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Comparison of cytokeratin 20 RNA and angiogenin in voided urine samples as diagnostic tools for bladder carcinoma

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Abstract

Background: We evaluated the diagnostic efficacy of urinary angiogenin (ANG) and cytokeratin 20 (CK-20) mRNA in comparison with voided urine cytology in the detection of bladder cancer patients.

Objectives and methods: A total of 97 Egyptian patients provided a single voided urine sample for ANG, CK-20 and cytology before cystoscopy. Of the 97 cases, 63 were histologically diagnosed as bladder cancer; 33 with transitional cell carcinoma (TCC) and 30 with squamous cell carcinoma (SCC), whereas the remaining 34 had benign urological disorders. A group of 46 healthy volunteers were also included in this study. Voided urine was centrifuged and the supernatant was used for estimation of ANG by EIA and confirmed by Western blotting (WB). The urine sediment was used for cytology and RNA extraction. CK-20 RNA was detected by RT-PCR.

Results: The best cutoff value for ANG was calculated by a ROC curve as 322.7 ng/mg protein. The median urinary ANG level in bladder carcinoma, benign urological disorders and healthy volunteer groups was: 802.7, 425 and 33 pg/mg protein, respectively. The positivity rate for urinary CK-20 mRNA of the control, benign and malignant groups was 0%, 2.9% and 82.3%, respectively ($P = 0.000$); while the rates for ANG were 11.6%, 54.8% and 75.4%, respectively ($P = 0.000$). There was no significant difference in positivity rates of CK-20 and ANG with respect to sex, smoking, schistosomiasis, urine cytology, tumor grade, tumor stage, hematuria or pus cells. The overall sensitivity and specificity were 71.4% and 90% for voided urine cytology, 75.4% and 70.3% for ANG, and 82.3% and 98.8% for CK-20. Combined sensitivity of voided urine cytology with ANG and CK-20 together (98.2%) was higher than either the combined sensitivity of voided urine cytology with ANG (96.5%) or with CK-20 (91.6%) or than that of the biomarker alone. We demonstrated significant positive correlation between CK-20 positivity with age ($P = 0.043$) and nodal involvement ($P = 0.037$); however, there was no significant correlation between CK-20 and ANG with the other clinicopathological parameters.

Conclusions: Our data indicate that CK-20 and ANG in voided urine had higher sensitivities compared to voided urine cytology. However, when specificity was considered, CK-20 alone had superior sensitivity and specificity compared to ANG and voided urine cytology.
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Introduction

Second to prostatic carcinoma, bladder carcinoma is the most frequent malignant tumor of the urinary tract and the second most common malignancy of the genitourinary system [1]. The early diagnosis of bladder cancer is central

to its effective treatment [2]. Superficial bladder cancer can be successively treated in most cases without the need for more aggressive surgical therapies. Screening for bladder cancer in patients who present to the urology clinic with symptoms of microscopic or gross hematuria or other irritative voiding symptoms is currently done with urinalysis, urinary cytology and office cystoscopy [3].

Cystoscopy is invasive, relatively expensive and uncomfortable for the patient. Cytology and flow cytometry have been assessed as diagnostic tools to replace cystoscopy but their sensitivities are not sufficiently high enough to detect well or moderately differentiated tumors [4].

Cytokeratins are major components of the intermediate filaments of epithelial cells and at least 20 different cytokeratins can be distinguished in human epithelia. The cytokeratins are the intermediate filament proteins characteristic of epithelial cells [5]. In human cells, some 20 different cytokeratin isotypes have been identified. Epithelial cells express between 2 and 10 cytokeratin isotypes and the consequent profile which reflects both epithelial type and differentiation status may be useful in tumor diagnosis [6].

The transitional epithelium or urothelium of the urinary tract shows alterations in the expression and configuration of cytokeratin isotypes related to stratification and differentiation. In transitional cell carcinoma, changes in cytokeratin profile may provide information of potential diagnostic and prognostic significance [7]. However, perhaps the most important recent finding is the demonstration that a normal CK-20 expression pattern can be used to make an objective differential diagnosis between transitional cell papilloma and carcinoma [8]. These findings emphasize the possibility that CK-20 may be a specific biomarker for detecting bladder cancer in voided urinary specimens.

To date, numerous studies have demonstrated that several angiogenesis regulators circulate in the blood and may function as endocrine factors in cancer patients. There is a possible clinical value in evaluating circulating angiogenesis regulators, mainly basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF), ANG, pleiotrophin, thrombospondin (TSP) and endostatin (ES) in cancer patients [9].

Angiogenin (ANG) is a polypeptide with a molecular size of 14.1 kDa and was originally isolated from conditioned medium of the HT29 human colon carcinoma cell line. It is one of the most potent angiogenic factors, and can induce blood vessel growth in the chick embryo chorioallantoic membranes and the rabbit cornea [10]. This protein has 35% amino acid sequence identity with human pancreatic ribonuclease and displays ribonucleolytic activity. Several investigators have reported the enhanced expression of ANG in human carcinoma cell lines and malignant tissues [11,12]. In addition, it has been clearly demonstrated in the various experimental models that the growth of ANG-secreting tumor cells is inhibited by the suppression of ANG function [13–16].

In the current study, the expression of CK-20 in cells isolated from urine was investigated by RT-PCR and the levels of ANG in urine supernatant was measured by EIA in patients with bladder carcinoma, benign urological disorders and healthy volunteers to evaluate the diagnostic efficacy of these molecular markers in detection of bladder cancer.

Materials and methods

Patient population

A prospective analysis was performed on 97 Egyptian patients admitted to the Urology Department, Faculty of Medicine, Ain Shams University Hospital, Egypt, between September 2002 and November 2003, after giving informed consent. All patients provided a single voided urine sample and cytological tests for the urine sediment were performed before cystoscopy. Cystoscopy was done for all patients as the reference standard for identification of bladder cancer. Biopsy of any suspicious lesion was performed for histopathological examination. Of the 97 cases, 63 were histologically diagnosed as bladder cancer (mean age ± SD: 55.9 ± 8.8; range: 40–78); 33 with transitional cell carcinoma (TCC) and 30 with squamous cell carcinoma (SCC), whereas the remaining 34 patients (mean age ± SD: 49.3 ± 15.6; range: 17–79) had benign urological disorders (cystitis [8], stones [17], senile enlargement of prostate [6] and combined disorders [3]). A group of 46 healthy volunteers (mean age ± SD: 22.7 ± 9, range: 17–50) was also included in this study. Bladder mucosal biopsy or transurethral resection of bladder tumor was performed when clinically indicated. Tumor staging and grading was determined according to TNM and World Health Organization classification [17].

Collection of samples

Sera and voided urine was obtained from all these groups before they received any treatment and before they underwent surgery. Approximately 50–100 ml of morning voided urine sample was collected. The sera were used for detection of schistosomiasis antibodies by Cellogent ® Schistosomiasis H kit supplied by Dade Behringwerte AG (Marburg, Germany).

Cytological preparation and RNA extraction

Each urine sample was centrifuged for 5 min at 2000 rpm. Using a pipette, half the sediment was transferred onto a glass slide forming two smears then the slide was quickly immersed in 95% ethanol for fixation, then stained with modified Papanicolaou stain [18] and examined for the presence of malignant cells by the same pathologist. The remaining part of the sediment was processed for RNA extraction according to the manufacturer's instructions by

Purescript RNA isolation Kit (Gentra Systems, USA) using a proprietary-modified salt precipitation procedure in combination with highly effective RNase inhibitors [19].

Microscopic analysis of centrifuged urine for white blood cells (pus) and RBCs (hematuria)

The number of white blood cells (WBCs) and red blood cells (RBCs) were counted per high power field. Up to 12 WBCs or RBCs is considered normal, more than 12 and up to 50 is considered moderate, and more than 50 is considered severe. We correlated the count of WBCs and RBCs to the marker positivity levels.

RT-PCR for CK-20

Was done according to our optimized protocol using RT-PCR beads (Amersham Pharmacia Biotech, USA). Briefly, single-stranded complementary deoxyribonucleic acid (cDNA) was synthesized by random priming of 1–5 μg total RNA using 1 μl of reverse transcriptase for 1 h at 42°C. After heating at 94°C for 5 min, the amplification reaction was carried out with 25 pmol of each primer [a sense primer which lies in exon 1 (5′-CAGACACACGGTGAAC-TATGG-3′) and antisense primer which lies in exon 3 (5′-GATCAGCTTCCACTGTTAGACG-3′)] [20] with denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min for 32 cycles followed by incubation at 72°C for 5 min. The amplified CK-20 cDNA of 370 bp was separated on a 2% solution of agarose gel and visualized by ethidium bromide staining (Fig. 1). The PCR conditions were performed in a PCR thermocycler (Hybaid limited, UK).

Detection of Angiogenin (ANG) by EIA

Quantitative determination of the human angiogenin (ANG) concentration in urine was done by a solid phase
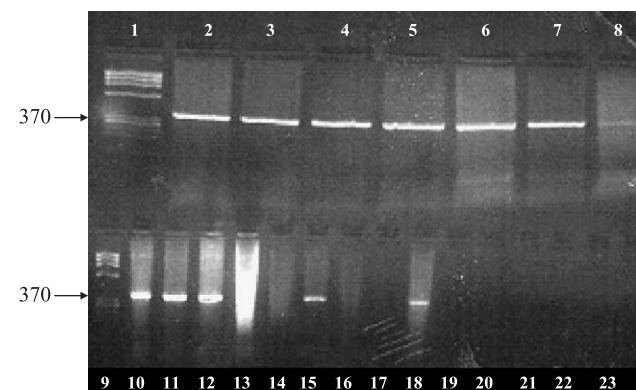

Fig. 1. RT-PCR product analysis of CK20 (370-bp band) by agarose gel electrophoresis and ethidium bromide staining. Lanes 1, 9: molecular weight markers. Lanes 2–12, 15, 18: urine samples from bladder carcinoma patients; and lanes 13,14,16,17: urine samples from benign urological lesions and 19–23 normal urine samples.
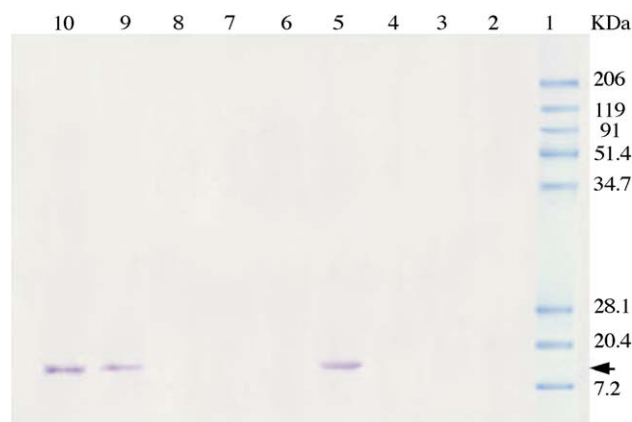

Fig. 2. SDS-PAGE of voided urine supernatant. Note that samples from malignant bladder patients in lanes 5, 9 and 10 show a single band of approximately 14 kDa, which corresponds to the molecular mass of ANG protein. The intensities of the band varied according to ANG concentration. Lane 1 contains molecular mass standards.

EIA (Quantikine, R&D system, USA). This assay employs the quantitative sandwich enzyme immunoassay technique [21]. Briefly, a monoclonal antibody specific for ANG has been pre-coated onto a microplate. Two hundred microliters of standards and samples were pipetted into the wells and incubated for 1 h at room temperature. Any ANG present is bound to the immobilized antibody. After washing any unbound substances, 200 μl of ANG conjugate (an enzyme-linked polyclonal antibody specific for ANG) was added to the wells and incubated for 1 h at room temperature. Following a wash to remove any unbound antibody-enzyme reagent, 200 μl of a substrate solution was added to the wells and incubated for 20 min at room temperature and protected from light. The color develops in proportion to the amount of ANG bound and color development was stopped with 50 μl of stop solution added to each well with the intensity of the color measured using a microplate reader set to 450 nm and read again at 540 nm. The readings at 540 nm were subtracted from the readings at 450 nm. A standard curve was created using computer software to determine ANG concentrations that are expressed in pg/ml. The protein concentration (mg/ml) in urine was determined by Bradford's [22] method using bovine serum albumin as a calibrator. The ANG concentration in urine was then expressed as pg ANG/mg protein.

Detection of angiogenin (ANG) protein by Western blotting (WB) technique

Western blots were performed according to Sambrook et al. [19] and adapted by us as follows: we used 12% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis to separate 20 μg sample protein of urine supernatant. The gels were transferred to nitrocellulose (NC) filters in Tris–glycine buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 7.4) for 1 h at 60 V. NC sheets were washed and the unoccupied binding sites were saturated with
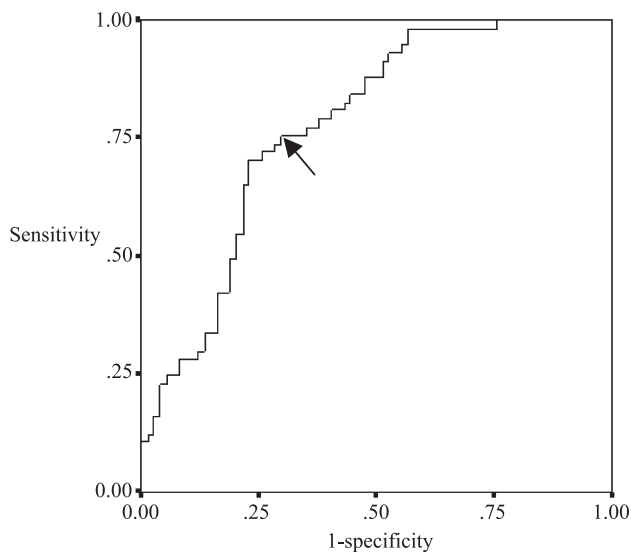
Fig. 3. ROC curve analysis for ANG to calculate the best cutoff to discriminate between nonmalignant and malignant groups. The arrow denotes a best cutoff point of 322.7 ng/mg protein at which sensitivity = 75.4% and specificity = 70.3%. Area under the curve (SE) equals 0.775 (0.04), 95% confidence limits range = 0.697–0.854, $P = 0.000$.

blocking solution (Chromogenic Western blotting kit, Biorad-Roche Diagnostics, GmbH, Germany) for 1 h at 37°C; the sheets then were incubated with 0.1 μg/ml of either antihuman angiogenin monoclonal antibody (MOAB) (R&D Systems, USA) or normal mouse IgG serum (negative control) overnight at 4°C. The membranes were washed with Tris buffer saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.5). The antibodies that bound to the NC membrane were visualized by incubation with anti-mouse IgG-alkaline phosphatase conjugate for 90 min at room temperature (RT). Finally, the filters were incubated with alkaline phosphatase substrate solution (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium in 0.1 M Tris buffer) at RT until the developed bands were of desired intensity. By comparing the resulting developed NC with others in which normal mouse IgG serum was substituted for ANG MOAB, the ANG band was identified (Fig. 2).

Statistical analysis

The threshold value for optimal sensitivity and specificity of ANG was determined by a receiver operating characteristics (ROC) curve, which was constructed by calculating the true-positive fraction (sensitivities %) and the false-positive fraction (100-specificity %) of ANG at several cutoff points [23]. The ROC curve can be used to select a cutoff for the diagnostic test that maximizes the sensitivity and minimizes the false-positive rate. The non-parametric Mann–Whitney rank sum U test and Kruskal–Wallis test were used for the statistical comparison of the variables between the various groups. The positivity rates were compared by chi-square test. 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, Chicago, IL) on an IBM personal computer.

Results

The benign and healthy normal groups were combined in a nonmalignant group and the best cutoff value for ANG was calculated by the ROC curve as 322.7 ng/mg protein. The area under the curve can range from 0.5 to 1, and diagnostic tests that approach 1 indicate a perfect discriminator. In our study, the area under the curve for ANG was 0.775 (Fig. 3). There was a statistically significant difference in the level of ANG between the three study groups (chi-square = 58.6, $P = 0.000$). The median urinary ANG levels in bladder carcinoma patients, benign urological disorders patients and healthy volunteers were 802.7, 425 and 33 ng/mg protein, respectively, and the mean rank was 86.39, 78.5 and 29.97 ng/mg protein, respectively ($P = 0.000$), Table 1.

The positivity rates for CK-20 mRNA in the voided urine samples of the control, benign and malignant groups were 0 (0%), 1/34 (2.9%) and 51/63 (82.3%), respectively (chi-square = 98.83, $P = 0.000$); while positivity rates for ANG were 5/43 (11.6%), 17/31 (54.8%) and 43/57 (75.4%), respectively (chi-square = 40.37, $P = 0.000$) (Table 2).

As shown in Table 3, the overall sensitivity and specificity were 71.4% and 90% for voided urine cytology ($n = 63$), 75.4% and 70.3% for ANG ($n = 57$), and 82.3% and 98.8% for CK-20 ($n = 62$). Combination of the three diagnostic methods gave the highest sensitivity (98.2%), while combination of voided urine cytology with ANG gave 96.5% and voided urine cytology with CK-20 was 91.6%.

There was no significant difference in positivity rates of CK-20 and ANG with respect to sex, smoking, schistosomiasis, urine cytology, tumor grade, tumor stage, hematuria or presence of pus. However, we noticed a significant

Table 1
Angiogenin in voided urine in the three study groups

Groups	Normal	Benign	Malignant
Angiogenin (pg/mg protein)			
Mean ± SEM	116 ± 28.5	1070.3 ± 203.7[a]	1869.5 ± 388.4[b]
Median	33	425	802.7
95th percentile	644	3965.5	10140.4
Range	0.65–850	25–4363.6	24.6–15862.1
Mean rank	29.97	78.5	86.39
Statistics (Kruskal–Wallis test)	chi-square = 58.55, $P = 0.000$		

No statistically significant difference between benign and malignant groups by Mann–Whitney U test, $Z = 1.096$, $P = 0.273$.
[a] Statistically significant different compared to the normal group by Mann–Whitney U test, $Z = 5.621$, $P = 0.000$.
[b] Statistically significant different compared to the normal group by Mann–Whitney U test, $Z = 7.217$, $P = 0.000$.

Table 2
Positivity rates for studied parameters in the three study groups

	Angiogenin greater than 322.7 pg/mg protein (%)	Positive CK-20 RT-PCR (%)	Positive voided urine cytology (%)
Normal	5/43 (11.6%)	0 (0%)	0 (0%)
Benign	17/31 (54.8%)	1/34 (2.9%)	8/34 (23.5%)
Malignant	43/57 (75.4%)	51/62 (82.3%)	45/63 (71.4%)
	chi-square = 40.37, P = 0.000	chi-square = 98.83, P = 0.000	chi-square = 61.655, P = 0.000

association between CK-20 with age and lymph nodal involvement (Table 4).

Discussion

Cystoscopy and bladder biopsy remain the primary methods in the diagnosis of bladder carcinoma. Screening as well as follow-up of patients for bladder malignancy is usually performed by urinary cytology, which is highly sensitive in the recognition of poorly differentiated urothelial carcinomas but has poor sensitivity in well-differentiated bladder tumors [24]. Although the sensitivity for tumors of low grade has been controversial less than 10–70% for low-grade transitional cell carcinomas, 40–80% for high-grade tumors [25], these sensitivities are similar to those obtained in this work.

Proteomic profiling of urine has been suggested as a diagnostic test for bladder cancer [26]. In addition, many other biochemical and genetic markers have been discovered that could be used to diagnose bladder cancer with fair sensitivity and specificity (reviewed in Ref. [27–29]). Among the various markers reviewed, the average published sensitivity and specificity for Brad tumor antigen "BTA" is 68% and 66%; BTA-TRAK, 71% and 62%; nuclear matrix metalloproteinase-22 "NMP22", 64% and 71%; telomerase, 74% and 89%; hyaluronic-hyaluronidase "HA-HAase", 91% and 86%; immunocyt, 68% and 79%; fibrinogen/fibrinogen degradation products "F/FDP", 68% and 86%; multicolor fluorescence in situ hybridization "FISH", 84% and 90%; cytokeratins, 76% and 84%; metalloproteinases, 60% and 80%; p53 mutation, 30% and 100%; microsatellites, 89% and 100%; calreticulin, 73% and 86%.

Cytokeratin 20 is a member of a family of cytoskeletal-associated intermediate filaments and is normally expressed in the umbrella cell layer of the bladder [30]. The CK-20 amplification band (370 base pairs) was obtained with mRNA extracted from transitional cell carcinoma cells of a bladder tumor. Sensitivity of the method was 91% whereas specificity was 67% without false-positive results in the healthy control group [31]. The same results were reported by Buchumensky et al. [32] with higher specificity (74.1%).

Detection of CK-20 mRNA by RT-PCR in urine seems to contribute an additional method for detection of bilharzial bladder carcinoma. In the present study, CK20 RNA was detected in 82.3% of the bladder carcinoma group and in one case of the 34 benign group (2.9%), while the CK-20 test was negative for all of the 46 healthy volunteers. Degenerative change or exfoliation of bladder cancer cells can cause release of CK-20 into urine [33], which explains the marked differences among malignant, benign and normal groups in our study. The overall sensitivity, specificity, PPV, NPV and accuracy were 82.3%, 98.8%, 98.1%, 87.8% and 91.6%, respectively. Our data show that the sensitivity and specificity of CK-20 is significantly higher (P < 0.05) than that of urinary cytology (82.3% and 98.8% vs. 71.4% and 90%, respectively). We found some cases of discordance, in which cytology was negative but CK-20 was positive since some samples contain very few cancer cells, which may be insufficient for cytological evaluation but detectable by CK-20 RT-PCR. Bonner et al. [34], by using quantitative fluorescence image analysis, could detect with the human eye as few as 2/10,000 cells in a voided urine sample. Buchumensky et al. [32] by using the RT-PCR technique assumed that the CK-20 test is capable of detecting as few as 1/1,000,000 transitional cell carcinoma cells in voided urine sample. Thus, CK-20 test may be advantageous, particularly for the screening of low-grade cancers. Combination of cytological examination with urinary CK-20 improved the sensitivity of both to 91.6% for detection of bladder cancer.

In the present work, 11 false-negative results were detected among the malignant group. One possible explanation for this finding is simply the degradation of CK-20

Table 3
Overall sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of angiogenin, CK-20 and voided urine cytology when tested independently or in combination

Parameter	Sensitivity %	Specificity %	PPV %	NPV %	Accuracy %
Cytology	71.4	90	84.9	80	81.2
ANG	75.4	70.3	66.2	78.8	72.5
CK-20	82.3	98.8	98.1	87.8	91.6
ANG + Cytology	96.5	64.9	67.9	96	78.6
CK-20 + Cytology	91.6	88.8	86.4	93.4	90.1
ANG + CK-20	94.7	61.4	71.1	92.1	78.1
ANG + CK-20 + Cytology	98.2	62.2	66.7	97.9	77.9

Table 4
Positivity rate of angiogenin and CK-20 in relation to clinicopathological factors in patients with bladder carcinoma group

Clinicopathological factors	Angiogenin greater than 322.7 pg/mg protein (%)	Positive CK-20 by RT-PCR (%)
Age		
≤50 years	17 (81)	20 (71.4)
>50 years	26 (72.2)	31 (91.2)
	P = 0.460	P = 0.043
Sex		
Male	32 (76.2)	37 (82.2)
Female	11 (73.3)	14 (82.4)
	P = 1.00	P = 1.00
Smoking		
Neg.	16 (76.2)	20 (87)
Pos.	27 (75)	31 (79.5)
	P = 1.00	P = 0.516
Schistosomiasis		
Neg.	1 (50)	2 (100)
Pos.	42 (76.4)	49 (81.7)
	P = 0.434	P = 1.00
Cancer type		
TCC	23 (76.7)	26 (78.8)
SCC	20 (74.1)	25 (86.2)
	P = 1.00	P = 0.519
Grade		
II	36 (75)	44 (84.6)
III	7 (77.8)	7 (70)
	P = 1.00	P = 0.363
Stage		
I and II	13 (76.5)	17 (89.5)
III and IV	30 (75)	34 (0.79)
	P = 0.906	P = 0.323
Nodal status		
Neg.	20 (74.1)	24 (72.7)
Pos.	23 (76.7)	27 (93.1)
	P = 1.00	P = 0.048
Urine pus cells/HPF		
0–12	14 (70)	18 (78)
13–50	21 (75)	23 (82.1)
Over 50	8 (88.9)	10 (90.9)
	P = 0.549	P = 0.665
RBCs/HPF		
0–12	38 (80.9)	43 (82.7)
13–50	3 (50)	4 (80)
Over 50	2 (50)	3 (75)
	P = 0.120	P = 0.925
Cytology		
Neg.	10 (62.5)	12 (70.6)
Pos.	33 (80.5)	39 (86.7)
	P = 0.183	P = 0.155
CK-20		
Neg.	8 (72.7)	–
Pos.	35 (76.1)	–
	P = 1.00	–

All P values were calculated using chi-square test.

RNA in urinary sediment samples. Moreover, Jiang et al. [35] suggested that CK-20 expression is limited to a subset of urothelial carcinomas. Even in the same tumor, CK-20 expression differs in different cell populations and the percentage of CK-20-positive cells varied from case to case.

Liebert et al. [36] reported that CK-20 is expressed in transitional cell carcinoma (TCC) but was generally absent in squamous tumors of multiple tissue types, including squamous cell carcinoma (SCC) of the bladder. However, Gee et al. [25] found that CK-20 is expressed in bladder tumors from Egyptian patients including TCC and SCC. In the present study, there was no significant difference in CK-20 expression in both tumor types; 78.8% in TCC and 86.2% in SCC. Presence of CK-20 in SCC implies that this molecular marker is also expressed when urothelial cells are transformed to squamous phenotype [37]. So, CK-20 is a suitable test for bilharzial bladder carcinoma in either TCC or SCC.

Jiang et al. [35] found that the lymph node metastases from urinary bladder carcinomas showed immunoreactivity for CK-20. The obtained data showed a positive correlation between urinary CK-20 and nodal status ($P < 0.05$). However, the current results showed no correlation between urinary CK-20 and tumor grade or tumor stage. Since RT-PCR is a sensitive method which is able to detect expression of cytokeratins in a few cells, it is no wonder that it is not suitable for evaluation of grading and staging. The advantage of the method depends on the capability to detect tumors regardless of size, staging and grading. Further studies on superficial bladder tumors will show whether a CK-20 RNA urine test will be useful for screening and/or prognosis of carcinoma of the bladder.

Many investigators have reported that various angiogenic factors including basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and hepatocyte growth factor (HGF), pleiotrophin, thrombospondin (TSP) and endostatin (ES), play crucial roles in the progression of urothelial carcinomas [38–41]. However, nothing has been reported about the significance of human angiogenin (ANG) in the urine of bladder cancer patients, although ANG is one of the potent angiogenic factors. Previous studies demonstrated that ANG is strongly expressed in the tumor tissue and is present in high levels in the serum of patients with invasive urothelial carcinoma compared with healthy volunteers and superficial carcinoma patients [21]. Moreover, the association between ANG and cancer progression and poor outcome in bladder cancer tissue has also been documented [42].

Therefore, in the current study, we examined the levels of ANG in voided urine of healthy volunteers and patients with bladder carcinoma by sandwich EIA, confirmed by Western blotting. The ANG level was higher in patients with bladder carcinoma ($P = 0.000$) than that of the healthy volunteers. From the ROC curve, a cutoff value for ANG was calculated (322.7 ng/mg protein) and 75.4% of the malignant group was positive for ANG. The sensitivity of ANG is slightly higher than that of urinary cytology (75.4% vs. 71.4%) while the specificity is lower (70.3% vs. 90%).

This phenomenon could be because ANG is actually involved in angiogenesis during carcinogenic processes and might, therefore, reflect active cell migration [15]. Another

aspect to consider is that although a tumor can be superficial and still localized to the bladder wall, it might nevertheless be aggressive and thus releasing relative high amounts of ANG directly into urine. These findings suggest that urinary ANG could be used as a new diagnostic biomarker for patients with bladder carcinoma.

The present findings demonstrated no significant correlation between ANG and any of the studied clinicopathological factors. On the other hand, Miyake et al. [21] reported that serum ANG level was associated with a poor prognosis and that serum ANG was an independent prognostic predictor for urothelial carcinoma patients. Further studies are warranted to examine urinary ANG levels as a prognostic factor in bladder carcinoma patients.

Combination of cytological examination with urinary ANG improved the sensitivity to 96.5% for detection of bladder cancer. Combination of the cytological examination, urinary CK-20 and urinary ANG improved the sensitivity up to 98.2%, although it lowered the specificity to 62.2%. A direct comparison between urine cytology, urinary CK-20 and urinary ANG revealed that CK-20 has the highest sensitivity and specificity. However, because several malignant patients were detected exclusively by ANG, the use of ANG in addition to other markers could improve the number of patients detected substantially.

In conclusion, we demonstrated that CK-20 mRNA and ANG had higher sensitivities compared to voided urine cytology. CK-20 alone had the highest sensitivity and specificity. The combined use of markers increased the sensitivity of cytology from 71.4% to 98.2% but at the expense of specificity.

Acknowledgments

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