Evaluation of IGF2, KRT14, and KRT20 as Urinary Biomarkers in Patients with Bladder Cancer

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Abstract

Background: Many researchers have tried to identify bladder cancer biomarkers to reduce the need for cystoscopy. The aim of this study was to identify and measure appropriate transcripts in patient urine to develop a non-invasive screening test.

Methods: From February 2020 to May 2022, 49 samples were obtained from Velayat Hospital, Qazvin University of Medical Sciences, Qazvin, Iran. Twenty-two samples were obtained from bladder cancer patients and 27 from bladder cancer-free subjects. RNA was extracted from participant samples, quantitative RT-PCR was performed, and TNP plots were used to assess IGF2 (NCBI Gene ID: 3481), KRT14 (NCBI Gene ID: 3861) and KRT20 (NCBI Gene ID: 54474) expression. For UCSC Xena analysis, Dataset ID: TCGA-BLCA was used to compare transitional cell carcinoma (TCC) and normal samples for survival rates.

Results: IGF and KRT14 were more greatly expressed in patient urine samples than in those of the normal group. However, KRT20 expression did not significantly differ between the two groups. IGF2 had 45.45 and 88.89% sensitivity and specificity, respectively, for detecting TCC in urine samples while KRT14 had 59 and 88.89% sensitivity and specificity, respectively. Also, these results infer that overexpression of IGF would be prognosticators of poor TCC outcomes.

Conclusions: Our study showed that IGF2 and KRT14 are overexpressed in bladder cancer patient urine, and IGF2 could be a potential biomarker for poor prognoses in TCC.

Keywords: Biomarkers, Diagnosis, Genes, Liquid biopsy, Urinary bladder neoplasms.

Introduction

Bladder cancer is the most expensive cancer per patient, partly because of frequent cystoscopy to monitor for recurrence. Currently, the gold standard for bladder cancer diagnosis is cystoscopy, which is invasive and relatively expensive. This test also causes discomfort and complications in patients. In addition, it is estimated that cystoscopy may fail to detect 10-20% of papillary lesions and 50% of smooth lesions, and in some patients, is not associated with a specific result. For this reason, cytology,

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which is non-invasive, is often performed along with cystoscopy. However, cytology is relative insensitive and not highly accuracy for low-grade tumors (1-3). Therefore, accurate urinary biomarkers that can identify recurrence and prognosis, or measure the invasion level can both reduce costs and help patient recovery. Due to its constant contact with the bladder, urine may contain bladder cancer biomarkers (4).

Several biomarkers have been approved by the Food and Drug Administration (FDA) along with cystoscopy to diagnose patients with suspected bladder cancer and monitor recurrence. However, these markers are not currently widely used in the clinic. The major limitation of these tests is specificity, which is influenced by other bladder problems including bladder stones, benign prostatic hyperplasia, and obstructive uropathy, which cause false positive results. For example, ureteral stents lead to 100% false positive results in nuclear matrix protein (NMP) 22 and BTA stat tests. BTA stat and BTA TRAK tests measure human complement factor H-related protein (hCFHrp) and complement factor H. Because these factors are abundant in blood, they lead to positive results in hematuria patients regardless of bladder tumors. Up to two years after treatment with intravesical bacillus Calmette-Guerin (BCG), false positive results are seen in the BTA test, which limits the use of this test in monitoring recurrence (5).

Some existing tests lack sensitivity and fail to detect low-grade tumors, resulting in failure to identify cases (1). Bladder cancers, like other cancers, are highly heterogeneic, which result from various pathways and lead to various disease subgroups. It seems the best screening method for this disease is to use markers of each pathway and subgroups that can cover various disease types and increase screening sensitivity and specificity (6, 7). To date, many researchers have tried to identify bladder cancer markers to diagnose the disease in time to provide appropriate treatment and reduce costs and patient worries. The aim of the present study was to identify and measure appropriate transcripts in patient urine to develop a non-invasive screening test.

Materials and Methods

From February 2020 to May 2022, 49 samples were obtained from individuals at Velayat hospital, Qazvin University of Medical Sciences, Qazvin, Iran. Twenty-two bladder cancer patients (mean age of 61.3 ± 14.9 years) and 27 healthy control subjects (mean age 63.23 ± 8.9 years) were enrolled in this study.

All participants were men. The inclusion criteria for the control group were no history of bladder cancer until the time of participation in the study and the absence of disease symptoms, including the presence of obvious intermittent hematuria without pain with the passage of a clot, or two or more different clinical laboratory diagnoses of microscopic hematuria with five or more red blood cells in the urine in each field. Inclusion criteria for the patient group included age over 35, exposure to known and approved risk factors for bladder cancer including tobacco or opium-derived compound use, occupational exposure to aromatic amines, and obvious painless intermittent hematuria with the passage of a clot, or two or more than two different clinical laboratory diagnoses of microscopic hematuria with five or more red blood cells in the urine in each field, and not undergoing treatments related to bladder cancer such as Bacillus Calmette–Guérin injection, chemotherapy, or radiotherapy, over the past two years.

First-morning urine samples were obtained before the cystoscopy and biopsy. All urine specimens were stored at 4 °C for a maximum of 4 h. Urine samples were centrifuged at 800 G for 10 min at 4 °C. Urine cell pellets (UPCs) were treated with TriPure isolation reagent (Roche, Germany) and stored at −80 °C for a maximum of one month before testing. The clinical diagnoses were pathologically confirmed. Participants suspected of having bladder cancer were identified after referral and necessary investigations, including urine tests and cystoscopy. Identified tumors were graded and staged.

As ethical consideration, all participants received a detailed description of the aim and procedures of this study and gave written informed consent. The Ethics Committee of Tehran University of Medical Sciences approved our study protocol. All tests were performed at no cost to the subjects.

RNA Extraction, Assessment of Integrity, and cDNA Synthesis

TriPure isolation reagent (Roche, Germany) was used to isolate total RNA from whole UPCs according to the manufacturer's protocol. The quality and purity of each RNA sample were measured with a NanoDrop ND-2000 Spectrophotometer (Biotek, Epoch™ Spectrophotometer System) and RNA sample integrity was analyzed by electrophoresis on 1.0% agarose gel. RNA samples were stored at −80 °C for further analyses. Subsequently, the Easy™cDNA Synthesis kit (Parstous, Iran, cat#A101161) was used to synthesize cDNA in a total volume of 20 μl according to the manufacturer's protocol.

Quantitative Real-Time PCR Analysis

The relative gene transcript levels were quantified on a Corbett Rotor-Gene RG-6000 Real-Time PCR Analyzer using RealQ Plus 2x Master Mix Green (Ampliqon). Each PCR was performed using an initial denaturation step at 95 °C for 1 min followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing at the appropriate temperature (Table 1) for 45 s. All experiments were performed in duplicate and the GAPDH gene was used as normalizer. The primer sequences are shown in Table 1.

Table 1. Primer sequences and PCR conditions used in the quantitative RT-PCR.

Primer	Sequence	Amplification size (bp)	Annealing temperature (°C)
IGF2	F: 5'-CGTGCTTCCGGACAACTTCC -3'	197	60
	R: 5'-CTTGGGTGGGTAGAGCAATC -3'		
KRT20	F: 5'-CTG AGG TTC AAC TAA CGG AGC TG -3'	173	62
	R: 5'-AAC AGC GAC TGG AGG TTG GCT A -3'		
KRT14	F: 5'-GGAGATGATTGGCAGCGTGGAG -3'	151	60
	R: 5'-AGAACTGGGAGGAGGAGAGGTG -3'		

TNM Plot and UCSC Xena Analysis

TNMplot21 (https://tnmplot.com/analysis/) has 56,938 multilevel quality-controlled samples. The extraction of the sources was from the GTEx, GEO, TCGA, and TARGET databases (8). This study used RNA-Seq data to analyze and include paired tumor and adjacent normal tissues and compare gene expression between transitional cell carcinoma (TCC) and normal samples. UCSC Xena supplies integrative online visualization of original cancer genomics datasets from TCGA, ICGC, TCGA Pan-Cancer Atlas, and the GDC. Xena covers various genomic fields, including genes, genomic elements, and any genomic area for both coding and non-coding genome fragments.

Dataset ID: TCGA-BLCA was used to compare TCC and normal samples, and for survival analysis (9).

Statistical Analysis

Statistical software GraphPad Prism 9 with the use of Welch's t-test, was used to analyze data. The significance level for all tests was less than 0.05. The receiver operating characteristic (ROC) curve was designed to appraise the propriety of gene expression measures for discrimination of tumoral and nontumoral samples. The Youden index (j) was used to obtain the greatest distinction between sensitivity (true-positive rate) and 1 - specificity (false-positive rate).

Results

Demographic & Clinicopathological Data

In this study, gene expression was evaluated on urinary samples of 22 patients with bladder cancer and 27 normal individuals. Nine patients had high grade and 13 had low grade TCCs based on the World Health Organization system.

Differential Expression Analysis

KRT14 and IGF2 expression were significantly greater in patient than in control UPCs (Fold differences (FDs) = 7.64; P= 0.030 and 8.05 and 0.047, respectively). KRT20 expression did not differ significantly between the two groups (FD = 1.519; P= 0.67) (Fig. 1).
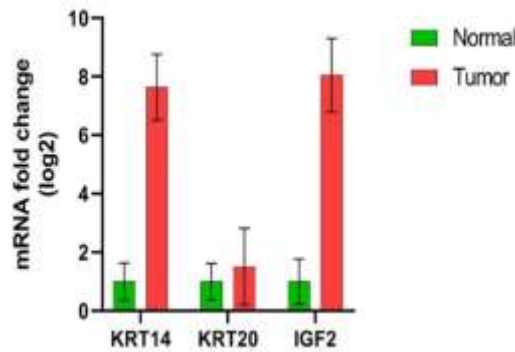

Fig. 1. Fold difference (mean ± SEM) observed with mRNA expression values. Total RNA was isolated from urine cell pellets and cDNAs for IGF2, KRT14, and KRT20 were synthesized. cDNAs were amplified by PCR on a Corbett Rotor-Gene RG-6000 Real-Time PCR Analyzer.
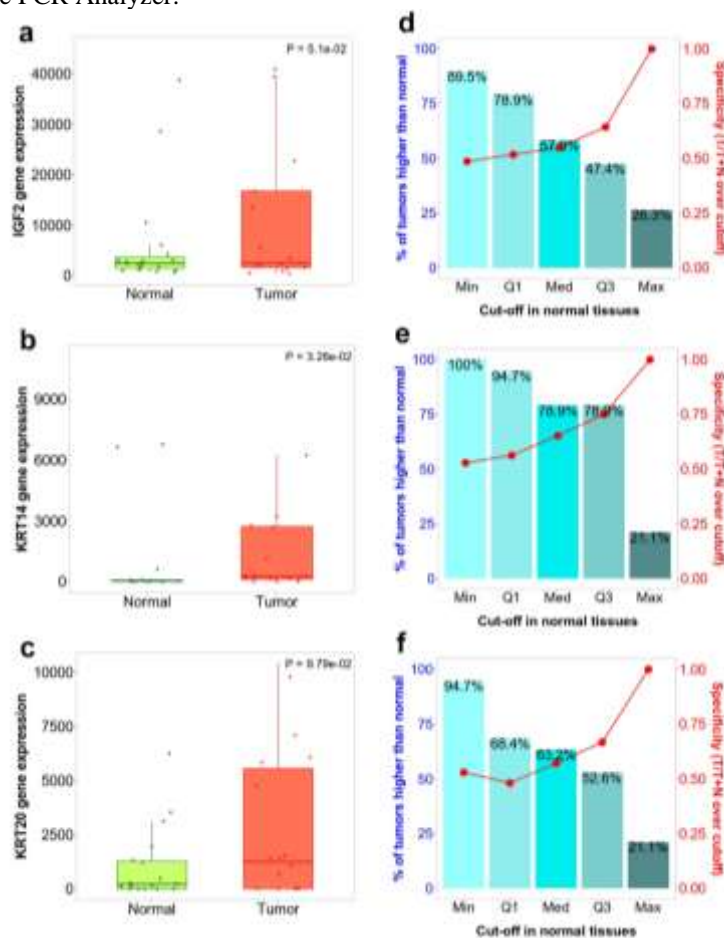

Fig. 2. Boxplots of IGF2 (a), KRT14 (b), and KRT20 (c) expression in TCC when comparing paired normal and tumor gene RNA-seq. The bar charts of IGF2 (d), KRT14 (e), KRT20 (f), represent the proportions of urinary samples in TCC that show greater expression of the particular gene compared to control group at each of the quantile cutoff values (minimum, 1st quartile, median, 3rd quartile, maximum).

Sensitivity and Specificity

The ROC curve analyses of gene expression in UCPs showed IGF2 had 45.45% and 88.89% sensitivity and specificity, respectively, for detecting TCC (P= 0.05). KRT14 had 59 and 88.89% sensitivity and specificity, respectively (P= 0.03) (Table 2 and Fig. 3). The online analysis by TNMplot provides a graphical representation of sensitivity and specificity at the major cutoff values (minimum, Q1, median, Q3, and maximum) (Fig. 2).

Table 2. Receiver operating characteristics (ROC) curve analysis of gene expression in urinary cell pellet samples.

gene	Estimate criterion	AUC	J†	Sensitivity	Specificity	P value‡
KRT14	< 6.930	0.6751	139.89	50	88.89	**0.03**
IGF2	< 6.430	0.6633	135.34	45.45	88.89	**0.05**

†Youden index.
‡Significance level p (area = 0.05).
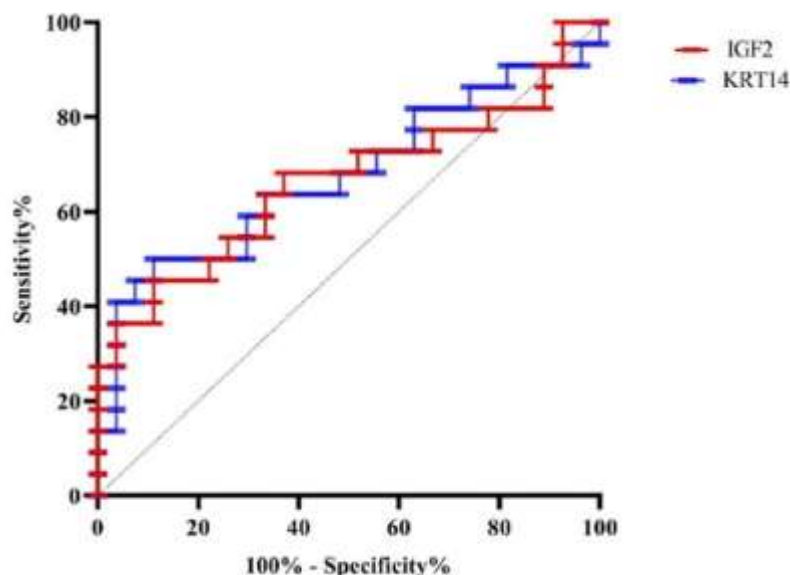AUC: Area under curve.

Fig. 3. The results of Receiver operating characteristics (ROC) curve analysis of performance of IGF2 and KRT14 transcript levels in urinary for differentiation of tumoral and non-tumoral samples.

Effects of IGF2, KRT14, and KRT20 Expression on Prognosis

IGF2, KRT14, and KRT20 expression were compared between the two groups, and the relation of their expression to patient prognosis was evaluated using the Kaplan Meier study by USUC Xena. We found that IGF2 was overexpressed in patient urine, leading to poor patient prognoses (Fig. 4a, log rank = 4.006, P= 0.04535). However, KRT14 (Fig. 4b, log rank = 3.474, P= 0.06233) and KRT20 (Fig. 4c, log rank = 1.191, P= 0.2750) overexpression were not significantly associated with low overall patient survival.
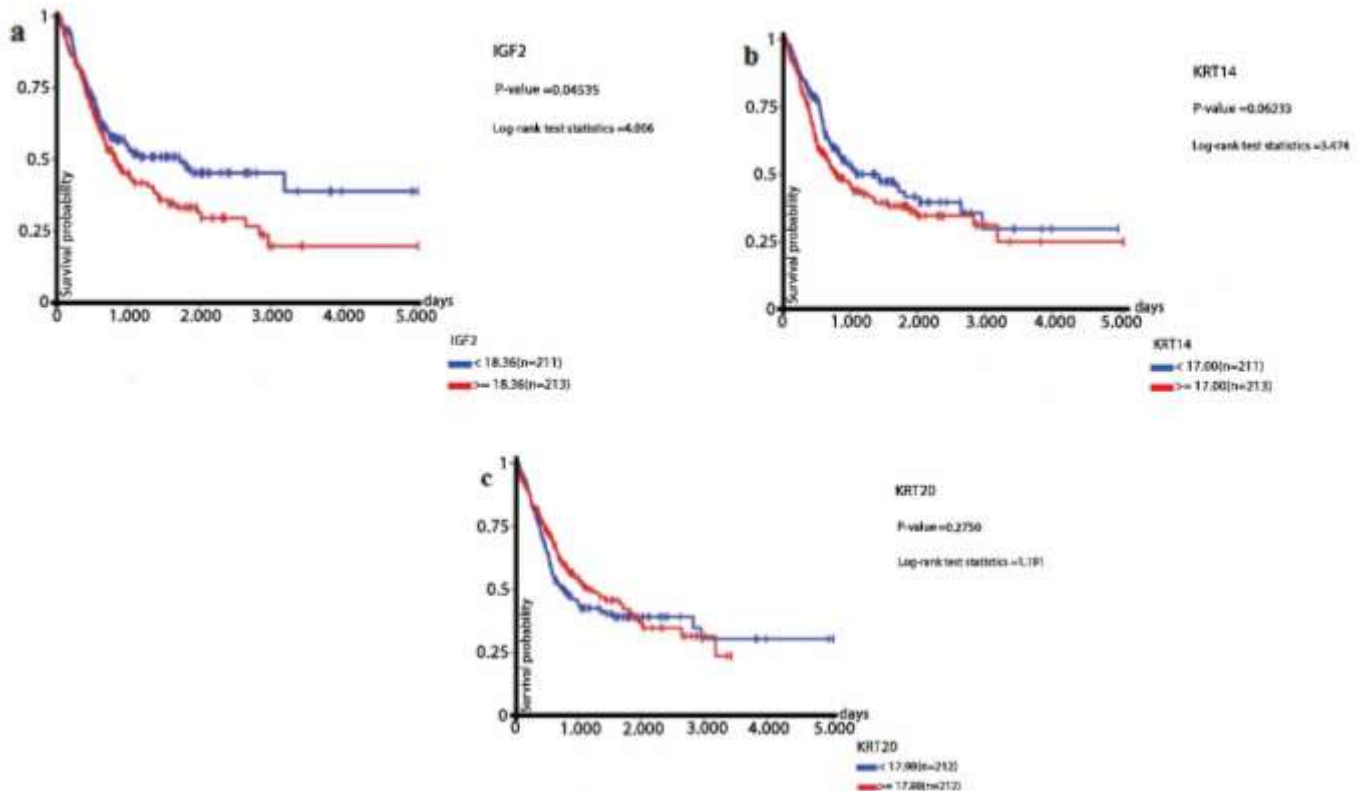
Fig. 4. Effects of the over-expression of IGF2 (a), KRT14 (b) and KRT20 (c) on overall survival in TCC patients. Kaplan–Meier Plotter, was used to analyse survival-time data and evaluate the prognostic significance of mRNA expression by USUC Xena.

Discussion

The first, most common and most important symptom of bladder cancer is hematuria, which is present in more than 90% of patients. This can be seen with the naked eye or diagnosed with a urine test. The presence of blood in the urine can be continuous or periodic depending on the severity of the cancer, and in most cases is painless. The gold standard for bladder cancer diagnosis is cystoscopy and biopsy (10). All adult patients with gross hematuria and all patients aged 35 years and older with microscopic hematuria should undergo cystoscopy evaluation. Flexible cystoscopy in the office to diagnose bladder cancer has the same results as rigid endoscopy. Although cystoscopy has excellent sensitivity and specificity for detecting large papillary tumors, it is less reliable for detecting small papillary tumors and carcinoma in situ (CIS). Urine cytology is also a standard diagnostic test to help diagnose bladder cancer. The contemporary sensitivity and specificity

of urinary cytology for diagnosing bladder cancer are 31- 62 and 94- 100%, respectively. Although urine cytology is a good marker in diagnosing bladder cancer, it is seriously weak in diagnosing low-grade cancers (11).

Urine-based biomarkers have been developed to complement standard diagnostic modalities for bladder cancer diagnosis and monitoring. Non-invasive tests with better sensitivity than urine cytology have been proposed as favorable alternatives to expensive and uncomfortable cystoscopy. Several urine-based biomarkers have been developed with greater sensitivity than urine cytology. Nuclear matrix protein 22 is a member of a protein family that shape the structure of the cell nucleus and is 20-fold overexpressed in malignant urothelial cells. The sensitivity and specificity of NMP22 are 0.69 and 0.77, respectively. UroVysion is a test that detects aneuploidy of chromosomes 3, 7, and 17, and homozygous loss of the 9p21 locus in exfoliated urothelial cells. A positive test is

defined as five or more urinary cells with an increase of two or more chromosomes, 10 or more cells with an increase of a single chromosome, or homozygous deletion of 9p21 in more than 20% of exfoliated cells. The overall sensitivity and specificity of fluorescence in situ hybridization (FISH) are 0.63 and 0.87, respectively. A negative FISH is associated with benign cytological changes and is used as a reflex test in an atypical cytology. In patients with atypical cytology and negative cystoscopy, the 3-year survival rate without recurrence increases from 34% in FISH-positive conditions to 67%. FISH is relatively insensitive for detecting low-grade bladder tumors, and no consensus exists on the criteria that can be used to evaluate abnormal cells (11).

The bladder tumor antigen assay (BTA) includes detection of two basement membrane antigens; hCFHrp and complement factor H using monoclonal antibodies, and the FDA has approved them for the diagnosis and follow-up of bladder cancer. The BTA stat test is a dipstick-based qualitative point-of-care test. The sensitivity and specificity of qualitative BTA in 22 studies were 0.64 and 0.77, respectively. The high rate of false positives in both BTA assays can be attributed to cross-reactivity with red blood cells because complement factor H is present in high concentration in serum, and therefore associated with the high rate of false positive results in hematuria. Immunocyte is a cellular adjunct for urinary cytology that uses fluorescently-labeled antibodies against three antigens that are specific for bladder cancer on urothelial cells (12). In the studies, the sensitivity and specificity of Immunocyte were both 0.78. Immunocyte depends on the operator, has high inter-observer differences, and poor compatibility (11, 13).

Many studies have been conducted to investigate the muscle-invasive bladder cancers (MIBCs). Non-muscle-invasive bladder cancers (NMIBCs) are difficult to detect, and most of the available tests are not sufficiently sensitive to identify these cancers in the lower grades. Therefore, studies were conducted to identify markers in NMIBC patients. Hedegaard et al. in 2016 divided NMIBC patients into three groups and proposed paths for their development and progress (14). Montalbo et al. identified eight genes in NMIBC patient urine that could be used as markers to diagnose these patients. These were ANXA10, IGF2, KIFC3, KRT20, LCN2, MAGEA3, RPS21, and SLC1A6 (2). Robertson et al, analyzed 412 muscle-invasive bladder cancers and reported IGF2 as an NMIBC gene and that KRT14 and KRT20 overexpress in basal squamous and luminal subtypes, respectively (15).

The results showed that IGF2 is overexpressed in urinary bladder cancer patient urine and could be a potential effective prognostic biomarker of poor outcomes in TCC. This result is similar to those found in some previous studies. In 2017, Salomo et al. evaluated urinary IGF2 transcript quantitation for the non-invasive bladder cancer diagnosis and found that relative transcript levels were significantly elevated 188-fold in patient urine compared to controls (P< 0.001). In their study, the sensitivity and specificity were similar to those of urine cytology (16). Recently, El-Abd et al. evaluated IGF2 expression in urine from 50 bladder disease patients and 20 controls. They found that urinary cancer patients had greater urinary IGF2 expression than normal individuals (17). Other studies found that IGF2 has a critical role in signaling in bladder cancer aggressiveness (18) and that IGF2 expression in urine significantly correlated with tumor recurrence and poor prognosis (19). The results of TNM analysis showed that IGF2 was not significantly expressed in patients with urinary bladder cancer. There is some evidence supporting the role of gender in the incidence and prognosis as well as molecular pathways in bladder cancer. It is suggested to investigate molecular pathways and genes involved in bladder cancer in male and female patients separately. In the present study, all the participants were male, but in the TNM analysis, the gender of the subjects was not specified and in USUC Xena analysis, 72.7%

of participants were male and 27.3% were female (20, 21).

This study showed that KRT14 is overexpressed in bladder cancer patient urine but is a not an effective prognostic biomarker for TCC outcomes. In 2022, Ingenwerth et al. evaluated the prognostic value of cytokeratin KRT14 in TCC by immunohistochemistry and RT-qPCR. They found that KRT14 and some other markers are significantly overexpressed in high-grade and muscle invasive TCC (MIBC) compared to low-grade and NMIBC patient tissues. They also showed that disease-specific survival rates were lower in patients with high KRT14 mRNA expression and KRT14 protein expression was significantly greater in high-grade than in low-grade TCC (22). This finding contradicted our results, possibly due to differences between IHC and urine samples.

In our study, KRT20 was not significantly overexpressed urinary bladder cancer patient urine, nor was it a good prognostic biomarker for TCC. This finding was similar to that of Ramirez-Backhaus et al., which found no differential clinical result in patients who underwent cystectomy in micro metastasis or lymph node involvement according to their KRT20 expression in pathology samples (23). It is possible that KRT20 is not specific to bladder cancer but is found in other epithelial tumors as well.

In 2022 Schulz et al. compared the sensitivity and specificity of cystoscopy, urine cytology, and some urinary biomarkers in TCC detection. They reported that the highest sensitivity and specificity of 97 and 93%, respectively, was for AssureMDx, while the sensitivity and specificity of urine cytology was 48% and 86%, respectively (24). As with cytology, it is important to report the performance of the desired markers in the various bladder cancer grades and stages. Van Kessel et al., for example, reported the better performance of AssureMDx in stages T1 and above, compared with Ta. Another important matter is that some marker results are based on

limited number of studies, such as AssureMDx only based on study by Van Kessel et al. (25).

In our study the sensitivity and specificity of IGF2 were 45.45 and 88.89%, respectively, for detecting TCC in urine samples, which was comparable to urine cytology. The sensitivity and specificity of KRT14 were 59 and 88.89%, which was greater than those of urine cytology and BTA-Stat. Although these variables for IGF2 and KRT14 are lower than some new tests, including NMP22, UroVysion, and Bladder EpiCheck (24), these genes could be used in combination with other tests to improve diagnostic accuracy.

Our study is not free from limitations. The small sample size and loss of comparison with other markers are two main drawbacks of our study. On the other hand, positives are that our study was performed in a laboratory independent from the original, and with a homogeneous population. Further multicentral studies on these markers with larger sample sizes are suggested to identify the satisfactory cut-off point or consider the combination of these biomarkers with cytology or other markers.

Our study showed that IGF2 and KRT14 are overexpressed in urine of patients with urinary bladder cancer and IGF2 could be a potential biomarker for poor prognoses in TCC.

Conflicts of Interest
The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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