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Using of Telomerase Enzyme in Urine as a Non invasive Marker for Cancer Bladder Detection

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Abstract

Background: Urinary bladder cancer is one of the major health problem all over the world. Cystoscopy remains the gold standard for identifying bladder cancer but it is invasive and expensive, therefore, a simple, non invasive test for detecting bladder cancer would be helpful. Several biomarkers for bladder cancer have been used, but no single marker has been accurate and conclusive.

Aim: The current study aimed to measure telomerase enzyme in urine as a useful non invasive marker for detection of bladder cancer.

Methods : Forty eight patients (39 males and 9 females) were included, They are complaining of urinary symptoms and undergo cystoscopy with biopsy of bladder lesions and histopathological examination. They were divided into groups: Group I: 16 patients (11 males and 5 females) have benign urologic conditions. Group II: 32 patients (28 males and 4 females) have proven bladder cancer patients underwent transurethral resection of bladder tumor or cystoscopy with biopsy of bladder lesions. Also, 15 apparently healthy volunteers with matched age and sex with patients were served as a control group. All subjects were submitted to laboratory estimation of the following in urine: urinary creatinine, urine cytology, telomerase enzyme in urine by telomerase PCR and complete urine examination.

Results : The results of this study revealed that a highly significant increase in the frequency of cytolological positive cases for tumor cells in malignant group than each of benign group and healthy subjects, while no significant difference was detected between benign group and healthy subjects. The frequency of telomerase in urine was significantly higher in malignant group than each of benign group and healthy subjects, while no significant difference was detected between benign group and healthy subjects. The telomerase activity has sensitivity of 90.6% for diagnosis of cancer bladder with 93.7% for specificity and PPV was 96.6%, NPV was 83.3% and diagnostic accuracy of 91.6%. While, urine cytology gives a sensitivity of 68.8%, specificity of 87.5%, PPV of 91.6%, NPV 58.3% and diagnostic accuracy of 75%. When combined tests were used the sensitivity raised to 96,8%, and specificity reached to 100%, PPV was 96.6%, NPV was 94.1% and diagnostic accuracy increased to 97.9%.

Conclusion: the urinary assay of telomerase could be used as non invasive test to identify the bladder cancer patients and distinguish them from normal subjects and patients with benign tumor of urinary bladder. The low cost of this test may help to be implicated as non invasive screening of bladder cancer.

Key words : Cancer Bladder ,Telomerase , PCR

Introduction

Several important risk factors have been identified for bladder cancer including cigarette smoking, exposure to chemicals and the presence of chronic inflammation like belharziasis (Boon & Drijver, 1986). Bladder cancer has a high incidence matched by a tendency to recur, necessitating close and regular follow up (Saad *et al*., 2001). Although the incidence of cancer bladder is increasing, methods of diagnosis have changed little during the last few decades (Pirtskalaishvili *et al*., 1999).

Cytoscopy and urine cytopathology identifies the presence of abnormal malignant cells, which are shed into the urine (Thomas *et al*., 1999). These methods are expensive, invasive, time consuming and have low sensitivity, however, its ability to detect low grade bladder cancer is limited (Saad *et al*., 2001). Researchers discovered markers with practical diagnostic potential among them are cytokeratines (Eissa *et al*., 2002), and molecular markers as telomerase enzyme (Muller ,2002).

Telomerase is an enzyme that adds specific DNA sequence repeats ("TTAGGG" in all vertebrates) to the 3' ("three prime") end of DNA strands in the telomere regions, which are found at the ends of eukaryotic chromosomes. The telomeres contain condensed DNA material, giving stability to the chromosomes. The enzyme is a reverse transcriptase that carries its own RNA molecule, which is used as a template when it elongates telomeres, which are shortened after each replication cycle. There are some indicators that telomerase is of retroviral origin (Witzany, 2008).

Most normal cells do not possess telomerase mechanism , but almost all cancer cells acquire, to overcome their mortality and extend their lifespan *(Granger et al*., 2002). As divisions are being counted, events occur on the cellular and molecular level, which may either delay or hasten growth arrest. As humans age, a particular concern is the accumulation of events that lead to the progression of cancer *(Granger et al*., 2002).

The role of telomerase is to reconstitute the telomeres, such action is turned off in normal cells, however telomerase is activated in malignant cells making them immortal. Telomerase activity could be detected in most common cancers and thus could be used as a molecular marker in bladder cancer (Saad *et al*., 2001), where measurement of telomerase in the cell sediments from voided urine for diagnosis of bladder cancer is promising (Sanchini *et al*., 2004). The level of telomerase activity is generally influenced by the fraction of cells in the proliferative pool. Shortened telomeres and high telomerase activity almost always correlates with disease severity in hematologic neoplasia such as relapsed leukemia and high-grade lymphomas, indicating that measurement of telomere length and telomerase activity might be useful to monitor disease condition ***(Ohyashik et al., 2002*)**.

This study aimed to assess usefulness of using a new molecular marker namely telomerase enzyme in urine as a non invasive marker in the detection of urinary bladder cancer as comparing to conventional urine cytology.

Subjects and Methods

Forty eight patients (39 males and 9 females) were included, they attending the outpatient clinic and inpatient ward of National Institute of Urology & Nephrology and Urology department of Al Zahraa University Hospital, their ages ranged from 42-75 years. They were complaining of urinary symptoms and undergo cystoscopy with biopsy of bladder lesions and histopathological examination.

Also, 15 apparently healthy volunteers with matched age and sex with patients were served as a control group.

The patients were divided into groups:

Group I: 16 patients (11 males and 5 females) have benign urologic conditions.

Group II: 32 patients (28 males and 4 females) have proven bladder cancer patients underwent transurethral resection of bladder tumor or cystoscopy with biopsy of bladder lesions.

All subjects were submitted to the following laboratory investigations:

Serum sample for kidney function tests (urea, creatinine, serum sodium and potassium).

Urine collection and preparation: the voided urine samples (retained at least for 3 hours in the urinary bladder, but not the morning sample) were collected from all subjects into two sterile containers. One contained 20 ml for urine cytology, the other container 10 ml used for complete urine examination and urinary creatinine estimation and 50 ml used to determination of telomerase enzyme activity

1- Detection of telomerase enzyme by PCR:

Fifty ml of was centrifuged at 10000 x g for 10 minutes. The supernatant was removed and the precipitated urothelial cells pellet was suspended and washed with phosphate buffered saline (PBS) and re-centrifuged and the pelleted cells were stored at -80 °C to be used in the telomerase PCR assay.

Telomerase enzyme activity in urine was detected by Telomerase PCR ELISA kit (Roche, Cat. no. 12013789001) supplied by (Roch diagnostic Gmbh-Germany). The kit is designed for highly qualitative detection of telomerase activity in cell extracts from cell cultures and other biological samples using telomeric repeat amplification protocol developed by Kim *et al*., (1994). It utilizes a biotinylated primer for immobilization within the ELISA microtiter plate and allows highly specific amplification of elongation products combined with non-radioactive detection following an ELISA protocol (Muller, 2002). Detection was done according to the manufacturer's instructions. In the first step telomerase adds telomeric repeats (TTAGGG) to the 3' end of the biotin-labeled synthetic Pl-TS-primer. In the second step, these elongation products are amplified by PCR using the primers P1-TS and P2, generating PCR products with the telomerase-specific 6 nucleotide increments. An aliquot of the PCR product is denaturated and hybridized to a digoxigenin-(DIG)-labeled, telomeric repeat-specific detection probe. The resulting product is immobilized via the biotin labeled primer to a streptavidin-coated microtiter plate. The immobilized PCR product is then detected with antibody against digoxigenin (anti-DIG-POD) that is conjugated to peroxidase. Finally, the probe is visualized by virtue of peroxidase metabolizing TMB to form a coated reaction product. Samples were regarded telomerase positive if the difference in absorbance readings at 450nm and 690nm was higher than 0.2 units.

2- Complete urine examination:

Macroscopic and microscopic examinations were done to assess the presence of RBCs, pus cells, crystals, albumin and casts .

3- Cytological examination of urine samples was performed according to Boon and Drijver (1986). Samples were centrifuged and the supernatants were completely discarded. The pellets were smeared on 3 smears and left for air drying, fixation was done then the smears were stained using Haematoxylin-Eosin stain.

Statistical analysis:

Data were coded and summarized using SPSS (statistical package for Social Sciences) version 12.0 for Windows. Qualitative variables were described using frequency and percentage. Comparison between groups was done using Chi square (X^2) test for qualitative variables To assess the diagnostic value of using parameters, the following indices were calculated: sensitivity, specificity, positive predictive value (PPV) which means the probability of diagnosis and negative predictive value (NPV) that is the probability of excluding the diagnosis. P value <0.05 was considered statistically significant.

Results

Tables (1) shows a highly significant increase in the frequency of cytolological positive cases for tumor cells ($p<0.001$) in malignant group than each of benign group ($p<0.001$) and healthy subjects ($p<0.001$), while no significant difference was detected between benign group and healthy subjects ($p>0.05$). However, complete urine examination, kidney function tests, or urine creatinine revealed no significant difference between benign and malignant groups (data not mentioned in the table).

As regard the frequency of telomerase enzyme activity in urine, it was significantly higher in malignant group and each of benign group ($p<0.001$) and healthy subjects ($p<0.001$), while no significant difference was detected between benign group and healthy subjects ($p>0.05$) (Table 2).

The telomerase activity has sensitivity of 90.6% for diagnosis of cancer bladder with 93.7% for specificity and PPV

was 96.6%, NPV was 83.3% and diagnostic accuracy of 91.6%.

The urine examination for cytology gives sensitivity of 68.8%, specificity of 87.5%, PPV of 91.6%, NPV 58.3% and diagnostic accuracy of 75%.

When combined tests were used the sensitivity raised to 96,8%, and specificity reached to 100%, PPV was 96.6%, NPV was 94.1% and diagnostic accuracy increased to 97.9% (Table 3).

Table (1) The frequency of urine cytology in the studied groups and controls

	Benign cases (No=16)	**Malignant cases** (No=32)	**Healthy controls** (No=15)
Urine Cytology:			
+ve	2 (12.5%)	22 (68.8%)	0 (0%)
-ve	14 (87.5%)	10 (31.2%)	15 (100%)
Test of significance	X^2=15.6 $P^{(a)}$ <0.001**	X^2=19.7 $P^{(b)}$ <0.001**	X^2=2.4 $P^{(c)}$ >0.05

$P^{(a)}$ = comparison between groups malignant and benign, $P^{(b)}$ = comparison between groups malignant and control, $P^{(c)}$ = comparison between groups benign and control.

Table (2) The frequency of telomerase activity in the studied groups and controls

	Benign cases (No=16)	**Malignant cases** (No=32)	**Healthy controls** (No=15)
Telomerase activity:			
+ve	1 (6.3%)	29 (90.6%)	0 (0%)
-ve	15 (93.7%)	3 (9.4)	15 (100%)
Test of significance	X^2=20.5 $P^{(a)}$ <0.001**	X^2=25.8 $P^{(b)}$ <0.001**	X^2=0.0 $P^{(c)}$ >0.05

$P^{(a)}$ = comparison between groups malignant and benign, $P^{(b)}$ = comparison between groups malignant and control, $P^{(c)}$ = comparison between groups benign and control.

Table (3) The significance of using telomerase activity and urine cytology (n=48)

Term	**Telomerase activity**	**Urine Cytology**	**Combined tests**
Sensitivity	90.6%	68.8%	96.8%
Specificity	93.7%	87.5%	100 %
PPV	96.6%	91.6%	96.8%
NPV	83.3%	58.3%	94.1%
Accuracy	91.6%	75%	97.9 %

PPV = positive predictive value, NPV= negative predictive value .

Discussion

Bladder cancer is the second most common malignancy affecting the genitourinary system and is one of the most common tumors in Egypt (Sabaa *et al.*, 2002), where the incidence of transitional cell carcinoma represent 37.9% from all histopathologic types.

Telomerase is an RNA dependant ribonucleoprotein complex that acts as a reverse transcriptase which utilizes sequence of its RNA component as a template for de novo synthesis of telomeric

DNA sequences (Masutomi *et al*., 2000).

Although cystoscopy is sensitive in the detection of bladder cancer, it is invasive, expensive and uncomfortable for patients. Urine cytology has been the most commonly used marker for transitional cell carcinoma, although useful cytology is hampered by subjectively among cytopathologist and relative insensitivity for moderate and well differentiated tumors (Burchardt *et al*., 2000). Currently, a variety of markers have been developed among of these, there are telomerase enzyme (Dettlaff-Pokora *et al*., 2005).

Forty eight patients were included in this study, they were divided into 2 groups, 16 patients have benign urologic conditions and 32 patients have proven bladder cancer patients as well as 15 healthy control subjects.

This study revealed that a highly significant increase in the frequency of cytolological positive cases for tumor cells in malignant group than each of benign group and healthy subjects, while no significant difference was detected between benign group and healthy subjects. Moreover, the frequency of telomerase activity in urine was significantly higher in malignant group than each of benign group and healthy subjects, while no significant difference was detected between benign group and healthy subjects. Also, Li *et al*., (2002) stated that the telomerase activity in urine of patients with urothelial carcinoma increased significantly as compared to the control group.

Dettlaff-Pokora *et al*., (2005) mentionned that 88% human bladder cancers had telomerase activity in the sediments from voided urine of patients with superficial bladder carcinoma. In case of muscle-invasive tumors, telomerase activity was found in 93% of urine sediments. Enzyme activity was not detected in control urine sediments. In contrast to all the previous results, a study done by Linn *et al.,* (1997) showed that all urine samples of 12 patients with bladder cancer tested for telomerase activity were found to be negative. This could be explained by false negative results, as high protein concentration and Taq polymerase inhibitors may be present, also RNAase or proteases may degrade the RNA template or catalytic domain of the telomerase enzyme (Liu and Loughlin, 2000).

In the current study, telomerase activity give a sensitivity of 90.6% for diagnosis of cancer bladder with 93.7% for specificity and positive predictive value (PPV) was 96.6%,negative predictive value (NPV) was 83.3% and diagnostic accuracy of 91.6%. Comparable results were reported by some investigators, the sensitivity and specificity were 77% & 85%, respectively as reported by Eissa *et al* (2003), also a study reported by Glas *et al.,* (2004) stated that telomerase sensitivity and specificity were 75% & 86% respectively.

While, in our study, urine cytology gives a sensitivity of 68.8%, specificity of 87.5%, PPV of 91.6%, NPV 58.3% and diagnostic accuracy of 75%. In contrast to Wu *et al*., (2001) study, in which urine cytology only yielded a sensitivity of 31% in the detection of bladder cancer and the study done by Boman *et al.,* (2002) in which cytology had 42% sensitivity at 97% specificity. The sensitivity of this study was higher than that reported by Eissa *et al*., (2002) where the sensitivity and specificity were 44% and 100% respectively for voided urine cytology. Urine cytology sensitivity was 13.3% as detected by Bartoletti *et al*., (2005), while the specificity reported for cytology was 94% as stated by Van Rhijn *et al. (*2005).

Surprisingly in our study, when combined tests were used the sensitivity raised to 96,8%, and specificity reached to 100%, PPV was 96.6%, NPV was 94.1% and diagnostic accuracy increased to 97.9%.

Finally, it could be concluded that telomerase enzyme activity had the highest sensitivity and specificity for the diagnosis of bladder cancer than using of urine cytology. The using of combined markers give 100% specificity. The low cost of telomerase activity in urine may help to be implicated as non invasive screening of bladder cancer.

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استخدام إنزيم تليموريز بالبول كدلاله للكشف عن سرطان المثانة

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قسم الباثولوجيا الاكلينيكيه, المعهد القومي للكلي والمسالك البوليه* بالقاهره و اقسام الباثولوجيا الإكلينيكية** والمسالك البولية*** كليه طب بنات الازهر , وقسم الباثولوجي# بطب القاهره

يعتبر سرطان المثانة أحد أهم المشاكل الصحية في جميع أنحاء العالم . ويعتبر منظار المثانة هو المقياس الذهبي لتشخيص سرطان المثانة . ونظرا لصعوبته وارتفاع تكلفته فإن البحث عن اختبار بسيط وغير مكلف يساعد بدرجة كبيرة في تشخيص سرطان المثانة . يوجد عدة علامات حيوية لسرطان المثانة تم استخدامها ولكن لا توجد علامة وحيدة دقيقة وحاسمة .

تهدف هذه الدراسة لاستخدام نشاط انزيم التليموريز في البول كتشخيص مبكر لسرطان المثانة وهو اختبار غير مكلف .

وقد تضمنت هذه الدراسة 48 مريضا (39 ذكور و 9 إناث) يشتكون من اعراض بالمسالك البولية وتم تقسيم الحالات كالاتي

المجموعة الأولى : 16 مريض (11 ذكر و 5 اناث)عندهم اعراض بالمسالك البوليه وليست سرطانية

المجموعة الثانية : 32 (28 ذكر و 4 اناث) يعانون من سرطان المثانة

المجموعة الثالثة : 15 اصحاء كمجموعة ضابطة

تم اختبار البول و كريتنين البول ونوع الخلايا ونشاط التليموريز بواسطة سلسله التفاعل الجزيئي وكانت النتائج كالاتى

زيادة هامة جدا في نشاط التيلموريز في حالات سرطان المثانة الخبيثة عن المجموعة الحميدة و المجموعة الضابطة ولا يوجد اختلاف مهم بين المجموعة الحميدة والمجموعة الضابطة

فكان نشاط تليموريز لة حساسية بنسبة 6و90 % لتشخيص سرطان المثانة مع 7و93% محددة و القيمة الأجابية التنبوئية 6و96 % والقيمة التنبوئية السلبية 3و83 % ودقة تشخيصة 6و 91 % بينما الخلايا السرطانية في البول تعطي حساسية 8و68 % وتحديدها 5و78 % و و القيمة الأجابية التنبوئية 6و91 % و والقيمة التنبوئية السلبية 3و58 % ودقة تشخيصة 75 %

وعند قياس نشاط التليموريز و الخلايا السرطانية في البول معا ارتفعت الحساسية فى التشخيص الى 8و96 % و تحديد 100 %

لذلك نوصي بقياس نشاط التيلموريز كاختبار للتميز بين مرض سرطان المثانة عن الأمراض الغير سرطانية للمثانة باعتبارة منخفض التكليف ودقتة العالية .