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Effect of pathogenic bacteria on reliability of CK-19, CK-20 and UPII as bladder cancer genetic markers: A molecular biology study

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ABSTRACT

Objectives: To evaluate the effect of the presence of pathogenic bacteria in urine on three bladder cancer genetic markers (Cytokeratin 19, Cytokeratin 20 and Uroplakin II mRNA), and to evaluate the reliability of each urine marker separately.
Methods: Voided urine samples from 20 bladder cancer patients, 15 patients with urinary tract infection patients and 10 healthy volunteers were collected. Isolation and identification of bacteria was performed followed by determination of antimicrobial susceptibility of isolates. Evaluation of CK-19, CK-20 and UPII mRNA in urine by RT-PCR was carried out.
Results: The most frequent organism isolated was *Escherichia coli* followed by *Klebsiella pneumoniae*. The overall sensitivity and specificity were 47.37% and 68.42%% for CK-19, 57.89% and 100% for CK-20 and 63.1% and 100%for UPII. Combined sensitivity and specificity of CK-20 and UPII biomarkers together was higher than that of each biomarker alone or even more than that of the three combined biomarkers.
Conclusions: E. coli is the most predominant bacteria isolated from bladder cancer patients. Both CK-20 and UPII have different expression levels for both benign and malignant cases. Combined use of UPII and CK-20 may be a promising noninvasive tool for the detection of bladder cancer in urine for patients who have both symptoms of UTI and cancer.

1. Introduction

Bladder cancer (BC) is the most common cancer of the urinary tract with around 380,000 new cases and 150,000 deaths per year worldwide [1]. It has a five times higher prevalence among men than women, and the median age at diagnosis is 65 years [2]. Risk factors associated with the development of BC include carcinogens in tobacco smoke, exposures of occupational origin, urinary tract infections, drinking tap water and certain drugs such as cyclophosphamide. Bladder cancer can sometimes cause microscopic or gross hematuria

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or other irritative voiding symptom such as urination more often than usual and pain or burning during urination. These symptoms are also more likely to be caused by a benign condition such as urinary tract infection and bladder stones.

Urinary tract infection (UTI) is one of the commonest infections to affect humans. Under normal circumstances the urinary tract is sterile and infection develops only when bacterial virulence overcomes normal host defense mechanisms. UTI is most commonly bacterial, but fungal, viral and parasitic infections can occur [3]. Several authors around the world have been reported the Gram negative bacteria of *Escherichia coli* and *Klebsiella* spp. being the most frequent organisms causing UTIs [4–6]. It is estimated that 20%–25% of all human cancers are caused by chronic infection and inflammation. *E. coli* infection might play a role in bladder cancer development, as cancer might be mediated by activation of NF-κB family of transcription factors, that regulates transcription of pro-inflammatory cytokines genes, adhesion molecules and the expression of several pro-survival genes resulting in inhibition of apoptosis and increased inflammation [7]. This also may be due to, small amounts of nitrosamines, which may be produced during infection and could initiate neoplastic or pre-neoplastic changes in the urothelium.

Screening for bladder cancer, in patients with previous symptoms to look for different substances or cancer cells in the urine is currently done with urinalysis, urinary cytology and cystoscopy [8].

Urinalysis is performed to check for blood in the urine or hematuria. However, up to 25% of bladder cancer patients may not have hematuria, even when they have a known bladder tumor [8,9]. Urine cytology is a microscopic examination for urine samples to search for any cancer or pre-cancer cells. Cytology has low sensitivity and specificity, particularly for low-grade tumors, its results are not available immediately and are interpreter dependent [10]. Cystoscopy is a technique that enables visualization of the bladder lining and biopsy of suspicious lesions for histopathological diagnosis and staging, but it is invasive, relatively expensive and uncomfortable for the patients [11].

The gold standard for initial clinical diagnosis of BC remains cystoscopic examination of the bladder coupled with voided urine cytology. High attention is focused on bladder cancer early detection and follow up using urine markers. The marker must be sensitive and specific to detect free cancer cell in urine and quantitatively describing cancer progression [10–14].

Cytokeratins are epithelium-specific intermediate filaments, expressed in various combinations, depending on the epithelial type and degree of differentiation, which may be useful in tumor diagnosis. One of these is cytokeratin-19. "CK-19" which is expressed in epithelium of the bladder. CYFRA 21-1, which measures fragments of cytokeratin 19, has even been used as a prognostic and diagnostic marker for transitional cell carcinoma of the bladder [15]. Another cytokeratin is "CK-20", which is expressed in urothelium of the bladder cancer patients. It can be considered as a marker of urothelial differentiation [8]. However, despite the fact that malignant cells generally retain the intermediate filaments of the progenitor, normal cells do not express the CK-20 gene. These findings emphasize the possibility that CK-20 is a specific marker for detecting bladder carcinoma [15]. On the other hand, Uroplakins are the integral protein subunits of the urothelial plaques. One of these uroplakins is uroplakin II (UP-II). It is readily detected by immunohistochemistry in urothelial carcinomas, but not in non-urothelial tumors [16,17]. UPII expression is not strictly correlative with low pathological grade, despite its being the terminal differentiation products of normal urothelium [18]. So, UP-II can be an excellent marker particularly when combined with other urothelium-restricted markers in the differential diagnosis of bladder cancer [19].

In our work we focused on three urine markers "cytokeratin-20, cytokeratin-19 and Uroplakin II", and the effect of pathogenic bacteria on their reliability.

2. Materials and methods

2.1. Urine collection

Two early morning voided urine samples of about 50–100 ml were collected from 20 bladder cancer and 15 UTI patients admitted to the Urology and Nephrology Center, Mansoura University and from ten normal volunteers. These samples were collected in sterile tightly locked containers after aseptic precautions during obtaining the samples. One sample was collected for bacterial culture, the other was obtained for quantification of gene expression using Real Time PCR.

2.2. Bacterial identification and antimicrobial susceptibility

The collected samples were cultured on C.L.E.D agar and blood agar media (OXOID, ENGLAND). Smears of the isolated bacteria were stained with gram stain and were examined for morphology and arrangement. Bacterial identification and characterization of the most dominant isolates from the samples using automated VITEK 2 according to manufactory instructions were carried out (bioMerieux, Marcy I'Etoile, France) [20].

The identification card for gram-negative bacilli (ID-GNB card) was done by a 64-well plastic card containing 41 fluorescent biochemical tests, including 18 enzymatic tests for amino peptidases and oxidases. The card was inserted in the VITEK 2 reader-incubator module (incubation temperature, 35.5 °C), and was automatically subjected to a kinetic fluorescence measurement every 15 min. The results were interpreted by the ID-GNB database after the incubation period about 3 h. According to Shaaban, Ghozlan [21] the antimicrobial susceptibility tests of the identified bacterial isolates were carried out using VITEK 2 antibiotic susceptibility panel cards.

2.3. Real time PCR

Urine sample was centrifuged for 10 min at 400 × g in cold conditions (2–8 °C). The sediment was preserved in 200 μl RNA Later solution (Qiagen Sciences, Maryland, USA) at −196 °C for further RNA extraction [22]. Total RNA was extracted from urine cells for all the samples according to the protocol employed Trizol (Invitrogen, USA). Then RNA purification using ZYMO Research urine RNA isolation kit (Zymo Research, USA). Purified RNA was stored at −196 °C. RNA concentration

was quantified using Nano Drop (Thermo scientific, USA) and its integrity was determined by 1.5% agarose gel electrophoresis and ethidium bromide staining. Only samples that were observed two RNA characteristic bands and were not degradable were used for real-time PCR (RT-PCR).

1 μg of total RNA was converted into single-stranded complementary deoxyribonucleic acid (cDNA) by random priming using RT First strand kit (Qiagen Sciences, Maryland, USA). Gene expression was performed separately for CK19, CK-20 and UPII using quantitative polymerase chain reaction (qPCR). PCR analysis. *B-actin* was used as housekeeping gene. Each amplification was performed for 40 cycles; a cycle profile consisted of denaturation at 94 °C for 20 s, annealing at 58 °C for 30 s, extension at 60 °C for 30 s. Sequences of the primers used are designed from NCBI as shown in Table 1.

Amplifications were performed by using a 25 μL total reaction volume contains 25 pmole of each primer, 3 μL of cDNA template, 12.5 μL 2× SYBR Green Rox Master Mix (Qiagen Sciences, Maryland, USA) and adjusted volume by nuclease-free water. The plate was inserted in real time thermal cycler (ABI PRISM 7000, Applied Biosystem, California, USA). For each sample, the procedure was carried out in triplicate. A mathematical model introduced by Pfaffl was used for the relative quantification of target genes [23].

2.4. Statistical analysis

Studying the difference of gene expression for urine samples between the three groups, and the relation between bacterial barcodes for samples and percentage of gene expression was performed. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) (SPSS, Chicago, IL, USA). ROC and Kaplan–Meier analysis were used to determine the cut off level of CK-19 CK-20 and UPII mRNA expression. The nonparametric Mann Whitney rank, Pearson, Spearman, Anova, Kruskal and sum U test were used for the statistical comparison of the variables between the various groups. The level of significance was determined to be less than 0.05.

3. Results

Among 20 bladder cancer patients, there were ten and three patients infected with *E. coli* and *Klebsiella pneumonia*, respectively. Out of the 15 patients with UTI, 13 were infected with *E. coli* and 2 with *Klebsiella pneumoniae*. Antibiotic susceptibility results indicated that all *E. coli* isolates were sensitive to imipenem and amikacin (resistance 0%). The resistance rates of other antibiotics were: amoxicillin/clavulanate 88% (24/27), trimethoprim/sulphamethoxazole 81% (22/27), ciprofloxacin 77% (21/27), cefotaxime and ceftazidime 62% (17/27), nitrofurantoin 40% (11/27), piperacillin/tazobactam 19% (5/27). All *K. pneumoniae* isolates were sensitive to imipenem, amikacin and piperacillin/tazobactam 100% (5/5), while the resistance rates were 60% (3/5) for cefotaxime, ceftazidime, trimethoprim/sulphamethoxazole, ciprofloxacin and ciprofloxacin. The resistance rate was 40% (2/5) for amoxicillin/clavulanate.

The expression levels for CK-19, CK-20 and UPII mRNA in the voided urine samples of the bladder tumor were 9/19 (47%), 11/19 (58%), and 12/19 (63%), respectively as shown in Table 2. There was statistical significance ($p < 0.05$) in UPII and CK20 expression level between groups; while there was no statistical significance ($p > 0.05$) in CK19 expression level between groups. So, presence of pathogenic bacteria may affect the reliability of CK19 marker for bladder cancer diagnosis, but has no effect on UPII and CK20 results. Fig. 1 shows different expression levels of *B-actin*, CK-19, CK-20 and UPII mRNA in study groups.

3.1. Sensitivity and specificity of CK-19, CK-20 and UPII RT-PCR

As shown in Table 3, the overall sensitivity and specificity were 47.37% and 68.42% for CK-19, 58% and 100%for CK-20, and 63.1% and 100% for UPII. The sensitivity and specificity of the combined use of CK-20 and UPII were (79%) and (100%), respectively. The latter rates were higher than those for each marker alone and were also higher than the rates for the combination of the three markers. The negative predictive value was 60%, 100% and 100% for CK-19, CK-20 and UPII, respectively. The positive predictive value was 56.5%, 70.3% and 73.08% for CK-19, CK-20 and UPII. Combined use of CK-20 and UPII gave the highest negative predictive value and positive predictive value.

3.2. Bacterial infection and gene expression

Effect of bacterial infection on the ck19, CK20 and UPII mRNA level in bladder cancer patients is shown in Table 4. The level of gene expression was higher in non-infected BC patients than infected BC patients. With respect to bacterial infection, p value for UPII mRNA level was >0.05, while p value for CK-19 and CK-20 mRNA levels were <0.05. So with respect to bacterial infection, there was no significant difference in level of UPII expression in BC patients, while there was statistical significance in the level of CK19 and CK20 expression in bladder cancer patients.

Table 1 – List of gene-specific primers in RT-PCR.

Genes	Forward primer	Reverse primer	An.temp (°C)	(bp)
CK-19	TGCGGGACAAGATTCTTGGT	TCTCAAACTTGGTTCGGAAGTCA	58	102
CK-20	CTGATGCAGATTCGGAGTAACA	TCTCTCTTCCAGGGTGCTTAAC	58	162
UPII	GCATACCAGGTGACAAACCTC	GTTCCTTCGAGGGAGTGTGG	58	120
B-Actin	AGGCACCAGGGCGTGAT	GCCCACATAGGAATCCTTCTGAC	58	51

Table 2 – The positive rates for CK-19, CK-20 and UPII mRNA among study groups.

groups	CK19			CK20			UPII		
	BC	UTI	N	BC	UTI	N	BC	UTI	N
N	19	12	7	19	12	7	19	12	7
SE	0.358	0.152	0.079	5.32	0.00	0.0	8.759	0.063	0.0
Median	0.07	0.06	0.01	0.680	0.00	0.00	3.10	0.01	0.00
p Value	0.58			0.015			0.008		

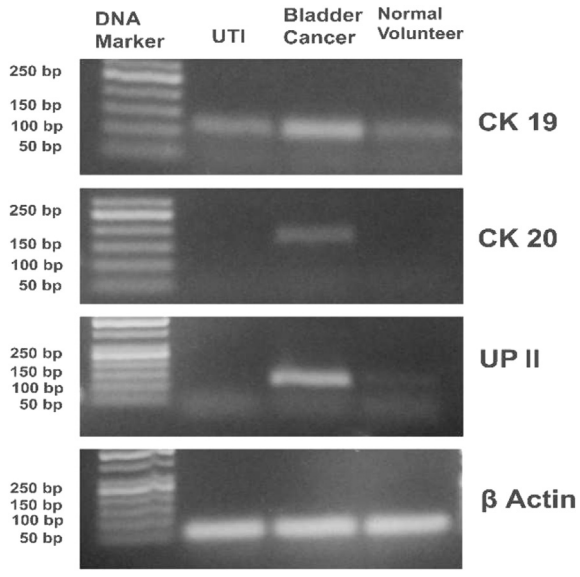

Fig. 1 – Different expression levels of *β-actin*, CK-19, CK-20 and UPII mRNA in study groups.

3.3. *Bacterial infection and tumor progression*

To test the correlation between bacterial infection and tumor progression, tumor grade and stage were compared with respect to infection as it is shown in Table 5. We found that bacterial infection was correlated to high tumor grade.

Table 5 – Correlation between bacterial infection and tumor grade and stage.

	T1	T2	T3,4	GI	GII	GIII
Infected	1	4	5	0	0	9
Non-Infected	5	2	2	1	5	4
p Value	0.80			0.031		

According to p value, with respect to bacterial infection, there was no statistically significant difference ($p > 0.05$) between tumor stages, while the difference was significant ($p < 0.05$) between tumor grades.

3.4. *Correlation between tumor grade and level of gene expression*

Considering the different tumor stages, the median expression of the three genes was elevated in the higher tumor stages and lower tumor grades. With respect to tumor stages, there was no statistical significance ($p > 0.05$) in the expression level of CK-19 and UPII, however a statistical significance ($p < 0.05$) was disclosed in expression level of CK-20 as shown in Table 6.

There was no statistically significant differences in the expression level of the three markers with respect to tumor grades (Table 7).

There was a significant difference in level of CK20 expression in bladder cancer patients among different tumor stages (Tables 6 and 7).

Table 3 – Sensitivity and specificity for separate and combined use of CK-19, CK-20 and UPII expression levels.

	Sensitivity	Specificity	Negative predictive value	Positive predictive value
CK20	57.89%	100%	100%	70.3%
CK19	47.37%	68.42%	60%	56.5%
UP II	63.1%	100%	100%	73.08%
CK20 + UP II + CK19	78.95%	68.42%	71.43%	76.47%
CK19 + CK20	73.68%	68.42%	70%	72.22%
UP II + CK19	63.16%	68.42%	66.67%	65%
Ck20 + UPII	78.95%	100%	100%	82.61%

Table 4 – Correlation between bacterial infection and gene expression level among bladder cancer patients.

Groups	CK19		CK20		UPII	
	Infected	Non infected	Infected	Non infected	Infected	Non infected
N	10	9	10	9	10	9
SE	0.04	0.6	1.2	10.8	2.4	16.1
Median	0.05	0.67	0.44	6.2	1	7.5
p Value	0.027		0.008		0.110	

Table 6 – Correlation between tumor grade & level of gene expression.

Grades	CK19		CK20		UPII	
	GII	GIII	GII	GIII	GII	GIII
N	6	13	6	13	6	13
SE	1.0	0.06	4.02	6.2	20.5	16.1
Median	0.37	0.07	3.12	0.68	34.20	3.1
p-Value	0.368		0.831		0.481	

4. Discussion

It's a well-established fact that gram-negative bacteria of *E. coli* and *K. pneumoniae* are the most common pathogenic bacteria causing UTI, a finding that entirely agree with our results. The current results also pointed out that *E. coli* is the most common uropathogenic bacteria, which infects bladder cancer patients. This finding copes with the suggestion of El-Mosalamy, Salman [7] that urinary bladder infection by *E. coli* may play a major additive and synergistic role during bladder carcinogenesis. It is known that inflammation facilitates initiation and progression of malignancy through production of inflammatory oxidants [24]. In our study we found a significant correlation between tumor grade and bacterial infection, as bacterial infection was more associated with high grade tumors.

Results of antimicrobial susceptibility pattern of *E. coli* isolates revealed that *E. coli* isolates were highly sensitive to Imipenem and Amikacin (100%). However, high resistance rate was reported for amoxicillin/clavulanate, followed by trimethoprim/sulphamethoxazole and then other drugs. Debnath, Das [25] reported that the *E. coli* resistance rates for nitrofurantoin, amikacin, amoxycillin/clavulanic, ciprofloxacin and piperacillin/tazobactum were 4%, 13%, 17%, 50% and 50%, respectively. Therefore, these results are somewhat different from these of the present study. The possible reason for this disagreement may be due to the difference in geographical location, population response or misuse of antibiotic in our locality.

All isolates of K. *pneumoniae*, were highly sensitive to Imipenem, Amikacin and Piperacillin/tazobactam, while the resistance rates were 60% for Cefotaxime, Ceftazidime, trimethoprim/sulphamethoxazole, Cipro-floxacin and Ciprofloxacin and were 40% for Amoxicillin/clavulanate. This result goes hand in hand with that of Vakilwala and Trivedi [26] who reported that the resistance rate for Ceftazidime, Ciprofloxacin and Trimethoprim/sulphamethoxazole were 60%; 48% and 80%, respectively.

Early diagnosis of bladder cancer is mandatory because any delay in treatment has been shown to affect progression. At present, cystoscopic examination and urine cytology remain the primary methods for the initial evaluation of the lower urinary tract lesions to rule out bladder cancer. However, cystoscopy is invasive and may be ineffective in patients with an indwelling catheter or active inflammation due to the presence of abnormal appearance of the bladder mucosa, whereas cytology has low sensitivity in low-grade papillary disease. Additional lab-based markers are needed to aid in the evaluation of these lesions [8]. Genetic marker development represents a promising direction for the diagnosis of bladder cancer [27]. Highly sensitive real-time RT-PCR has already shown its ability to differentiate between malignant and benign tissue and also to determine the stage and grade of tumor tissue [28].

CK-19 is expressed in normal urothelium and can be measured in the urine when urothelial cells are exfoliated. The current study showed that CK-19 is expressed in all group members. We determined cutoff value which gives the highest sensitivity and specificity by the ROC curve. The sensitivity of CK-19 mRNA level was 47.37% and specificity was 68.42%. The obtained sensitivity agrees with the study of Hassanien, Nossier [29] that showed a sensitivity of CK-19 of 31%, however the specificity was higher (100%). Other studies have shown sensitivities ranging from 43% to 79% and specificities ranging from 68% to 88% for CK-19 mRNA and protein [30,31]. The high false positive rates of around 40% indicate that pathogenic bacteria may affect the reliability of considering CK-19 as a marker for bladder cancer detection. Our results showed that CK-19 mRNA level was significantly different among BC patients with or without bacterial infection, while no correlation was found between urinary CK-19 expression level and tumor grade or tumor stage. The latter finding disagrees with the results of El-Salahy [15], where he found significant correlation between CK-19 expression level and tumor grade and stage.

RCK20 are intermediate filaments expressed in epithelial cells. In our study, CK20 is only expressed in BC group, with no false positive results. This absolutely agrees with immunological and northern blot studies found that CK20 expression was restricted primarily to gastrointestinal tissue, urothelial cell carcinoma and merkel cells [32]. In the current study, there was a statistically significant difference in CK-20 mRNA level among BC, UTI and control groups. This finding is

Table 7 – Correlation between tumor stage and level of gene expression.

Groups	CK19			CK20				UPII		
	T1	T2	T3,T4	T1	T2	T3	T4	T1	T2	T3, T4
N	6	6	7	6	6	7		6	6	7
SE	0.77	0.021	1.09	3.03	0.27	50.5		16.6	4.57	46.6
Median	0.25	0.06	1.48	4.4	0.68	50.5		1.8	3.11	53
p-Value	0.43			0.04				0.266		

consistent with the results of Guo, Luo [32]. Other studies have shown sensitivities ranging from 65% to 90% and specificities ranging from 67% to 90% for CK-20 mRNA [27]. On the other hand, some authors reported that CK-20 has a very low specificity [33]. Our results demonstrated that there was a statistically significant decrease in CK-20 mRNA level in BC patients with respect to bacterial infection and there was a significant correlation of CK-20 expression with tumor stage. Similarly, El-Salahy [15] reported a significant correlation between CK-20 mRNA expression and tumor stage. However, there was no correlation with the tumor grade in the present study, a finding which is not coping with the results of El-Salahy [15] but is coping with Pu, Wang [8].

Uroplakins (UPs) are integral membrane proteins that are synthesized as the major differentiation products of mammalian urothelium. Olsburgh, Harnden [17] expression of UPII genes is highly specific to urothelium and they suggested that the tight differentiation-restricted expression of uroplakin genes in normal urothelium is lost following malignant transformation.

UPII was detected in BC and UTI patients as they both cause exfoliation of epithelia cells into urine, while it was not detected in normal group.

We found the overall sensitivity of UPII was 63.1% and specificity was 100%. Its sensitivity in transitional cell carcinoma was 90% in our study. This copes with the suggestion that UPII is a highly specific marker for human TCC [17]. These results agree with those of (Kurahashi, Hara [34]) who reported that UPII mRNA was reliable in early detection of bladder cancer metastasis by testing its level in tissue and blood. We found no significant correlation between UPII mRNA expression level and tumor grade or stage.

In the current study, the combined use of UPII and CK-20 gave a highest sensitivity (78%) and specificity (100%), than the combination of the three markers together or each marker alone. This implies that the combined use of the UPII and CK-20 may be a reliable tool for detecting bladder cancer.

4.1. *Conclusion*

E. coli are the most predominant bacterial isolates among bladder cancer patients. CK-19 urine marker has low specificity for bladder cancer detection. But CK-20 and UPII urine markers show high specificity as they have different expression level in benign and malignant states. Combined use of UPII and CK-20 could represent a promising noninvasive tool for the detection of bladder cancer in urine for patients who have both symptoms of UTI and cancer. So further studies – with large individuals number – on the reliability of the combination of these two markers are recommended.

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