Journal of Clinical and Biomedical Sciences

Review Article

Deregulated molecular genetic pathways in urinary bladder cancer with the importance of molecular biomarkers in diagnosis and follow-up- A comprehensive and updated review.

Sudarshana Basu[1*], Nirmalya Banerjee[2], Sourav Kumar Nandi[3], Soumen Das[4], Kalpesh Parmar[5], Soma Mukhopadhyay[6]

1. Scientist, Department of Molecular Biology, Netaji Subhash Chandra Bose Cancer Research Institute, 3081, Nayabad, Kolkata-700094, West Bengal, India.
2. Consultant histopathologist and lab in-charge, Department of Pathology, First floor, Netaji Subhas Chandra Bose Cancer Hospital. 3081, Nayabad,Kolkata-700094, West Bengal, India
3. Research Fellow, Department of Molecular Biology, Netaji Subhash Chandra Bose Cancer Research Institute, 3081, Nayabad, Kolkata-700094, West Bengal, India
4. Chief Consultant, Department of Surgical Oncology, Second floor, Netaji Subhas Chandra Bose Cancer Hospital. 3081, Nayabad, Kolkata-700094, West Bengal, India
5. Assistant Professor, Department of Urology. Nehru Hospital. Post Graduate Institute of Medical Education and Research, Chandigarh-160012, India.
6. Managing Trustee and HOD, Department of Molecular Biology, Netaji Subhash Chandra Bose Cancer Research Institute, 3081, Nayabad, Kolkata-700094, West Bengal, India.

Abstract

This article reviews and compares various currently available non-invasive, molecular biomarker-based tests for bladder cancer (BC) detection, and evaluates other potential molecules that may be used for BC diagnosis and surveillance. Currently, urinary cytology and periodic cystoscopies are clinically recommended for the diagnosis and monitoring of BC. Though highly specific, urinary cytology lacks sensitivity, whereas cystoscopies are invasive tests that are uncomfortable for the patient. Molecular biomarkers associated with BC progression form the basis for various available non-invasive tests. Urinary proteins like NMP22, MCM5 and Human complement factor-H related protein (BTA) are detected by ELISA/Immunochromatographic assay devices (NMP22, BTA, ADXBLADDER). Chromosomal abnormalities reported in BC -aneuploidy in chromosomes 3, 7, and 17 and deletions in chromosome 9- are detected by the Fluorescence in situ Hybridization based UroVysion test. Tumor antigens like sulfated mucin glycoproteins and glycosylated CEA help to light up urothelial carcinoma cells exfoliated into urine (uCyt+). Mutations in TERTp, FGFR3, KRAS, HRAS, TP53, CDKN2A, ERBB2 and PIK3CA contribute to BC progression and these are detected in tumor DNA by RT-qPCR, NGS and/or Sanger sequencing-based assays (Uromonitor, UroSEEK, AssureMDX). mRNA levels of genomic markers frequently deregulated in BC like IGFBP5, HOXA13, MDK, CDC2, CXCR5, IGF2, ABL1, CRH, UPK1B and ANXA10 are assesed by CxBladder and Xpert Bladder Cancer Monitor. BC associated changes in DNA methylation are detected by real time PCR and NGS based assays (EpiCheck, UroMark, AssureMDX). These tests have not yet been formally indicted into clinical practice but can serve as sensitive indicators for early diagnosis, disease monitoring, and treatment response in BC.

Keywords: bladder cancer, biomarkers, non-invasive tests, molecular events in bladder cancer, genetic changes in bladder cancer.

***Corresponding Author**
Dr. Sudarshana Basu
Scientist, Department of Molecular Biology, Netaji Subhash Chandra Bose Cancer Research Institute, 3081, Nayabad, Kolkata-700094, West Bengal, India.
Mobile No : 09836076351
E-mail: sudarshanabasu77@gmail.com
Conflict of Interest: None
Financial Aid: Nil

Introduction

Urinary Bladder Cancer (BC) is the 10th most common cancer globally, and though less prevalent in India, its incidence is increasing due to environmental factors like smoking and exposure to potentially mutagenic compounds that are filtered into the urine by the kidneys. The urinary bladder is an organ located in the lower abdomen that collects the urine

received from the kidneys till micturition. Urothelial cells lining the inner surface of the bladder and urinary tract are continually exposed to various environmental carcinogens like aromatic amines, polycyclic aromatic and chlorinated hydrocarbons generated by smoking or occupational exposure. Unsurprisingly, 90% of BC cases arise in these urothelial cells of the bladder or the urinary tract. Localized tumors that have not yet invaded the smooth muscle layer carry excellent prognosis. However, if the smooth muscle is involved, survival rates drop significantly.

1.1 Classification, Staging, and Grading

Two distinct subtypes pathologically characterize BC—non-muscle-invasive carcinoma (NMIBC) and muscle-invasive carcinoma (MIBC)—depending on whether or not the tumor has invaded the bladder muscle layer. The first classification of urothelial tumors divided them according to grades, with G1 being the lowest and G3 being the highest.[1] This classification was updated in 2004 and more recently in 2016, with there being essentially two grades of lesions- low grade and high grade. Low grade encompassed the earlier G1 and part of G2, and high grade lesions comprised the more aggressive lesions of G2 and G3. Abnormal growth lesions of low malignant potential were characterized as Papillary Urothelial Neoplasms of Low Malignant Potential (PUNMLP) and grouped as G1 tumors.[1]

World Health Organization classification (fourth edition) of tumors of the urothelial tract[2] further stratified non-invasive urothelial tumors depending on the extent of invasion (Figure 1). Ta and T1 tumors are tumors that have invaded till the lamina propria. Ta tumors are papillary carcinomas that grow towards the bladder's luminal portion but not into the bladder wall. T1 tumors grow into the connective tissue layer in the bladder wall but have not crossed the lamina propria. These tumors may be low grade or high grade depending on cellular features. Carcinoma In situ (CIS) is a type of NMIBC composed of flat cells growing in the bladder wall's inner lining. Though non-invasive, this lesion is of a higher grade, and its associated risk to the patient and molecular features are similar to MIBC.

Tumors extending through the bladder muscle layer are high grade tumors classified as MIBC. They are classified from T2 to T4, based on the degree of invasion through the muscular layer, invasion of perivesical tissue, or invasion of adjacent tissues/organs.

NMIBC is more common at detection with the majority of patients of BC in stage Ta (70%), T1 (20%), or CIS (10%). However, recurrence occurs at least once (80%), and for some (30%), the disease will progress into MIBC.[3]

1.2 Genetic and molecular events in BC

BC is characterized by a high mutation rate of 7.68 mutations per MB within coding regions, with aneusomies, deletions, and amplifications affecting almost all chromosomes.[4] However, drivers of this high mutation rate are not known conclusively. A near diploid karyotype with few genomic rearrangements usually characterizes NMIBC. Genetically MIBC is aneuploid with many structural alterations.[5]

The deletion of chromosome 9 appears to be the first step in the pathogenesis of BC, as it is prevalent in both hyperplasia and dysplasia.[6] The candidate tumor suppressor genes usually affected by this deletion are Cyclin-dependent kinase inhibitor 2A (CDKN2A), Cyclin-dependent kinase inhibitor 2B (CDKN2B), Patched 1 (PTCH1), Deleted in bladder cancer 1 (DBC1), and Tuberous sclerosis 1 (TSC1).[5] Deletions in chromosomes 8p, 2q, and 5q are usually associated with aggressive disease. Loss of heterozygosity of 9p, homozygous deletion of CDKN2A, and loss of expression of p16 in NMIBC predict reduced recurrence-free survival.[5]

Comparative Genomic Hybridization and LOH analysis have identified other copy number changes and allelic loss observed in BC.[5] The high number of somatic mutations are dominated by C:G>T:A transitions in the context of TpC dinucleotides. These mutations are characteristic of mutations caused by the APOBEC (Apolipoprotein B mRNA editing enzyme) family of cytidine deaminases.[7] Additionally, APOBEC3B was overexpressed in all BC cases examined, suggesting a significant role for APOBEC3B mediated mutagenesis in BC.[4]

Telomerase Reverse Transcriptase (TERT) elongates telomeres at the ends of chromosomes. Since telomere shortening acts as a mitotic clock, activation of TERT is crucial for tumor cells' continued growth. TERT promoter mutations located -124 and -146 base pairs (bps) upstream of the transcription start site of TERT have been detected in up to 80% of BC cases independently of stage and grade and is associated with both NMIBC and MIBC pathways of urothelial tumorigenesis.[8,9] These mutations constitute an early event in oncogenesis and are not present in inflammatory or urinary infections. Thus, detection of TERTp mutations can

serve as early biomarkers for diagnosing and surveillance of BC.[10, 11]

Another alternative telomerase activating mechanism in BC involves methylation of the TERT promoter in a region described as TERT hypermethylated oncological region (THOR).[12] THOR hypermethylation has been found to coexist in BC with TERTp mutations. This coexistence has significant prognostic value as it is a dynamic process that aids disease progression in both TERTpWT and TERTpMUT NMIBC.[13]

The TCGA cohort study of MIBC cases observed that mutations in specific gene pairs are mutually exclusive, whereas other gene pairs show co -occurrence of mutations.[4,7] This sheds light on the molecular pathology of urinary BC. The mutually exclusive alterations belong to CDKN2A and TP53 (Tumour protein P53), CDKN2A and RB1 (Retinoblastoma protein), TP53 and MDM2 (Mouse double minute 2 homolog), and FGFR3 (Fibroblast growth factor receptor 3) and RB1 gene pairs. Co-occurrence of mutations occurs in TP53 and RB1 and the FGFR3 and CDKN2A genes. Additionally, FGFR3 and RAS (Rat Sarcoma) alterations are mutually exclusive events as RAS is a downstream effector of FGFR3 and so co-occurrence of mutations in both is a redundant event in the FGFR3 signaling cascade.[14]

Amplification of chromosome 6p22 has been frequently seen in MIBC and high-grade NMIBC.[15] High expression of CDKAL1 (CDK5 Regulatory Subunit Associated Protein 1 Like 1), E2F3 (E2F transcription factor 3), and SOX4 (SRY-Box Transcription Factor 4) is seen in patients with the chromosomal 6p22 amplification.[15] Tumor Protein 53 (TP53) regulates the cell cycle, thereby preventing uncontrolled cellular growth and proliferation. Mutation or deletion of TP53 is observed in most cases of CIS and MIBC.[5,16] According to TCGA cohort data, 89% of MIBC cases have an inactivated TP53 cell cycle pathway with either TP53 mutations or MDM2 amplification or overexpression.[7]

Deregulated activation of the PI3K/AKT/ mTOR pathway also leads to increased cellular proliferation, tumorigenesis, and tumor progression in MIBC. This deregulation may be caused by activating mutations in receptor tyrosine kinase genes like ERBB2 (Erb-B2 Receptor Tyrosine Kinase 2), ERBB3 (Erb-B2 Receptor Tyrosine Kinase 3), and FGFR3; or by silencing PTEN (Phosphatase and tensin homolog) and TSC1 (Tuberous sclerosis 1), which are negative regulators of this pathway. Downstream kinases like AKT/PKB (Ak strain transforming/ Protein kinase B) and PI3KCA (Phosphatidylinositol-4,5-bisphosphate 3-kinase) may also be mutated in MIBC.[17]

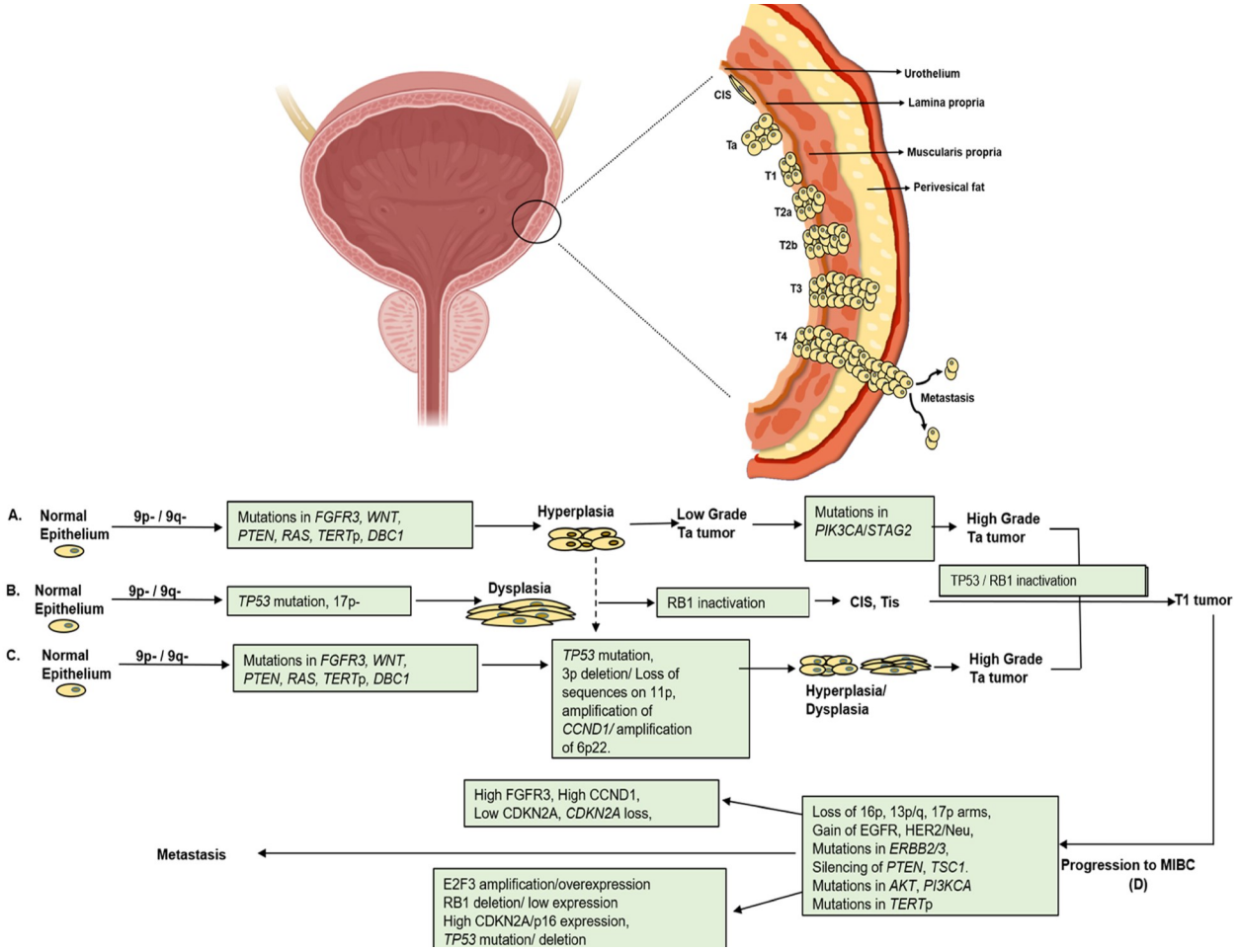
1.3 Genetic basis of progression

The genetic and molecular pathways involved in the histological progression of BC have been schematically represented in Figure 1. Since the deletion of chromosome 9 is prevalent in both hyperplasia and dysplasia, it is the first step leading to carcinogenesis in both NMIBC and MIBC. NMIBC usually develops via the FGFR3/RAS pathway, whereas MIBC progresses predominantly due to the deregulation of the TP53/RB1 pathway.[18] FGFR3 alterations are predominantly rare in CIS and MIBC, except luminal MIBC that appears to be enriched with FGFR3 activating mutations.[19]

A histological progression of NMIBC would begin as either hyperplasia or dysplasia. Urothelial hyperplastic cells with mutations in the FGFR3/ RAS pathway develop into non-invasive papillary tumors with a high recurrence rate.[14,20,21] Additional mutations in PI3KCA/ STAG2 (Stromal antigen 2) lead to high-grade Ta tumors, which may progress to the T1 stage after CDKN2A inactivation or TP53 /RB1 inactivation.[17] Activation of the FGFR3/ RAS pathway is usually associated with favorable clinical outcomes in pT1 tumors.[17,22] Dysplasia develops due to mutations in TP53. The loss of RB1 in dysplastic cells leads to the T1 stage via CIS (Tis).[17]

NMIBC can recur retaining the same alterations, stage, and grade, or progress into MIBC through the acquisition of multiple additional alterations such as the loss of RB1, acquisition of CDKN2A mutations, loss of 16p, 13q/p, or 17p, gain of epidermal growth factor receptor (EGFR) function, and amplification of cell cycle genes particularly Cyclin D1 (CCND1).[6,23] Overall, two different molecular pathways appear to be operative in urothelial carcinoma progression in MIBC. One is defined by high FGFR3 and CCND1 expression, low CDKN2A expression, often associated with CDKN2A loss. The other pathway is defined by E2F3 amplification/ overexpression, RB1 deletions/ low expression, and high CDKN2A/p16 expression. [24-26] Additional mutations in genes of the PI3K/AKT/ mTOR pathway and somatic TERT promoter mutations may further supplement this sequence of histological progression.[17]

Figure 1: Potential pathways in molecular and histological progression of Bladder Cancer (BC).



BC is characterized by broadly two distinct subtypes: non-muscle invasive BC (NMIBC) and muscle invasive BC (MIBC). Stages Ta and T1 are non-muscle invasive whereas T2-T4 are muscle invasive. Carcinoma in situ (CIS) is a non-muscle invasive (NMI) high-grade tumor that presents as a flat urothelial lesion. BC progression occurs by 3 pathways depicted as (A), (B) and (C). Chromosome 9 deletion is usually the first step in tumorigenesis.[6] This is followed by mutations in the FGFR3/RAS pathway (A and C) and/ or the TP53/RB1 pathway (B and C).[18] Low grade Ta carcinomas with recurrent PIK3CA/STAG2 mutation develop into high-grade Ta carcinomas.[17] Progression to T1 occurs due to TP53/ RB1 inactivation. Progression from T1 to T2 (MIBC) is initiated by various genomic alterations.[6,23] Two different molecular pathways appear to be operative in MIBC progression (D). One is defined by high FGFR3 and CCND1 expression, low CDKN2A expression, often associated with CDKN2A loss. The other pathway is defined by E2F3 amplification/ overexpression, RB1 deletions/ low expression, and high CDKN2A/p16 expression.[24-26]

1.4 BC diagnosis and follow-up

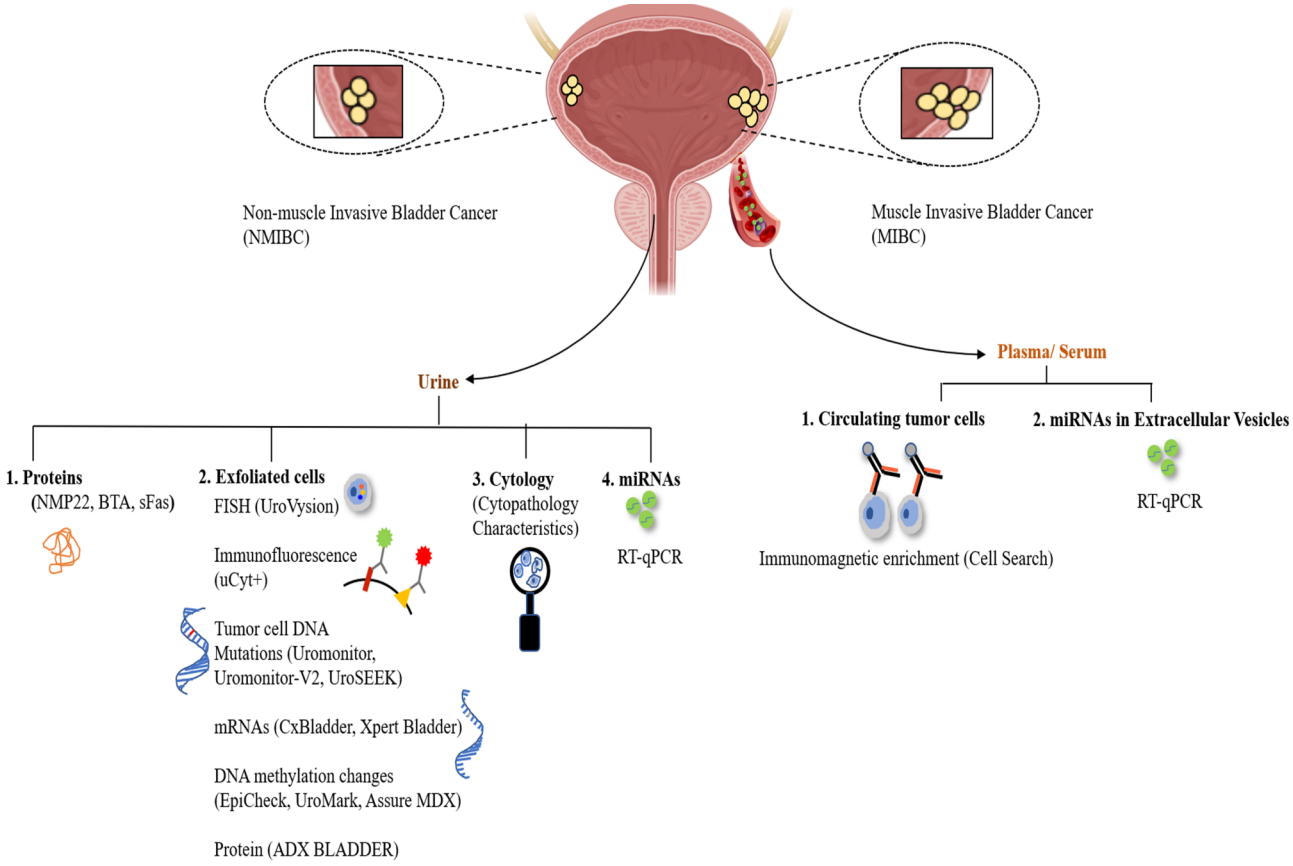
The majority of NMIBC tumors have a high rate of recurrence after primary tumor removal. Thus, life-long follow-up of BC cases is an essential aspect in disease management and calls for tests with parameters different from those used for the initial diagnosis. Initial diagnosis requires biomarker-based tests with high specificity (to avoid unnecessary cystoscopy and other invasive interventions), whereas follow up requires the detection of biomarkers with high sensitivity (so that there is no possibility of missing a recurrent case). The classical tests used for initial diagnosis and follow-up of BC are urine cytology and cystoscopy. Urine cytology is non-invasive, easy to perform, and inexpensive, with a detection specificity of up to 98% in high-grade carcinomas. Cytology was earlier thought to have good sensitivity for high-grade disease, with reported sensitivities of 38% - 84%.[27] However, recently conducted more extensive multicentric studies have demonstrated much lower sensitivities, with a prospective study of 1016 patients demonstrating an overall sensitivity of 22%- 13% for low-grade tumors, 23% for high-grade tumors, and 25% for high-risk

tumors.[28,29] The reason for this large variability in multicentric studies is that cytology requires a skilled uro-pathologist for interpretation. Hence, cytology cannot be relied upon as a stand-alone test in valuating BC since a negative cytology report may not exclude the presence of a tumor in the urinary tract. Also, atypical cytology reports have resulted in the use of cytology in follow-up becoming increasingly questionable.

Cystoscopy, though more reliable than urine cytology in BC diagnosis, is unsuitable for primary screening because of its invasiveness and costs. Serial cystoscopies done for follow-up may cause discomfort and distress to patients. Furthermore, the accuracy of cystoscopy in diagnosis depends on the clinician's ability. Hence, it may be falsely positive or negative due to the presence of small areas of tumor or carcinoma in situ (CIS) that may be difficult to recognize. Thus, there is a need for new urine biomarkers for BC diagnosis and follow-up surveillance, which will detect BC with high specificity during initial diagnosis and be sensitive enough to identify early recurrence and prevent disease progression to higher grades. Such biomarker-based tests should be non-invasive, cost-effective, accurate with a high degree of sensitivity and specificity, rapid, easy to administer, and interpret by clinicians.

In this review, we have summarized and critically reviewed potential BC biomarkers that aim to detect changes in cellular targets such as proteins/ peptides, antigens/metabolites, genomic, epigenetic, or transcriptomic materials in serum and urine samples (Figure 2). We also identify some emerging molecules that may be used for diagnosis and follow-up in BC patients.

Figure 2: Targets for biomarker analysis in Bladder Cancer and their starting bio-material.



Summary of various techniques and biomarkers that detect genomic, transcriptomic, epigenetic or protein alterations in serum or in urine samples for the diagnosis and surveillance of BC. Proteins, exfoliated tumor cells, microscopic detection of the cytopathological characteristics of cells, and RT-qPCR based detection of miRNAs within extracellular vesicles may be used as urinary biomarkers. Immunomagnetic enrichment of circulating tumor cells or RT-qPCR based identification of miRNAs in extracellular vesicles may serve as diagnostic biomarkers from plasma/ serum.

2. Method of Search

To understand the pathogenesis of Bladder Cancer and the genetic and molecular events contributing to it, various reviews and research articles were studied to get a comprehensive idea of disease progression. A systematic literature search was performed using PubMed and Google Scholar databases to identify validation studies and reports evaluating established and potential BC biomarkers till October 2020. The keywords used for the literature search were: (bladder cancer OR urothelial cell carcinoma) AND (detection OR diagnosis OR surveillance) AND (biomarker OR assay). The literature search yielded 59 articles out of which 50 articles were included in the study. For each biomarker-based test, entries received were divided in to 3 parts: (i) information on the biomarkers and the test- how it is done and the technique employed to detect the biomarker, (ii) validation studies performed for the biomarker based test with sensitivity and specificity values and (iii) potential advantages and disadvantages for each test.

Inclusion criteria- All studies had ≥20 patients in both bladder cancer and control cohorts and reported sensitivity and/ or specificity and/ or receiver operating characteristics (ROC) curve. Validation studies with larger cohorts were preferred. Literature discussing only human studies were considered.

"Exclusion criteria – Comments, letters to editor, non -relevant articles, Articles not written in English".

3. Biomarkers for Bladder Cancer diagnosis and follow-up

3.1 NMP 22 Protein Test

NMP22 is a nuclear mitotic protein involved in the distribution of chromatin to daughter cells during mitosis. This protein is present at low levels in the urine of healthy individuals; however, BC patients may have up to 25-fold higher levels.[30] The apoptosis of urothelial cells releases the protein into the urine, where it may be detected by two commercially available FDA-approved tests known as NMP22 BC Test and NMP 22 BladderChek.[3] The NMP22 BC Test is a quantitative laboratory-based sandwich ELISA that utilizes antibodies which recognize two different epitopes of the protein. The NMP22 BladderChek test is a qualitative, point-of-care test that involves adding four drops of urine to a proprietary immunochromato graphic-assay device, from which the results can be read 30-50 minutes later.

The sensitivity of the NMP22 tests varies from 47% to 100% and is more sensitive in detecting low grade and low stage BC than cytology alone (sensitivity of 38%).[3] However, these studies mostly involved patients without a history of BC. The NMP22 assay sensitivity increases with an increase in tumor size, grade, and stage. Since recurrent tumors are often smaller than primary tumors, measurement of NMP22 alone may not be sufficient to detect BC recurrence. Also, NMP22 assays show less specificity than urine cytology (98%) due to higher false-positive rates (33%-50%) in patients with urolithiasis, inflammation, benign prostatic hyperplasia, or urinary tract infections.[31] The reported specificity of the quantitative NMP22 immunoassay varies between 60 to 90%, depending on the cut-off value used.[3] Although the manufacturers' recommended cut-off value is 10U/ml, variable limits ranging from 3.6 U/mL to 27 U/mL have been applied, depending on the optimum sensitivity and specificity determined by the receiver operating curve. Also, the typical values for NMP22 levels in urine differ between men and women, with women having a higher protein level than age-matched men.[31,32]

The FDA has approved both NMP22 assays for detection and surveillance of BC. Both tests are inexpensive, non-invasive, and easy to administer and have the potential of avoiding discomfort, risk, and expense associated with a cystoscopy.

3.2 Bladder Tumor Antigen (BTA) Test

The Bladder Tumour Antigen test is a urine test that detects bladder tumor-associated antigen (human complement factor H-related protein). Bladder tumor cells produce human complement factor H-related protein (hCFHrp), which is released into patients' urine as the tumor invades the stroma. The protein may be detected using the BTA stat and the BTA TRAK tests. The BTA stat is a qualitative immunochromatographic assay device that can detect hCFHrp in patients' urine and can be performed at the point-of-care. BTA TRAK is a quantitative ELISA test that is performed in a laboratory.

In a study conducted by Raitanen et al., voided urine samples were obtained from 501 patients before cystoscopy and tested for cytology and BTA stat.[33] The overall sensitivity for BTA stat as compared to cytology was 56% and 19.2%, respectively. However, the specificity of BTA stat was 85.7%, as opposed to 98.3% in the case of cytology. They concluded that the BTA stat test could replace routine cytology as it was more sensitive in patients with low-grade disease.[33] Similar values were obtained by Banos et al. and verified an overall sensitivity that was superior to cytology (73.62% vs. 61.7%), but with lower specificity (83.33% vs. 92.36%).[34] The BTA stat test's sensitivity was also found to increase with an increase in tumor grade

and stage.[34] However, in another study conducted by Murphy et al.[35] only 29% of random voided urine samples collected from patients with a history of BC were found to be positive for the BTA qualitative test.

The main disadvantage of the BTA tests appears to be its low specificity as opposed to that of cytology. Complement factor H (CFH) is produced and secreted by kuppfer cells, hepatocytes, vascular endothelial cells, and platelets. Thus, any disease that causes the endogenous protein to leak into the bladder can yield a positive result. Lower specificity has been observed in patients with urinary tract infections, urinary calculi, nephritis, renal stones, renal cancer, cystitis, benign prostatic hyperplasia, hematuria, and proteinuria.[31] Also, CFH is present in human serum at high concentrations (0.5mg/ml), so the BTA tests may yield false-positive results in benign conditions that cause hematuria.[31] These tests should only be used when information is available for the clinical evaluation of the patient and alongside other diagnostic procedures, but not for screening. The FDA has approved both tests for use in the management of BC in combination with cystoscopy for the follow-up of NMIBC patients.

3.3 UroVysion

UroVysion utilizes Fluorescent in situ Hybridization (FISH) and involves probing exfoliated cells in urine with four fluorescent, centromeric, chromosome-enumeration probes. It is a multitarget, multicolor test that detects aneuploidy in chromosomes 3, 7, and 17; and the loss of the 9p21 locus.[36] The criteria for defining a positive UroVysion test involves observation of ≥ 5 cells which have gained two or more chromosomes, ≥ 10 cells with a gain of one chromosome, or ≥ 20% of cells with a loss of the 9p21 locus.[31] However, Bubendorf et al. concluded that not all FISH aberrations are equally important and that there are no universally accepted criteria for a positive FISH result.[37]

Lokeshwar et al. have reviewed numerous case-control studies that report a sensitivity between 69% and 87% with reduced sensitivity in the detection of low grade (36% to 57%) and low stage (62% to 65%) tumors.[38] However, high-grade and high-stage tumor detection showed higher sensitivity (83% to 97%) with 100% sensitivity in detecting CIS.[31] Chromosomal aberrations detected by UroVysion FISH may also indicate other primary tumors like those of the renal pelvic, ureteral transitional cell carcinoma, prostatic carcinoma, and others. Hence, this test is not specific for the detection of BC. A comparative study of UroVysion FISH and urine cytology in BC detection indicates a higher sensitivity than cytology (62% vs. 29%), but lower specificity (89% vs. 97%). The authors recommend that UroVysion only be used in high-risk patients, especially those with an ambiguous urine cytology result.[39] An interesting study conducted by Skacel et al. demonstrated that false-positive FISH test results with ambiguous cytology in the backdrop of a negative concurrent bladder biopsy could predict transitional cell carcinoma (biopsy-proven) within 12 months following the date when the sample tested by FISH was obtained.[40]

The FDA has approved the UroVysion test to diagnose BC in patients presenting hematuria and monitor patients with a history of BC. Its primary disadvantage is that it requires the use of fluorescent probes and visualization of results under a fluorescent microscope. So, it is expensive to perform and requires highly specialized equipment and experienced personnel for reliable operation.

3.4 uCyt+

The uCyt+ assay is an immunocytologic test based on the visualization of tumor-associated antigens in exfoliated urothelial carcinoma cells using monoclonal antibodies. The test uses fluorescein-labeled antibodies directed against sulfated mucin glycoproteins and a Texas red– linked monoclonal antibody against glycosylated forms of high molecular carcinoembryonic antigens (CEA). This assay, like UroVysion, requires a high-quality fluorescence microscope and a cytocentrifuge. Even though the assay can be performed easily in the laboratory, training of personnel, experience, and regular quality control is essential. Also, the microscopic examination of slides is time-consuming and is observer-dependent.

uCyt+ assays report a sensitivity that varies between 38% and 100%, and the specificity ranges from 75% to 90%.[38] A longitudinal study by Schmitz-Drager et al. evaluated uCyt+ and cytology performance for low-grade NMIBC tumor (pTa G1-2) follow-up. uCyt+ presented a high specificity of 82.8%, which dropped to 67% in hematuria presenting cases. Urine cytology, on the other hand, had a specificity of 94.9%. The sensitivity of uCyt+ was higher in primary (92.3%) than in recurrent (65%) disease, probably because of the smaller size of the recurrent tumors. Sensitivity was superior to cytology, which was 30.3%.[41] The FDA has approved this assay for surveillance in patients with a history of BC.

3.5 Cell Search

The CellSearch CTC kit identifies, isolates, and enumerates circulating tumor cells of epithelial origin (CD45-, EpCAM+, and cytokeratins 8, 18+,

and/or 19+) from peripheral blood. Zhang et al. performed a meta-analysis to assess the relevance of circulating tumor cell (CTC) detection in the prognosis and diagnosis of BC. The detection of CTCs showed a significant correlation with tumor stage, grade, metastasis, and regional lymph node metastasis. The CTC detection assays had a sensitivity and specificity of 35% and 97%, respectively.[42]

3.6 CxBladder

CxBladder is a suite of non-invasive, urine-based tests that detect and measure the mRNA levels of five genomic markers (Insulin-Like Growth Factor Binding Protein 5- IGFBP5, Homeobox A 13- HOXA13, Midkine-MDK, Cell Division Cycle Protein 2 Homolog- CDC2, and C-X-C motif Chemokine Receptor 5- CXCR5) by RT-qPCR in a single voided urine sample. The CxBladder suite of tests is easy to use and clinically validated even though they have not obtained FDA approval.

The CxBladder suite of tests has three different modalities for low-risk patients with hematuria (CxBladder Triage), patients with a higher risk of BC (CxBladder Detect), and for surveillance of patients with a history of BC (CxBladder Monitor). The expression of the five genomic markers is measured in the Detect modality, while data from these genomic markers and clinical variables (age, gender, frequency of macrohematuria, and smoking history) are considered in its Triage clinical modality.[43] The CxBladder tests showed a sensitivity of 82% and specificity of 85%.[44] Cxbladder was also able to distinguish between low grade, stage Ta urothelial carcinoma, and more advanced urothelial carcinomas with a sensitivity of 91% and a specificity of 90%.[44]

3.7 Xpert Bladder Cancer Detection

The Xpert Bladder Cancer Monitor uses the Cepheid GeneXpert Instrument Systems to measure the expression of five mRNA targets (Tyrosine-protein kinase ABL1- ABL1, Corticotropin Releasing Hormone- CRH, Insulin-Like Growth factor 2- IGF2, Uroplakin 1B- UPK1B, Annexin A10- ANXA10) in a single voided urine sample using a self-contained cartridge by real-time PCR. Xpert detection shows a sensitivity of 76% and a specificity of 85%.[45] Thus it outperforms cytology with regard to sensitivity even in low-grade and pTa tumors, with comparable specificity.[46]

3.8 Uromonitor and Uromonitor-V2

Hotspot mutations in TERT promoter and FGFR3 are the most frequent somatic alterations in BC. These mutations tend to occur more frequently together than perchance, and so the combination of both constitutes a reliable biomarker for monitoring NMIBC recurrence.[47,48] Based on the detection of these reliable biomarkers, researchers from IPATIMUP, Porto, Portugal, (Institute of Molecular Pathology and Immunology of the University of Porto) developed the ultrasensitive Uromonitor test kit. This test kit utilizes a urine filtering system to trap and concentrate cells exfoliated to urine and allows long-term storage of cells until analysis. The exfoliated cells collect in the filter, which is then sent at room temperature to the laboratory. Mutations in DNA isolated from exfoliated tumor cells are detected based on a highly sensitive, custom-made, multiplex real-time allelic discrimination assay using lock nucleic acid (LNA) competitive probes for TERTp alterations and a modified competitive allele-specific real-time detection PCR for FGFR3 alterations. This test is capable of detecting trace amounts of TERTp (c.1-124C > T and c.1-146C > T) and FGFR3 (p.R248C and p.S249C) mutations in a minimal quantity of tumor cells out of a pool of mostly unaltered cells.[49] Thus, this method is more sensitive than Sanger Sequencing and shows cost and time-effectiveness in contrast to next-generation sequencing (NGS)- based methods.

The Uromonitor test was validated in a multicentric study for NMIBC recurrence detection and presented a sensitivity of 73.5%, a specificity of 93.2%, and overall good performance across tumor stage and grade.[49] The Uromonitor test may potentially replace cytology as it showed better performance alone (42.9% cytology sensitivity vs. 73.5% Uromonitor sensitivity); or as an adjunct to cystoscopy (100% sensitivity and 88.6% specificity vs. 86.7% sensitivity).[49] The Uromonitor screening could directly substitute cystoscopy in cases where cystoscopy cannot be performed, or maybe alternated with cystoscopy for regular long-term follow-up of patients exhibiting low-risk, low-grade lesions.[49]

Uromonitor-V2 added KRAS hotspot mutation (codon 12 and codon 61) detection in addition to TERTp and FGFR3 alterations. This test achieved an improved 100% sensitivity, 83.3% specificity, a Positive Predictive Value (PPV) of 66.7%, and a Negative Predictive Value (NPV) of 100% in NMIBC follow-up recurrence detection.[49] Both Uromonitor and Uromonitor-V2 are CE certified for BC surveillance.

3.9 UroSEEK

UroSEEK is a parallel-sequencing based assay that detects BC through genetic analysis of tumor DNA present in exfoliated cells of urine.

UroSEEK analyses and detects three components that constitute typical biomarkers of BC. They are- a) intragenic mutations in regions of ten genes (FGFR3- Fibroblast growth factor receptor 3, TP53- tumor protein p53, CDKN2A- cyclin-dependent kinase inhibitor 2A, ERBB2- Erb-B2 Receptor Tyrosine Kinase 2, HRAS- Harvey rat sarcoma viral oncogene homolog, KRAS- Kirsten Rat Sarcoma virus, PIK3CA- Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha, MET - Mesenchymal Epithelial Transition Proto-Oncogene Receptor Tyrosine Kinase, VHL- von Hippel-Lindau tumor suppressor, and MLL- mixed-lineage leukemia 1) detected by NGS b) alterations in TERT promoter detected by Sanger Sequencing and (c) detection of aneuploidy by PCR of L-1 retrotransposons.[50] The test aims to complement cytology in the detection of BC, rather than substitute it. UroSEEK was effective in guiding disease management in cases of atypical cytology.[51]

Validation studies show that UroSEEK adds to the sensitivity of cytology; from 43% to 95% in the early detection cohort, from 25% to 71% in the Surveillance cohort, and from 10% to 75% in the upper tract urothelial cancer (UTUC) cohort.[50] UroSEEK alone (when a positive in either of the three described assays was considered as positive) showed a sensitivity of 95% and a specificity of 93%. As in other tests, cytology was more specific than UroSEEK in the detection cohort (100% vs. 93%).[50]

Equivalent results were obtained for UroSEEK in another validation study.[51] They reported high sensitivity and specificity (96% and 88%, respectively) and a strong negative predictive value of 99% in the early detection setting. However, reduced results were obtained for the surveillance cohort with a sensitivity, specificity, and negative predictive value of 74%, 72%, and 53%, respectively. The main advantage of UroSEEK appears to be the lead time obtained due to early diagnosis (6 months prior to clinical diagnosis) observed in both early detection and surveillance of BC.[51]

3.10 EpiCheck

The Bladder Epicheck test is a urine-based assay that uses DNA methylation changes associated with BC in a panel of 15 genomic biomarkers. DNA methylation markers known to be altered in BC are detected by real-time PCR and analyzed by a probability algorithm developed by EpiScore, providing a probability range (0 to 100) indicating the overall methylation level of exfoliated cells in the urine sample. The test is considered positive if the EpiScore is ≥60. It is used to monitor tumor recurrence in conjunction with cystoscopy in patients previously diagnosed with BC.[52]

A multicenter, prospective, blinded clinical trial in NMIBC follow-up patients presented an overall sensitivity, specificity, NPV, and positive predictive value of 68.2%, 88.0%, 95.1%, and 44.8%, respectively.[53] Upon excluding low-grade Ta recurrences, the sensitivity increased to 91.7%, and the NPV was 99.3%. Comparison of Bladder Epicheck with urinary cytology revealed a higher sensitivity with a lower specificity than cytology (62.3% vs. 33.3% and 86.3% vs. 98.6%, respectively).[52] However, the test performed better in specificity than cystoscopy and histology combined. The Epicheck test is costly and technically challenging in execution.

3.11 UroMark

Feber and colleagues developed a targeted bisulfite next-generation sequencing assay to diagnose BC from voided urine samples. They performed a genome-wide methylation profiling of DNA isolated from a cohort of 86 patients with muscle invasive BC and 30 patients with normal urothelium. Based on their studies, they defined a biomarker panel of 150 CpG loci, which was marketed under the brand name of UroMark.[54] Although this panel was predominately from high-grade disease, more than 98% of the alterations present were confirmed in low-grade disease. The UroMark assay is a next-generation bisulfite sequencing assay and analysis pipeline for the detection of BC from urinary sediment DNA. In clinical validation cohorts of 107 BC patients and 167 non-cancer individuals, the UroMark assay presented an AUC of 97%, a sensitivity of 98%, and a specificity of 97%.[54]

3.12 AssureMDX

AssureMDX is a two-pronged assay that combines the detection of epigenetic status of genes Orthodenticle Homeobox 1 (OTX1), One Cut Homeobox 2 (ONECUT2), and Twist-related protein 1 (TWIST1) with the detection of somatic mutations in FGFR3, TERT, and HRAS for the detection of BC in urine. It utilizes methylation-specific PCR to assess the DNA methylation status and next-generation sequencing to determine the mutational status of the given genes.[55] This assay was designed to predict BC risk in hematuria patients; high-risk patients would then undergo more invasive tests such as cystoscopy. Internal validation presented with 97% sensitivity, 83% specificity, and 99% negative predictive value (AUC= 0.92).[55] External validation study reported a sensitivity reaching 93%, specificity 86%, and overall negative predictive

value of 99%, leading to a potential 77% reduction in unnecessary diagnostic cystoscopy.[56]

3.13 ADXBLADDER

ADXBLADDER is based on the detection of MCM5 (Minichromosome Maintenance Complex Component 5) in urine sediment pellet. The protein MCM5 is essential for DNA replication in actively-dividing cells. In normal urothelium, cells of the basal proliferative compartment express MCM5. However, in urothelial carcinoma, MCM5-positive cells are present throughout the urothelium and exfoliated into the urine. MCM5 is a reliable biomarker in BC diagnosis, with specifically high sensitivity for high-risk disease (high-grade, pT1 and above, and carcinoma in situ [CIS] tumors).[57,58] A multicentre, prospective, blinded study conducted by Gontero et al. showed that ADXBLADDER could detect both low- and high-grades of NMIBC recurrence. It was more sensitive than cytology at detecting recurrent bladder tumors for all sub-types-low-grade, high-grade, CIS, and non-pTa low-grade tumors.[59] ADXBLADDER presented a sensitivity of 51.9%, a specificity of 66.4%, and an NPV of 92%, with a sensitivity of 44.1% and 58.8% for low-grade and high-grade recurrences, respectively. The sensitivity of cytology in their study was 16.7%, specificity was 98%, and NPV was 90.7%, with a sensitivity of 17.6% for both low-grade and high-grade recurrences.[59] The ADXBLADDER is a simple and fast ELISA test. An optical density reading greater than the predefined cut-off is considered positive, making it reliable and less prone to variability.

4. Other Potential Markers for BC Diagnosis And Surveillance

4.1 Protein markers

Studies show elevations in urinary levels of α1-antitrypsin, apolipoprotein E, vascular endothelial growth factor (VEGF), carbonic anhydrase 9 (CA9), angiogenin, interleukin 8 (IL-8), and Matrix metallopeptidase 9 (MMP-9) in BC.[60-62] Out of these, the combination of α1-antitrypsin and apolipoprotein E (AUC 0.9399, 91% sensitivity, 89% specificity); VEGF (83% sensitivity and 87% specificity, AUC= 0.886) and IL-8 (AUC= 0.79) appear to be the most promising biomarkers. A custom-made multiplex immunoassay analyzed a 10-protein biomarker panel (Interleukin 8, Matrix Metallopeptidase 9, Matrix Metallopeptidase 10, Angiogenin, Apolipoprotein E, Syndecan 1, α-1 Antitrypsin, Plasminogen activator inhibitor-1, Carbonic anhydrase 9, and Vascular Endothelial Growth Factor A) in voided urine samples of Japanese BC patients collected before cystoscopy. Urinary biomarkers were significantly elevated in BC, with better prediction accuracy in high grade and muscle-invasive tumors. The panel presented an overall AUC of 0.895 with sensitivity and specificity of 84.8 and 80.6%.[63]

Urinary soluble Fas appears as an independent predictor of BC recurrence and invasiveness in patients with a history of non-muscle invasive bladder transitional cell carcinoma.[64] NMIBC cases with a higher stage or grade report higher urinary sFas levels, which could be significantly correlated with recurrence incidence in NMIBC patients.[65] Thus, urinary sFas may serve as a predictor of tumor recurrence but needs to be further studied in larger cohorts before being used as a definitive prediction tool.

The Lewis X is a blood group antigen normally absent from urothelial cells in adult individuals but expressed in transitional cell tumors regardless of status, grade, or stage. Immunostaining of the Lewis X antigen in exfoliated urothelial cells from voided urine presented sensitivity and specificity values from 79.8 to 85% and from 80 to 86.4%, respectively.[32] Immunocytology of Lewis X antigen is independent of tumor status and particularly sensitive for detecting low-grade carcinomas and predicting tumor recurrence and/or progression.[66]

The glycosaminoglycan Hyaluronic acid (HA) and its degrading enzyme hyaluronidase (HAase) are important markers of cancer detection, previously shown to be elevated in BC patient urine.[67] In a study conducted by Hautmann et al., 261 BC patients, 252 patients without BC, 71 BC tissue specimens, and 12 normal bladder tissues were analyzed to assess the accuracy of HA and HAase as BC tumor markers.[68] Their study presented a sensitivity of 91% and specificity of 84% in the detection of BC and proved equally sensitive for monitoring tumor recurrence.

4.2 miRNA markers

miRNAs are 20-22 nt long non-coding regulatory RNAs that fine-tune gene expression by binding to the 3'-UTR of target mRNAs and cause either repression or degradation.[69] These tiny regulators control diverse biological processes, as most mammalian genes are under miRNA regulation.[69] Deregulation of miRNA biogenesis and function is known to lead to various human pathologies, including cancer. miRNAs are present in tumor tissue and have been reported in various body fluids like urine, blood, saliva, and peritoneal fluid.[70] The acquisition of urine to detect miRNA biomarkers in BC is an easy and non-invasive way to predict the disease's local stage due to its direct contact with the

tumor tissue in the urinary tract.[71] In 2010, Hanke et al. published the first report on the higher expression of miR-126, miR-182, and miR-199a in the urine of BC patients, raising the possibility of using urinary miRNAs as diagnostic tools.[72] There have been numerous reports of miRNA signatures that may be used as biofluid markers of urological tumors. However, there exists no clear consensus regarding a definitive panel that may be translated into commercial kits for BC detection. Furthermore, most of these studies report suboptimal AUC, sensitivity, and specificity values negating their implementation in clinical practice. In a study of 43 urine samples (34 BC,9 non-smoker healthy volunteers), Andreu et al. reported significant downregulation of miR-375 in high-grade BC and significant upregulation of miR-146a in low-grade BC.[73] An increase in urinary levels of miR-146a-5p in BC patients (as compared to healthy controls) has also been reported by Sasaki et al.[74] They found that elevated urine miR-146a-5p reduced to normal levels after transurethral resection, suggesting the miRNA's tumor origin. Thus, miR-146a might be a promising candidate as a non-invasive diagnostic marker of BC. Zhang et al. devised a direct PCR Method (qRT-PCR-D) to quantify cell-free miR-155 in urine without RNA extraction. Cell-free miR-155 detected by RT-qPCR-D distinguished NMIBC patients from cystitis patients and healthy donors with 80.2% sensitivity and 84.6% specificity.[75] The expression of miR-155 could be correlated with tumor stage and grade, and reduced levels were observed after transurethral resection.

A notable study exploiting serum miRNAs as diagnostic tools was conducted by Jiang and colleagues. They reported that an expression panel of two upregulated (miR-152 and miR-148b-3p) and four downregulated (miR-3187-3p, miR-15b-5p, miR-27a-3p, and miR-30a-5p) miRNAs showed an AUC of 0.899 with a sensitivity of 90.00, 84.85, and 89.36% for Ta, T1, and T2-T4 stages respectively. Additionally, upregulation of miR-152 and downregulation of miR-3187-3p was statistically associated with worse recurrence-free survival.[76]

5. Concluding remarks

Biomarkers for BC should be selected such that they (a) can reduce the need or frequency of invasive procedures, (b) can detect recurrent tumors which are frequently small in size, (c) can detect tumors before progression to invasive disease and (d) predict effective treatment response. Though highly specific, urinary cytology is limited by reduced sensitivity for low-grade tumors. Cystoscopy is an instrument-mediated invasive test, and therefore, repeated cystoscopies conducted regularly as follow-up cause discomfort and pain to patients. Cystoscopy can also show decreased sensitivity in cases of Cis or non-papillary lesions. Hence, the above-described biomarkers and assays are being explored in non-invasive settings, especially for the early detection of low-grade and low-stage BC and recurrence of NMIBC. A brief description of the available tests with sensitivity and specificity values of each have been summarized in Table 1.

Current FDA-approved commercially available tests, excepting CellSearch, show higher sensitivity than cytology but are less specific. CellSearch detects CTCs in plasma/serum with higher specificity than cytology but it is not a sensitive test. Hence CellSearch should be used in conjunction with clinical information from other sources for appropriate disease management. The available non-FDA approved tests like ADXBLADDER, CxBladder, XPERT BC, UroMark, UroSEEK, Uromonitor/ Uromonitor-V2, EpiCheck and AssureMDX are also more sensitive than cytology with sensitivity values ranging from 51.9% to 100%. UroSEEK, UroMark, Uromonitor and Uromonitor-V2 are highly specific, with specificity values either comparable to or more than cytology. UroSEEK shows reduced performance in the surveillance cohort and hence should be used in conjunction with cytology. UroMark, Uromonitor and Uromonitor-V2 are good candidates for BC detection as they show high sensitivity and specificity and can detect low-grade tumors. Clinicians can decide on the applicability of these tests based on the patient's context and limitations.

The other potential molecules that may be exploited as BC biomarkers include various proteins and miRNAs. Biomarker panels comprising of proteins such as Interleukin 8, Matrix Metallopeptidase 9, Matrix Metallopeptidase 10, Angiogenin, Apolipoprotein E, Syndecan 1, α-1 Antitrypsin, Plasminogen activator inhibitor-1, Carbonic anhydrase 9, and Vascular Endothelial Growth Factor A may be used to develop non-invasive BC detection tests. Published studies on miRNAs deregulated in BC yield encouraging findings, but there is a lack of consensus among them which explains the absence of miRNAs from commercial biomarker panels. The journey from bench-to-bedside for these molecules requires prospective validation in larger cohorts, before they may be incorporated into routine clinical practice.

Table 1: Performance parameters and characteristics of available commercial kits for Bladder Cancer diagnosis and surveillance.

Name (Commercially available kits/ procedures)	FDA Approval/CE Mark	Starting Sample	Biomaterial used	Technology	Biomarker Analysed/ detected	Performance Sensitivity	Performance Specificity	References for Sensitivity and Specificity	Advantages	Disadvantages
Cytology	Yes	Urine	Exfoliated	Giemsa and H&E staining	Cell Phenotype	40.8% 22%	92.8% 87%	Freifeld et al. 2019 (28) Lotan et al. (29)	1. Highly specific for BC detection 2. Easy to perform, inexpensive	1. Low sensitivity for BC tumours 2. Interpretation based on skills of uro-pathologist
NMP22 BC test/Bladder Check	Yes/Yes	Urine	Protein	ELISA/ Immunochromatogr aphic assay device	NMP22 protein	47% to 100%	60% to 90%	Xylinas et al. 2014 (3)	1. Sensitivity igher than cytology 2. Easy to perform, Inexpensive	1. Not sensitive enough to detect small, recurrent tumours 2. Less specificity in patients with urinary tract diseases
Bladder Tumor Antigen BTA stat/BtA TRAK	Yes/ Information not available	Urine	Protein	Immunochromatogr aoguc assay device/ ELISA.	Biadder Tumour Antigen (Human complement factor H-related protein)	56%	85.70%	Raitanen et al. 2008 (33)	1. Sensitivity higher than cytology in low grade disease 2. Easy to perform, can be done at point-of-care	1. Specitivity less than cytology 2. Can give false-positive reswults 3. Not suitavle for screening
UroVysion	Yes/Yes	Urine	Exfoliated cells	Fluorescent in situ Hybridization (FISH)	aneuploidy in chromosomes 3.7 and 17 and the loss of the 9p21 locus	69% to 87% 62%	89%	Lokeshwar et al 2005 (38) Dimashkier et al. 2013 (39)	1. More sensitive than cytology 2. Comparable specificity to cytology	1. can also indicate other primary tumors 2. Reduced sensitivity in detection of low grade, low stage tumors
uCyt+	Yes/ Information not availabele	Urine	Exfoliated	Immunofluorescence	Sulfated mucin glycoproteins, glycosylated CEA.	38% to 100%	75% to 90%	Lokeshwar et al 2005 (38)	1. Superior sensitivity tahan cytology	1. Reduced specificity in low grade NMIBC and hematuria presenting cases 2. Reduced sensitivity in recurrent tumours
Cell Search	Yes/Yes	Plasma/ Serum	Circulating tumour cells	Immunomagnetic enrichment	Cell surface proteins (CD45-,EpCAM+, & cytokertins8. 18+&/or19+)	35?%	97%	Zhang et al. 2017 (42)	1. Shows correiation with tumor status 2. Higher specificity than cytrology	1. Low sensitivity
CxBladder	No/No	Urine	Exfoliated cells	RT-qPCR	mRNA(IGF, HOXA, MDK,CDC & IL8R),clinical information	82%	85%	O Sullivan et al. 2012 (44)	1. Higher sensitivity than cytology 2. Comparable specificity with cytology	1. Not FDA approved/No CE Mark
Xpert Bladder Cancer Detection	No/Yes	Urine	Exfoliated cells	RT-qPCR	mRNA (ABL1, CRH, IGF2, UPK1B,ANXA10)	76%	85%	Valenberg et al. 2017 (45)	1. Higher sensitivity than cytology 2. Comparable specificity with cytology	1. Not FDA approved
Uromonitor & Uromonitor-V2	No/Yes	Urine	Exfoliated cells	Real time PCR	Multations in tumor cell DNA (TERTP,FGFR3 & KRAS)	Uromonitor-73.5% Uromonitor-V2-100%	Uromonitor-93.2% Uromonitor-V2-83.3%	Batista et al 2019 (49)	1. Higher Sensitivity than cytology 2. Good performance across tumor stage & grade	1. Not FDA approved
UroSEEK	No/No	Urine	Exfoliated cells	Parallel Sequencing based assay (NGS, Saner sequencing and PCR)	Mulations in tumor cell DNA (FGFR3, TP53, CDKN2A, ERBB2, HRAS, KRAS, PIK3CA, MET, VHL, MLL. TERTp, aneuploidy)	95%	93%	Spriner et al. 2018 (50)	1. Higher sensitivity than cytology, adds to the sensitivity of cytology when used in complement to it	1. Cannot to used as a substitute to cytology 2. Reduced sensitivity & specificity in surveillance cohorts
EpiCheck	No/Yes	Urine	Exfoliated cells	Real time PCR	DNA methylation changes	68.20%	88.00%	Witjes et al. 2018 (53)	1. Higher sensitivity than cytology	1. Low sensitivity for low-grade & Ta recurrent tumors. 2. Lower specificity than cytology
UroMark	No/No	Urine	Exfoliated cells	Bisulfite next generation sequencing assay	DNA methylation changes	98%	97%	Feber et al. 2017 (54)	1. High sensitivity & specificity 2. Biomarkers detected also present in low grade disease	1. Not FDA approved/No CE Mark
AssureMDX	No/No	Urine	Exfoliated cells	Methylation specific PCR with NGS	DNA methylation status (OTX1. ONECUT2, AND TWIST1) with mulations (FGFR3, TERT & HRAS)	97%	83%	van Kessel et al. 2018 (55)	1. High sensitivity 2. Can predict BC risk in hematuria presenting cases	1. Lower specificity than cytology 2. Not FDA approved/No CE Mark
ADXBLADER	No/Yes	Urine	Exfoliated cells	ELISA	MCM5	51.90%	66.40%	Gontero,P.et al. 2020 (59)	1. More sensitivie than cytology across all subtypes of BC 2. Simple & fast test	1. Lower specificity than cytology 2. Not FDA approved

References

1. Compérat EM, Burger M, Gontero P, Mostafid AH, Palou J, Rouprêt M, et al. Grading of Urothelial Carcinoma and The New "World Health Organisation Classification of Tumours of the Urinary System and Male Genital Organs 2016". Eur Urol Focus 2019; 5: 457–466.
2. Humphrey PA, Moch H, Cubilla AL, Ulbright TM, Reuter VE. The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs-Part B: Prostate and Bladder Tumours. Eur Urol 2016; 70: 106-119.
3. Xylinas E, Kluth LA, Rieken M, Karakiewicz PI, Lotan Y, Shariat SF. Urine markers for detection and surveillance of bladder cancer. Urol Oncol 2014; 32:222-9.
4. Cancer Genome Atlas Research Network, Comprehensive molecular characterization of urothelial bladder carcinoma. Nature 2014; 507:315–322.
5. Knowles MA, Hurst CD. Molecular biology of bladder cancer: new insights into pathogenesis and clinical diversity. Nat Rev Cancer 2015; 15: 25-41
6. Hartmann A, Schlake G, Zaak D, Hungerhuber E, Hofstetter A, Hofstaedter F. Occurrence of chromosome 9 and p53 alterations in multifocal dysplasia and carcinoma in situ of human urinary bladder. Cancer Res 2002; 62: 809-18.
7. Robertson AG, Kim J, Al-Ahmadie, H. Bellmunt J, Guo G, Cherniack AD, et al. Comprehensive Molecular Characterization of Muscle-Invasive Bladder Cancer. Cell 2017; 171:540–556. e25.
8. Rachakonda PS, Hosen I, de Verdier PJ, Fallah M, Heidenreich B, Ryk C, et al. TERT promoter mutations in bladder cancer affect patient survival and disease recurrence through modification by a common polymorphism. Proc Natl Acad Sci U S A 2013; 110: 17426-31.
9. Allory Y, Beukers W, Sagrera A, Flández M, Marqués M, Márquez M, et al. Telomerase reverse transcriptase promoter mutations in bladder cancer: high frequency across stages, detection in urine, and lack of association with outcome. Eur Urol 2014; 65: 360-6.
10. Kurtis B, Zhuge J, Ojaimi C, Ye F, Cai D, Zhang D, et al. Recurrent TERT promoter mutations in urothelial carcinoma and potential clinical applications. Ann Diagn Pathol 2016; 21: 7-11.
11. Kinde I, Munari E, Faraj SF, Hruban RH, Schoenberg M, Bivalacqua T, et al. TERT promoter mutations occur early in urothelial neoplasia and are biomarkers of early disease and disease recurrence in urine. Cancer Res 2013; 73: 7162-7.
12. Lee DD, Leão R, Komosa M, Gallo M, Zhang CH, Lipman T, et al. DNA hypermethylation within TERT promoter upregulates TERT expression in cancer. J Clin Invest 2019; 129: 223-229.
13. Leão R, Lee D, Figueiredo A, Hermanns T, Wild P, Komosa M, et al. Combined genetic and epigenetic alterations of the TERT promoter affect clinical and biological behavior of bladder cancer. Int J Cancer 2019; 144: 1676-1684.
14. Jebar AH, Hurst CD, Tomlinson DC, Johnston C, Taylor CF, Knowles MA. FGFR3 and Ras gene mutations are mutually exclusive genetic events in urothelial cell carcinoma. Oncogene 2005; 24: 5218-25.
15. Bellmunt J. Stem-Like Signature Predicting Disease Progression in Early Stage Bladder Cancer. The Role of E2F3 and SOX4. Biomedicines 2018; 6:85.
16. Esrig D, Spruck CH, Nichols PW, Chaiwun B, Steven K, Groshen S, et al. p53 nuclear protein accumulation correlates with mutations in the p53 gene, tumor grade, and stage in bladder cancer. Am J Pathol 1993; 143:1389-97.
17. Inamura K. Bladder Cancer: New Insights into Its Molecular Pathology. Cancers (Basel) 2018; 10 (4):100.
18. Bakkar AA, Wallerand H, Radvanyi F, Lahaye JB, Pissard S, Lecerf L, et al. FGFR3 and TP53 gene mutations define two distinct pathways in urothelial cell carcinoma of the bladder. Cancer Res 2003; 63: 8108-12.
19. Choi W, Porten S, Kim S, Willis D, Plimack ER, Hoffman-Censits J, et al. Identification of distinct basal and luminal subtypes of muscle-invasive bladder cancer with different sensitivities to frontline chemotherapy. Cancer Cell 2014; 25 152 -65.
20. Kompier LC, Lurkin I, van der Aa MN, van Rhijn BW, van der Kwast TH, Zwarthoff EC. FGFR3, HRAS, KRAS, NRAS and PIK3CA mutations in bladder cancer and their potential as biomarkers for surveillance and therapy. PLoS One 2010; 5: e13821.
21. Billerey C, Chopin D, Aubriot-Lorton MH, Ricol D, Gil Diez de Medina S, Van Rhijn B, et al. Frequent FGFR3 mutations in papillary non-invasive bladder (pTa) tumors. Am J Pathol 2001; 158: 1955-9.
22. van Rhijn BW, van der Kwast TH, Liu L, Fleshner NE, Bostrom PJ, Vis AN, et al. The FGFR3 mutation is related to favorable pT1 bladder cancer. J Urol 2012; 187: 310-4.
23. Martin-Doyle W, Kwiatkowski DJ. Molecular biology of bladder cancer. Hematol Oncol Clin North Am 2015; 29: 191-203, vii.
24. Breyer J, Wirtz RM, Erben P, Worst TS, Stoehr R, Eckstein M, et al. High CDKN2A/p16 and Low FGFR3 Expression Predict Progressive Potential of Stage pT1 Urothelial Bladder Carcinoma. Clin Genitourin Cancer 2018; 16: 248-256.e2.

25. Sjödahl G, Lövgren K, Lauss M, Patschan O, Gudjonsson S, Chebil G, et al. Toward a molecular pathologic classification of urothelial carcinoma. Am J Pathol 2013; 183: 681-91.
26. Worst TS, Weis CA, Stöhr R, Bertz S, Eckstein M, Otto W, et al. CDKN2A as transcriptomic marker for muscle-invasive bladder cancer risk stratification and therapy decision-making. Sci Rep 2018; 8: 14383
27. Lotan Y, Roehrborn CG. Sensitivity and specificity of commonly available bladder tumor markers versus cytology: results of a comprehensive literature review and meta-analyses. Urology 2003; 61: 109–18
28. Freifeld Y, Lotan Y. Effect of blue-light cystoscopy on contemporary performance of urine cytology. BJU Int 2019; 124: 251–7.
29. Lotan Y, O'Sullivan P, Raman JD, Shariat SF, Kavalieris L, Frampton C. Clinical comparison of noninvasive urine tests for ruling out recurrent urothelial carcinoma. Urol Oncol 2017; 35: 531.e15–22
30. Miyanaga N, Akaza H, Ishikawa S, Ohtani M, Noguchi R, Kawai K, et al. Clinical evaluation of nuclear matrix protein 22 (NMP22) in urine as a novel marker for urothelial cancer. Eur Urol 1997; 31:163-8.
31. Lee OJ, Kang HW, Yun SJ. Urine Cytology and Urinary Biomarkers. In: Ku JH, editor. Bladder Cancer. Academic Press: Elsevier; 2018.p67-81.
32. Dal Moro F, Valotto C, Guttilla A, Zattoni F. Urinary markers in the everyday diagnosis of bladder cancer. Urologia 2013; 80: 265- 275.
33. Raitanen MP, FinnBladder Group. The role of BTA stat Test in follow-up of patients with bladder cancer: results from FinnBladder studies. World J Urol 2008; 26:45-50.
34. Gutiérrez Baños JL, del Henar Rebollo Rodrigo M, Antolín Juárez FM, García BM. Usefulness of the BTA STAT Test for the diagnosis of bladder cancer. Urology 2001; 57: 685-9.
35. Murphy WM, Rivera-Ramirez I, Medina CA, Wright NJ, Wajsman Z. The bladder tumor antigen (BTA) test compared to voided urine cytology in the detection of bladder neoplasms. J Urol 1997; 158: 2102-6.
36. Sarosdy MF, Schellhammer P, Bokinsky G, Kahn P, Chao R, Yore L, et al. Clinical evaluation of a multitarget fluorescent in situ hybridization assay for detection of bladder cancer. J Urol 2002; 168:19504.
37. Bubendorf L, Grilli B, Sauter G, Mihatsch MJ, Gasser TC, Dalquen P. Multiprobe FISH for enhanced detection of bladder cancer in voided urine specimens and bladder washings. Am J Clin Pathol 2001; 116: 79–86.
38. Lokeshwar VB, Habuchi T, Grossman HB, Murphy WM, Hautmann SH, Hemstreet GP, et al. Bladder tumor markers beyond cytology: International Consensus Panel on bladder tumor markers. Urology 2005; 66: 35-63.
39. Dimashkieh H, Wolff DJ, Smith TM, Houser PM, Nietert PJ, Yang J. Evaluation of urovysion and cytology for bladder cancer detection: a study of 1835 paired urine samples with clinical and histologic correlation. Cancer Cytopathol 2013; 121: 591-7.
40. Skacel M, Fahmy M, Brainard JA, Pettay JD, Biscotti CV, Liou LS, et al. Multitarget fluorescence in situ hybridization assay detects transitional cell carcinoma in the majority of patients with bladder cancer and atypical or negative urine cytology. J Urol 2003; 169: 2101-5.
41. Schmitz-Dräger C, Bonberg N, Pesch B, Todenhöfer T, Sahin S, Behrens T, et al. Replacing cystoscopy by urine markers in the follow-up of patients with low-risk non-muscle-invasive bladder cancer? An International Bladder Cancer Network project. Urol Oncol 2016; 34: 452–9.
42. Zhang Z, Fan W, Deng Q, Tang S, Wang P, Xu P, et al. Oncotarget 2017; 8:59527-59538.
43. Darling D, Luxmanan C, O'Sullivan P, Lough T, Suttie J. Clinical Utility of Cxbladder for the Diagnosis of Urothelial Carcinoma. Adv Ther 2017; 34: 1087-1096.
44. O'Sullivan P, Sharples K, Dalphin M, Davidson P, Gilling P, Cambridge L, et al. A multigene urine test for the detection and stratification of bladder cancer in patients presenting with hematuria. J Urol 2012; 188: 741-7.
45. Valenberg FV, Bridge J, Mayne D, Beqaj S, Sexton W, Lotan Y, et al. Validation of a mRNA-based urine test for bladder cancer detection in patients with hematuria. Eur Urol Suppl 2017; 16: e190–e191.
46. Pichler R, Fritz J, Tulchiner G, Klinglmair G, Soleiman A, Horninger W, et al. Increased accuracy of a novel mRNA-based urine test for bladder cancer surveillance. BJU Int 2018; 121:29-37.
47. Hosen I, Rachakonda PS, Heidenreich B, de Verdier PJ, Ryk C, Steineck G, et al. Mutations in TERT promoter and FGFR3 and telomere length in bladder cancer. Int J Cancer 2015; 137: 1621-9.
48. Critelli R, Fasanelli F, Oderda M, Polidoro S, Assumma MB, Viberti C, et al. Detection of multiple mutations in urinary exfoliated cells from male bladder cancer patients at diagnosis and during follow-up. Oncotarget 2016; 7: 67435-67448.
49. Batista R, Vinagre J, Prazeres H, Sampaio C, Peralta P, Conceição P, et al. Validation of a Novel, Sensitive, and Specific Urine-Based Test for Recurrence Surveillance of Patients With Non-Muscle-

Invasive Bladder Cancer in a Comprehensive Multicenter Study. Front Genet 2019; 10: 1237.
50. Springer SU, Chen CH, Rodriguez Pena MDC, Li L, Douville C, Wang Y, et al. Non-invasive detection of urothelial cancer through the analysis of driver gene mutations and aneuploidy. eLife 2018; 7: e32134.
51. Rodriguez Pena MDC, Springer SU, Taheri D, Li L, Tregnago AC, Eich ML, et al. Performance of novel non-invasive urine assay UroSEEK in cohorts of equivocal urine cytology. Virchows Arch 2020; 476:423-429.
52. Trenti E, D'Elia C, Mian C, Schwienbacher C, Hanspeter E, Pycha A, et al. Diagnostic predictive value of the Bladder EpiCheck test in the follow-up of patients with non-muscle-invasive bladder cancer. Cancer Cytopathol 2019; 127: 465–469.
53. Witjes JA, Morote J, Cornel EB, Gakis G, van Valenberg FJP, Lozano F, et al. Performance of the Bladder EpiCheck Methylation Test for patients under surveillance for non–muscle-invasive bladder cancer: Results of a multicenter, prospective, blinded clinical trial. Eur Urol Oncol 2018; 1: 307–313.
54. Feber A, Dhami P, Dong L, de Winter P, Tan WS, Martínez-Fernández M, et al. UroMark-a urinary biomarker assay for the detection of bladder cancer. Clin Epigenetics 2017; 9: 8.
55. van Kessel KE, Van Neste L, Lurkin I, Zwarthoff EC, Van Criekinge W. Evaluation of an Epigenetic Profile for the Detection of Bladder Cancer in Patients with Hematuria. J Urol 2016; 195: 601-7.
56. van Kessel KE, Beukers W, Lurkin I, Ziel-van der Made A, van der Keur KA, Boormans JL, et al. Validation of a DNA Methylation-Mutation Urine Assay to Select Patients with Hematuria for Cystoscopy. J Urol 2017; 197: 590-595.
57. Stoeber K, Swinn R, Prevost AT, de Clive-Lowe P, Halsall I, Dilworth SM, et al. Diagnosis of genitourinary tract cancer by detection of minichromosome maintenance 5 protein in urine sediments. J Natl Cancer Inst 2002; 94: 1071-9.
58. Dudderidge T, Stockley J, Nabi G, Mom J, Umez-Eronini N, Hrouda D, et al. A Novel, non-invasive Test Enabling Bladder Cancer Detection in Urine Sediment of Patients Presenting with Haematuria-A Prospective Multicentre Performance Evaluation of ADXBLADDER. Eur Urol Oncol 2020; 3: 42-46.
59. Gontero P, Montanari E, Roupret M, Longo F, Stockley J, Kennedy A, et al. Comparison of the performances of the ADXBLADDER test and urinary cytology in the follow-up of non-muscle-invasive bladder cancer: a blinded prospective multicentric study. BJU Int 2020; Aug 3. doi: 10.1111/bju.15194. Epub ahead of print.
60. Urquidi V, Goodison S, Ross S, Chang M, Dai Y, Rosser CJ. Diagnostic potential of urinary α1-antitrypsin and apolipoprotein E in the detection of bladder cancer. J Urol 2012; 188: 2377-83.
61. Urquidi V, Goodison S, Kim J, Chang M, Dai Y, Rosser CJ. Vascular endothelial growth factor, carbonic anhydrase 9, and angiogenin as urinary biomarkers for bladder cancer detection. Urology 2012; 79:1185.e1-6.
62. Urquidi V, Chang M, Dai Y, Kim J, Wolfson ED, Goodison S, et al. IL-8 as a urinary biomarker for the detection of bladder cancer. BMC Urol 2012; 12: 12.
63. Goodison S, Ogawa O, Matsui Y, Kobayashi T, Miyake M, Ohnishi S, et al. A multiplex urinary immunoassay for bladder cancer detection: analysis of a Japanese cohort. J Transl Med 2016; 14: 287.
64. Svatek RS, Herman MP, Lotan Y, Casella R, Hsieh JT, Sagalowsky AI, et al. Soluble Fas--a promising novel urinary marker for the detection of recurrent superficial bladder cancer. Cancer 2006; 106: 1701-7.
65. Yang H, Guo Y, Wang Z, Wang Z. [Value of detection of preoperative urinary soluble Fas expression in predicting the recurrence of non-muscle invasive bladder cancer]. Zhong Nan Da Xue Xue Bao Yi Xue Ban 2016; 41: 24-9. Chinese.
66. Golijanin D, Sherman Y, Shapiro A, Pode D. Detection of bladder tumors by immunostaining of the Lewis X antigen in cells from voided urine. Urology 1995; 46: 173-7.
67. Lokeshwar VB, Obek C, Soloway MS, Block NL. Tumor-associated hyaluronic acid: a new sensitive and specific urine marker for bladder cancer. Cancer Res 1997; 57: 773-7.
68. Hautmann SH, Schroeder GL, Civantos F, Duncan RC, Gnann R, Friedrich MG, et al. Hyaluronsäure und Hyaluronidase. Zwei neue Blasenkarzinommarker [Hyaluronic acid and hyaluronidase. 2 new bladder carcinoma markers]. Urologe A 2001; 40: 121-6. German.
69. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009; 136: 215-33.
70. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. Clin Chem 2010; 56: 1733-41.
71. Huang X, Liang M, Dittmar R, Wang L. Extracellular microRNAs in urologic malignancies: chances and challenges. Int J Mol Sci 2013; 14: 14785-99.
72. Hanke M, Hoefig K, Merz H, Feller AC, Kausch I, Jocham D, et al. A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder

cancer. Urol Oncol 2010; 28: 655-61.
73. Andreu Z, Otta Oshiro R, Redruello A, López-Martín S, Gutiérrez-Vázquez C, Morato E, et al. Extracellular vesicles as a source for non-invasive biomarkers in bladder cancer progression. Eur J Pharm Sci 2017; 98: 70-79.
74. Sasaki H, Yoshiike M, Nozawa S, Usuba W, Katsuoka Y, Aida K, et al. Expression Level of Urinary MicroRNA-146a-5p Is Increased in Patients With Bladder Cancer and Decreased in Those After Transurethral Resection. Clin Genitourin Cancer 2016; 14: e493-e499.
75. Zhang X, Zhang Y, Liu X, Fang A, Wang J, Yang Y, et al. Direct quantitative detection for cell-free miR-155 in urine: a potential role in diagnosis and prognosis for non-muscle invasive bladder cancer. Oncotarget 2016; 7: 3255-66.
76. Jiang X, Du L, Wang L, Li J, Liu Y, Zheng G, et al. Serum microRNA expression signatures identified from genome-wide microRNA profiling serve as novel noninvasive biomarkers for diagnosis and recurrence of bladder cancer. Int J Cancer 2015; 136: 854-62.