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The Potential Role of Telomerase Reverse Transcriptase Promoter Mutations as Non-Invasive Urinary Biomarkers in Bladder Cancer Detection: Is the New Droplet Digital Assay (*ddPCR*) Reliable to be Clinically Used?

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Abstract

Background: Bladder cancer is the second most common cancer of the genitourinary system. The main components of disease follow-up include periodic cystoscopy and urinary cytology. Due to the aggressive nature of cystoscopy, the researchers are looking for less invasive tests.

Purpose: There is no accepted screening test for bladder cancer. Recently, Telomerase Reverse Transcriptase gene mutations have been hailed as the most common bladder cancer mutations.

UroMuTERT® and *ddPCR* tests evaluate the mutations in the urinary DNA. This study aims to evaluate the validity of these tests for clinical use and mass screening.

Patients and Methods: In this cross-sectional study, patients with bladder cancer and healthy controls were evaluated. The patients underwent a complete transurethral resection of tumor. Urine samples were examined for urinary cytology, *ddPCR* (Droplet Digital PCR test) and UroMuTERT® PCR test. The UroMuTERT® test measures *C228T* and *C250T* mutations, and *ddPCR* test, also measures two other mutations, *C228A* and *CC242-243TT*.

Results: 31 primary or recurrent bladder cancer cases and 50 controls were evaluated. *C228T* and *C250T* mutations were the most common mutations. Sensitivity and specificity of *ddPCR* and UroMuTERT® tests were calculated to be 64.5% and 90%, respectively. In addition, the diagnostic sensitivity of *ddPCR* test increased in high-grade tumors and MIBC tumors.

Conclusion: TERT promoter mutations could be a way for detection of bladder cancer. The available evidence does not allow clinical use. Although these biomarkers will be helpful to detect the presence of tumor, they cannot replace the cystoscopy. They may be helpful to reduce or delay cystoscopies.

Keywords: Bladder cancer; Urinary biomarkers; Non-invasive detection; Telomerase; Somatic mutations; TERT promoter mutation, Tumor recurrence

Introduction

The urothelial tissue of the bladder is covered by transitional cells that have the ability to develop into a variety of benign and malignant tumors. Urothelial carcinoma is the seventh most common cause of neoplasms worldwide [1]. These cancers are highly heterogeneous in clinical features and have led to changes in genetic characteristics, clinical features, grading, and staging [2].

More than 60% of bladder cancers and half of all deaths from bladder cancer occur in less developed regions of the world [3]. The fastest incidence rates are also found in underdeveloped countries. The Middle East region has a high incidence of bladder cancer. Iran is one of the most common regions of bladder cancer, in 2018, a total of 23,291 cases of bladder cancer were reported in Iran [4]. Thus, bladder cancer is the most common cancer in men in Kerman province (the second province in terms of prevalence), while according to recorded statistics is the fifth cancer in Iran [5,6].

Risk factors for bladder cancer include old age, male gender, smoking, and occupational exposure to carcinogens [7,8] is also more exposed to environmental toxins [9].

Bladder cancer has the highest lifetime cost per patient among all cancers. [10]This is mainly due to the possibility of high recurrence of the disease and therefore the need for long-term follow-up. Follow-up usually includes periodic diagnostic cystoscopy, urinary cytology, and periodic imaging of the upper urinary tract [11].

Painless hematuria is the primary symptom in 85% of patients with bladder tumors, and microscopic hematuria occurs in almost all patients [12]. Fifty percent of patients with Gross hematuria have a probable cause, with 20 percent reporting urologic malignancy and 12% reporting bladder tumors [13]. All

patients with Gross hematuria should undergo a complete urological evaluation. Approximately 9–18% of the general population will have a degree of hematuria during their lifetime [14]. All patients with hematuria should undergo a complete urological evaluation.

Microscopic hematuria is usually asymptomatic and the potential risk of bladder cancer in these patients is 4.1% and the risk of upper extremity malignancy with a hematuria assessment of 0.2-0.7% [15]. Complete evaluation of hematuria for bladder cancer includes diagnostic cystoscopy, urinary cytology, upper urinary tract imaging (mainly CT scan of the abdomen and pelvis) and Prostate-Specific Antigen (PSA) blood test [16]. The American Urological Association recommends that cystourethroscopy be performed for all patients with Gross hematuria, patients with microscopic hematuria older than 35 years, and those with a risk factor less than 35 years [17].

Today, the primary diagnosis and follow-up methods for bladder cancer include urinary cytology, ultrasonography, CT-scan, and cystoscopy. The main diagnostic tests for bladder cancer include diagnostic cystoscopy and biopsy. According to the latest guidelines of the European Urological Association, Wight Light Cystoscopy (WLC) using hard or flexible endoscopes is still the gold standard of diagnosis [18]. White light cystoscopy has the desired sensitivity and specificity for bladder papillary tumors. , But is too weak to diagnose CIS (in situ carcinoma) tumors.

Other promising technologies include Narrow Band Imaging (NBI), which uses light wavelength differences in deep tissue penetration and may be more sensitive to the detection of small papillary and CIS tumors. Several studies, including a prospective randomized clinical trial, have now suggested that adding NBI to WLC could improve cancer diagnosis. However, the significant problem with NBI is its low specificity (60 to 85%) in these experiments [19]. Bladder cancer is usually initially monitored by endoscopic resection, and their high recurrence rate requires lifelong follow-up [20].

After removal of a bladder tumor through the urethra (TURBT), patients are divided into two groups: Non-Muscle Invasive Bladder Cancer (NMIBC) or Muscle Invasive Bladder Cancer (MIBC). At the time of diagnosis, the majority of cases (75%) are NMIBCs (CIS, Ta, T1), which are mostly Low-Grade (LG). Low-grade tumors are rarely fatal but recur locally with varying and unpredictable rates.

Literature Review

In organ-limited MIBCs, radical cystectomy and pelvic lymphadenectomy or chemotherapy for localized tumor control are indicated because poor prognosis necessitates a radical approach. Because patients with NMIBC have a favourable prognosis, the bladder can be preserved in these patients. Treatment includes TURBT followed by intravesical injections according to disease risk classification. The risk of recurrence (31-78%) or progression (1-45%) is significant for patients with NMIBC. Therefore, regular follow-up visits over a period of at least 1 year are required for low-risk patients and up to 5 years for moderate-risk patients, and lifelong follow-up is required for high-risk patients. Cystoscopy is also the gold standard for following up on these patients. After cystoscopy, patients may experience symptoms of urinary irritation and even severe complications (e.g. sepsis). In addition to being an invasive and sometimes painful procedure, cystoscopy is expensive and time consuming and reduces the quality of life [21]. In a study, complications such as pain during urination, frequent urination and macroscopic hematuria were observed in 50, 37 and 19% of cases, respectively [22].

Urine cytology analysis is considered as a non-invasive and cost-effective option alongside cystoscopy and is widely used as a screening test. Urinary cytology is a non-invasive method for diagnosing bladder cancer by identifying abnormal urinary cells in the urine or contents of the bladder lavage [23].

The average cost of a cystoscopy is about $ 206 and the cost of non-invasive urinary cytology is about $56 [24].

For NMIBC patients, the goal is to identify and treat recurrences to prevent progression to MIBC. Currently, the only method is an accurate follow-up, which consists of cystoscopy with or without biopsy and urinary cytology. Guidelines from the European and American Urological Association still recommend cystoscopy and cytology for follow-up [25,26]. Less invasive methods have become more popular in recent years. Because the goal of screening is to detect early-stage disease, new markers associated with the biological and clinical behaviour of bladder cancer may help improve staging, prognosis, and choice of treatment options (i.e., predicting response to treatment) and serve as targets for new therapeutic approaches and follow-up responses.

Researchers now want to look at less invasive tests for cancer. Bladder cancer is one of the malignancies that researchers have discovered the most non-invasive tests. Due to the constant contact of urine with cancer cells, urinary sample use can potentially detect or rule out a recurrence of the disease before it can be visually identified. Urine biomarkers in the follow-up phase after initial diagnosis have three goals: To reduce the number of invasive tests while still detecting early relapse, to rule out relapse, to detect progression to the Muscle Invasive Bladder Cancer (MIBC), and finally to predict Response to therapeutic methods [27].

Researchers developing and researching urinary biomarkers are looking for a test with high sensitivity and high negative predictive value. This target profile is given special attention in the disease follow-up scenario because the purpose of these tests is to reduce the number or avoid cystoscopy. In order to replace cystoscopy, the urinalysis test must be well performed and interpretable at all grades and stages of the disease. In contrast, the cost of tests based on urinary biomarkers in bladder cancer is much lower than cystoscopy. Therefore, it has the potential to be easily implemented for lower cost bladder cancer follow-up strategies.

Several urinary biomarkers have been studied for these purposes, but those that use genetic material such as DNA and RNA appear to have the highest hope for clinical use Genetic alterations, including abnormal DNA methylation Changes in chromatin regeneration and abnormal expression of cell RNAs

are common occurrences in bladder cancer and can trigger and induce cancer. Accordingly, these genetic changes are now being used as potential biomarkers for the disease.

It should be noted that recurrent tumors are generally detected earlier than primary tumors. Accordingly, recurrent tumors are smaller and less tumor DNA is expected in urine samples, which may lead to lower test sensitivity in mutant analysis [28].

Several genetic mutations and polymorphisms have been discovered in bladder cancer, and many attempts have been made to find a urine biomarker test to replace urinary cytology. Most of these cases have sufficient sensitivity but poor specificity, which leads to false positive results and necessitates the need for further diagnostic tests.

However, patients reported that a urinary biomarker test required at least 90% sensitivity to replace conventional cystoscopy [29] none of the currently available urinary markers fully detect this sensitivity. Therefore a combination of cystoscopy with urinary marker testing in the current situation is a good choice for diagnostic procedures in patients with bladder cancer.

To date, several urinary biomarkers have been approved by the FDA in the follow-up of NMIBC patients. However, the tests performed were not sufficient to determine their accuracy and clinical utility [30].

Telomerase is a DNA polymerase that synthesizes telomeres at the ends of chromosomes. When the telomere is critically shortened, the cells enter a phase of permanent growth [31]. Telomerase Reverse Transcriptase (TERT) expression and telomerase activity are detectable in most (up to 90%) human malignancies [32]. Several tumors, including bladder cancer, show telomerase overactivity, which protects the chromosomes of cancer cells and thus causes them to die. Given that TERT mutations are the most common Genetic mutations in cancers have received a great deal of attention in recent years. Mutations in the TERT gene have been identified in several human neoplasms, including melanoma, glioma, thyroid cancer, and bladder cancer [33].

TERT gene mutations appear to be the most common somatic mutations in bladder tumors (60-85% of all cases) [34]. Mutations usually occur at the same frequency regardless of stage or grade, not related to prognosis. TERT promoter mutations were not associated with age, sex, or smoking. Existence of TERT gene mutations in bladder cancer subtypes such as Small cell carcinoma [38], Nested-variant, Glandular differentiation cancer [35] and Bladder squamous cell carcinoma is also shown.

This high frequency of TERT point mutations has made this gene a very attractive target for the detection of bladder tumors [36]. And combined tests for both the TERT and FGFR3 mutations may be more sensitive. A combination of these markers increases the sensitivity of relapse detection [37].

The aim of this clinical trial was to evaluate the clinical efficacy for diagnosing bladder cancer using the non-invasive UroMuTERT test. The UroMuTERT® test is a new PCR (Single-plex) test to detect local mutations in the telomerase promoter (TERTp) in DNA from cells in the urine. This test targets *C228T* and *C250T* mutations and has reported the highest sensitivity in diagnosing the bladder cancer. The test result was independent of the stage and grade of tumor and was independent of the first incidence of malignancy or tumor recurrence.

This test has also been reported to be valuable in the diagnosis of NMIBC tumors. Thus, the sensitivity of 68% and the specificity of 98% in the diagnosis of primary tumor in the study of France and Portugal [38]. In this study, the sensitivity of the test for the diagnosis of low grade tumors was significantly higher than the cytological test.

The high sensitivity of the test is important for the diagnosis of bladder cancer, because patients who test negative are not re-examined afterwards, and therefore the presence of cancer must be ruled out with great certainty [39]. To follow up the disease after diagnosis, the requirement for high test sensitivity will depend on the method of follow-up [40]. The follow-up procedure, in which cystoscopy is completely replaced by a urinary mutation test, requires high sensitivity for testing. However, the follow-up method, in which cystoscopy is performed alternately with delay depending on the result of the urinary biomarker mutation test, also makes the sensitivity of the urine test somewhat less possible, because false-negative results in cystoscopy after an acceptable time interval can be seen. High specificity is important in both diagnostic methods because false-positive results lead to unnecessary cystoscopies [41-43].

Due to the lack of non-invasive tests such as UroMuTERT® test in Iran and the increasing popularity of physicians to diagnose malignant and fatal diseases using non-invasive, easy tests, faster and cheaper treatment, this research project detection and approval of the mentioned tests and, if possible, use or further research is designed to prepare and manufacture such tests in the country.

Methods and Materials

Patients with previously known history of Low-grade and High-grade bladder cancer (in the follow-up period with or without a history of chemoradiotherapy) or those referred to Shahid Bahonar Hospital in Kerman for the first time with a definitive diagnosis of bladder tumor for TURT examination and surgery. They entered the research study as a patient group. The control group consisted of people with no history of bladder cancer that were candidates for prostatectomy or referred for treatment of kidney stone surgery by PCNL (Percutaneous Nephrolithotomy). This study was conducted as a pilot. Due to the high cost of the test and at the request of the International Agency for Research on Cancer (IARC), the study was piloted by 50 people in each group. It should be noted that eligible individuals over the age of 18 were selected. All demographic and clinical characteristics and specific records were collected and documented from patients and controls according to the designed questionnaire.

First, midstream urine samples from 51 definitive patients with bladder cancer with a previous history of Low-grade and

High-grade bladder cancer [in the follow-up period] or people who were diagnosed with a bladder tumor for the first time for TURT examination and surgery were collected. Ethical consent was obtained to participate in the research project. Control urine samples were collected from 53 healthy individuals with no history of bladder cancer who were candidates for prostatectomy or referred for PCNL kidney stone surgery and were stored in special containers. Then the patients and the control group underwent diagnostic white light cystoscopy as a diagnostic standard in the urology operating room. After confirming the presence of any mucosal lesion or new tumor lesion or obvious tumor recurrence in the bladder, patients underwent transurethral tumor resection (TURT) and samples were collected. Finally, the samples were referred to a pathologist to determine the type of tumor or malignancy, grade and stage.

Discussion

Urine samples were prepared and identified in separate test tubes of 15 cc Falcon Conical tube and containers for urinary cytology test for each person after special preparation stages in Iran for each person. These 15 cc tubes, combined with approximately 14 cc of complete urine +1 cc of conditioning buffer (980uL) specially prepared by Zymo (The Quick-DNATM Urine Kit) for up to one month Transferred to a freezer at -80°C, frozen and stored. This buffer made it possible to store cells and DNA in urine for a month at room temperature. By combining urine and buffer, it became impossible to separate cell DNA and supernatant cell free DNA. A specific identifier was redefined for each urine and cytology specimen, and the cytology specimens were referred to a pathologist within a maximum of 12 hours in accordance with laboratory protocols. Only the researchers knew the nature of the samples and the names of the patients and whether they were sick or healthy.

Finally, urine samples were sent to the IARC Institute in France for analysis by UroMuTERT® test via maintaining dry temperature by freezing. The results of the test were given as blind feedback and after classification were analyzed by the researchers. The UroMuTERT® test measured two mutations, *C228T* and *C250T*, and the newer *ddPCR* test, in addition to the above mutations, also measured two other mutations, *C228A* and *CC242-243TT*, which were performed on the samples at the request of the IARC Institute.

Conclusion

Given the high prevalence of TERT mutations in bladder cancer and the high sensitivity of the test, especially in the early diagnosis of this cancer in our study, we also believe that TERT promoter mutations in urine can be a promising way for early diagnosis or Recurrence detection in patients with bladder cancer. However, the available evidence for urinary biomarkers of the TERT mutation for the diagnosis of bladder cancer does not currently allow clinical use. The combination of different molecular markers, such as urinary biomarkers, TERT mutation and DNA methylation, may provide a better option for non-invasive diagnosis of bladder cancer, so future studies should also focus on identifying these biomarkers. To accelerate future clinical use, prospective studies with larger sample sizes with sequential sampling methods with matched control groups are necessary.

Recalling the high recurrence rate of bladder tumors and the multiplicity of patient cystoscopies and the high costs and complications, it seems that although these biomarkers will be very helpful in diagnosing the presence of a tumor, they can still not be replaced by cystoscopy in general. They can only reduce or delay cystoscopies.

Given the potential therapeutic applications, it should be noted that the TERT promoter region, which often carries TERT promoter mutations in bladder cancer and is not present in normal non-cancerous bladder cells, could potentially be the target of bladder anticancer therapy, including immunotherapy, based on this mutation in the future.

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