



## Review – Bladder Cancer

# Urinary Markers in Bladder Cancer

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### Abstract

**Objectives:** Many markers for the detection of bladder cancers have been tested. Almost all urinary markers reported are better than cytology with regard to sensitivity, but they score lower in specificity. The purpose of this review is to highlight the most important urinary biomarkers studied and reported recently.

**Methods:** Literature on bladder cancer markers has been reviewed regularly in the last few years. In the current review we have tried to summarise the most recent literature of urinary markers.

**Results:** The results of this review show that the first-generation urinary markers did not add much to urinary cytology. The current generation of markers is promising but larger clinical trials are needed. The future of marker development is bright with new techniques emerging, but the perfect marker is still to be found.

**Conclusion:** Currently, no single marker can yet guide us in surveillance and lower the frequency of urethrocytostcopy.

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## 1. Introduction

Bladder cancer is the second most common malignancy affecting the urinary system. Approximately 90% of urothelial tumours are urothelial cancers (UCs). The remaining tumours are squamous cell cancers or adenocarcinomas. Small-cell carcinomas account for less than 1%. The spectrum of bladder cancer includes non-muscle-invasive, muscle-invasive, and metastatic disease, each with its own specific typical behaviour, prognosis, and treatment. Patients are diagnosed and monitored with

urethrocytostcopy, cytology, and imaging of the upper urinary tract. Although considered the gold standard, urethrocytostcopy can miss certain lesions, in particular small areas of carcinoma in situ (CIS). Besides this limitation, urethrocytostcopy is invasive for the patient and it is expensive. Cytology, the second gold standard, has a median sensitivity of only 35% and median specificity of 94% [1], although it is useful for detecting high-grade tumours and CIS. The low sensitivity in low-grade cancer, however, has limited clinical relevance. Inflammatory conditions of the bladder,

for example infection or instillation, can confound the results of cytology [59].

The limitations of cytology and the invasiveness of urethrocytostcopy for detecting bladder cancer have generated interest in other noninvasive diagnostic tools. The search for detectable urinary markers began with the testing of urine for diabetes. Since then medical science has evolved and, during the last decade, the development of new biomarkers has given us new tests in the detection and follow-up of bladder cancer.

## 2. Methods

The purpose of this review is to highlight the most important urinary biomarkers studied and reported recently. For that purpose we looked at recently published reviews and performed a PubMed search using the search terms “bladder neoplasm,” “urothelial cell carcinoma,” and “urinary markers.”

### 2.1. Fluorescence in situ hybridisation

As in other forms of cancer, bladder cancer also shows chromosomal anomalies. With fluorescence in situ hybridisation (FISH) techniques, chromosomal anomalies can be detected in exfoliated bladder cells. A currently commercially available test, the UroVysion™ Bladder Cancer Kit (Vysis Inc, Downers Grove, IL, USA), has probes for chromosome 3, 7, and 17, and a locus-specific probe for 9p21.

In various case-control studies, as recently summarised by Lokeshwar [2], the sensitivity of FISH varies between 69% and 87%. All studies reported a low sensitivity of FISH to detect low-grade (36–57%) and low-stage (62–65%) tumours, but FISH has high sensitivity to detect high-grade and high-stage tumours (83–97%). The detection of CIS is close to 100%. The specificity of FISH is high (89–96%) and is comparable to cytology. The limited performance of FISH in low-grade or low-stage tumours is not consistent. Jones [3] also recently reviewed literature regarding the role of FISH in bladder cancer surveillance. He concluded that FISH outperformed conventional cytology across all stages and grades in all published reports. Notably, cytology detected only 67% of CIS versus 100% detection by FISH. Marin-Aguilera et al [4] found in their study higher overall sensitivity for FISH versus cytology (70.3% vs. 35.1%). In this study the significant difference was maintained when non-muscle-invasive UC detection was broken down into low-grade and high-grade tumours. In contrast with these two studies, Moonen [5] found no improvement of FISH over cytology in the diagnosis of recurrent non-muscle-invasive bladder cancer in a study including 64 patients with biopsy-proven UC. Sensitivity and specificity were, respectively, 39.1% and 89.7% for FISH, 40.6%, and 89.7% for cytology.

A potential advantage of FISH is its ability to detect occult diseases not visible on urethrocytostcopy. Many authors note that a false-positive FISH test can predict future recurrence within 3 to 12 mo in 41–89% of patients [6–8]. Veeramachaneni

et al [9] concluded in a cohort study that a positive FISH test may indicate frank neoplastic urothelial transformation, or it may merely be an indicator of unstable urothelium. Specificity of FISH, therefore, may be underestimated because of this phenomenon and explains why FISH performs differently in different patient populations (eg, less well in surveillance compared with detection of primary tumours). Another advantage of FISH is that it is unaffected by Bacillus Calmette-Guérin (BCG) therapy and therefore can also be used for surveillance in patients who have been treated with intravesical BCG [10]. A disadvantage is that this test is labour-intensive and requires a clear learning curve before it can be used reliably.

In conclusion, because of the workload and the high costs of the FISH test, its clinical use still is low. However, most authors agree that FISH is better than cytology, although the test might have limited sensitivity to detect low-grade tumours. The fairly high false-positive rate is explained by some to reflect the potential of the FISH test to predict future recurrences.

### 2.2. Microsatellite analysis

Microsatellites are highly polymorphic, short, tandem DNA repeats found in the human genome. Two types of microsatellite alterations can be found in many cancers: loss of heterozygosity (LOH), an allelic deletion, and somatic alteration of microsatellite repeat length [11]. In bladder cancer, most mutations are in the form of LOH [11,12]. Microsatellite alterations in exfoliated urine are detected by a polymerase chain reaction (PCR) using DNA primers for a panel of known microsatellite markers.

In various studies overall sensitivity and specificity of MSA ranged from 72% to 97% and 80% to 100%, respectively [2]. In contrast to conventional cytology it appears that microsatellite analysis (MSA) has the ability to detect low-grade and low-stage disease as accurately as high-grade and high-stage disease [13]. Frigerio et al [14] found that the combined use of cytology and LOH analysis had high sensitivity for identifying primary tumours and had the ability to detect almost all recurrent diseases in voided urine. They found sensitivity of 72% for grade 1–2 tumours and 96% for grade 3 tumours.

In conclusion, MSA has good overall sensitivity and specificity, but this test is complex and expensive, and therefore not used in daily clinical practice. Currently two multicentre trials are being performed.

### 2.3. ImmunoCyt™

Immunocytology is based on the visualisation of tumour-associated antigens in urothelial carcinoma cells using monoclonal antibodies. Three fluorescently marked antibodies label two mucinlike proteins and a high-molecular-weight form of carcinoembryonic antigen. After this process the cells are examined under a fluorescent microscope. Sensitivity varies between 38.5% and 100% [15–18]. ImmunoCyt (Bostwick Labs) shows specificity between 73% and 84.2% [15–17]. A prospective study in which 942 patients were enrolled showed 298 patients with histopathologically proven UC. The results were encouraging: Sensitivity was 79.3% for

grade 1 tumours, 84.1% for grade 2, and 92.1% for grade 3, and specificity was 72.5% [19]. Schmitz-Dräger et al [18] recently found high sensitivity and good specificity in a population of 189 patients with microhaematuria. They found bladder cancer in 8 patients and only one tumour of low malignant potential was missed. However, taken into account the small number of tumours in these studies, more data are needed.

In conclusion, sensitivity of ImmunoCyt is good, but it fails in comparison with conventional cytology with regard to specificity. Another aspect is the high interobserver variability and the need for constant quality control.

#### 2.4. Telomerase

Telomeres are repetitious sequences at the end of chromosomes that protect genetic stability during DNA replication. There is loss of telomeres during each cell division, which causes chromosomal instability and cellular senescence. Bladder cancer cells express telomerase, an enzyme that regenerates telomeres at the end of each DNA replication and therefore sets the cellular clock to immortality. Determination of telomerase activity is a PCR-based technology and must be performed in specialised laboratories. Overall sensitivity and specificity of the telomerase assay, as reported by Lokeshwar [2], were between 70% to 100% and 60% to 70%, respectively. In a systematic review Glas et al [20] showed that telomerase had the best sensitivity (75%) compared with the other markers, including cytology. Specificity, however, was lower than that of cytology. A more recently conducted case-control study [21] on 218 men showed an overall sensitivity of 90% and specificity of 88%, which increased to 94% for individuals younger than 75 yr. The same phenomenon was noted by Bravaccini [22]: In a case-control study conducted in 212 women, sensitivity was 87% and specificity 66%. A breakdown analysis as a function of age showed a higher assay accuracy in women younger than 75 years (sensitivity, 91%; specificity, 69%) compared with older women (sensitivity, 64%; specificity, 59%). The authors noted that a possible explanation for this effect is the higher number of viable and telomerase-positive nonurothelial cells, such as epithelial cells from the lower genital tract or inflammatory elements, in urine of the elderly.

In conclusion, telomerase seems to have good sensitivity but lacks sufficient specificity. Moreover, test results can be influenced by inflammation and age. These disadvantages make it a suboptimal test for detection of bladder cancer.

#### 2.5. BTA-TRAK™ and BTA-stat™

BTA-TRAK and BTA-stat (Alidex Inc, Redmond, WA, USA) are both versions of the bladder tumour antigen assay that measures complement factor H-related protein in urine. BTA-stat is an immunoassay that can be performed “on bench” within several minutes. BTA-TRAK is a quantitative test that is performed in a laboratory. The literature recently reviewed by van Rhijn et al [1] showed a sensitivity that was slightly higher than that of cytology, but specificity was much lower. BTA-stat had a median sensitivity of 70% (range, 24–89%) and median specificity of 75% (range, 52–93%). For BTA-TRAK, median

sensitivity was 69% (range, 57–79%) and median specificity of 65% (range, 48–95%). These tests can also be false-positive in patients with inflammation, infection, or haematuria [1].

In conclusion, the use of BTA-TRAK and BTA-stat is limited because of their low specificity and the false-positive test results in patients with benign genitourinary conditions.

#### 2.6. Hyaluronic acid and hyaluronidase

Hyaluronic acid (HA) is a glycosaminoglycan and a normal component of tissue matrices and body fluids. In tumour tissues, elevated HA is mostly localised to tumour stroma, in bladder carcinoma HA is found in tumour cells, and elevated HA levels have been shown in urinary samples of bladder cancer patients [23]. The concentration of HA is also associated with tumour metastases [24]. Hyaluronidase (HA-ase) is an enzyme that cleaves HA into fragments. Three HA-ase genes have been identified and bladder tumour-derived HA-ase was shown to be the HYAL1 type [23]. HA-ase levels are elevated in bladder tumour tissue, and an increase is correlated with tumour grade [25]. In a study by Lokeshwar et al [23], it was shown that blocking HYAL1 expression in a bladder cancer cell line results in a 4-fold decrease in cell growth rate, suggesting that HYAL1 expression by tumour cells is required for cell proliferation. They also mentioned that HYAL1 plays a role in promoting the invasive potential of bladder tumour cells and is not elevated in low-grade tumours. Both the HA test and HA-ase test are enzyme-linked immuno sorbent assays (ELISAs). The HA test detects bladder cancer regardless of tumour grade and, as mentioned above; the HA-ase test preferentially detects grade 2 and 3 bladder tumours [26]. Sensitivity of the combined HA-HA-ase test varies between 83% and 94% [2,27–30]. The overall specificity varies between 77% and 93.4% [26–30]. In a study by Passerotti et al [31], urine samples taken from 83 patients (22 controls and 61 diagnosed positive for bladder cancer) showed a sensitivity of 92.9% and a specificity of 83%. In a few comparative studies, HA-HA-ase were shown to be better than other markers, including cytology [27–30].

In conclusion, HA-HA-ase is a very promising marker that deserves further studies. The test has high sensitivity to detect both low- and high-grade/stage tumours.

#### 2.7. Nuclear matrix protein 22

Nuclear matrix protein 22 (NMP22) is a nuclear matrix protein and is an important regulator of mitosis. In tumour cells the nuclear mitotic apparatus is elevated and NMP22 is released from cells in detectable levels. The first NMP22 test was a quantitative ELISA test. The newer ImmunoCyt™ (Freiberg, Germany) is a point-of-care assay that uses monoclonal antibodies in a lateral flow strip to detect NMP22, with a cut-off value of 10 U/ml. Grossman et al [32] investigated the capability of this test in detecting malignancy in 1331 patients with risk factors of bladder cancer. They found sensitivity of 55.7% and specificity of 85% for NMP22 compared with 15.8% and 99.2% for cytology. In a subsequent study they examined whether NMP22 could improve detection of recurrences in 668 patients. Sensitivity of NMP22 in this study was 49.5% and specificity was 87.3%. NMP22 detected eight malignancies that

were not detected by urethrocystoscopy. The combination of NMP22 and urethrocystoscopy identified 99.0% of all malignancies versus 91.3% with urethrocystoscopy alone. Voided cytology did not significantly increase the sensitivity of urethrocystoscopy [33]. These studies taken into account seem to indicate that NMP22 performs less well in surveillance compared with primary detection of bladder cancer, but still has a better sensitivity than cytology. In a recent study [34], NMP22 was compared with photodynamic diagnosis (PDD) as the gold standard. The authors found sensitivity of 65% and specificity of 40% for NMP22, and 44% and 78%, respectively, for voided cytology. Washed cytology, however, had sensitivity of 75% and specificity of 62%. The authors concluded that, as validated by PDD, NMP22 is not recommended for surveillance in daily clinical use. Another point of debate is the cut-off value for the NMP22 test. Shariat et al [35] assessed the variability in the diagnostic performance of NMP22 for detecting recurrence and progression in a population of 2871 patients. The manufacturers recommended cut off of 10 U/ml detected 57% of cases, with a 19% false-positive rate. The authors found a substantial degree of heterogeneity in the diagnostic performance of NMP22 applied to populations from different institutions, and stated that there is no clearly defined NMP22 cut off at which to recommend urethrocystoscopy.

In conclusion, the current NMP22 point-of-care test is easy to perform, with sensitivity better than cytology and reasonable specificity. NMP22 is also sensitive in low-grade tumours and seems unaffected by BCG therapy.

## 2.8. BLCA-4

Six nuclear matrix proteins that are specifically expressed in bladder cancer have been identified. One of them is BLCA-4. Overexpression of BLCA-4 seems to increase the growth rate in cells and also causes cells to express a more tumorigenic phenotype [36]. BLCA-4 is analysed with ELISA and has a reported sensitivity between 89% and 96.4%, and specificity between 95% and 100% [37,38]. Currently a large multicentre trial is being performed.

BLCA-1, another nuclear matrix protein expressed in bladder cancer, showed sensitivity of 80% and specificity of 87% in a study involving 25 patients with bladder cancer and 46 controls. A limitation of this study was the small number of low-grade tumours [39].

In conclusion, BLCA-4 seems to have good sensitivity and specificity for detecting bladder cancer, but a larger trial is needed to confirm this observation. There is as yet not much evidence on the performance of BLCA-1 in detecting bladder cancer.

## 2.9. Cytokeratins

Cytokeratins are intermediate filaments; their main function is to enable cells to withstand mechanical stress. In humans 20 different cytokeratin isotypes have been identified. Cytokeratins 8, 18, 19, and 20 have been associated with bladder cancer [40]. The Urinary Bladder Cancer (UBC) test detects cytokeratin 8 and 18 fragments in urine. The sensitivity of the UBC test varies from 35% to 79% and depends on tumour grade

and stage [2], but UBC tests were inferior to voided cytology in test quality [2,41]

CYFRA 21-1 is a soluble fragment of cytokeratin 19, is analysed with ELISA, and is measurable in serum and urine. In one study [42], abnormal serum levels of CYFRA 21-1 in patients with bladder cancer were seen in only patients with metastatic disease. In another study [43], abnormal CYFRA 21-1 levels also showed a significantly worse overall median survival, and correlated with response to systemic treatment. In an early study, Pariente et al [44] found an optimal cut-off value of 4 ng/ml for urinary CYFRA 21-1. In a prospective study [45], urine samples of 325 patients were examined, including 107 patients under surveillance after transurethral resection of bladder cancer. This study found an optimal cut-off concentration for the detection of primary bladder tumours of 4.9 ng/ml. This cut off resulted in sensitivity of 79.3% and specificity of 84%. These authors also found increased concentrations of CYFRA 21-1 after instillation with BCG, even years after treatment. A recent study by Fernandez-Gomez et al [46] showed sensitivity of 43% and specificity of 68% at a cut-off value of 4 ng/ml. Lowering the cut-off point to 1.5 ng/ml increased sensitivity to 73.8% but decreased specificity to 41%. Specificity increased, excluding all patients treated with pelvic radiotherapy, with urinary tract infections (UTIs) or urethral catheterisation and intravesical instillation within the 3 previous months. With a cut-off value of 4 ng/ml, sensitivity increased to 80.2%.

Cytokeratin 20 is not truly a soluble marker, and requires processing of exfoliated cells and performance of reverse transcriptase-polymerase chain reaction (RT-PCR) [47].

In conclusion, CYFRA 21-1 seems to be the best cytokeratin for use as a urinary marker for bladder cancer. But it shows a disappointing performance in low stage-bladder cancer, and CYFRA21-1 levels are strongly influenced by benign urological diseases and intravesical instillations.

## 2.10. Survivin

Survivin is a member of the family of proteins that regulate cell death, the so-called inhibitor of apoptosis family. Its overexpression inhibits extrinsic and intrinsic pathways of apoptosis [48]. Survivin is expressed during foetal development but not in terminally differentiated adult tissues [49]; however, it is one of the most commonly overexpressed genes in cancer [47]. In bladder cancer, survivin is expressed in urine, and its expression is associated with disease recurrence, stage, progression, and mortality [50]. RT-PCR provides a diagnostic tool to detect survivin messenger RNA (mRNA) in urine. In recent literature sensitivity and specificity between 64% to 94% and 93% to 100%, respectively, have been noted [48,51,52]. In a study by Schultz et al [53], survivin mRNA expression helped to distinguish between patients with primary Ta urothelial cell carcinoma and long or short recurrence-free intervals. Survivin identified 71.4% and 69.6% of the patients with long or short recurrence-free periods, respectively.

In conclusion, survivin is a very promising marker with good sensitivity and specificity, and also deserves further study. Survivin seems predictive for recurrence and can be helpful in preventing unnecessary urethrocystoscopies.

### 2.11. Growth factors

Numerous growth factors are described in literature. Epidermal growth factor receptor (EGFR), vascular endothelial growth factor, tumour necrosis factor alpha, type 1 insulin-like growth factor receptor, fibroblast growth factor receptor-3, and heparin-binding epidermal growth factor-like growth factor are just a few of them. Members of the EGFR family, a type I tyrosine kinase growth factor receptor, are involved in various forms of cancers and serve as prognostic markers or therapeutic targets [54]. EGFR is considered one of the most important oncogenes in bladder cancer development [55]. Notably, a recent study with archival tissue by Litlekalsoy et al [56] showed a time-dependent pattern of biological features in UC. In the 1930s, these tumours tended to have a high proportion of high-molecular-weight cytokeratin and EGFR-positive cases, combined with more metastases and shorter life span. Seventy years later there is a tendency toward the opposite pattern, indicating the different environmental and carcinogenic influences that account for the development of bladder cancer.

In conclusion, the usage of growth factors bears promise, but their use is mainly study-based and prognostic. No large trials have been conducted yet; therefore, no clinically usable urinary markers have been identified.

### 2.12. Proteomics

Proteomics is the study of structure and functions of proteins, and plays an important role in the search for cancer biomarkers. Innumerable proteins can be found in human urine; changes in excretion rates of specific proteins/peptides can have predictive value in early diagnosis of bladder cancer. In a training set of 30 archived urine samples from bladder cancer patients and 30 urinary samples from healthy volunteers, urine was analysed via ProteinChip technology and computer-based data mining. Bladder cancer was segregated from controls with a sensitivity of 80% and

specificities of 90–97% [57]. Munro et al [58] obtained duplicate proteomic profiles from 227 subjects (118 UCs, 77 healthy controls, and 32 controls with benign urological conditions). They used linear mixed effect models to identify peaks that are differentially expressed between models and within UC subgroups. From this group, 130 profiles were randomly selected in an initial test set ( $n = 43$ ) and an independent validation set ( $n = 43$ ). UC was predicted with 71.7% sensitivity and 62.5% specificity in the initial set, and with 78% sensitivity and 65.0% specificity in the validation set.

In conclusion, proteomics offer many possibilities in future diagnostics. However, at this moment, there are no clinical usable assays for early detection of bladder cancer and/or surveillance.

## 3. Conclusion

The development of urinary markers for the detection of bladder cancers is a dynamic field. Therefore it is not possible to review all known markers, simply because of the numerous markers that are available and under investigation.

As known, cytology has a good sensitivity for detecting high-grade tumours. Detection of low-grade tumours is poor, but the clinical relevance of this is probably limited. Finally, cytology is highly operator-dependent. So the perfect marker should have high sensitivity and high specificity, must have no interobserver variability, and must be easy to perform. Overall, almost all mentioned urinary markers are better than cytology in relation to sensitivity, but they do score lower in specificity. The perfect test is an on-bench test that gives result after a few minutes. The NMP22BladderCheck assay is such a test with somewhat better sensitivities than cytology, and is easy to perform. BTA-stat is

**Table 1 – Sensitivity and specificity with advantages and disadvantages of urinary markers**

Marker	Sensitivity (%)	Specificity (%)	Advantage	Disadvantage
FISH	69–87	89–96	Unaffected by BCG	Labour intensive and expensive
MSA	72–97	80–100	Also detection of low grade tumour	Complex and expensive
Immunocyt	38.5–100	73–84.2		High interobserver variability
Telomerase	70–100	60–70	Sensitivity	Influenced by inflammation and age
BTA-TRAK	24–89	52–93		Influenced by benign genitourinary conditions
BTA-stat	57–79	48–95	On bench test	Influenced by benign genitourinary conditions
HA-HA-ase	83–94	77–93.4	Also detection of low grade tumour	Needs further study
NMP22	49.5–65	40–87.3	Unaffected by BCG and detection of low grade tumour	No clearly defined cut-off value
BLCA-4	89–96.4	95–100	Sensitivity and specificity	Needs further study
CYFRA 21-1	43–79.3	68–84		Influenced by benign genitourinary conditions and instillations
Survivin	64–94	93–100	Sensitivity and specificity	Needs further study

FISH, fluorescence in situ hybridisation; BCG, Bacillus Calmette-Guérin; MSA, microsatellite analysis; HA-HA-ase, hyaluronic acid and hyaluronidase; NMP22, nuclear matrix protein 22; CYFRA 21-1, cytokeratin 19 fragment.

also an on-bench test but scores lower than cytology. Many other tests have high workload and are more expensive. Telomerase, BTA-TRAK, and CYFRA21-1 are influenced by benign urological conditions, which influences specificity and makes these tests less usable in daily clinical practice. The value of the ImmunoCyt test is also limited by its specificity and variability. MSA is promising because of its good sensitivity/specificity and its ability to also detect low-grade/stage tumours, but the test is complicated. Survivin and HA-HA-ase also have good sensitivity and specificity, but further studies are needed for both. Proteomics seem to be a great pool from which new markers will certainly evolve.

Table 1 gives sensitivities and specificities with potential advantages and disadvantages of the urinary markers described in this review.

In conclusion, the first-generation urinary markers did not add much to urinary cytology. The current generation of markers is promising but larger trials are needed. The future of marker development is bright and new techniques are emerging, but the perfect marker is still to be found. As soon as we have good markers, an important issue will be the financial cost–benefit ratio, which has not been studied sufficiently yet. Currently, no single marker can guide us in surveillance and lower the frequency of urethroscopy. Whether use of a set of markers will be the answer will have to be studied.

### Conflicts of interest

Dr Witjes received a lecture honorarium from Matritech in 2007.

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