)	67.11	188	70.38	205.5
SCC without bilhariziasis(n=21)	34.53	114	28.75	117
TCC with bilhariziasis(n= 24)	35.63	115	40.37	123
TCC without bilhariziasis(n=23)	35.24	112	26.18	115
Statistics	test =30.32		test =45.8	

aSignificant difference in VEGF levels at p< 0.001 (using Kruskal-Wallis analysis)

A total of Sixty-one patients with late stage had significantly elevated both tissue and urinary VEGF levels compared to fifty-nine patients with early stage (Table 3). Accordingly, in contrast to low grade bladder tumor, high VEGF expression levels were found in patients with high grade (grades low-versus high p <0.001). Regarding lymph node status, 45% of bladder cancer patients were lymph node involvement, whereas 55% were lymph node-negative. Although patients with LN-involvement had increased tissue VEGF levels compared to those with LN-negative, the difference between lymph node status and VEGF failed to reach statistical significance. On the contrary, significant difference was observed between urinary VEGF and positive LN-involvement.

Correlation between VEGF levels and pathological types based on bilhariziasis: Increased VEGF levels were observed in SCC patients infected with bilhariziasis (n= 52) compared to non bilharzial SCC ones (n=21). However, no significant difference between TCC patients infected with bilhariziasis (n= 24) compared to non bilharzial TCC ones (n= 23) (Table 4, Fig. 2). On the other hand, our results show significant difference in tissue and urinary VEGF levels between SCC patients infected with bilhariziasis and their TCC counterpart.

DISCUSSION

Angiogenesis, an essential event for tumor growth, is regulated by various angiogenic factors. One of the most important angiogenic factors is vascular endothelial growth factor (VEGF)[8]. Since the results rely upon the quality of the technique, we used in this study EIA to determine VEGF level because qualitative immunological detection provides better quality control, particularly to reproducibility, in addition to the fact that EIA is an easier and less time-consuming technique that would enable us to detect VEGF value. The performance of VEGF using EIA has been evaluated and shown to be reliable for the quantitation of VEGF in minimal amounts of samples (100µl of cell lysates and urine) obtained from bladder cancer patients.

Increased VEGF levels in tissue have been reported by many investigators[15,16], similarly increased urinary VEGF levels have been detected in bladder cancer patients[17]. However, nothing has been reported about whether there is correlation between them, although it is one of the potent angiogenic factors[18]. Therefore, in the current study, we examined the correlation between tissue and released urinary VEGF levels of individual bladder cancer patients. Our results revealed significant association between them; indicating that VEGF level in tissue is crucial for the final urinary VEGF levels as this angiogenic factor in urine is derived from the tumor.

TCC of bladder is the fifth and the fourth most common solid malignancy in the United States[1], however, SCCs are uncommon in developed countries, where they are mostly seen in elderly people or with chronic cystitis[19]. Our study reported significantly higher VEGF levels in SCC compared to TCC types. Previously, it was reported that SCC components are more genetically unstable and had alterations not present in TCC cases[20]. Accordingly, it is possible to postulate that SCCs of the bladder stimulates angiogenesis by directly secreting angiogenic substances or by activating and releasing angiogenic compounds stored within the extracellular matrix[21].

Bladder cancer is a major health problem in Egypt accounting for 29.85% of all malignant diseases from our central registry[2]. The two major types of bladder cancer in Egypt are balharzial bladder cancer and non-balharzial. The present study included 77 bladder cancer patients infected with balhariziasis and revealed high VEGF levels compared to non- balharzial cases. The non- balharzial type is similar to the western type but the balharzial bladder cancer type is more commonly squamous cell carcinoma. In our squamous cell carcinoma group; 71% SCC cases were infected with balhariziasis and showed significant increase in VEGF levels compared to their counterparts of TCC type (51%). More recently, balharzial bladder cancer was found to be positively correlated with over-expression of Bcl-2 (apoptotic marker)[22] that enhance the angiogenesis process; in particular Bcl-2 over-expression induced an increase of VEGF protein secretion as reported by Biroccoi *et al*[23]. From these published findings and our current results, we may hypothesized that molecular changes occurring in bladder cancer patients infected with bilhariziasis can undergo the phenotypic (angiogenic) switch therefore are able to induce phenotypic changes in endothelial cells, leading to angiogenesis. However, further studies with balharzial bladder cancer patients should be aimed at exploring this possible mechanism by providing in vivo evidence for an association between long-term bilhariziasis and increased VEGF levels.

Also, we were interested in whether VEGF levels correlate with classical clinicopatholgical parameters. Investigating VEGF levels in bladder tissue and urine, we found that VEGF elevated significantly in late stage compared with early ones. This could result from the direct angiogenic effect of the growth factor as well as induction of proteolytic enzymes such as urokinase-type plasminogen activator in endothelial cells[24]. Similarly, higher levels of VEGF were observed significantly ($p<0.001$) in high grade compared to low grade bladder tumors, suggesting that VEGF production increases as tumors become more anaplastic. Shinoda[25] and Tuttle *et al*[26] observed similar findings in other tumors. Moreover, our results revealed significant association between increased urinary VEGF levels and positive LN-involvement. Association between increased VEGF levels with late-stage, high-grade and LN involvement in bladder cancer patients indicates that VEGF may play a role in the invasion and metastasis of cancer[17] and may serve as an indicator of tumor progression and future recurrence.

In conclusion, we have demonstrated a consistent association between increased VEGF levels in tissue and its released level in urine of bladder cancer patients using EIA method strengthening the evidence that urinary VEGF may be valuable as molecular marker for monitoring bladder cancer patients. Additionally, our data suggest that bilharizial angiogenesis may be in a part due to induce neoplastic changes in a potent endothelial cell-inducing agent i.e. VEGF. However, further studies with bilhariziasis patients should bring further information about the possible mechanism for the association between bilhariziasis and increased VEGF levels.

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