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REVIEW ARTICLE

Markers for Early Diagnosis and Post-operative Recurrence Monitoring of Bladder Cancer

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Abstract: The diagnosis and management of bladder cancer (BC) are high complex due to cancer heterogeneity among patients. Thus, biomarkers play pivotal roles in the diagnosis, prognosis determination, and planning of therapeutic intervention of BC. With years of research and discovery, many candidate markers for BC have emerged. The alterations of nucleosides, proteins, post-translational modifications, and cells are the candidate markers for early diagnosis and post-operative recurrence monitoring of BC. This review mainly discusses the recent progresses in the proteins and nucleosides markers for diagnosis and recurrence monitoring of BC. In the detection of BC, some potential nucleoside-based markers have been reported, including *telomerase reverse transcriptase* (*TERT*) gene, microsatellite, and chromosome instability, whereas the protein markers include bladder tumor antigen, nuclear matrix protein family (Nuclear matrix protein 22), fibrin/fibrin degradation product, and aberrantly glycosylated integrin α3β1. Besides, the performance of diagnostic methods based on these markers are reviewed. The sensitivity and specificity of candidate markers and detection methods of BC are compared. In summary, this review provides invaluable information about the early diagnosis and recurrence of BC, which guides the development and improvement of novel markers for early diagnosis and post-operative recurrence monitoring of BC in future.

Keywords: Bladder cancer, Biomarker, Diagnosis, Recurrence, Aberrantly Glycosylated Integrin α3β1

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1 Introduction

Bladder cancer (BC) is one of the cancers with the highest economic burden worldwide[1]. Although vast amount of diagnosis and therapeutic interventions have been implemented to improve clinical outcome, a sharp increase in the cases of BC is still seen in recent years[2]. Therefore, there is an urgent need to address these issues to alleviate the burdens on the health-care resources allocated to BC.

Conventionally, dipstick detection of hematuria, which is the most common symptom of primary BC, is the classical method used in the

diagnosis of BC[3]. The improvement of diagnostic research has led to the conception of cystoscopy and urine exfoliative cytology, which are the most commonly used diagnostic methods for BC in this decade. Cystoscopy and biopsy procedures involve the insertion of cystoscope into the bladder and the collection of samples for pathological examination. Despite its high sensitivity, this procedure is invasive and could cause some complications such as pain, bleeding, and urinary tract infections[4,5]. Furthermore, misdiagnosis using this method is also likely to occur due to the misdetection of bladder tumors at unpredictable positions. Conversely, urine exfoliative cytology, which involves the examination of cancer cells under a microscope after the collection of samples by physicians, is a non-invasive, simple, and inexpensive method for BC diagnosis. However, certain limitations, such as low sensitivity and specificity, were reported[4,6]. In view of the above, highly effective techniques for BC detection are warranted.

Advances in genomic approaches such as next-generation sequencing and bioinformatic analysis have provided a deep insight into the molecular pathogenesis behind the development, progression, metastasis, and recurrence of BC[7]. This enables the development of the marker-based diagnostic method that aids in the diagnosis through deduction based on the expression of markers. Nevertheless, marker-based diagnostic method is limited by the lower specificity as compared to cytology-based method. Besides, marker-based diagnostic method is unable to differentiate between urothelial carcinoma and inflammations[8]. These shortcomings are the culprit of high false-positive results in BC detection.

Despite its limitations, marker-based diagnostic method is still praised for its non-invasiveness, low cost and simple procedure in BC detection. In this review, we discuss various BC markers which are commonly used in the BC diagnosis. In addition, the performance and some perspectives on the improvement of BC marker-based diagnostic methods are also reviewed.

2 Telomerase

Telomerase is a ribonucleoprotein complex composed of *telomerase reverse transcriptase* (*TERT*), telomerase RNA component, and telomerase-related protein. It synthesizes DNA at the end of chromosome and confers immortality for cell. In addition, telomerase plays an important role in maintaining genome integrity, telomere stability, cell activity, and proliferation capacity. Activity of telomerase is inhibited in healthy individuals and reactivated in cancer patients[9,10]. However, the somatic mutations of *TERT* affecting the telomerase activity appear to be very uncommon in cancer[11]. Until 2013, two studies reported frequent mutations in the promoter of *TERT* in melanoma[12,13]. Furthermore, the recurrent somatic mutations in the *TERT* promoter were also uncovered in hepatocellular carcinoma (59%) as well as cancers of the central nervous system (43 – 51%), bladder (59 – 66%), thyroid (follicular cell-derived tumors, 10%), skin (melanoma, 29 – 73%), etc.[14-17]. The promoter luciferase assay revealed that the alteration of *TERT* promoter resulted in a two- to four-fold increase in telomerase expression *in vitro*[12,13]. Thus, the mutation of *TERT* promoter and the elevated telomerase activity could be the potential biomarkers of cancer, in which the elevated telomerase activity is a promising therapeutic target of cancer[18].

In a panel of 23 BC cell lines, Borah *et al.* found frequent incidence of the −124C >T mutation, and less frequent incidence of the −146C >T mutation[19]. The study also reported that *TERT* promoter mutation was closely related to high expression level of *TERT* mRNA, TERT protein, telomerase activation, and telomere length[19]. Besides, the elevated expression of *TERT* mRNA was closely related to decreased BC-specific survival[19]. In addition, analyses of the relationship between *TERT* mRNA expression and disease-specific survival of BC patients were conducted in two cohort studies[20,21]. The detection of *TERT* promoter mutations and expression hold promise for the detection of BC, and it would be highly attractive if the detection could be detected in the urine for the diagnosis of BC[22].

In a study that analyzed *TERT* promoter mutations and mRNA in tumor and urine samples from 182 Han Chinese patients with BC,

it was found that 47.8% of the tumors harbored *TERT* promoter mutation and *TERT* mRNA was detected in 93.9% of the urine samples derived from the BC patients before operation[23]. Moreover, Stasik *et al.* found that 77% of the urinary sediment DNA samples and 63% of the cell-free DNA samples from BC patients had *TERT* promoter mutation[24]. In a nested case–control study, urinary *TERT* promoter mutations were detected in 14 out of 30 pre-clinical BC (sensitivity 46.7%) and none of the controls (n = 101, specificity 100.0%)[25]. These data laid the basis for BC diagnosis that depends on *TERT* expression or promoter mutation in the urine samples.

Taken together, we believe that *TERT* promoter mutation can increase telomerase activity through specific regulatory mechanisms. Furthermore, urinary *TERT* promoter mutations is a promising non-invasive biomarker for the early detection of BC[26]. Notwithstanding the evidence, more investigations are still needed for further evaluations on the potential use of *TERT* promoter mutation and telomerase activity in BC detection.

3 Microsatellite marker

Microsatellite markers are highly polymorphic short tandem which were composed of 2 – 6 nucleotide tandem repeats in DNA sequences of human genome. Microsatellite markers of BC mainly include microsatellite instability and loss of heterozygosity (LOH)[27].

Legrand *et al.* performed microsatellite analysis on urine samples of 43 patients with superficial transitional cell carcinoma of bladder and 42 healthy controls[28]. The results showed that 39.5% of the patients displayed LOH and 86.0% of them displayed allelic losses[28]. Besides, the total number of microsatellite alterations was related to patient's gender, age, stage and grade of tumor, and European Association of Urology classification. Interestingly, microsatellite alterations in the short arm of chromosome 9 were the most common (35%). The alterations of locus 17p13.1 were closely related to high-stage and high-grade BC. The sensitivity and specificity of urine LOH detection were 39.3% and 100%, respectively, whereas the sensitivity and specificity of allelic losses detection were 88% and 73.8%, respectively. This study demonstrated that LOH is highly specific and can be used as a complementary tool for diagnosis of non-muscle invasive BC.

According to a previous study, 12 best performing LOH markers with few stutter peaks and a constant ratio between peaks heights (D8S1109, D8S1125, D8S1130, D9S252, D9S299, D9S304, D9S752, D9S1118, D11S1981, D11S1999, D17S969, and G10693) were identified from 49 common BC microsatellite markers[29]. Subsequently, the LOH markers were tested on DNA from 104 primary bladder tumors and 102 recurrent bladder tumors. The study found that 45 out of 104 bladder tumor tissues (43.3%) did not have LOH according to the results of 12 LOH markers tested while the remaining 59 bladder tumor tissues (56.7%) contained at least one LOH marker. In urine samples from 102 recurrent BC patients, 43 out of 102 bladder tumor tissues (42.2%) did not have LOH according to the results of 12 LOH markers tested while the remaining 59 bladder tumor tissues (57.8%) contained at least one LOH marker. The sensitivity and specificity of the 12 LOH markers in the detection of recurrent BC were 57.8% and 100%, respectively.

Taken together, microsatellite markers are the potential indicators of BC development and recurrence, obliterating the need for blood sample. However, more in-depth investigations are needed to determine its validity and suitability for muscle invasive BC and prognosis determination of BC in the future.

4 Chromosomal instability

The development of BC was highly associated with chromosomal instability. The previous studies found that unstable chromosomal sites of BC are mainly located on chromosomes 1, 3, 7, 9, 11, and 17, and more importantly, homozygous deletion of 9p21 locus is the most common genetic change in bladder urothelial carcinoma[30-32].

A commercial fluorescence *in situ* hybridization (FISH) kit called UroVysion®

(Abbott Laboratories, USA) was successfully developed to target the above-mentioned unstable chromosomal sites for early diagnosis of BC. This detection kit is developed on the basis of *in situ* hybridization between fluorescently labeled DNA probe and DNA sample. The fluorescence signals are measured through the use of fluorescence microscope, thereby detecting chromosomal or genetic abnormality of cells, tissues, and body fluids.

Petrov *et al.* used UroVysion® kit to examine bladder irrigation fluids from 50 primary BC patients who were categorized according to tumor stage and grade before transurethral resection of bladder tumor[33]. Results showed that the sensitivities of FISH for different stages such as Ta, T1, and T2 of BC were 81.5%, 91.7%, and 100%, respectively, and the sensitivities for different grades such as G1, G2, and G3 of BC were 70%, 100%, and 100%, respectively[33]. The results concluded that the detection rate of FISH was significantly higher for stage T2 cells than for the cells in stages Ta and T1. Besides, the detection rate was significantly higher for G3 grade than for G1 grade and G2 grade. In addition, number of signals from each chromosome increased with tumor stage and grade. Therefore, FISH is a highly sensitive method for early diagnosis of BC in addition to its additional benefit in the prediction of tumor morphological features before surgery.

In a study comprising 1045 patients with post-operative BC and 790 patients with hematuria, the results showed that sensitivity, specificity, positive predictive value, and negative predictive value of FISH test were 61.9%, 89.7%, 53.9%, and 92.4%, respectively, and 29.1%, 96.9%, 64.4%, and 87.5%, respectively, for urine exfoliative cytology[34]. These results signified that FISH technique is superior to urinary exfoliative cytology in the detection of BC. Thus, for patients whose diagnosis can hardly be confirmed by urinary exfoliative cytology, FISH is an appropriate choice of detection method. However, due to high false-positive rate and lack of standardized positive diagnostic criteria, its application in early diagnosis and post-operative recurrence monitoring of BC is limited.

5 Bladder tumor antigen (BTA)

BTA, also known as human complement factor H-related protein, is a polymer complex released during bladder tumor growth. Composed of 16 – 165 kDa polypeptides, its structure is similar to human complement factor H. BTA can allow bladder tumor cells to escape from host immune attack by interfering the complement pathway and thereby render them selective growth advantage. Elevated urine BTA level has provided invaluable information for early diagnosis of BC.

The commonly used BTA detection methods are BTA STAT (a qualitative test) and BTA TRAK (a quantitative test). Both methods aid in the diagnosis of BC by detecting BTA level in urine using monoclonal antibodies. Miyake *et al.* used BTA STAT (Bion Diagnostic Sciences, Inc., Redmond, Washington)[35] and BTA TRAK (Polymedco Inc. Cortlandt Manor, NY, USA) to measure BTA levels in urine samples collected from 64 BC patients with hematuria and 62 control subjects with hematuria[36]. The study results showed that positive rate in BC patients was 72%, but 47% of patients in control subjects also showed positive results as well, implying the possibility of false-positive results. In the same study, the sensitivity and specificity of BTA detection for BC were 72% and 53%, respectively. Thus, BTA detection is helpful for early diagnosis of BC, but it is susceptible to false-positive result due to hematuria[36]. Therefore, the selection of BTA as a diagnostic method remains debatable and more research are warranted to develop a BTA-based diagnostic tool with higher sensitivity and specificity.

6 Nuclear matrix protein 22 (NMP22)

As a member of nuclear matrix protein family, NMP22 acts as a scaffold for nuclear structure within nucleus. Mostly, it regulates the central dogma of molecular biology, such as DNA replication, transcription, and the regulation of gene expression. In general, the level of NMP22 protein in urine samples of BC patients is significantly higher than that of healthy individuals. Therefore, NMP22 can be used

as an auxiliary method for early diagnosis and post-operative recurrence monitoring of BC.

At present, commercial kit known as Alere NMP22® BladderChek® Test is widely available and can be directly applied for clinical testing[37]. Balci *et al.* performed NMP22® BladderChek® Test (Matritech, Newton, MA, USA) and urine exfoliative cytology on specimens collected from 160 patients with non-muscle invasive BC[38]. The results showed that sensitivities of the NMP22® BladderChek® Test and urine exfoliative cytology were 66.7% and 46.7%, respectively, while there specificities were 81.0% and 98%, respectively. Sensitivity, specificity, positive predictive value, and negative predictive value of the combination of two methods were 73.3%, 79%, 67.7%, and 83.2%, respectively[38]. These findings suggested that NMP22 diagnostic method was more sensitive, but on the other hand, less specific than conventional urinary exfoliative cytology. Combination of the two methods did not significantly improve the accuracy of early diagnosis and recurrence monitoring of BC. In spite of the advantages of NMP22 testing such as non-invasiveness, simple and rapid procedures, and low cost, the accuracy of this test could be compromised by the false-positive results due to benign conditions such as urinary tract infection, urinary tract stone, and hematuria that causes elevated urinary level in NMP22. Hosseini *et al.* emphasized that NMP22 screening was well-suited for the diagnosis of superficial BC, especially for low-risk and intermediate-risk superficial BC, but its clinical application is limited to some extent due to low specificity[39].

7 Fibrin/fibrin degradation product (FB/FDP)

FB/FDP is protein fragment produced following degradation of fibrin or fibrinogen by plasmin[40]. Bladder tumor cells can increase the permeability of peripheral blood vessels by producing large amount of vascular endothelial growth factor, causing increased leakage of fibrinogen, and its conversion into fibrin. Later, fibrin is further degraded into FDP by plasmin[41]. Thus, an increase in FB/FDP is often observed or detected in the urine of BC patients[42].

Schmetter *et al.* recruited 192 patients with a history of BC in a prospective multicenter study and compared the detection of BC of AuraTek FDP rapid immunoassay device (PerImmune Inc., Rockville, Maryland, USA)[43] with urinary cytology and hemoglobin dipstick[11]. The sensitivity of AuraTek FDP assay was significantly higher (68%) than urinary cytology (34%) or hemoglobin dipstick (41%) in the detection of BC. Furthermore, AuraTek FDP assay had a sensitivity of 100% for muscle-invasive BC (T2-T4). In another prospective study, Topsakal *et al.* assessed the use of FB/FDP by Accu-Dx test (Intracel Co., Ltd, Rockville, Maryland, USA)[44] in the detection of BC[45]. Following examination of urinary specimens of 97 patients in which 69 of them were diagnosed with BC, positive rate of FB/FDP detection (69.6%) was significantly higher than that of urine exfoliative cytology (44.9%). However, specificity (67.9%) was lower than that of urine exfoliative cytology (96.4%)[45]. For tumors with higher tumor stage and grade, FB/FDP had a higher positive rate (Ta: 50%, T2 and above: 100%; G1: 42.9%, and G3: 94.1%). However, there were false-positive results in six cases of cystitis and the overall detection accuracy was 69%. To sum up advantages and disadvantages, Accu-Dx test is a simple, rapid, reproducible, and non-invasive method to detect BC yet limited to its relatively low accuracy and specificity. Although it hardly can substitute cystoscopy biopsy method, Accu-Dx test can assist cystoscopy in diagnosis or follow-up examination of BC patients.

8 AG-α3β1 (antigen of BCMab1)

As a new member of integrin family, integrin α3β1 is a heterodimer composed of non-covalently associated α and β subunits, which mediates cell-extracellular matrix and cell-cell adhesion. In an elegant study, Li *et al.* developed mouse monoclonal antibodies using human BC cell line T24 as immunogen, and successfully identified BCMab1, a highly specific monoclonal antibody against BC[46]. By affinity chromatography with BCMab1 and gel filtration, aberrantly glycosylated integrin α3β1 (AG-α3β1) was identified as an antigen

recognized by BCMab1, and this antigen was also established as a novel biomarker of BC[46]. In addition, AG-α3β1 was examined in 123 human tumor tissues and 56 normal tissues through immunohistochemistry, and it specifically expresses on membrane of human bladder tumor cell rather than other tumor tissues or normal tissues[46]. Furthermore, the expression of AG-α3β1 is significantly higher in histologically high-grade and invasive BC than that of low-grade, superficial, or non-invasive BC[46]. More importantly, the data of an 80-month follow-up of 69 BC patients indicated that BC patients with high expression of AG-α3β1 had significantly worse prognosis than those with low expression of AG-α3β1[46]. These results indicate that AG-α3β1 can be used as a specific marker for BC and has broad application prospects in early diagnosis and recurrence monitoring of BC.

9 Other markers for early diagnosis and recurrence monitoring

In recent years, a large number of BC markers have been reported and most of which have demonstrated certain clinical value for early diagnosis and post-operative recurrence monitoring of BC. In addition to those discussed above, BC markers also include hyaluronic acid (HA) and hyaluronidase (HAase)[47], Lewis X[48], survivin[49], BC specific nuclear matrix protein 4 (BLCA-4)[50], human uroplakin 3A (UPK3A)[51], and cytokeratin 20 (CK20)[52] (**Table 1**). Besides, the development of detection methods also improve the diagnosis and post-operative recurrence monitoring of BC, as evidenced by the establishment of Karyometric analysis (Quanticyt)[53], ImmunoCyt (Diagnocure Inc., Que Inc., QuQu)[54], BTA STAT (Bion Diagnostic Sciences, Inc., Redmond, Washington)[35,55], and telomeric repeat amplification protocol (TRAP) assay based on TRAPezeTM kits (Appligene Oncor®, Gaithersburg, USA)[56] (**Table 2**). Despite the potential of these markers in the diagnosis of BC, further investigations are needed to evaluate the regulation of these markers in the development, progression, and recurrence of BC.

Table 1. Sensitivity and specificity of potential biomarkers in the detection of bladder cancer.

Marker	Sensitivity (%)	Specificity (%)	Reference
NMP22	49.5 − 92.1	66.0 − 87.3	[11-13]
TERT	84.8 − 95.0	84.0 − 100.0	[18,21-23]
HA	61.0 – 83.1	53.6 − 90.1	[47]
HAase	81.5	83.8	[47]
HA/HAase	88.1 − 94.0	63.0 − 84.4	[47]
Lewis X	79.8 − 84.0	80.0 − 86.4	[48]
Survivin	75.0	100.0	[49]
LOH	60 − 97.0	93.0	[43-45]
BLCA-4	89 − 96.4	95.0 − 100.0	[50]
UPK3A	83.0	83.0	[51]
CK20	78.0 − 87.0	56.0 − 80.0	[52]
AG-α3β1	>95	>95	[46]

NMP22, Nuclear matrix protein 22; TERT, Telomerase reverse transcriptase; HA, Hyaluronic acid; HAase, Hyaluronidase; LOH, Loss of heterozygosity; BLCA-4, Bladder cancer specific nuclear matrix protein 4; UPK3A, Human uroplakin 3A; CK20, Cytokeratin 20; AG-α3β1, Aberrantly glycosylated integrin α3β1

Table 2. Sensitivity and specificity of detection methods of bladder cancer.

Marker	Sensitivity (%)	Specificity (%)	Reference
Cytology	12.2 – 79.0	78.4 – 99.4	[12,18-20,42,57]
Quanticyt	42.1 – 69.0	67.9 – 87.0	[53]
ImmunoCyt	66.7 – 84.9	62.0 – 84.7	[54]
FISH	69.0 – 92.1	89.0 – 94.5	[41,42,57]
BTA STAT	50.0 – 70.0	67.0 – 78.0	[9,10,55]
TRAP	77.4 – 90.0	88.0 – 93.5	[56]

FISH, Fluorescence in situ hybridization; TRAP, Telomeric repeat amplification protocol

10 Combined use of multiple BC markers

Cystoscopy coupled with biopsy remains the gold standard in the diagnosis of BC in the clinical setting. However, this technique is dwarfed by its invasiveness, limited observation field, and relatively low sensitivity which are the major defects of cystoscopy for BC detection. Besides, clinical observation and decisions can be inaccurately made if bleeding occurs. Moreover,

precancerous lesions and small lesions may be missed out during pathological examination. For this reason, a vast number of new BC markers which have great potential for early diagnosis and recurrence monitoring of BC have emerged. However, the current detection methods for BC that employ the application of only a single marker lack of specificity or sensitivity; therefore, this type of detection method is currently unable to substitute cystoscopy coupled with biopsy. In some cases, especially when cystoscopy and biopsy are not suitable for the patients, the application of a set of multiple tumor markers is expected to be an effective approach to diagnosis.

For example, Li *et al.* used FISH, NMP22® BladderChek® test and liquid-based cytology (LBC) to examine the urine samples collected from 37 healthy controls and 138 patients with urinary diseases, in which 104 of them were diagnosed as bladder urothelial carcinoma[57]. In this study, the positive rates of FISH and NMP22® BladderChek® were 85.7% and 61.9%, respectively, while the false-positive rates were 37.5% and 50.0%, respectively. Besides, the sensitivities of LBC, FISH, and NMP22® BladderChek® in the detection of BC urothelial carcinoma were 73.1%, 86.5%, and 67.6%, respectively. Compared with single method, combined use of three detection methods was more sensitive (96.7%), but the specificity was slightly reduced. Thus, the combination of multiple BC markers could enhance the sensitivity of BC detection. Nevertheless, this area still warrants further research.

In sum, many biomarkers could be applied in BC detection and diagnosis. Unfortunately, no single potent biomarker could satisfy the standard of clinical requirements. Future research would focus on the novel biomarkers and detection technologies to improve the specificity and sensitivity.

11 Conclusion

Although numerous BC markers for early diagnosis, recurrence monitoring, and prognosis are currently available, to improve the efficiency and accuracy, there is always a need for discovery of more new markers and improvement of diagnostic methods. The rationale behind the development of more effective marker-based diagnostic tools is justified by the current limitations of the diagnostic tools, including low sensitivity and specificity, high cost of marker screenings, and reliability of the diagnostic tools. The emergence of more specific markers and efficient diagnostic technology will assist in improving the prediction of tumor response, targeted therapy, and prognosis of BC. The knowledge about BC markers is essential for improving the clinical outcome of BC through the improvement of diagnosis and personalized treatment.

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Conflicts of interest

The authors declare that they have no potential conflicts of interest.

Author contributions

C.L. and Z.Y. conceived the idea of this review. C.L., Z.Y. and N.Z. wrote the paper. Z.S., S.B., M.S., L.Y., J.L., X.L. and C.Y. revised the paper.

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