Telomerase Activity, Cytokeratin 20 and Cytokeratin 19 in Urine Cells of Bladder Cancer Patients

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ABSTRACT

Aim of the Study: This work aims to search for markers suitable for the screening of bladder cancer, which should be specific, sensitive, reproducible, non-invasive and at acceptable cost.

Patients and Methods: The study included 50 patients diagnosed as bladder cancer (35 TCC, 15 SCC) of different stages and grades, 30 patients with various urothelial diseases, besides 20 apparently healthy subjects of matched age and sex to the malignant group. A random midstream urine sample was collected in a sterile container for the determination of telomerase by RT-PCR, keratin 19 by ELSA CYFRA 21-1 IRMA kit, keratin 20 by RT-PCR and immunohistochemical staining, and urine cytology.

Results: For all parameters (telomerase, K19, K20 and cytology) the malignant group was significantly different from both the benign and the control groups. None of the four studied parameters was correlated to the stage of the disease, and when it comes to grade, only K19 showed a significant positive correlation with grade both in TCC and SCC. When ROC curves for all parameters were compared, K19 had the largest area under the curve, and then comes K20.

Conclusion:

• K19 may be used as a biological marker for the diagnosis of bladder cancer. K19 could not be used for differential diagnosis of different types of bladder cancer, meanwhile it could be a marker for differentiation that decreases in less differentiated tumors.
• As a tumor marker, K20 reflects inability to differentiate tumor type or grade in TCC, while in SCC of the bladder it is correlated with the grade.
• As a method, RT-PCR is superior to immunostaining for the detection of bladder cancer, meanwhile K20 immunohistochemistry (IHC) results were much better than urine cytology as a bladder cancer screening test.

INTRODUCTION

In Western populations, bladder cancer is the 4th most common malignant neoplasm in men and the 8th in women. It occurs mostly due to smoking and exposure to aromatic amines and is a disease of the old with mean age of 75 years. It accounts for 6% and 2% of all cancers in males and females respectively, with a male/female ratio of 3:1 [1]. About 90% of all bladder tumors are Transitional Cell Carcinomas (TCC), 5% are Squamous Cell Carcinomas (SCC) and the remainders are rare tumors as adenocarcinoma, carcinosarcoma and sarcomatoid lesions [2].

In Egypt, bladder cancer is the first site of incidence for males accounting for 40.1% of all cancer cases, while in females it accounts for 14.4%, ranking second with a male/female ratio of 5:1 [3]. Incidence rates for Alexandria, standardized according to the World Standard Population were 192/1,000,000/year for males and 36/1,000,000/year for females. Bladder cancer death rates in Egypt are the highest for males (108/1,000,000/year) and the fifth for females (23/1,000,000/year) [4].
In Egypt, bilharzial infection has always been looked at as the primary cause of bladder cancer. Schistosoma-associated bladder cancer has a clinicopathologic pattern that differs from that seen in Europe and America. The peak age of diagnosis is 50.5 years. Most tumors are presented as bulky masses with deep infiltration into the bladder wall. The majority is of the SCC type representing 59% of the cases. However, there is a recent trend towards a relative increase in TCC frequency.

The gold standard for bladder cancer screening is still urinary cytology, as it is non-invasive, safe and inexpensive. Although it is highly specific, the results are not reproducible and the interpretation is highly dependent on the skill of the operator. In addition, it exhibits variable sensitivities depending on tumor grade and cannot exclude the presence of malignancy. This calls for the search for other markers for the screening of bladder cancer which should be specific, sensitive, reproducible, non-invasive and at acceptable cost.

Telomeres are protective structures that cap the ends of eukaryotic chromosomes and are composed of both repeated DNA elements and specific DNA-binding proteins. Telomeres are essential for maintaining genomic stability and loss of normal telomere function can lead to end-to-end fusions and chromosome loss by exonuclease digestion. Degradation of telomeres appears to constitute a signal with which a cell is no longer able to undergo cell division. Telomerase, an RNA-dependent DNA polymerase, maintains telomeric DNA. Telomerase synthesizes telomeric DNA sequences and almost universally provides the molecular basis for unlimited proliferative potential. Telomerase activity was found to be absent in most normal human somatic cells but present in over 90% of cancerous cells and in vitro-immortalized cells.

In humans, over 85% of malignant tumors express telomerase activity whereas most somatic tissues do not. Increased telomerase activity strongly correlates with increased malignant potential and stage, in addition, genomic instability associated with loss of telomere sequences correlates with a late stage in the development of colonic carcinomas. Telomerase especially helps in certain tumor types, where indeterminate cases are common, with the general specificity of 91% and sensitivity of 85%.

Telomerase-based methods can be applied in body fluids and washes, making sample collection easier. This, however, might pose the difficulty of sampling error and the presence of infiltrating lymphocytes or other cells, giving false positive results. For detection of bladder carcinoma in urine specimens, telomerase proved far more sensitive than cytology or other available screens. The sensitivity of telomerase RT-PCR assay ranged from 75.6% to 94%, compared with 34% to 51% for routine cytology and the specificity ranged from 72% to 100%.

Cytokeratins (40-70 kDa) are the major structural proteins in the cytoplasm of epithelial cells and their derivatives. They can be subdivided into two major types, I (acidic, K9-K20) and II (neutral / basic, K1-K8). Keratin filaments are built from lateral and longitudinal interactions involving type I-II heterodimers. Each type of epithelial cells synthesizes at least one type I and one type II keratin, which co-polymerize into filaments. The organization of keratin filaments and their association with plasma membranes suggest that their principal function is structural - to reinforce cells and to organize cells into tissues. Keratin filaments are characterized by tissue-specific expression patterns from early embryogenesis onwards, suggesting that these proteins are important in defining tissue structure and potential function. Keratins also influence the availability of regulatory molecules such as apoptosis-inducing factors, heat shock proteins or signaling molecules and so, affect the sensitivity of cells to proliferative and apoptotic stimuli and play a role in cellular stress responses and drug resistance.

Keratin 19 (K19) is a type I keratin protein of 40 kD and is the smallest member of the family. K19 is one of the first keratins expressed early during development. Because of its early expression it has been assumed that K19 has an important function during embryogenesis. Many premalignant and malignant tissues display K19 expression, such as dysplasia and adenocarcinoma of the lung, breast, pancreas, stomach and colon.
Keratin 20 (K20) is the most recently identified type I keratin protein of 46 KD, which shows a limited pattern of expression in normal tissues [22]. K20 is co-expressed along with K18 and K19 in the same intestinal cell types, which suggests that these three type I keratins may have redundant or complementary functions [23].

The expression of K20 in urothelium was restricted to superficial 'umbrella' cells even in the presence of severe inflammation. Only malignancy induced alteration in K20 expression pattern. It has been suggested that the pattern of K20 immunohistochemical staining is a useful adjunct to morphology in the diagnosis of urothelial dysplasia, since only malignant cells will show K20 immunostaining [24]. Because of the lack of immunological cross-reactivity with other cytokeratins, K20 has become an important tool for delineating the origin of metastatic human carcinomas arising from an unknown primary source [25]. Recently, several reports have suggested the use of a reverse transcription-polymerase chain reaction technique for the detection of K20 expression in exfoliated urine cells as a useful, noninvasive diagnostic test for urothelial carcinoma and premalignant urothelial lesions [26-27].

SUBJECTS AND METHODS

Subjects:
The study included 100 subjects classified into three main groups:

I- Malignant Group: Includes 50 patients, diagnosed as bladder cancer patients and confirmed by pathological examination of the biopsies [28]. This group was further divided according to the type of tumor into two main subclasses:

1- Transitional cell carcinoma (TCC) patients: 35 patients of grades I, II and III.
2- Squamous cell carcinoma (SCC) patients: 15 patients of grades I, II and III.

II- Benign Group: Includes 30 patients of matched age and sex as the malignant group, with various non-malignant urothelial diseases including pyelonephritis and cystitis with and without stone formation.

III- Control Group: Including 20 apparently healthy people of matched age and sex as the malignant group.

All the patients of the malignant and benign groups involved in the study were chosen from those admitted to the Urology Department in the Faculty of Medicine, Alexandria University. All the patients of groups I and II were subjected to thorough clinical examination and history taken.

Methods:
Specimen collection: A random midstream urine sample was collected from each patient in a sterile container. Each sample was divided into four aliquots: (1) 100 µl aliquot was separated collectively for bacterial culture, (2) 1 ml aliquot stored at -80ºC until use for the measurement of keratin 19, (3) 10-15 ml aliquot for urine cytology and keratin 20 immunostaining, and (4) the remaining urine sample used for RT-PCR of telomerase and keratin 20. For aliquot 3, the cells shed in urine were separated by centrifugation at 4000xg for 10 minutes then slides were made for urine cytology and immunostaining. For aliquot 4, cells shed in the urine were separated by centrifugation at 4000xg at 4ºC for 10 minutes then washed in PBS then the cell pellet was stored at -80ºC until RT-PCR was performed.

For all the specimens collected of all groups, the following was carried out:

1- RT-PCR: For the detection of telomerase and keratin 20 expression. The method involves:

1- Extraction of mRNA. The cells were dissolved in TRIzol® Reagent (Invitrogen Life Technologies, U.S.A.), which is a mono-phasic solution of phenol and guanidium isothiocyanate. RNA was isolated according to the manufacturer’s instructions and quantified spectrophotometrically.

2- RT-PCR. Reverse Transcription and PCR were carried out by a single-tube, single-step Ready-to-Go RT-PCR Beads (Amer sham Biosciences, U.S.A.), which utilize Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase to synthesize first strand cDNA from RNA. For each of the three cDNAs to be amplified: telomerase, keratin 20 and Glyceraldehyde phosphate dehydrogenase (GPDH); a pair of primers (GenScript Corporation, U.S.A.) were used as forward and reverse primers, their sequences are shown in Table (1).
Electrophoresis of PCR products. RT-PCR products were separated by electrophoresis on 2% agarose gel prepared in Tris Acetate EDTA electrophoresis buffer.

II- IRMA: The soluble fragment of Keratin 19 was measured by the ELSA CYFRA 21-1 Immunoradiometric kit (SIS Bio International, Schering S.A., France), which is a solid-phase “sandwich” immunoradiometric assay.

III- Urine cytology: The slides were fixed in 96% ethyl alcohol and subjected to Hema-toxylin and Eosin Staining.

IV- Immunohistochemical staining: Slides of exfoliated cells sedimented in urine were prepared, fixed and stained with mouse anti-keratin 20 antibody and visualized by Anti-polyvalent/HPR/DAB Detection System (Spring Biosciences, Fremont, CA).

V- Bacterial culture: 10µl of urine were inoculated into blood agar and MacConkey agar plates, incubated for 24h in 37ºC then the colonies were identified using API system. Viable count was done by the method of Miles and Misra [29].

RESULTS

The age and sex distribution of all studied groups were matched and are presented in Table (2). The male: female ratio was 3:1 and the age Mean ± Standard Deviation (M±SD) were 55.9±9.4, 58.5±7.1 and 57.7±6.2 years for the malignant, benign and control groups respectively. Table (3) represents the distribution of stages and grades in different subgroups of the malignant group, which shows that most of the patients of the malignant group are of grades II and III (40% and 38% respectively) and stages 1 and 2 (30% and 40% respectively).

Fig. (1) represents examples for the RT-PCR results of (a) 253bp band of K 20, (b) 145bp band of telomerase and (c) 208bp band of GP-DH. In Table (4) data presenting all four studied parameters are listed. In all parameters (K19, K20, telomerase and cytology), the malignant group was significantly different from both the benign and the control groups. In K19 and telomerase, the benign group was also significantly different from the control group. None of the four-studied parameters was correlated to stage of the disease. When it comes to grade, only K19 showed significant positive correlation with grade both on TCC and SCC, as shown in Table (5). Also there was no significant difference in any of the four parameters when different tumor types (TCC and SCC) are compared (Table 5).

Table (6) represents the difference between infected and non-infected patients, and it shows that only K19 is affected by the presence of urinary bladder infection as reflected by the significant difference that exists between infected and non-infected patients. Table (7) repre-
Mohamed I. Morsi, et al. presents the sensitivities and specificities and the relative optimum cutoff values deduced from the ROC curves for the studied parameters.

Fig. (2) represents the ROC curve of K19, K20 and telomerase. Fig. (3) shows positive cytoplasmic K20 staining of malignant cells in urine cytology in a case of TCC and the H&E staining of the same case. Fig. (4) shows positive deep cytoplasmic staining with K20 antibody of malignant cells in urine cytology in a case of SCC and the H&E staining of the same case.

Fig. (1): RT-PCR results for (a) 253bp band of K20, (b) 145bp band of telomerase and (c) 208bp band of GPDH.

Fig. (2): ROC curve of K19, K20 and telomerase.

Fig. (3): Positive cytoplasmic K20 staining of malignant cells in urine cytology in a case of TCC. (K20 IHC: 100).

In set: Malignant cells in the same case of TCC in urine (H&E: 100).
**Fig. (4):** Positive deep cytoplasmic staining of malignant cells in urine cytology in a case of SCC (K20 IHC: 100).

In set: Malignant cells in the same case of SCC in urine (H&E: 100).

**Table (2):** Age and sex distribution of the studied groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Male : Female ratio</th>
<th>Age (M ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malignant</td>
<td>50</td>
<td>3:1</td>
<td>55.9±9.4</td>
</tr>
<tr>
<td>Benign</td>
<td>30</td>
<td>3:1</td>
<td>58.3±7.1</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>3:1</td>
<td>57.7±6.2</td>
</tr>
</tbody>
</table>

**Table (3):** Grade and stages distribution of the malignant histologic subgroups.

<table>
<thead>
<tr>
<th>Type</th>
<th>Grade</th>
<th>Stage</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
<td>III</td>
</tr>
<tr>
<td>TCC (35)</td>
<td>8 (22.9%)</td>
<td>14 (40.0%)</td>
<td>13 (37.1%)</td>
</tr>
<tr>
<td>SCC (15)</td>
<td>3 (20%)</td>
<td>6 (40%)</td>
<td>6 (40%)</td>
</tr>
<tr>
<td>Total (50)</td>
<td>11 (22%)</td>
<td>20 (40%)</td>
<td>19 (38%)</td>
</tr>
</tbody>
</table>

**Table (4):** Percent positivity of all parameters and the difference between all groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>K19</th>
<th>K20</th>
<th>Telomerase</th>
<th>Cytology % positive</th>
<th>Bacterial infection % positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M ± SE (ng/ml)</td>
<td>% &gt;UL</td>
<td>RT-PCR M ± SE (AU)</td>
<td>% &gt;UL</td>
<td>IHC % positive</td>
</tr>
<tr>
<td>Malignant</td>
<td>54.4±5.4*#</td>
<td>45/50 (90%)</td>
<td>27.5±1.6*#</td>
<td>40/50 (80%)</td>
<td>29/50 (58.0%)</td>
</tr>
<tr>
<td>TCC</td>
<td>52.8±6.6*#</td>
<td>30/35 (85.7%)</td>
<td>27.8±2.6*#</td>
<td>27/35 (77.1%)</td>
<td>22/35 (62.8%)</td>
</tr>
<tr>
<td>SCC</td>
<td>58.5±7.5*#</td>
<td>15/15 (100%)</td>
<td>28.4±3.1*#</td>
<td>13/15 (86.7%)</td>
<td>7/15 (46%)</td>
</tr>
<tr>
<td>Benign</td>
<td>19.6±1.4*</td>
<td>27/30 (90%)</td>
<td>13.8±0.86</td>
<td>5/30 (16.7%)</td>
<td>20%</td>
</tr>
<tr>
<td>Control</td>
<td>5.3±0.3</td>
<td>0/20 (0%)</td>
<td>14.3±1.5</td>
<td>2/20 (10%)</td>
<td>0%</td>
</tr>
</tbody>
</table>

*: Compared to the control group.  #: Compared to the benign group.  Significant when p<0.05.

% > upper limit = Number of patients more than 8.1ng/ml for K19, 20 AU for K20 and 30 AU for telomerase.
DISCUSSION

Bladder cancer has always received much attention in Egypt due to its high prevalence, with high mortality rates and the distinct pattern that was different from that reported in western countries. However, some recent reports suggested that the view of bladder cancer in Egypt is dramatically changing, reporting a much lower incidence of bladder SCC in favor of TCC and an overall increase in the mean age of incidence closing to that reported in Western countries [30].

Data from the current study agree much with these recent reports. We found that the prevalence of bladder SCC had decreased to less than 10% of all bladder cancer with TCC rates rising in response. The mean age of the malignant group was higher than that previously reported for our area (55.9 vs 50 years) but still lower than that reported in western populations. Also the male:female ratio was similar to that reported in Western bladder cancer (3:1).

Cystoscopic evaluation forms the basis of bladder cancer diagnosis and staging. Cystoscopy has a number of disadvantages as the discomfort, risk of urinary tract infection, pain and cost. In addition, it fails to detect dysplasias and carcinoma in situ (CIS). All this makes cystoscopy unsuitable for screening [1].

The golden marker for bladder cancer screening is still urinary cytology, as it is non-invasive, safe and inexpensive. It depends on the microscopic examination of urine sediments, to search for the probable presence of malignant cells [30]. Our results for urine cytology is comparable to those reported in literature. The specificity of this method was 100%, and when it came to sensitivity, it was only 50%.

The very low sensitivity of urine cytology renders its results inconclusive when it is used for screening for the presence of bladder cancer. All this calls for the search for new non-invasive markers for the detection of bladder cancer which should be specific, sensitive, reproducible, uninvasive and at acceptable cost.
Keratin 19:

Cytokeratins are water insoluble intermediate filaments that are expressed by epithelial cells and are used as markers of epithelial differentiation. Tumor cells may be shed in urine of bladder cancer patients, during necrosis and lysis of these cells, keratin 19 is degraded and released as soluble fragments that may be measured [31].

In our study, the upper limit of K19 in urine was estimated by 8.1ng/ml, that is the “Mean+2SD” of the control group, i.e. cut off value, K19 had an over all sensitivity of 90%. For TCC, the sensitivity of K19 assessment was 85.7%, slightly higher than the results obtained by Pariente et al. [32] and Sanchez-Carbayo et al. [33] who reported sensitivities of 83.8% and 80% respectively for bladder TCC. The specificity reported in literature was 67.2%, which was higher than that obtained in our study 46%, despite their lower cut off value (4ng/ml) [32]. Meanwhile, Dittadi et al. [34] reported a much lower sensitivity of 75% but a much higher specificity of 95% for the same assay.

The significant difference of the TCC and SCC subgroups from the benign and the control groups suggests the validity of K19 as a tumor marker of bladder cancer but there was no difference between the two types of the malignant group, which suggests that K19 could not be used for differential diagnosis of different types of bladder cancer.

There was a statistically significant regression of K19 levels with tumor grade in both TCC and SCC, an observation that reflects that K19 is a differentiation marker, that decreases in less differentiated tumors.

Several reports suggested that bacterial infection, a prevalent companion to bladder cancer, could influence the K19 results, an effect that is probably mediated by inflammatory reactions [31,32]. This remark is in agreement with our observation that a statistically significant difference in K19 levels exists between infected and non-infected patients.

Keratin 20:

K20 was measured by RT-PCR and by immunohistochemical staining of tumor cells shed in urine of bladder cancer patients. RT-PCR method as expected is reflecting much higher sensitivity and specificity than immunostaining. RT-PCR had a general sensitivity of 80% and specificity of 86%, while immunostaining showed 58% sensitivity and 80% specificity. In the study conducted by Buchumensky et al. [35] a much higher sensitivity and lower specificity for RT-PCR have been reported (sensitivity was 91% and specificity was 67%).

On the other hand, K20 immunostaining reflected better results than urine cytology with a higher sensitivity (58% vs 50%), especially in low-grade tumors, where cytology failed to detect malignancy, immunostaining gave better results.

K20 overexpression was more prevalent in SCC patients (86.7%) than in TCC patients (77.1%). These results were in agreement with the results of El Salamy, [36] who reported a similar profile with higher prevalence of K20 overexpression in SCC than in TCC. However, contradictory results were reported by Gee et al. [37] who claimed all SCC bladder tumors to be lacking K20 expression.

There was no statistically significant difference between TCC and SCC of the bladder, which reflects the inability of K20 as a tumor marker to differentiate the two types.

K20 expression showed no correlation with the grade of the disease in bladder TCC, on the other hand, in SCC, it was significantly correlated to grade. Others had reported a correlation of K20 with grade in TCC [35,38] a correlation that was missing in our study, and in SCC [34] in agreement with our work.

In a study by Golijanin et al. [39] that aimed to evaluate K20 immunostaining for bladder cancer diagnosis, it resulted in 81.6% sensitivity which was much higher than the sensitivity reported in our study (58%) and 77% specificity that was close to that recorded in the current study (80%).

When comparing RT-PCR and immunostaining of K20 as techniques for bladder cancer detection, immunostaining has two major advantages of lower cost and high specificity, opposed by non-reproducibility, low sensitivity and inherent technical risks as background staining which could result in false positive
results. On the other hand, RT-PCR carries the advantages of high sensitivity and reproducibility with the disadvantage of high cost.

**Telomerase:**

In our study, telomerase was expressed in a high percent of the malignant group, with an overall sensitivity of 82% and specificity of only 66%. The sensitivity and specificity detected in our study were much lower than those reported in literature, where sensitivity ranged from 86% to 95.7% and specificity from 90% to 100% [40-43].

In our results, there was a high positive prevalence in patients of the benign group, which greatly reduced the specificity of the assay. This high positive rate in the benign group is most probably due to haematuria and inflammation, since leukocytes, whether from blood in urine or from inflammatory reaction at the bladder, express telomerase and that expression is most probably is the source of the positive results rather than the epithelial bladder cells.

**ROC Curve analysis:**

From the ROC curves of all the parameters that were assayed in the study, we can see that K19 is the best of all the parameters assayed with the largest area under the curve comprising 88.0%. At an optimum cutoff value of 18.5ng/ml, K19 assay would have the optimum sensitivity of 80.4% and specificity of 88.0%. Closely following was K20 with 86.9% area under the curve. At an optimum cutoff value of 21.5 AU, it had a sensitivity of 80.4% and a specificity of 78.0%.

Telomerase was not proven to be a good diagnostic marker of bladder cancer with the least area under the curve of 76.9% and at an optimum cutoff value of 73 AU, it displayed a sensitivity of 76.5% and a specificity of only 76.0%. The low specificity of telomerase is mainly attributed to the high false positive rate in the patients of the benign group, which is most probably due to the presence of telomerase-positive inflammatory cells in urine.

In conclusion, K19 and K20 are the best candidates as screening tests for bladder cancer, representing the highest sensitivity and specificity. Meanwhile, telomerase although was a sensitive enough marker, it reflected a high false positive rate that could put at risk its validity for screening of bladder cancer. K20 immunostaining gave much better results than standard urine cytology, so K20 IHC can replace urine cytology as a bladder cancer screening test.

**REFERENCES**


