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Diagnostic Evaluation of Urinary Angiogenin (ANG) and Clusterin (CLU) as Biomarker for Bladder Cancer

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***Abstract*—**Bladder carcinoma is an important worldwide health problem. Both cystoscopy and urine cytology used in detecting bladder cancer suffer from drawbacks where cystoscopy is an invasive method and urine cytology shows low sensitivity in low grade tumors. This study validates easier and less time-consuming techniques to evaluate the value of combined use of angiogenin and clusterin in comparison and combination with voided urine cytology in the detection of bladder cancer patients. This study includes malignant (bladder cancer patients, n= 50), benign (n=20) and healthy (n=20) groups. The studied groups were subjected to cystoscopic examination, detection of bilharzial antibodies, urine cytology, and estimation of urinary angiogenin and clusterin by ELISA. The overall sensitivity and specificity were 66% and 75% for angiogenin, 70% and 82.5% for clusterin and 46% and 80% for voided urine cytology. Combined sensitivity of angiogenin and clusterin with urine cytology increased from 82 to 88%.

***Keywords*—**Angiogenin, Bladder Cancer, Clusterin, Cytology.

I. Introduction

BLADDER Cancer represents a global health problem. It is the seventh common human cancer. The American Cancer Society's estimates that, there would be 72,570 new cases of bladder cancer about 54,610 in men and 17,960 in women and 15,210 deaths from bladder cancer about 10,820 in men and 4,390 in women in 2013 [1]. In Egypt, Bladder cancer has been attributed to *Schistosoma* infection, a major risk factor for squamous cell carcinoma (SCC). Recently, transitional cell carcinoma (TCC) incidence has been increasing while SCC has declined [2].

Combination of cystoscopy and urine cytology is considered to be the ‘‘gold standard’’ for identification of bladder tumors [3]. However, Cystoscopy is the reference standard for diagnosis of bladder cancer patients. The procedure can be uncomfortable and can lead to problems in compliance [4]. Cytology of voided urine is the most established noninvasive method in the diagnosis and follow-up in patients with a history of bladder cancer and is used as an adjunct to cystoscopy [5]. Although it is a convenient noninvasive test, it has high sensitivity in high-grade tumors but low sensitivity in low-grade tumors [6]. Because of the low sensitivity of urine cytology, the invasive nature of cystoscopy, and the high cost, efforts have been put forth to find urinary biomarkers that would be noninvasive, simple, efficient, and objective and have high sensitivity and specificity [7].

Angiogenin (ANG) is a 14 KDa, non-glycosylated polypeptide so named for its ability to induce new blood vessel growth. Accumulating evidence indicates that the angiogenic activity of ANG is related to its ability in regulating ribosomal RNA (rRNA) transcription [8]. Angiogenin plays an important role in angiogenesis of urinary bladder cancer which initiates cell migration and aids in proliferation and differentiation of endothelial cells. Since it may have a role in the development and evolution of carcinomas, it is a particularly interesting molecule to study as a potential tumor marker and / or prognostic indicator for urinary bladder cancer [9].

Clusterin (CLU) is 80 KDa, a multifunctional secretory glycoprotein. Clusterin is found in all body fluids. Clusterin can modulate cell-cell and/or cell-matrix interactions, and has a variety of functions including transporting lipoproteins, the inhibition of complement-mediated cell lysis, regulation of survival/apoptosis, tissue remodeling and tumor genesis [10]. Clusterin has a potential oncogenic role in the development and/or progression of several human cancers including prostate cancer [11], breast carcinoma [12], lung [13], and colon [14] as well as urinary bladder cancer [15]. Few reports do, however, suggest decreased clusterin levels in specific cancers, including esophageal squamous cell carcinoma [16], prostate [17], and pancreatic cancer [18].

We supposed that the combined analysis of these two markers with different functional molecular targets could improve the general sensitivity and specificity of bladder cancer diagnosis in urine. In this study, we evaluated expression of angiogenin (ANG) and clusterin (CLU) mRNA in voided urine of patients with bladder cancer, benign urological disease and healthy volunteers. The value of combining use of the two biomarkers alone or with voided urine cytology was also evaluated.

II. Materials and Methods

A. Patients Database

Between October 2011 and October 2012, 70 subjects admitted to the national cancer institute in Egypt, were included in the study after giving informed consent. All

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patients provided a single approximately 50– 100ml morning voided urine sample for the urine sediment before cystoscopy. Of the 70 subjects, 50 were histologically diagnosed as bladder cancer patients (mean age ±SD: 61.56 ± 9.79; range: 44-91). Whereas the remaining 20 patients (mean age ± SD: 64.1 ±9.86; range: 36–80) suffering from hematuria due to non-neoplastic causes as control (urinary tract infections, stones, benign prostate hyperplasia and combined disorders). A group of 20 healthy volunteers (mean age ± SD: 50.05 ± 8.3; range: 35–65) was also included in this study. All subjects except 20 healthy volunteers underwent cystoscopy as a reference standard for detection of bladder cancer, and all tumors or suspicious lesions were resected for histopathological examination. The final diagnosis of bladder cancer was based on histological examination. Tumor staging and grading was determined according to TNM and World Health Organization classification [19].

B. Sample Collection and Cytological Preparation

Blood and urine samples were collected and transported to the laboratory on ice. Blood samples were centrifuged and sera were separated, and stored at -80°C. Voided urine samples were collected and separated by centrifugation at 2500-4000rpm for 15-20min and separated into urine supernatants and urine pellets. Then, supernatants were divided into aliquots and stored at -80°C while pellets were preserved in a protease inhibitor cocktail and were stored at -80°C. Part of each pellet was applied on a slide, dried in air, fixed with 95% ethanol, stained by Papanicolaou stain and sent to the pathologist for cytology examination to detect malignant cells. Urine cytology was carried out in the cytopathology laboratory at oncology diagnostic unit by an expert pathologist.

C. Quantitative Determination of Bilharzial Antibody

Bilharzial antibodies were measured in all patients ' serum by using indirect haemagglutination test IHA, Schistosomiasis FUMOUZE Kit, LEVALLOIS-PERRET CEDEX/FRANCE.

D. Enzyme-Linked Immune Sorbent Assay for Angiogenin and Clusterin

The levels of human Angiogenin (Cat # CSB-E04498h), and human Clusterin (Cat# CSB-E09121h) were measured in urine supernatant samples using enzyme-linked immune sorbent assays (ELISA). The assays were conducted according to the manufacturer's instructions. Calibration curves were prepared using purified standards for each protein assessed. Curve fitting was accomplished by either linear or four-parameter logistic regression following manufacturer's instructions.

E. Statistical Assay

The threshold value for optimal sensitivity and specificity of angiogenin and clusterin were determined by a receiver operating characteristics (ROC) curve. The cutoff that maximized the sum of sensitivity and specificity for discrimination between malignant and nonmalignant (benign and normal) groups was chosen. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for angiogenin, clusterin, and cytology were calculated using the 2x2 contingency table. For simultaneous evaluation of the two markers, the result was considered positive when any marker of the two markers was positive and negative for diagnosis of tumor when all markers were negative. The positive rates were compared by chi-square test. The nonparametric Kruskal–Wallis test was used for the statistical comparison of the variables between the various groups. The level of significance was determined to be less than 0.05. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) (SPSS version 20, Chicago, IL, USA).

III. Results

A. Distribution of Evaluated Urinary Markers among the Studied Group

Referring to the ROC curve, the best cutoff value for angiogenin was 145pg/ml, whereas the area under the curve was 0.803 (Fig. 1). The best Cutoff value for clusterin was 15ng/ml. whereas the area under the curve was 0.817 (Fig. 2). As shown in (Table I), angiogenin positivity rate (≥ 145pg/ml) was detected in 66% of the malignant group compared with 30% in benign group and 20% in healthy subjects (P <0.001). Clusterin positivity rate (≥15ng/ml) was detected in 70% of the malignant group compared with 30% in benign group and 5% in healthy subjects (P <0.001). Urine cytology was detected in 46% of the malignant group, 40% in benign group and 0% in healthy subjects. Another statistical method (Kruskal-Wallis test) was used and significant difference between malignant group and non malignant group was found for both angiogenin and clusterin (P <0.001) (Table II).

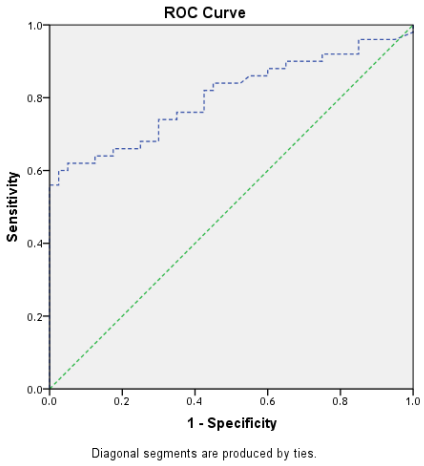

Fig. 1 Roc Curve for Angiogenin

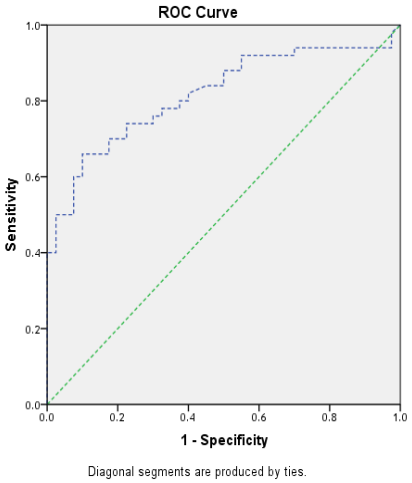

Fig. 2 Roc Curve for Clusterin

TABLE I
COMPARISON OF ANGIOGENIN (ANG), CLUSTERIN (CLU), AND URINE CYTOLOGY AMONG NORMAL, BENIGN, AND MALIGNANT GROUP USING CHI-SQUARE TEST

Urine markers	Malignant group (n=50) (%)	Benign group (n=20) (%)	Normal group (n=20) (%)	Chi-square X^2	*P* value
Angiogenin					
No. of positive cases (≥145 pg/ml)	33 (66%)	6 (30%)	4 (20%)	15.373	0.000
No. of negative cases (<145 pg/ml)	17 (34%)	14 (70%)	16 (80%)		
Clusterin (ng/ml)					
No. of positive cases (≥15 ng/ml)	35 (70%)	6 (30%)	1 (5%)	27.121	0.000
No. of negative cases (<15 ng/ml)	15 (30%)	14 (70%)	19 (95%)		
Cytology					
No. of positive cases	23 (46%)	8 (40%)	0 (0%)	13.739	0.001
No. of negative cases	27 (54%)	12(60%)	16(100%)		

TABLE II
COMPARISON OF ANGIOGENIN (ANG), CLUSTERIN (CLU), AND URINE CYTOLOGY AMONG NORMAL, BENIGN, AND MALIGNANT GROUP USING KRUSCAL WALLIS TEST

Urine markers	Malignant group (n=50)	Benign group (n=20)	Normal group (n=20)	Chi-square X^2	*P* value
Angiogenin (pg/ml)					
Mean Rank	57.63	32.20	28.48		
Median	307.45	45.46	27.46	24.460	0.000
Range	1233.86	289.58	196.16		
Clusterin (ng/ml)					
Mean Rank	58.17	35.23	24.10		
Median	38.19	10.535	5.22	28.278	0.000
Range	748.75	56.88	29.57		

B. Markers Positivity in Relation to Different Clinicopathological Factors

On comparing the positivity rates for angiogenin and clusterin in relation to different clinicopathological factors in the malignant group. Urinary angiogenin was related to the stage of bladder carcinoma where significantly higher positivity rate was associated to invasive stage (II–III) (100%) than superficial stage (0–I) (43.33%) at P <0.05. Urinary angiogenin was related to disease grade of bladder cancer where significantly higher positivity rate was associated to grade III (100%) than grade II (87.5%) and grade I (0%) at P <0.05. Urinary angiogenin positivity rate was significantly higher in smoker patients with bladder cancer (92.3%) than non-smoker patients (37.5%) at P <0.05. Urinary clusterin was related to disease stage of bladder cancer where significantly higher positivity rate was associated to invasive stage (II–III) (95%) than superficial stage (0–I) (53.33%) at P <0.05. Urinary clusterin was related to disease grade of bladder cancer where significantly higher positivity rate was associated to grade III (100%) than grade II (70.83%) and grade I (42.85%) at P <0.05. Urinary clusterin positivity rate was significantly higher in non-smoker patients with bladder cancer (91.66%) than smoker patients (50%) at P <0.05 (Table III and IV).

TABLE III
POSITIVITY RATE FOR ANGIOGENIN (ANG) AND CLUSTERIN (CLU) IN RELATION TO DIFFERENT CLINICOPATHOLOGICAL FACTORS IN THE MALIGNANT GROUP

Parameter	Total no. of patients	No. of Angiogenin positive patients (%)	No. of Clusterin positive patients (%)
Pathological Type			
SCC	7	7 (100%)	7 (100%)
TCC	43	26 (60.46%)	28 (65.11%)
P value		0.041	0.062 NS
Grade			
I	14	0 (0%)	6 (42.85%)
II	24	21 (87.5)	17 (70.83%)
III	12	12 (100%)	12 (100%)
P value		0.000	0.007
Stage			
0	14	2 (14.28%)	7 (50%)
I	16	11(68.75%)	9 (56.25%)
II	10	10(100%)	10(100%)
III	10	10(100%)	9 (90%)
P value		0.000	0.016
Smoking			
Smoker	26	24 (92.3%)	13 (50%)
Non Smoker	24	9 (37.5%)	22 (91.66%)
P value		0.000	0.019
Bil			
Negative Bil	37	21 (56.75%)	26 (70.27%)
Positive Bil	13	12 (92.3%)	9 (69.23%)
P value		0.02	0.994 NS
Cytology			
Negative Cytology	27	15 (55.55 %)	17 (62.96%)
Positive Cytology	23	18 (78.26%)	18 (78.26%)
P value		0.091 NS	0.239 NS
Gender			
Male	38	21 (55.26%)	26 (68.42%)
Female	12	12 (100%)	9 (75%)
P value		0.004	0.665 NS

TABLE IV
POSITIVITY RATE FOR ANGIOGENIN (ANG) THE LEVEL OF ANGIOGENIN (ANG) AND CLUSTERIN (CLU) GROUPS IN RELATION TO DIFFERENT CLINICOPATHOLOGICAL FACTORS IN THE MALIGNANT GROUP

Parameter	Total no. of patients	Angiogenin concentration (pg/ml) Mean Rank	Clusterin concentration (ng/ml) Mean Rank
Pathological Type			
SCC	7	33.64	39.29
TCC	43	24.17	23.26
P value		0.111 NS	0.007
Grade			
I	14	7.82	13.39
II	24	28.10	25.54
III	12	40.92	39.54
P value		0.000	0.000
Stage			
0	14	10.39	16.25
I	16	27.59	19.38
II	10	35.95	36.65
III	10	32.85	37.10
P value		0.000	0.000
Smoking			
Smoker	26	33.88	32.10
Non Smoker	24	16.42	18.35
P value		0.000	0.001
Bil			
Negative Bil	37	22.26	24.81
Positive Bil	13	34.73	27.46
P value		0.008	0.573 NS
Cytology			
Negative Cytology	27	25.33	21.26
Positive Cytology	23	25.74	30.48
P value		0.915 NS	0.026
Gender			
Male	38	23.88	23.16
Female	12	30.63	32.92
P value		0.162 NS	0.043

C. Overall Sensitivity, Specificity, PPV, and NPV of Each of the Investigated Bladder Cancer Marker either Alone or in Combination

At the best cutoff for angiogenin (≥ 145pg/ml), the sensitivity was 66% and the specificity was 75%. At the best cutoff for clusterin (> 15 ng/ml), the sensitivity was 70% and the specificity was 82.5%. The sensitivity was raised to reach 82% when angiogenin was combined with clusterin and the specificity decreased to 67.5%. This sensitivity was raised to 88% when angiogenin was combined with clusterin and urine cytology and the specificity decreased to 55% (Table V).

TABLE V
OVERALL SENSITIVITY, SPECIFICITY, PPV, AND NPV OF EACH OF THE INVESTIGATED BLADDER CANCER MARKER EITHER ALONE OR IN COMBINATION

Parameter	Sensitivity	Specificity	PPV	NPV
ANG	66%	75%	76.74%	63.82%
CLU	70%	82.5%	83.33%	68.75%
Cytology	46%	80%	74.19%	54.23%
ANG+CLU	82%	67.5%	75.92%	75%
ANG + Cytology	76%	62.5%	71.69%	67.56%
CLU + Cytology	80%	65%	74.07%	72.22%
ANG + CLU + Cytology	88%	55%	70.96%	78.57%

IV. DISCUSSION

In the present study, in an attempt to improve the sensitivity and the specificity for diagnosis of bladder carcinoma, the authors evaluated the efficiency of detection of angiogenin and clusterin in supernatant of voided urine sample by ELISA. Different combinations of the two markers and urine cytology were tried to achieve the highest sensitivity and specificity. In the current study, cytology results revealed a sensitivity of 46% and a specificity of 80% between the malignant and the nonmalignant groups.

Human angiogenin, expressed in both normal and malignant cells, is a potent angiogenic factor that is believed to initiate cell migration and aid in cellular proliferation. Angiogenin binds to a cell surface and these complex results in plasmin generation, which directly degrades the extracellular matrix facilitating cell migration and invasion [9]. When we compared the correlation between the positivity rate of urinary angiogenin and the different clinicopathological factors in the malignant group, Eissa et al. found that angiogenin was highly expressed in patients with bladder SCC vs. bladder TCC (76% vs. 73%) [9]. In our study angiogenin was also highly expressed in SCC vs. TCC of bladder cancer (100% vs. 60.46%) with significance vale (p= 0.041).

Urquidi et al. analyzed the supernatant of voided urine sample from a cohort of 127 consisted of 64 subjects with active urinary bladder cancer and 63 subjects without active bladder cancer, history of bladder cancer, gross hematuria, urolithiasis or urinary tract infection. The median urinary angiogenin levels in bladder cancer patients vs. benign subjects were 410.98pg/ml vs. 44.58pg/ml respectively. Urinary angiogenin had a respectable diagnostic capability; sensitivity of 67%, specificity of 68%, positive predictive value of 96% and negative predictive value of 74% [4]. In the current study, we were able to confirm these results with median urinary angiogenin levels of 307.45pg/ml vs. 45.46pg/ml in cancer vs. benign subjects, respectively. Urinary angiogenin had a respectable diagnostic capability; sensitivity of 66%, specificity of 75%, positive predictive value of 76.74% and negative predictive value of 63.82%.

Angiogenesis plays a central role in both local tumor growth and distant metastasis. Angiogenin appears to be a crucial stimulant for the angiogenic process to allow tumor growth beyond a few millimetres as well as the development of metastasis [20]. This was confirmed in our study, angiogenin was highly expressed in invasive stage (II–III) (100%) than superficial stage (0–I) (43.33%) at P <0.05. Moreover, angiogenin was highly expressed in grade III (100%) than grade II (87.5%) and grade I (0%) at P <0.05.

There are two different but related clusterin protein isoforms, a glycosylated form (secreted or cytoplasmic clusterin of 76–80kDa) as well as an nonglycosylated form (nuclear clusterin of 49kDa protein) that are coded by clusterin gene and are derived by alternative posttranslational processes, from the same precursor of 53kDa protein [21]. Data from in vivo and in vitro studies of clusterin in tumorigenesis have demonstrated that nuclear clusterin was predominantly expressed in normal mucosa of colon may act

as a proapoptosis protein, while cytoplasmic clusterin may function as an anti-apoptosis protein [14]. It is now accepted that the primary function of clusterin in distinct genetic backgrounds of cancer cells is antiapoptotic [22]. This antiapoptotic activity of clusterin may account for the genesis and biologically aggressive behavior of several cancer cells [23].

Stejskal and Fiala have examined clusterin concentrations of the urine in 43 patients with bladder cancer by using ELISA test and compared them with 50 patients with benign urological diseases. They found that urine clusterin were significantly higher in individuals with bladder cancer with sensitivity of 49% and specificity of 92%. They concluded that urine clusterin could be the possible laboratory marker of bladder cancer [24]. Hazzaa et al. used ELISA to measure the concentration of clusterin urine and serum. Clusterin was observed to increase from nontumor control to superficial low grade TCC to invasive high grade carcinoma in both urine and serum ($p<0.001$). The sensitivity and specificity of urine clusterin as a tumor marker for TCC of the bladder was found to be 87.1% and 96.7% respectively [10]. In the current study, we confirmed these results. The sensitivity and specificity of urine clusterin as a tumor marker for bladder was found to be 70% and 82.5% respectively.

Recent data indicate that progression towards high-grade and metastatic carcinoma leads to elevated clusterin levels and altered intracellular distribution of nuclear clusterin. Thus, the function of clusterin in tumors may be related to a pattern shift in its isoform production [25]. This was confirmed in the present study, we found that the mean expression level of clusterin mRNA in SCC specimens was higher than that in TCC (100% and 65.11%) but it was not significant with p value 0.061. Based on the semi-quantitative analysis of clusterin mRNA levels, we found that the clusterin expression level correlated significantly with pathologic stage i.e., overexpression of clusterin was more frequently detected in invasive stages (II and III) when compared to that in superfacial stages (0 and I) with (95% and 53.33%) respectively with p0.016. Moreover, we found that the clusterin expression level correlated significantly with tumor grade. It was higher in grade (III) (100%) than grade (I and II) (42.85% and 70.83%) respectively with p= 0.007. These results were in agreement with many studies which have also documented that increased expression of clusterin was involved in the development and progression of several types of carcinomas. Miyake et al. have reported a close relationship between the expression level of clusterin in TCC of the bladder by Northern blot analysis and pathologic stage and tumor grade when examining human bladder carcinoma specimens [26]. Similarly, Kruger et al. found that clusterin may be used, in addition to conventional and other immunohistochemical prognostic factors, as a supplementary tool to provide more prognostic information in patients undergoing cystectomy for muscle-invasive bladder cancer [15].

A direct comparison between urine cytology, angiogenin, and clusterin showed that clusterin had the highest sensitivity (70%), and the highest specificity (82.5%). Combination of urine cytology with angiogenin increases the sensitivity to (76%), combination of urine cytology and clusterin increases the sensitivity to (80%), and the combination of urine angiogenin and clusterin increases the sensitivity to (82%). Combined use of the three urine markers improved the sensitivity up to (88%) at the expense of specificity (55%).

In conclusion, urinary angiogenin and clusterin can be considered as potentially useful markers in detection of bladder cancer with two different molecular mechanisms as non invasive biomarkers where their combination gives high sensitivity. Moreover, combining the gold standard cytology with the previous markers gives the highest sensitivity and NPV. However, large multicentric studies should be carried out to prove the usefulness of these marker combinations. Moreover, further investigations are still necessary for easier markers analysis and a more economical application to the clinical outcome.

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