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Telomerase Reverse Transcriptase and miR-34a in diagnosis of non-muscle invasive bladder cancer

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ABSTRACT

Background: Management of high-risk non-muscle invasive bladder cancer is difficult, as no validated tool exists to predict risk of recurrence. Urine has the potential to contain a variety of molecular markers that may be associated with tumors.

Methods: The study included 3 groups: group 1comprised 100 patients diagnosed with non-muscle invasive bladder cancer **(**NMIBC). Each of Group 2 included 100 patients with other pathology rather than NMIBC. Group3 comprised 100 healthy persons. Group1 was subdivided into patients with and without recurrence. The patients with recurrent tumors were subclassified into single or multiple recurrences. Urinary markers: TERTand miR-34a were evaluated.

Results: Higher urinary TERT > 3.2 and lower urinary miR34a < 0.85 were detected significantly in NMIBC group compared to controls.

Conclusion: Urinary molecular biomarkers are reliable non-invasive tools for NMIBC detection and prediction of recurrence. Urinary TERT >3.2 and miR34a< 0.85 were significantly higher in patients with NMIBC in comparison with controls. Urinary TERT > 3.2 had the highest overall diagnostic accuracy for bladder cancer detection.

Introduction

Bladder cancer is one of the most common cancers worldwide with the urothelial cancer (UC) as the most predominant one **(1).** Of all newly diagnosed UC cases, approximately 80% are non-muscle invasive papillary tumors confined to the urothelium (CIS, Ta) or lamina propria T1**(2).** Despite the fact that most non-muscle invasive UCs can be successfully treated by transurethral resection of bladder tumor (TURBT), 70% of patients will suffer tumor recurrence after the initial treatment and 10-20% of those recurrent tumors canprogressto muscle invasive**(3).**

Non-muscle invasive bladder cancer (NMIBC) requires life-long follow-up by frequent cystoscopy which is considered as the most accurate method for monitoring of NMIBC. Nevertheless, it is an invasive technique that requires anesthesia and hospital admission for biopsy. Also, they are expensive and time consuming.

Urine has direct contact with bladder epithelial cells and metabolites released from UC cells may be detected in urine samples. Therefore, urine metabolomics are considered a promising approach for UC detection and marker discovery. Use of urinary biomarkers could potentially either predict disease recurrence before it becomes visually apparent or exclude its presence. Urinary biomarkers in the surveillance setting of NMIBC have several aims: to reduce the frequency of invasive testing while still detecting early disease recurrence , to exclude the presence of recurrent disease, to detect progression, and to predict response to therapies **(4)**. Several biomarkers in urine were used for monitoring of recurrence of NMIBC such as bladder tumor marker (BTM), telomerase and nuclear matrix protein (NMP)**(5)**.

Latestresearch studied Telomerase Reverse Transcriptase (TERT) in prediction of recurrence of NMIBC and authors concluded that TERT in urine was a reliable and dynamic predictor of recurrence in NMIBC and TERT positive-status was associated with recurrence in the subset of patients with negative cystoscopy**(6).**Authors of this study used only univariate analysis with no multivariate analysis, also indicating some reasons that introduced bias in data analysis. So, we aimed to reevaluate use of TERT to test its reproducibility of the previous results.

On the other hand, metabolic dysfunction has been implicated in a wide variety of human diseases including bladder cancer **(7).** Recent advances on technological and data analysis have enabled the characterizationof metabolites in biofluids. Metabolites, in general, such as taurine, carnitine and cholinergic compounds have been proposed as urinary markers for bladder tumors **(8)**. But, previous studies aiming at the identification of metabolomics biomarkers for UC diagnosis were limited by the use of heterogeneous cohorts of NMIBC and MIBC patients. Therefore, not fully representative of the target population **(9)**.

miR34a, which has been studied extensively in solid tumors, is known to be down-regulated in chronic lymphocytic leukemia, colorectal cancer, lung cancer, and several other types of cancer**(10)**. In the setting of UC, previous studies showed that downregulation of miR-34a had direct correlation with P53**(11)** and lower tissue level of miR-34a was associated with higher rate of bladder tumor recurrence **(12)**. Nevertheless, it is not known whether study of this marker in urine samples

would be of value in diagnosis and prediction of recurrence in NMIBC.

So we aimed at this study to evaluate the role of non-invasive urinary tumor markers in diagnosis of the presence and in prediction of NMIBC after initial endoscopic treatmentusing particular markers namely:TERT and urinary metabolite biomarker of miR-34a.

Materials and methods

Study population

This is a prospective study carried out between January 2015 and September 2019.The study included3 groups: group1 (study group) comprised 100 patients who were histologically confirmed as TCC with no evidence of muscle invasion after TURBT based on TNM classification of bladder cancer (2009) at the date of the initial TURBT. Clinico-pathological details were evaluated using the 2004 WHO criteria for determination of grade **(13)**. Group 2 (control non-healthy group) included 100 patients with other pathology rather than NMIBC and no history of previous malignancy as following: neurogenic bladder, overactive bladder, and vesical stone. Group 3 (control normal) included 100 healthy persons.Group 1was subdivided according to recurrence state and its frequency into patients with and without recurrence. Among patients with recurrence, we further classified them into those with single or multiple recurrences.

After TURBT, patients in group 1 were followed upby cystoscopy and urine cytology every 3 months for 2 years, then every 6 months for one year, and annually thereafter. The median duration of follow up in our study was 25 (3-46) months.Patients were defined as having “recurrence” if at any time the cancer reappeared and had histopathological findings of NMIBC after TURBT.Urine sampleswere obtained during the first TURBT and during cystoscopy before tissue biopsy for patients in group 1. Voided urine morning samples were obtained from patients of both control groups.

Determination of Telomerase Reverse Transcriptase (TERT) in urine samples using ELISA.

A ready-to-use microwell, strip plate Sandwich ELISA Kit (Telo TAGGG Telomerase PCR ELISA ^{PLUS}, Roche, Cat no. 12013789001) for analyzing the presence of the TERT was used, ELISA Kit target analytes in biological samples (urine). The samples could be used undiluted body. The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of TERT in urine samples. This ELISA kit uses Sandwich-ELISA as the method. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human TERT. Standards or samples are added to the appropriate micro ELISA plate wells and bound by the specific antibody. Then a biotinylated detection antibody specific for Human TERT and Avidin-Horseradish. Peroxidase (HRP) conjugate was added to each micro plate well successively and incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contain Human TERT, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color turned yellow. The optical density (OD) was measured spectrophotometrically at a wavelength. The OD value is proportional to the concentration of Human TERT. Calculation of the

concentration of Human TERT in the samples done by comparing the OD of the samples to the standard curve.

Determination of miR-34a in urine samples

Total RNA including miRNAs was extracted from urine cells using miRNeasy Mini Kit (cat.# 217004, Qiagen, Hilden, Germany) , then it reverse transcribed to cDNA in a final volume of 20 µl using the miScript Reverse Transcription kit (cat.# 218161,Qiagen, Hilden, Germany). qPCR assays were performed in triplicate measurements using the miScript SYBR-Green PCR kit (SYBR® Green PCR Kit (cat.# 218073,Qiagen, Hilden, Germany) and miScript primer assay for miR-34a (Qiagen) on the Rotor-Gene Q 5-Plex (Qiagen) according to the manufacturer's instructions. Results were normalized to RUN6-2 (Qiagen).The amplification profile was denatured at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min and 70 for 30°C s. The expression level of miRNA gene was represented by fold change, which was calculated using the equation $2^{-\Delta\Delta Ct}$.

Statistical Analysis

Continuous data were expressed as mean ± SD or median (range) according to the pattern of distribution. Univariate analysis of factors affecting bladder tumor recurrence and times of recurrence was done using independent sample T-test, Chi-square, and Mann-Whitney U tests as appropriate. In all biomarkers, we calculated the sensitivity, specificity, overall accuracy, positive and negative predictive values (PPV and NPV) compared to the gold standard. The receiver operating characteristics (ROC) curve was used to identify cut-off values for significant continuous variables in univariate analysis. Multivariate analysis was performed by calculation of the Hazard Ratio (HR) and Cox's regression analysis. All statistical tests were carried out using IBM ''SPSS'' statistics version 21, with a P-value of less than 0.05 was considered significant.

Ethics

Approval from institutional review board (IRB) was obtained and informed consents were taken from all participants. There was no conflict of interest and no funds were received. Patients were managed according to the principles of declaration of Helsinki.

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Results

Age and gender were comparable among patients of the 3 groups (**Table 1**). In the final histopathological examination of TURBT specimen of group 1: all patients had stage T1, 45% had GII and 55% had GIII papillary NMIBC.

Diagnosis of bladder tumor

Higher urinary value of TERT and lower urinary values of miR34a were detected in bladder cancergroup compared to controls ($p<0.0001$) (**Table2**).

Discussion

During the last decade the study of tumor markers for bladder cancer has grown markedly aiming for finding a non-invasive diagnostic method for this disease. As it is known

that, bladder tumor had a greater tendency to relapse and progress, regardless of complete TURBT and the addition of adjuvant intravesical therapy. Consequently, close follow up of those patients is needed. This elucidates the higher economic burden of this disease (14). Although cystoscopy is the mainstay for follow up and diagnosis, it is still an invasive technique.In clinical practice, cystoscopy is accompanied by urinecytology. This plays a more important role in the case ofhigh-grade tumors and carcinoma in situ (CIS) with low sensitivity in low grade tumors. Urine cytology is the most common non-invasive tool of monitoring of such patients. Several biomarkers in urine, are the result of chromosomal abnormalities, were used for monitoring of recurrence of NMIBC such as bladder tumor marker (BTM), telomerase and nuclear matrix protein (NMP)(15).

Telomerase Reverse Transcriptase (TERT) is an essential safeguard of genomic integrity, responsible for telomere maintenance. In aging or damaged cells, TERT activity is physiologically shutdown leading to shortened telomeres and induction of a form of cell death called senescence. Escaping senescence is a hallmark of cancer (16) and nearly all tumors develop genetic or epigenetic strategies to avoid elimination by increasing TERT activity(17). Two recurrent somatic mutations (C228T and C250T) have been identified in the TERT promoter in melanoma (18) as well as in various other tumors including bladder cancers(19, 20). TERT promoter mutations have previously been described at high frequencies across stages in malignant bladder tumors, but the prognostic value in urine is still unclear(21, 22).Recent researchstudied TERT in prediction of recurrence of non-muscle invasive bladder cancer. Authors showed that the overall sensitivity was 80.5% and specificity 89.8%, and was not greatly impacted by inflammation or infection. TERT remaining positive after initial surgery was associated with residual CIS. TERT in urine was a reliable and dynamic predictor of recurrence in NMIBC ($P<0.0001$). In univariate analysis, TERT positive-status after initial surgery increased risk of recurrence by 5.34 fold ($P<0.0004$). TERT positive-status was still associated with recurrence in patients with negative cystoscopy ($P<0.034$). The authors concluded that TERT mutations in urine might be helpful for early detection of recurrence in bladder cancer, especially in NMIBC (6). In our study, we found that higher level of urinary TERT >3.2 was detected in patients with bladder tumor in comparison of normal and non-healthycontrols with PPV of 99%. Also, higher urinary TERT level > 8.6 was associated with 1.4 higher rate of recurrence with the highest PPV for BC recurrence.

Metabolic dysfunction has been implicated in a wide variety of human diseases including bladder cancer (7).Recent advances on technological and data analysis have enabled the characterization of metabolites in bio fluids. Growth and division of tumor cells are associated with an increase in the activity of a variety of metabolic pathways. Recent studies reported that metabolites uniquely detected in urine samples from patients with bladder cancer, not those in the matched non-cancer controls, could be a useful biomarker for bladder cancer diagnosis and prognosis(23). Because the bladder serves as a temporary urine reservoir,analysis of urine metabolites could provide potentialcandidates of sensitive and specific biomarkers of bladder cancer.

MicroRNAs (miRNAs) are a conserved class of non-coding small RNAs that regulate gene and protein expression by binding to mRNA, leading to mRNA degradation or inhibition of translation(10). Either individually or as a cluster, the expression levels of miRNAs have been shown to be up-regulated or down-regulated in several cancers, including prostate cancer, breast cancer, lung cancer, medulloblastoma and bladdercancer (26, 27). Recently, the miR-34 family, including miR-34a, b, c has been found to be directly correlated with p53(11). Some evidences have shown thatreduced expression of miR-34a is involved in the initiation and progression of cancer and the functional activity of miR-34 indicates a potential role as a tumor suppressor(28, 29). Wang et al, in his study in vitro demonstrated the association between higher tumor recurrence and lower miR34a in tumor tissue(12). In our study, lower miR34a than 0.6 was associated with higher incidence of recurrence with PPV of 97.5%.

While this study had benefits as it abolish drawbacks of Descotes et al study(6)using TERT in prediction of recurrenceas they indicated some reasons that introduced some positive bias in the analysis of their data. On the other hand, in the present study we prospectively validate further data interpretation with a rich integrated database of metabolome, transcriptome, and proteome, as well as systematic bioinformatics approach to confirm the biological function of metabolite candidates in prediction of NMIBC recurrence. However, the study was limited in relatively small sample size.

To conclude Urinary molecular biomarkers are reliable non-invasive tools for NMIBC detection. Urinary TERT >3.2and miR34a< 0.85 were significantly higher in patients with NMIBC in comparison with controls.

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Tables legends:

Table 1: Patient's demographics

Variable	Study group (n=100)	Non-healthy control (n=100)	Healthy control (n=100)	P-value
Age (mean±SD)*	60.6±10	64 ± 8.2	57.8 ± 12	0.2
Gender (M/F)**	91/9	93/7	89/11	0.4

***ANOVA test **Chi-Square test.**
M= male & F= female.

Table 2: Urinary markers in bladder cancer patients and controls.

Variable	Study group (n=100)	Non-healthy control group (n=100)	Healthy control group (n=100)	P-value
Urinary Telomerase *(median & range)	13.3(3.2-20)	1.2(0.36-3.21)	0.4(0.12-0.94)	**<0.0001**
miR-34a **(mean ± SD)	0.5±0.2	1.2±0.1	1.1±0.1	**<0.0001**

***Kruskall-wallis test **One way ANOVA**

Table 3: ROC curve values of urinary markers in bladder cancer diagnosis

Variable	Cutoff point	AUC	Sensitivity%	Specificity%	PPV%	NPV%	P-value	Overall diagnostic accuracy%
Urinary Telomerase	3.2	1	100	100	99	89.4	<0.0001	98.3
miR-34a	0.85	0.993	96	100	65.5	48.1	<0.0001	51.5

Figure legends:

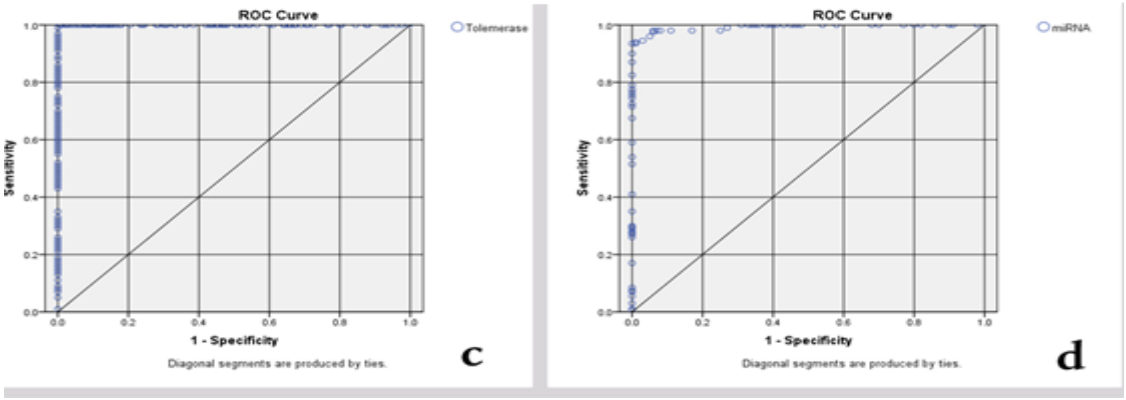

Figure 1: ROC curves for urinary biomarkers for diagnosis of NMIBC: c) TERT& d) miR-34a