

Prognostic Markers and Detection Approaches of Bladder Cancer

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Abstract: Bladder cancer (BC) is one of the most commonly diagnosed cancers in men worldwide. Numerous treatments of BC are available, but it is noteworthy that BC patients have heterogeneous responses to these treatments. Therefore, the prognostic markers, which are associated with clinical outcomes, are vital for formulating BC treatment plan. This review provides an overview of prognostic markers and their importance in early diagnosis and recurrence of BC. Apart from that, this review discusses the application of prognostic markers and their related population studies. A deeper understanding of these prognostic markers and their prognostic value would aid in designing treatment strategies, predicting response to treatment, and managing the clinical care for BC in future.

Keywords: Bladder cancer, Prognostic markers, Prognostic value

Received: September 27, 2021

Accepted: December 9, 2021

Published Online: December 22, 2021

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CITATION

Zhang N, Zhang F, Shi M, *et al*, 2021, Prognostic Markers and Detection Approaches of Bladder Cancer. *Cancer Plus*, 3(4):38-47.

DOI: 10.18063/cp.v3i4.273

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1. Introduction

Prognosis is the forecast of patients' future conditions and the outcome of a disease based on the conditions at the time of determining prognosis. A positive prognosis indicates a high possibility that the risk of disease will likely to reduce, while a negative prognosis predicts the deterioration of disease condition that would result in a low survival rate of patients. Therefore, the researchers, clinicians, and patients can be fully informed about the likely course of the disease according to information about prognosis. This will help in the arrangement of therapeutic interventions and management of clinical care to patients^[1].

Prognostic markers are biological characteristics that are objectively measured and evaluated to predict a response to a therapeutic intervention among patients with the same characteristic and outcome, such as recurrence of disease after primary treatment^[2]. Prognostic markers could predict the response to therapy, and the risk of metastasis and recurrence of bladder cancer (BC). Furthermore, prognostic markers can distinguish patients with BC according to the risk level, so as to formulate treatment strategies^[3]. Attributed by the advance in next-generation sequencing and bioinformatic analyses, our understanding of molecular signatures and pathomechanisms is becoming clearer. Hence, this inevitably refines the molecular characterization of prognostic markers.

Cells, proteins, nucleic acids, and DNA methylation are the most reported prognostic markers for BC. Cell-based prognostic markers mainly include circulating tumor cells (CTC)^[4] and exfoliated urothelial cells (EUC)^[5] which can be detected by CellSearch detection system^[6] and fluorescence *in situ* hybridization (FISH)^[7], respectively. Protein-based prognostic markers mainly include survivin^[8], epidermal growth factor receptor (EGFR)^[9], granulocyte-macrophage colony-stimulating factor (GM-CSF)^[10], and interleukin (IL)-2^[11] which can be detected mainly by enzyme-linked immunosorbent assay (ELISA)^[12] and chemiluminescence enzyme immunoassay (CLEIA)^[13]. On the other hand, nucleic

acid-based prognostic markers mainly include circulating cell-free DNA (cfDNA)^[14] and gene mutations^[15] which can be detected by real-time polymerase chain reaction (RT-PCR)^[16] and next-generation sequencing^[17]. The main detection method of DNA methylation is methylation-specific PCR^[18].

The prognostic markers of BC could be used to predict relapse. For example, UroVysion™ is a BC detection kit that applies multitarget FISH to detect aneuploidy for chromosomes 3, 7, and 17, and deletion of locus 9p21. In a multicenter, prospective study, Liem *et al.* found that 10 of 18 BC patients with a positive FISH test 3 months following transurethral resection of bladder tumor (TURBT) had a 4.0-4.6 times greater risk of developing a recurrence compared to patients with a negative FISH (7 of 48)^[7]. To investigate the relationship between CTC and outcomes of BC patients, Soave *et al.* collected blood samples from 226 BC patients preoperatively and applied CellSearch® system for CTC analysis. Using multivariable analysis, the presence of CTC was associated with disease recurrence, cancer-specific, and overall mortality in BC patients^[19]. Prognostic markers not only predict tumor recurrence but also aid in treatment strategy design and clinical outcome prediction. Recent literature suggested that high levels of IL-2, IL-6, IL-8, IL-10, and tumor necrosis factor (TNF)- α in urine correlated with the efficacy of bacillus Calmette-Guerin (BCG) treatment in BC patients^[20]. These results demonstrated that urinary protein markers could be applied in the prediction of treatment response of BC patients. Thus, prognostic markers play a crucial role in the treatment strategy design, clinical outcome prediction, and clinical care management of BC patients.

This review will provide an overview of prognostic markers and their importance in the regulation of BC. Apart from that, we will discuss the relevant clinical studies and systematic reviews to provide an understanding of the performance of prognostic markers in aiding the clinical management of BC. Furthermore, this review will introduce some approaches in the detection and evaluation of prognostic markers in BC patients. The identification of potent prognostic markers will help in tailoring effective treatment strategies, predicting clinical outcomes, and improving clinical management of BC in future.

2. Cell-based prognostic markers and detection methods for BC

2.1. Cell-based prognostic markers

2.1.1. CTC

CTC are the rare subsets of tumor cells that shed from primary or secondary tumor lesions into blood circulation. As the seed for metastases, CTC have similar antigenic and genetic properties as primary tumor cells. Numerous studies had shown that CTC are non-invasive and have real-time potential prognostic value for BC. The presence

of CTC was highly associated with the faster recurrence of BC. Since high risks of recurrence or progression of the tumor can be determined through CTC screening, early preventions and targeted treatment can be implemented^[21].

Rink *et al.* investigates the potential prognostic value of CTC in patients with advanced non-metastatic bladder urothelial carcinoma (UCB)^[4]. The CellSearch system was used to detect the presence of CTC in the blood samples of 100 UCB patients who had undergone radical cystectomy. The results showed that the risks of disease recurrence, cancer specificity, and overall mortality were significantly higher in CTC-positive patients. There was a consistency between CTC, primary tumor, and lymph node metastasis in all CTC-positive cases. To determine whether the presence of CTC can improve the prognosis of high-risk non-muscle invasive BC, Gazzaniga *et al.* designed a single-center trial in which 102 patients who met the requirements for urethral resection received BCG intravesical adjuvant immunotherapy^[22]. The CellSearch system was used to enumerate CTC derived from peripheral blood. In CTC-positive patients, both predicted time to first relapse and time to progression were reduced. Literature reported that CTC can be replanted at the primary tumor site and promote tumor growth and angiogenesis through the release of cytokines which was highly associated with local recurrence of tumor^[23]. Apart from that, CTC are highly associated with micrometastases and progression of BC, indicating that CTC have prognosis significance for BC. These studies further emphasize the role of CTC as a prognostic marker for tumor recurrence.

The application of CTC as a prognostic marker in BC is limited due to certain factors. First, a robust detection method with high sensitivity and specificity is required for the detection of a minute number of CTC in peripheral blood^[24]. Lack of highly specific tumor markers which distinguish tumor cells, inflammatory cells and other non-tumor components in blood in CTC detections also frequently leads to false-positive results. Furthermore, CTC detection technologies possess some drawbacks. For instance, the use of a quantitative PCR detection method is prone to contamination due to technical errors, and thus, only highly trained and experienced laboratory staff can perform the screening. Due to the heterogeneity of BC and other unknown factors, the CTC screening results could be biased^[25]. Although providing invaluable results, CTC screening through the CellSearch system is costly because of the antibodies used in CTC detection in the blood^[26]. There is an urgent need to overcome these limitations and develop a consistent, uniform, high sensitivity, and high specificity CTC detection system, thereby providing significant information on the prognosis of BC.

2.1.2. EUC

The diseased mucosal epithelial cells belong to the cells of the naturally shed epithelium. Due to the direct anatomical

relationship between the growth site of EUC and the production of urine, the presence of tumor cells can be preliminarily determined by examining the shed cells in urine.

Bonberg *et al.* analyzed the correlation between chromosomal copy number variation in urine shed cells and the development of BC using FISH^[5]. The results showed that the more accumulation of copy number variations in chromosomes 3, 7, and 17 and 9p21 in urine shed cells, the higher grade of BC. Bao *et al.* performed FISH on the urine samples of 42 patients with recurrent BC who underwent urethral resection and 24 patients with non-recurrent BC to analyze the relationship between chromosomal karyotype aberrations of EUC and recurrence of BC^[27]. The results showed that in the patients with grade 2 or 3 superficial BC, the aneuploidy rate of chromosomes 17 of those with recurrence was 64.3%, which was significantly higher than patients without recurrence (22.2%). In addition, the aneuploidy rates of chromosomes 7 and 17 in 14 of 42 cases of patient relapse that showed progression were 78.6% and 92.9%, respectively, which were significantly higher than the remaining 28 patients without progression (42.9% and 46.4%, respectively).

2.2. Detection methods for cell-based prognostic markers

2.2.1. Cell search detection system

At present, the CellSearch detection system is the only United States Food and Drug Administration-approved, semi-automated CTC detection system. Despite the capability in the enumeration and identification of CTC, this detection system is limited by the lack of uniformity in results acquisition due to the large heterogeneity of CTC^[6]. Scientists have developed varieties of technologies for improving the enrichment, isolation, and identification of CTC based on their biological and physical properties. At present, CTC are enumerated using the microfluidic chip and nanotechnology systems^[28]. After isolation, identification and analysis of CTC are carried out in genomic profiling and cytogenetic analysis^[29] that require next-generation sequencing, proteomic profiling, immunohistochemistry analysis, flow cytometry, and RT-quantitative PCR expression studies. Pertaining to their clinical significance, the potential use of CTC as liquid biopsy should be underscored, and the use of CTC detection systems would help facilitate cancer diagnosis, prediction of response to therapy, the discovery of drug targets besides proposing^[29,30].

2.2.2. FISH

FISH is an important non-radioactive *in situ* hybridization technique. According to the principle of base pairing, the probe with fluorescent substance is joined to the target DNA so that position of the target DNA can be directly

observed. Liem *et al.* used UroVysion™ to perform a FISH test on BC to assess whether FISH can be used to identify early recurrence of BC during BCG treatment^[7]. Bladder washouts from BC patients receiving BCG were collected at 3 time points (t_0 = week 0, before BCG; t_1 = 6 weeks after TURBT; t_2 = 3 months after TURBT) for FISH test. The results showed that 36 of 114 patients (31.6%) relapsed after 6 months. There was no significant association between positive FISH results and recurrence of BC during time t_0 or t_1 . A positive FISH result at time t_2 has a higher risk of recurrence, which was 4.0-4.6 times higher than those with negative results. Kim *et al.* counted CTC by immunofluorescence staining of vimentin and cytokeratin, and cultured CTC in mesenchymal stem cell growth medium for 16–18 days^[31]. The cultured CTC were analyzed using UroVysion FISH. This method was used to determine whether CTC in BC patients originated from BC. The results showed that among 27 patients, 9 patients had polysomy detected on chromosomes 3 and 7, and 16 patients had polysomy detected on chromosomes 3 and 17. Among the patients with chromosomal gain, 17 met the positive criteria for UroVysion FISH.

Cell-based prognostic markers hold promise for determining the likely clinical course of BC. Compared with tumor tissue samples, collection of blood samples is easy and less invasive, and it can be performed repeatedly. It is an ideal source for routine clinical testing. It is important to note that the CTC test results cannot confirm whether the patient's tumor is located in the bladder, but the examination of EUC can determine whether a tumor is present in the patient's urinary system. Examination of EUC can achieve a rapid diagnosis and high detection rate of cancer cells. However, the detection of EUC may result in misdiagnosis of BC, and it is difficult to determine the specific location of the tumor and make a clear classification of cancer cells.

3. Protein

3.1. Protein-based prognostic markers

3.1.1. Survivin

Belonging to the member of the inhibitor of apoptosis protein family, survivin is involved in the regulation of mitosis in the cell cycle, inhibition of apoptosis, and increase of cell proliferation^[32]. Although rarely expressed in normal tissues, survivin is highly expressed in tumor tissues, as supported by the fact that inhibition of apoptosis is the hallmark of cancer^[33].

Jeon *et al.* analyzed the expression of survivin and its effect on the prognosis of BC through a meta-analysis of 2165 muscle-invasive bladder tumors patients from 14 eligible articles^[8]. The hazard ratios (HR) for relapse-free survival, progression-free survival, cancer-specific survival, and overall survival (OS) were 1.81, 2.12, 2.01, and 1.53, respectively. In addition, sensitivity analysis

confirmed that when immunohistochemistry was used to determine survivin expression, survivin can reliably estimate the HR of recurrence-free survival (RFS), progression-free survival, cancer-specific survival, and OS. Senol *et al.* studied the expression of survivin in 147 patients with urothelial carcinoma (UC) and 113 non-muscle infiltrating UC (NMI-UC) by tissue chip and immunohistochemistry^[34]. The results showed that the positive expression of survivin was closely related to high T stage BC ($\geq T2$). During follow-up, BC recurrence and progression were observed in 47.6% and 29.9% of patients. The Kaplan–Meier analysis, coupled with a log-rank test, showed that high expression of survivin was significantly associated with low relapse-free survival rate (63.1%), progression-free survival rate (63.1%), and OS rate (63.1%). The RFS cycle and progression-free survival cycle were 27.01 months, 40.85 months, and 42.64 months, respectively. In the NMI-UC group, Cox regression analysis showed that high survivin expression was associated with tumor recurrence (HR = 3.876). Wang *et al.* analyzed the expression of survivin in 138 patients undergoing transurethral resection by immunohistochemistry^[35]. Kaplan–Meier method was used to estimate the RFS curve, and Cox regression model was used to analyze the relationship between survivin expression and clinical factors. The results showed that survivin could be used to determine the prognosis of NMIBC patients, and the expression of survivin was inversely correlated with the RFS.

3.1.2. EGFR

EGFR belongs to the ErbB family of receptor tyrosine kinases, which plays a role in ligand stimulation and activation of intracellular signaling cascades. These pathways relay signals from the cell surface and intracellular vesicle to nucleus, thereby regulating the expression of genes responsible for cell proliferation, survival, and differentiation. Mutation or overexpression of *EGFR* gene has been observed in various human cancers. Therefore, EGFR has become the target of various cancer therapies in the clinic^[36].

Di Maida *et al.* extracted RNA from BC samples and used it for gene expression analysis by RT-PCR to determine the role of EGFR in predicting disease recurrence and progression in non-muscle-invasive BC^[9]. The results showed that patients with high EGFR expression had a significantly lower RFS (27.9% vs. 58%), progression-free survival (75.9% vs. 90.2%), and cancer-specific survival (77.7% vs. 93.3%). Hashmi *et al.* performed EGFR immunohistochemical analysis on 126 patients with BC, and evaluated the relationship between EGFR expression and tumor grade or disease recurrence^[37]. The results showed that EGFR expression was significantly correlated with tumor grade; Kaplan–Meier curve showed that EGFR expression was significantly correlated with

tumor recurrence, but there was no significant correlation with the OS rate of patients. To guide individualized clinical treatment and determine the risk of recurrence of BC, Long *et al.* measured the expression levels of EGFR and Ki-67 in 320 patients with upper urinary tract urothelial cancer^[38]. After intravesical chemotherapy, the incidence rates of bladder upper urinary tract urothelial cancer recurrence decreased greatly in patients with negative EGFR staining. This suggested that EGFR-negative patients were more sensitive to intravesical instillation.

3.1.3. GM-CSF

GM-CSF is a hematopoietic growth factor, which has a broad impact on the survival, activation, and differentiation of myeloid populations, and participates in the regulation of bone marrow cell maturation. GM-CSF is mainly produced by activated leukocytes and exhibits a pro-inflammatory phenotype in macrophages^[39].

Jackson *et al.* measured the soluble immune molecule GM-CSF in the urine of 34 BC patients who received topical BCG vaccine and analyzed the results using traditional multivariate statistical methods and pattern recognition methods^[10]. The results showed that GM-CSF can be used as a predictor of the clinical outcome before BCG treatment in patients with superficial BC. To analyze the effects of GM-CSF and programmed cell death protein-1 (PD-1)/programmed death-ligand 1 (PD-L1) axis blockade on standard neoadjuvant chemotherapy in BC, Miyake *et al.* developed a syngeneic animal model of local recurrence with murine BC cell line MBT2 cells^[40]. Compared to neoadjuvant GC chemotherapy, the addition of mouse GM-CSF and anti-mouse PD-L1 antibody significantly enhanced the anti-tumor effect and prolonged the RFS and cancer-specific survival of tumor-bearing mice.

3.1.4. IL-2

IL-2 is a cytokine belonging to the chemokine family. IL-2 is mainly produced by activated T cells and can promote the growth, proliferation, and differentiation of lymphocytes. IL-2 can activate T cells and promote cytokine production.

Watanabe *et al.* collected urine samples of 20 patients with carcinoma of the bladder *in situ* who received BCG intravesical instillation after different infusion times^[11]. All samples were measured for cytokines using ELISA. The results indicated that IL-2, IL-6, IL-8, IL-10, interferon-gamma, and TNF-alpha were remarkably elevated in the eighth instillation as compared to the first instillation. The multivariate analysis showed that urinary IL-2 is an independent prognostic marker of treatment effect. In another study, 15 urine samples from BC patients receiving BCG were collected and the expression of IL-2 was measured by immunoassays^[41]. The results showed that 12 of 15 patients responded to BCG and 3 of 15 patients showed resistance to BCG. The expression of IL-2 was

elevated in 10 of 12 urine samples from BCG-responsive patients (83.3%). However, IL-2 was not observed in the three urine samples from BCG-resistant patients. Taken together, IL-2 is a potential predictor of BC patient's response to BCG therapy.

3.2. Detection methods for protein-based prognostic markers

3.2.1. ELISA

ELISA refers to a method that binds soluble antigen or antibody to a solid-phase carrier to form an antigen-antibody complex for quantitative detection of the molecule of interest. ELISA test on survivin in urine samples obtained from 111 BC patients and 133 controls without urinary system diseases revealed that survivin could be applied in the detection of BC, especially advanced BC^[12]. In addition, the combination of survivin ELISA and UBC[®] Rapid could elevate the sensitivity and specificity. In another study, the expression of survivin in the urine of 36 BC patients and 36 patients with benign diseases that were analyzed by ELISA indicated that the sensitivity and accuracy of detecting survivin by ELISA were 58.0% and 76.4%, respectively^[42]. Furthermore, an ELISA system based on horseradish peroxidase-labeled survivin-specific monoclonal antibodies were developed to detect survivin expression in serum samples, urine samples, and cancer tissues^[43]. The sensitivity and specificity of the method were 76.2% and 88.6%, respectively. Although ELISA assay could be applied in the detection of BC, it has defects of insufficient specificity and accuracy, which warrant the needs to combine this assay with other detection methods.

3.2.2. CLEIA

Yang *et al.* established CLEIA-based stepped magnetic particles, which was used to detect survivin in urine samples collected from 200 patients with BC and 114 healthy individuals^[13]. The results showed that this method can accurately detect survivin in urine with a detection limit of 0.949 ng/ml. In another study, the expression of survivin was detected in urine samples from 130 BC patients and 113 healthy controls by microplate magnetic chemiluminescence immunoassay^[44]. The expression of survivin in the urine was significantly higher in BC patients than that in healthy controls. Furthermore, the detection limit of this method is 0.83 ng/ml. When the concentration of survivin was 2.0884 ng/ml, the sensitivity and specificity were 86.9% and 61.9%, respectively.

3.2.3. Immunohistochemistry

Several studies showed that the combination of multiple BC markers helps to improve the accuracy of BC prognostic assessment. To determine whether p53, p21, pRB, p27, Ki-67, and survivin can be used as prognostic markers for BC, Wang *et al.* performed immunohistochemistry on

tissue microarrays using radical cystectomy specimen from patients with UC^[45]. In addition, immunohistochemistry has also been used to measure PTBP1 expression in patients with primary non-muscle invasive BC (NMIBC), and the result showed that PTBP1 expression was positively correlated with NMIBC progression, which was inconsistent with IHC staining results^[46]. Furthermore, the enhanced expression of PTBP1 was positively correlated with high rate of tumor recurrence and decreased survival time of NMIBC patients, which is an indicator of the poor prognosis of BC patients.

IL-2 and GM-CSF can be used to evaluate the clinical outcome of patients with BC after receiving BCG treatment. Survivin is tumor-specific and only expressed in tumor and embryonic tissues. EGFR plays an important role in the physiological processes of cell growth, proliferation, and differentiation. Both survivin and EGFR can predict the prognosis of BC patients. However, related results show that EGFR and survivin are not specific and sensitive enough as markers, respectively. It is advisable to consider combining the two markers to analyze the prognosis of patients with BC.

4. Nucleic acid

4.1. Nucleic acid-based prognostic markers

4.1.1. cfDNA

Circulating cfDNA can be found in peripheral blood after being released from normal cells and/or tumor cells. cfDNA derived from tumor cells is known as circulating tumor DNA (ctDNA)^[47]. Researchers employed PCR to examine ctDNA level in 640 patients with different types of cancer, including pancreatic cancer, ovarian cancer, colorectal cancer, BC, stomach cancer, breast cancer, and liver cancer. The investigators found that ctDNA levels in cancer patients were significantly higher than in normal individuals^[48,49], implying that ctDNA can be a potential prognostic marker in cancer diagnosis.

Compared with a tissue biopsy, the detection of cfDNA can easily reflect the genetic characteristics and heterogeneity of BC. Xu *et al.* calculated the ratios of *IQGAP3/BMP4* and *IQGAP3/FAM107A* in urine cfDNA of 103 NMIBC patients using RT-PCR, and compared the results with clinical data using Kaplan–Meier curve and Cox regression analysis to determine *IQGAP3/BMP4* and *IQGAP3/FAM107A* ratio in urine cfDNA and the prognosis of BC^[14]. Kaplan–Meier analysis showed that NMIBC patients with high *IQGAP3/BMP4* and *IQGAP3/FAM107A* ratios had poor progression-free survival. Multivariate Cox regression analysis showed that *IQGAP3/BMP4* ratio was associated with RFS (HR: 2.462) and progression-free survival (HR: 3.871). In a study comprising 68 patients with locally advanced BC, Christensen *et al.* identified patient-specific somatic mutations by whole-exome sequencing^[50]. The ctDNAs derived from 656 plasma DNA samples of the 68 patients before and after cystectomy or chemotherapy

were identified by ultra-deep sequencing. The presence of ctDNA correlated with decreased RFS and OS time of BC patients. Moreover, the overall recurrence rate of ctDNA-positive patients was 76% (13 of 17 patients). However, ctDNA-negative patients did not experience recurrence. These results showed that ctDNA is a feasible prognostic marker for relapse detection. For ctDNA-positive patients, changes in ctDNA during chemotherapy were associated with disease recurrence.

4.1.2. Genetic prognostic markers

Liu *et al.* used the cancer genome map to study the effect of *YWHAZ* amplification on *TP53* mutation^[15]. The analysis of 127 cases in the BC dataset from the Cancer Genome Atlas showed that patients with *TP53* mutation amplified by *YWHAZ* and patients with only *TP53* mutation showed significantly longer OS and disease-free survival. Kuang *et al.* determined the prognostic value of *BRCA2* in BC using data of BC patients from the Cancer Genome Atlas database^[51]. The results showed that the prognosis of the *BRCA2* mutant group was better than that of the non-mutant group, and the *P53* signaling pathway was highly enriched in *BRCA2*.

4.2. Detection methods for nucleic acid-based prognostic markers

4.2.1. RT-PCR

Brisuda *et al.* measured the volume of urine in 66 patients with BC and 34 controls and measured the concentration of urine cfDNA by RT-PCR^[16]. The results show that the determination of the total amount of urine cfDNA can distinguish the BC patients from controls with an area under the receiver-operating characteristic (ROC) curve of 0.725, and the positive and negative predictive values of the test are 90 and 45%, respectively.

4.2.2. FISH

Matsuyama *et al.* used FISH to test whether the copy number alterations of chromosomes 3, 7, 9p21, and 17 could predict the outcome of 118 patients with NMIBC^[52]. The results of multivariate analysis showed that the copy number alterations of chromosomes 3, 7, 9p21, and 17 were a prognostic factor for disease progression and the percentage of 9p21 loss (>12%) was an independent prognostic factor for recurrence. Thus, FISH analysis using UroVysion is a sensitive and non-invasive method to detect genetic alteration, which could be a powerful tool for determining prognosis of BC, particularly for the progression of NMIBC.

4.2.3. Next-generation sequencing (NGS)

Christensen *et al.* tested 65 plasma samples from patients with BC using the targeted NGS method^[17]. The test identified 24 of the 38 mutations originally identified in

multiple plasma samples using digital droplet PCR. cfDNA analysis of plasma samples continuously collected from patients with BC showed that recurrence can be detected earlier than radiography.

cfDNA is present in the peripheral blood of patients and is an easily accessible and repeatable prognostic marker. However, the detection of cfDNA is expensive and not conducive to clinical development. It is advisable to perform analysis on a combination of multiple prognostic genes. Since the prognostic gene combination has not yet been determined, the judgment of the prognosis of patients could be biased.

5. Methylation

5.1. DNA methylation as prognostic marker

Epigenetic modifications involve the heritable alteration in the gene expression without affecting the change in DNA sequences^[53]. One of the notable epigenetic modifications is DNA methylation. This process involves the addition of the methyl group on the DNA sequences through the DNA methyltransferase. DNA methylation prevents rapid cell proliferation and limits its expression capacity^[54]. Moreover, it causes changes in DNA conformation, DNA stability, chromatin structure, and DNA-protein interaction. These activities aim to maintain normal homeostasis in the human body and wide variety of biological activities^[53].

DNA hypermethylation is common in a variety of malignant tumors, including BC^[55]. Intriguingly, hypermethylation occurs frequently at the promoter regions. Consequently, this prevents the attachment of transcription factors in the promoter region and silences the expression of target genes. Normally, DNA hypermethylation in cancer results in the elevation of oncogene expressions and the repression of tumor suppressor genes^[53].

Meta-analysis showed that the methylation level of RAS-related domain family 1A (*RASSF1A*) promoter region is closely related to the prognosis of BC patients^[56]. Furthermore, methylation-specific PCR analysis was conducted to examine *RASSF1A* methylation in the tissue samples collected from 301 patients. With the overall detection rate of 33.6%, the methylation rate in muscle-invasive BC (46.1%) was significantly higher than that in NMIBC (25.8%), implying that *RASSF1A* methylation plays a role in NMIBC recurrence. In addition, *RASSF1A* methylation was positively correlated with the stage and grade of NMIBC. Apart from that, Kaplan–Meier survival analysis revealed that *RASSF1A* methylation was associated with shorter time to tumor progression in recurrent NMIBC, further supporting that *RASSF1A* methylation can be used as an independent risk factor to predict tumor progression^[57]. Therefore, *RASSF1A* methylation is generally considered a potential prognostic marker for recurrent NMIBC.

Wang *et al.* tested the methylation of 118 patients with urinary diseases and 30 volunteers (*EOMES*, *GDF15*, *NID2*, *PCDH17*, *POU4F2*, *TCF21*, and *ZNF154*), and

determined a feasible BC prognostic factor combination through the logistic regression model calculation^[58]. The results showed that the sensitivity and specificity of the *POU4F2/PCDH17* combination were 90.00% and 93.96%, respectively. Renard *et al.* performed methylation-specific PCR on DNA extracted from non-BC tissues and BC tissues to evaluate gene methylation^[59]. The results showed that *TWIST1* and *NID2* from the urine samples collected of BC patients were frequently methylated. The sensitivity of this two-gene panel (90%) is significantly higher than that of cytology (48%). The positive predictive value and negative predictive value of the two-gene panel were 86% and 95%, respectively.

5.2. DNA methylation detection method

5.2.1. Methylation-specific PCR

Wang *et al.* evaluated the relationship between BC and gene methylation by performing methylation-specific PCR on 112 patients with BC and 10 controls^[18]. The results showed that there were significant differences in the methylation status of *p14ARF*, *RUNX3*, *RARβ*, *DAPK*, and *HPPI1* in patients with BC and the control group. In addition, the area under the ROC curve (area under curve [AUC]) of the five genes was 0.936, and the sensitivity and specificity were 98.21% and 88.89%, respectively. Bayramov *et al.* performed methylation-specific PCR on samples from 65 patients with urothelial cancer and 35 controls to determine the methylation profiles of *CDH1* and *p14ARF* in tumor and urine samples and found that 95.4% and 78.5% of BC samples harbored the methylation of *CDH1* and *p14ARF*, respectively^[60]. The methylation frequencies of *CDH1* and *p14ARF* in urine samples were 68.8% and 72.9%, respectively. In addition, the sensitivity of methylation-specific PCR to detect the methylation status of *CDH1* and *p14ARF* (67.4% and 72.1%) was significantly higher than that of urine cytology (34.9%).

5.2.2. Pyrosequencing

Van der Heijden *et al.* analyzed the methylation status of *CDH13*, *CFTR*, *NID2*, *SALL3*, *TMEFF2*, *TWIST1*, and *VIM2* using pyrosequencing and found that the AUC of the trigene methylation classifier containing *CFTR*, *SALL3*, and *TWIST1* was 0.874^[61]. In the validation series, the AUC of the classifier was higher than cytology (0.741 vs. 0.696). Combining the methylation classifier with the cytology results, AUC of 0.86 was obtained in the validation set with a sensitivity of 96% and positive and negative predictive values were 56 and 92%, respectively.

6. Conclusions

This review focuses on the introduction of prognostic markers of BC and their detection methods. The detection of BC prognostic markers enables early diagnosis of BC and provides patients with effective treatment options^[62].

Although many prognostic markers of BC have been reported, many of them still could not be used for clinical prognosis independently. Therefore, the prognostic markers with the greatest value will provide the necessary information for prognosis and clinical management of BC in the future.

Acknowledgments

This work was supported by the Fundamental Research Funds for the Central Universities (No. buctrc201910), Beijing-Tianjin-Hebei Basic Research Cooperation Special Project (19JCZDJC65800(Z)) and the grant from the Ministry of Science and Technology of China (2010ZX09401-403).

Conflicts of interest

The authors declare 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. F.Z., M.S. revised the paper.

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