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Urine-Based Liquid Biopsy for Detecting Recurrence of Bladder Cancer

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Abstract

Bladder cancer represents a widespread malignant neoplasm globally, characterized by a notable rate of recurrence following the initial diagnosis and treatment. Traditional methods for identifying and monitoring the recurrence of bladder cancer include cystoscopy and cytological assessments. Nonetheless, the invasive nature of cystoscopy and the restricted sensitivity of urinary cytology in identifying low-grade tumors pose significant challenges. Presently, there is a deficiency of reliable biomarkers for the early detection and prognostic evaluation of bladder cancer. Urine serves as a rich reservoir of tumor-associated proteins, DNA, and RNA, which holds considerable clinical significance for detection purposes. However, the sensitivity and specificity of various biomarkers necessitate further validation. With advancements in detection methodologies and precision medicine, urinary biomarkers are anticipated to assume an increasingly crucial role in the clinical diagnosis and monitoring of bladder cancer recurrence.

Keywords

Bladder Cancer, Recurrence, Urine, Biomarkers

1. Introduction

Bladder cancer (BLCA) is a commonly encountered malignant tumor in clinical settings, ranking as the eighth most prevalent malignancy in China, with non-muscle invasive bladder cancer (NMIBC) being the predominant subtype [1]. However, approximately 15% of patients progress to muscle-invasive bladder cancer (MIBC), which is characterized by rapid progression, high metastasis rate, and high mortality, with a five-year survival rate of less than 50%. For patients with early-stage disease, surgical excision of the neoplastic tissue is a standard clinical intervention. However, due to the intricate and variable biological behavior ex-

hibited by bladder cancer, the likelihood of postoperative recurrence remains elevated [2]. Recent investigations into the risk factors associated with bladder cancer recurrence have unveiled considerable heterogeneity, and the efficacy of postoperative recurrence management remains constrained [3]. Currently recognized factors linked to bladder cancer recurrence and progression encompass tumor grade and stage. However, these parameters alone are insufficient to comprehensively predict recurrence and progression tendencies. Given the elevated recurrence rates associated with bladder cancer, surveillance cystoscopy is advised based on the initial tumor grade and stage. In severe instances, cystoscopy should be avoided within a three-month period [4]. Urine cytology serves as a non-invasive technique for detecting and monitoring urothelial bladder cancer within the urinary tract. While cytological evaluations demonstrate high specificity, their limited sensitivity constrains their utility in identifying common low-grade urothelial bladder cancer [4]. Recently, numerous urine biomarkers indicative of postoperative recurrence and progression of bladder cancer have been identified. This review aims to summarize these urinary biomarkers.

2. Urinary Protein-Based Assays

2.1. NMP22

Urinary nuclear matrix protein 22 (NMP22) is a nuclear mitotic protein released from apoptotic cells, playing a role in DNA recombination and replication [5]. The NMP22 test presents advantages in terms of sample collection and operational simplicity, with the potential to fundamentally transform approaches to bladder cancer diagnosis and monitoring [6]. The only two protein-based urinary assays that have received FDA approval and CE marking are NMP22, which is recommended in conjunction with cystoscopy for surveillance purposes, but not for initial diagnosis due to the risk of false positive results stemming from hematuria, urolithiasis, infectious diseases, and endoscopic interventions [7]. NMP22 is a nuclear matrix protein that is highly expressed in malignant urothelial cells and is released into urine by necrotic cells [8]. Both quantitative ELISA assays and qualitative tests are available for targeting NMP22. A systematic review assessing the clinical utility of urinary biomarkers indicated that NMP22 assays exhibited a pooled sensitivity of 69% (CI 50 - 85%) and a pooled specificity of 81% (46 - 93%) [9]. More recent studies have highlighted considerable variability among findings, with instances of notably low sensitivity values reported [10]. Research has demonstrated a sensitivity of 79% and specificity of 64% [11] for NMP22 testing in BLCA. BLCA diagnosis has been explored through various studies, revealing a sensitivity range of 11% to 85% and specificity between 69.6% and 100% [12] for NMP22 testing in detecting BLCA recurrence. In the present investigation, the sensitivity of NMP22 testing as a standalone method for identifying BLCA recurrence was determined to be 60.78%, with a specificity of 78.18%. It is clear that the sensitivity and specificity of NMP22 testing can fluctuate considerably across different research efforts, which can be attributed to variations in study populations,

designs, and numerous factors that influence NMP22 testing outcomes [13]. Some studies have indicated that the integration of NMP22 testing with exfoliated urothelial cells significantly enhances diagnostic sensitivity without a notable reduction in specificity. In high-grade bladder cancer (e.g., Grade 3), the NMP22 test (such as the BladderChek NMP22) demonstrates a high positive rate. One study reported that in patients with high-grade tumors, the BladderChek NMP22 achieved a positive rate of 68.4%, which was comparable to the NMP22 ELISA and urinary cytology (68.4% and 63.2%, respectively), indicating no significant differences among these methods in high-grade cancer detection [14]. Additionally, the study noted that the NMP22 test is relatively unaffected by contamination with red or white blood cells, enhancing its clinical utility. In contrast, the performance of the NMP22 test is more pronounced in low-grade bladder cancer (e.g., Grade 1). The same study found that the BladderChek NMP22 yielded a positive rate of 58.3% in low-grade tumors, significantly higher than that of the NMP22 ELISA (33.3%) and urinary cytology (8.3%) [14]. This suggests that the NMP22 test exhibits higher sensitivity in low-grade cancers, potentially addressing the limitations of traditional methods like cytology in detecting such tumors. Overall, in a cohort of 51 patients with urothelial carcinoma, the NMP22 test showed an overall sensitivity of 56.8%, further supporting its advantage in low-grade disease [14]. Other investigations have compared NMP22 with novel biomarkers; for instance, in low-grade non-muscle-invasive bladder cancer, the urine laminin-γ2 monomer demonstrated a higher area under the curve (AUC) than NMP22, indicating that NMP22 may underperform relative to certain emerging markers but still surpasses conventional approaches such as BTA or cytology [15]. In summary, the NMP22 test serves as a rapid and convenient screening tool, exhibiting favorable sensitivity in the graded diagnosis of bladder cancer, particularly for low-grade tumors, and can be utilized as an adjunct in clinical practice. Although NMP22 is also present in normal urothelial cells, which can lead to false-positive results and affect diagnostic specificity, the combined detection of exfoliated urothelial cells and NMP22 cannot replace cystoscopy in the postoperative follow-up of bladder cancer, but it can significantly improve the detection rate of positive cases. Consequently, this combined testing method can offer a more thorough and precise evaluation for bladder cancer patients prior to cystoscopy, thus holding substantial clinical relevance.

2.2. Urine Polypeptides

Schiffer *et al*. developed a model featuring four urine polypeptides capable of differentiating NMIBC from MIBC and offering timely indications of BLCA progression [16]. Additionally, a broad array of cytokines has been recognized as valuable targets for monitoring recurrence in NMIBC patients treated with Bacillus Calmette-Guerin (BCG). Research by Cai *et al*. established that the IL-6/IL-10 ratio serves as an independent predictor of recurrence [17]. Furthermore, baseline urine IL-8 concentrations were significantly associated with tumor recurrence fol-

lowing BCG treatment, indicating that higher urine IL-8 levels in NMIBC patients correlate with shorter tumor recurrence intervals [18]. Kamat *et al*. expanded on this by employing nine cytokines (IL-2, IL-8, IL-6, IL-1ra, IL-10, IL-12, TRAIL, and TNF-α) to formulate a model for tracking tumor recurrence in NMIBC patients undergoing BCG treatment [19], achieving an accuracy of 85.5%.However, these models are still in the research phase.

2.3. ALCAM

The activated leukocyte cell adhesion molecule (ALCAM), a cell adhesion molecule involved in tumor cell migration, has been shown to predict overall survival following cystectomy in bladder cancer patients [20]. Snell *et al*. demonstrated that urine HAI-1 and EpCAM could forecast prognoses in NMIBC patients and potentially inform treatment strategies for those at high risk for NMIBC [21]. Moreover, overexpression of Snail has been identified as an indicator of NMIBC recurrence [22]. Azevedo *et al*. utilized a dextran affinity glycoproteomic nanoplatform to pinpoint specific glycoproteins in the urine of patients with low-grade (LG) and high-grade (HG) NMIBC, finding that urine CD44 concentrations were significantly elevated in HG MIBC patients and correlated with unfavorable prognoses [23]. Research into serum or plasma proteins is less extensive compared to urine proteins, with most prognostically relevant proteins being cytokines [24]-[28]. For instance, the expression levels of soluble E-cadherin, MMP2, MMP7, endothelial inhibitors, TGF-β, and uPA have been associated with advanced pathological stages or poor prognoses. Elevated baseline levels of circulating IL-8 have been linked to adverse outcomes in patients receiving sunitinib or pazopanib [29] [30]. Comprehensive analyses of multiple cytokines may yield cytokine profiles that contribute to the prognostic evaluation of patients. Kumari *et al*. found that elevated levels of cytokines such as IL-1RA, IL-10, IL-4, IL-6, IP-10, TNF-α, PDGF, and VEGF correlated with poorer recurrence-free survival in bladder cancer patients [31]. The prognostic potential of markers such as EGFR, EpCAM, BTA, MMP2, tenascin-C, and cystatin B has also been examined [32]; however, none of these markers have undergone independent validation. Additionally, while urine EGFR may relate to bladder cancer prognoses, its concentrations are typically too low for practical measurement via ELISA, presenting a significant barrier to its clinical application and thus keeping it still in the clinical research stage.

3. Soluble Fas

Soluble Fas, a member of the tumor necrosis factor receptor family, plays a critical role in maintaining immune homeostasis and facilitating immune surveillance by activating inhibitory apoptotic signaling pathways. This mechanism consequently contributes to inflammation and the progression of cancer [33]. Research indicates that urinary levels of soluble Fas are significantly elevated in various malignancies, including bladder urothelial carcinoma and cervical cancer. Notably, patients with early-stage and low-grade tumors exhibit higher concentrations of sol-

uble Fas compared to those with advanced-stage and high-grade tumors [34]. Traditional enzyme-linked immunosorbent assay (ELISA) analyses suggest that urinary soluble Fas levels can serve as predictive biomarkers for bladder cancer recurrence and its advancement to more invasive stages. Although no statistically significant overall differences have been observed between urinary soluble Fas and NMP22 testing in bladder cancer patients, soluble Fas demonstrates superior specificity compared to NMP22 when sensitivity exceeds 75% [35]. Given the relatively lower specificity of urinary soluble Fas, there is a pressing need for the standardization of sample collection protocols and analytical methods to mitigate their potential impact on clinical diagnostics. Currently, diagnostic kits or methods based on Soluble Fas for bladder cancer detection have not received FDA approval or CE certification for routine clinical diagnosis. They remain primarily in the research phase.

4. BLCA-1

Initially identified by Getzenberg *et al.* in 1996 [36], BLCA-1 has become foundational for the early diagnosis of bladder cancer and the postoperative monitoring of recurrence. The assessment of urinary BLCA-1 levels provides a straightforward and convenient diagnostic approach for bladder cancer. Following cellular death, nuclear matrix proteins are released into the urine in a soluble form, resulting in detectable levels of BLCA-1. As tumor cells on the bladder wall undergo necrosis, a portion of BLCA-1 is released into the urine, leading to increased urinary BLCA-1 concentrations. Existing literature supports the notion that monitoring BLCA-1 levels is beneficial for evaluating bladder cancer recurrence [37]. The recurrence of bladder cancer is predominantly attributed to incomplete clearance of tumor factors or the distant metastasis of tumor cells. During recurrence, the rapid proliferation of tumor cells disrupts the original tissue architecture and results in compromised blood supply. To satisfy their metabolic demands for blood and oxygen, proliferating tumor cells secrete vascular endothelial growth factor, which accelerates angiogenesis and subsequently elevates BLCA-1 levels. Patients with bladder cancer typically exhibit heightened urinary BLCA-1 expression, and those experiencing postoperative recurrence demonstrate further increases in BLCA-1 levels. This heightened expression of BLCA-1 presents significant promise for achieving high diagnostic accuracy in bladder cancer detection and establishes it as a crucial marker for monitoring postoperative recurrence.

5. DNA Signatures

The ongoing advancement and refinement of liquid biopsy techniques have significantly broadened their applicability in tumor diagnosis and treatment. Prior studies have validated the considerable potential of circulating free DNA in various bodily fluids for tumor diagnosis [38]-[40]. The presence of circulating tumor DNA (ctDNA) in plasma has been linked to tumor recurrence and unfavorable prognoses [41] [42]. Research conducted by Bikenkamp-Demtroder and Chris-

tensen *et al*. [43] has shown that ctDNA can be detected in liquid biopsies from bladder cancer patients, with elevated ctDNA levels correlating with postoperative disease progression. As such, routine monitoring of ctDNA levels following surgery is clinically relevant for predicting tumor recurrence or progression in bladder cancer patients. Urine, as a non-invasive sample source, presents a viable option for detecting ctDNA levels [44] [45]. Testing for ctDNA in urine has the potential to emerge as an innovative approach for monitoring tumor recurrence or progression in postoperative bladder cancer patients. A positive detection of ctDNA in urine, particularly in instances where imaging and cystoscopy yield negative results, underscores the importance of this method in clinical practice. Abnormalities in ctDNA levels raise significant concerns regarding the potential recurrence or progression of tumors. Research conducted by Reinert *et al*. [42] has established that alterations in serum ctDNA can be identified prior to the clinical diagnosis of recurrence through imaging techniques. In a prospective study, Birkenkamp-Demtroder *et al*. [46] demonstrated that patients with bladder cancer who underwent radical cystectomy could be monitored for ctDNA presence before imaging confirmed tumor recurrence, achieving an average lead time of 101 days. Furthermore, a retrospective study by Birkenkamp-Demtroder *et al*. [43] indicated that increased ctDNA levels could be detected in the urine of patients with NMIBC several months prior to clinical evidence of tumor recurrence or progression. The clinical implementation of ctDNA testing in bladder cancer management faces several significant practical barriers. Firstly, the low abundance of ctDNA, particularly in early-stage or minimal residual disease settings, poses a considerable challenge for detection sensitivity [47]. This is further complicated by the heterogeneity of bladder tumors, which may lead to incomplete representation of the tumor's genomic landscape in circulating DNA [47]. Additionally, the lack of standardized methodologies for ctDNA extraction, analysis, and interpretation across different platforms remains a critical hurdle, potentially affecting the reproducibility and reliability of results [47]-[49]. The tumor-agnostic approaches, while promising, may miss specific mutations unique to individual patients, limiting their comprehensive application [50]. Furthermore, the integration of ctDNA monitoring into clinical decision-making requires validation through large-scale, prospective randomized controlled trials to establish its definitive utility in guiding perioperative therapies, an area where current evidence is still evolving [51]. Cost-effectiveness and accessibility of these advanced molecular assays also present practical constraints for widespread adoption in diverse healthcare settings [52]. This finding emphasizes the high specificity of urinary ctDNA monitoring in forecasting bladder tumor recurrence or progression in patients post-surgery but still require validation through large-scale clinical trials.

6. RNA Signatures

6.1. Cxbladder Detect and Monitor

The Cxbladder assay is a reverse transcription quantitative PCR test designed to

detect and quantify urinary mRNA levels of CDK1, MDK, HOXA13, IGFBP5, and CXCR2. There are two versions of this assay: Cxbladder Detect and Cxbladder Monitor. The former exhibits high sensitivity and specificity (82% and 85%, respectively) for detecting bladder cancer in individuals presenting with hematuria [53]. The Cxbladder Monitor version demonstrates significant sensitivity and negative predictive value (NPV) for identifying patients at low risk of recurrence who might not require repeated cystoscopy [54] [55]. Koya *et al*. illustrated that the inclusion of Cxbladder Monitor in clinical guidelines enabled the safe selection of patients who could be monitored through cystoscopy every two years [56]. Studies have indicated that Cxbladder, as a urinary biomarker, demonstrates a sensitivity ranging from approximately 0.57 to 0.82 and a specificity ranging from approximately 0.74 to 0.88 in the overall diagnosis of bladder cancer, though the number of related studies remains limited [57]. With respect to tumor grading, literature also suggests that sensitivity increases with higher tumor grade, implying that Cxbladder exhibits lower sensitivity and reduced accuracy in low-grade tumors, whereas its sensitivity is notably higher in high-grade tumors. Overall, the diagnostic performance of Cxbladder is inferior in low-grade tumors compared to high-grade tumors; however, these findings are constrained by methodological issues and statistical heterogeneity across studies.By reducing the frequency of cystoscopic examinations, this test has the potential to alleviate the economic burden associated with bladder cancer surveillance and enhance patient adherence to follow-up protocols.

6.2. Xpert Bladder Cancer Detect and Monitor

The Xpert® Bladder Cancer test is a urinary assay that evaluates the expression levels of five mRNA markers (ABL1, ANXA10, CRH, IGF2, and UPK1B) that may be overexpressed in bladder cancer, utilizing RT-PCR technology [58]. The test is conducted using the Gene Xpert System (Cepheid), which automates the processes of nucleic acid amplification and detection of mRNA targets, providing a rapid and straightforward assay [58]. Two types of assays have been developed: the first for initial diagnosis, and the second for ongoing surveillance [59]. The Xpert Detection assay has demonstrated an NPV of 99% (CI 98 - 100%) for high-grade tumors [59]. Numerous studies have indicated that active surveillance represents a safe and cost-effective alternative therapeutic strategy for patients with recurrent NMIBC [60]. D'Elia *et al*. conducted a prospective study involving 230 patients with 52 recurrences, revealing that Xpert BC Monitor achieved a sensitivity of 85.7% for high-grade tumors [61] [62]. Additionally, Pichler *et al*. noted a high sensitivity of 77% for low-grade disease [63]. In a recently published study, the Xpert Bladder Monitor exhibited an overall sensitivity of 74% and specificity of 80%, with sensitivities of 83% for high-grade and 63% for low-grade cancers [64]. Notably, Hurle *et al*. reported that the Xpert Bladder Monitor could prevent unnecessary cystoscopies without overlooking high-grade cancers, based on a cohort of 106 patients undergoing active surveillance when a cutoff value of <0.4 was

employed [65].

6.3. Exosomes lncRNA PCAT-1

Exosomes are bilayered vesicles found in various bodily fluids, including blood, urine, and saliva, and their formation is intricately linked to cellular processes [66]. Cells, as the fundamental units of the human body, secrete a variety of cellular factors, growth factors, and extracellular matrix proteins during their growth, thereby creating a conducive environment for tumor cell proliferation [67]. Research has demonstrated that exosomes, as a form of extracellular vesicle, can penetrate tumor cells and facilitate the transport of proteins and information between them, positioning exosomes as significant tumor biomarkers [68]. Recent studies on the content of urinary exosomes in NMIBC have revealed that the biomacromolecules encapsulated in urinary exosomes, particularly lncRNA, play a significant role in the prognostic evaluation of NMIBC [69]. LncRNAs constitute a category of RNA molecules that do not code for proteins, yet they are integral to various cellular processes such as proliferation, differentiation, and tumor progression [70]. Research conducted by Zhang *et al*. [71] highlighted the essential function of lncRNAs derived from extracellular vesicles in differentiating tumor cells from normal cells, thereby reflecting the pathological alterations and progression of the disease within tumor cells. One notable lncRNA, PCAT-1, initially identified as a transcription factor in prostate cancer, has been found by Guo *et al*. [72] to exhibit significantly increased expression levels in NMIBC, with its expression correlating closely with cellular proliferation and apoptosis. Furthermore, urinary extracellular vesicle lncRNA PCAT-1 serves as a critical biomarker for predicting recurrence in patients with NMIBC. However, as of now, research on its use as a diagnostic marker remains very limited, and it is still in the research phase.

7. Improved cytology

7.1. FISH

In the realm of enhanced cytology, fluorescence in situ hybridization (FISH) employs the principle of complementary base pairing, facilitating the specific attachment of labeled DNA probes to exfoliated urinary cells. This method enables the detection of bladder cancer through immunofluorescence staining. A study by Dimashkieh *et al*. [73] monitored patients diagnosed with bladder cancer, revealing a postoperative recurrence rate of 45.51%. The utilization of FISH for detecting bladder cancer recurrence yielded a positive detection rate of 57.75%. An examination of the medical histories of recurrent patients during follow-up indicated that FISH exhibited heightened sensitivity in identifying recurrence among patients presenting with hematuria compared to those with a prior history of urinary tract tumors, with sensitivity rates of 60% and 62.2%, respectively. The efficacy of FISH detection is contingent upon the preparation of cell smears, with results dependent on the number of detectable cells, which complicates the identification of

tumor recurrence. Additionally, the reliance on specialized equipment such as fluorescence microscopes and the need for proficient laboratory personnel limit the widespread application of this technique. FISH is a universal molecular cytogenetic technique. As a methodology, it is not a direct subject of certification by regulatory agencies.

7.2. Urovysion

Urovysion, a multitarget FISH assay, is performed on exfoliated cells obtained from voided urine, yielding a dichotomous response based on specific criteria, including chromosomal alterations (3, 7, 9, and 17) and morphological changes in cells [74]. Recent studies have reported a sensitivity range of 67% - 69% and specificity of 72% - 76%, although variability has been noted in other investigations, particularly in cases involving atypical urothelial cells, where sensitivity and specificity were recorded at 44% - 48% and 78% - 81%, respectively [75] [76]. Given the unique attributes of Urovysion, it has been employed in various clinical applications. In a follow-up context for assessing the recurrence risk of bladder cancer, Urovysion demonstrated effective performance, reporting recurrence rates of 16.5% (one positive test) and 33.3% (two positive tests) following transurethral resection of bladder tumor (TURBT) [77]. Moreover, Urovysion has proven beneficial in clarifying atypical urothelial cells identified in urinary cytology, detecting high-grade bladder cancer in 17.9% of cases [78]. Despite these promising findings, Urovysion exhibits lower sensitivity for low-grade tumors compared to Immunocyt [79]. Lastly, encouraging results have been observed in the application of Urovysion for detecting upper urinary tract urothelial cancer [57] [80]. UroVysion is a multi-target FISH-based assay employed for the diagnosis and surveillance of bladder cancer, which detects genetic alterations by identifying abnormalities in chromosomes 3, 7, and 17, as well as deletions at the 9p21 locus in exfoliated urinary cells [81]. Although widely utilized in clinical practice, its performance varies between low-grade and high-grade bladder tumors. In low-grade tumors, UroVysion exhibits relatively low sensitivity. For instance, one study reported a sensitivity of only 8% for UroVysion, compared to 62% for ImmunoCyt, suggesting inferior detection capability potentially due to fewer or atypical genetic changes in these tumors [79]. However, UroVysion maintains high specificity, approximately 90%, which is comparable to urine cytology [79]. Systematic reviews further indicate that while UroVysion's sensitivity in low-grade tumors shows improvement over urine cytology, the exact values differ across studies [82]. For high-grade tumors, UroVysion's sensitivity increases but remains limited, with reported values around 18% in contrast to 91% for ImmunoCyt [79]. Despite this, UroVysion demonstrates utility in predicting recurrence, as positive results in follow-up studies can anticipate relapse, particularly in high-grade cases [83]. Comparative analyses note an overall sensitivity of 67% - 69% and specificity of 72% - 76% for UroVysion, similar to urine cytology but without significant superiority [75]. Limitations in sensitivity may partly arise from in-

stances where high-grade tumors display tetraploid chromosome counts yet fail to meet UroVysion's positive criteria [75]. In summary, UroVysion offers high specificity and potential value in predicting recurrence for high-grade bladder cancer, but its sensitivity is suboptimal in both low-grade and high-grade tumors, showing no clear advantage over urine cytology [79] [82]. Future investigations should focus on optimizing this method to enhance its efficacy, particularly in low-grade neoplasms. The reliance of UroVysion fluorescence in situ hybridization technology on specialized equipment presents a significant practical barrier in clinical detection for bladder cancer. This is primarily reflected in its requirement for commercial UroVysion kits to perform fluorescence in situ hybridization analysis, which necessitates high-resolution fluorescence microscopes and specific DNA probe systems to detect chromosomal aneuploidies [83]. Such equipment-intensive characteristics may lead to substantial initial investment and maintenance costs, limiting its adoption in resource-constrained medical institutions. Additionally, the procedure demands strict adherence to manufacturer protocols and relies on well-trained technical personnel, thereby increasing operational complexity and the risk of variability [84]. Although this technique has demonstrated diagnostic potential in specific studies, its strong dependency on specialized equipment may hinder broader clinical application, particularly in primary care or remote settings. Cost-effectiveness analyses should be integrated to optimize implementation strategies. Unlike many urine biomarkers that are still in the research phase, Urovysion is a mature, commercially available diagnostic product that has been approved by regulatory agencies and is used globally in clinical practice.

7.3. Uromark

The Uromark assay represents a non-invasive urine test that analyzes a panel of 150 epigenetic alterations utilizing next-generation DNA sequencing (NGS) techniques and RainDrop bisulfite sequencing (BS-Seq), which involves microdroplet-based polymerase chain reaction (PCR) amplification. The utilization of bisulfite-converted DNA has shown significant advantages over traditional PCR techniques, particularly in the context of next-generation sequencing (NGS), which allows for the extraction and analysis of DNA from nearly all urine samples due to its minimal input requirements [85]. This assay has been developed and validated across various cohorts, encompassing a total of 274 patients, yielding impressive sensitivity and specificity rates of 95% and 96%, respectively [86]. Presently, two multicenter studies are underway to assess the efficacy of the Uromark assay in both initial diagnoses and recurrent bladder cancer (BC) [87] [88]. Uromark, as a promising detection method, has seen its related research published in academic journals, demonstrating its potential value in the diagnosis and monitoring of bladder cancer. However, transforming it into a mature diagnostic product for routine clinical use still requires the completion of large-scale clinical trials and a complex regulatory approval process.

7.4. ImmunoCyt

In 1997, Fradet *et al*. [89] pioneered the application of ImmunoCyt detection for the diagnosis of bladder cancer, which received approval from the U.S. Food and Drug Administration (FDA) for prognostic evaluation in 2000. This immunocytochemical detection technique employs three monoclonal antibodies to identify exfoliated urothelial cells present in urine. A meta-analysis conducted by He *et al*. [90] systematically reviewed data from seven studies involving 1602 subjects, focusing on the diagnostic accuracy of ImmunoCyt detection for prostate cancer. The findings revealed a sensitivity of 0.725 (95% confidence interval [CI] 0.683 to 0.765) and a specificity of 0.657 (95% CI 0.629 to 0.685). Although immunocytochemical detection exhibits high sensitivity, its relatively low specificity indicates its potential role as a supplementary tool for monitoring bladder cancer recurrence. When combined with other diagnostic methods, it may enhance the overall sensitivity and specificity of postoperative surveillance for bladder cancer. Future advancements could involve the identification of novel biomarkers to optimize ImmunoCyt detection.

8. Limitations

As previously discussed, although liquid biopsy has been extensively studied in the diagnosis and monitoring of bladder cancer, its clinical application still faces several limitations.

First, achieving uniform homogenization in the processing of liquid samples across different laboratories remains challenging, leading to variability in biomarker performance validation. Additionally, technological disparities among regions or laboratories further hinder the widespread clinical implementation of liquid biopsy.

Second, most prior studies have primarily used healthy individuals as controls, overlooking potential interference from non-malignant diseases. This may result in reduced specificity of biomarkers. Therefore, large-scale, prospective, multicenter studies with ample sample sizes are essential to validate the clinical utility of liquid biopsy and minimize biases inherent in case-control designs.

Different liquid biopsy biomarkers also present their own limitations. For instance, shed cells are scarce in blood and urine, increasing the risk of false negatives. Their clinical application depends on advancements in capture and identification techniques. Circulating tumor DNA (ctDNA) has a short half-life in bodily fluids, complicating the detection of mutations and methylation. Similarly, exosomal RNA is unstable and rapidly degraded, posing challenges for sample storage, transportation, and efficient isolation of bioactive components.

Moreover, various exosome isolation methods significantly impact the quantity and composition of exosomes and their cargo, underscoring the need for standardization in isolation protocols. Additionally, the high cost of exosome analysis necessitates improvements in current analytical approaches to enhance feasibility and reproducibility.

9. Conclusions and Future Perspective

In conclusion, the urinary biomarkers currently employed for monitoring bladder cancer recurrence include NMP22, soluble Fas, ImmunoCyt, BLCA-1,ctDNA, extracellular vesicle long non-coding RNA PCAT-1, and fFISH detection. While cystoscopy and biopsy remain the most reliable diagnostic approaches for detecting BLCA recurrence, their invasive nature often results in low patient acceptance and compliance, rendering them unsuitable for extensive screening in high-risk populations. Consequently, the integration of urine biomarkers as adjunctive tests can significantly improve the detection rates of BLCA recurrence, offering a safe, straightforward, and non-invasive diagnostic alternative. Currently, only a limited number of biomarkers exhibit higher sensitivity, albeit with lower specificity compared to cytology. Moreover, some of these biomarkers present challenges in terms of ease of implementation in clinical practice [91]. The initial biomarker introduced was NMP22 [92] [93]; however, given the diverse molecular alterations associated with bladder tumors, reliance on single biomarkers is suboptimal for effective BC detection. The advent of high-throughput technologies has facilitated the discovery of new biomarkers and the development of multiplex panels, with several research groups investigating the clinical advantages of such assays (e.g., gene methylation and RNA signatures) [94]-[98].

Currently, only a select few biomarkers are utilized in clinical practice for follow-up, with NMP22 and UroVysion being acknowledged in the European Association of Urology (EAU) and American Urological Association (AUA) guidelines, though they are not widely implemented in clinical settings. At this time, no biomarkers are available to predict therapeutic responses. Nevertheless, with the emergence of immunotherapeutic agents targeting programmed cell death protein 1 (PD-1) and programmed cell death ligand-1 (PD-L1), predictive biomarkers could prove particularly beneficial. The bladder's anatomical proximity to urine presents a unique opportunity for liquid biopsy applications, which hold promise for providing biomarkers for detection, prognosis, surveillance, and monitoring clinical outcomes post-treatment. There is an increasing body of evidence underscoring the necessity for clinical validation of newly identified urinary biomarkers, which could fulfill a significant need in patient evaluation. However, various challenges must be addressed, including issues related to low mutant allele fractions.

The stability of tumor DNA in urine, alongside the necessity for innovative technologies and appropriate sample storage and transport, remains a critical consideration. Furthermore, the sequencing and detection of tumor DNA are currently costly procedures, which may only prove cost-effective if they can facilitate alterations in therapeutic strategies or enable the identification of druggable targets. NMIBC is characterized by a high recurrence rate, with cystoscopy still recognized as the gold standard for diagnostic evaluation. Consequently, the clinical management of BLCA incurs significant expenses. Over the past decade, a variety of assays based on biomarker panels have been developed, primarily for patient

follow-up. Although several tests, including Urovysion and NMP22, have received FDA approval, their clinical application is limited due to low negative predictive values (NPV). In contrast, the Xpert Bladder Monitor, BladderEpiCheck, and CxBladder Monitor have demonstrated more favorable results, achieving NPVs exceeding 98%. Bladder tumors exhibit both cellular and molecular heterogeneity, both inter-tumoral and intra-tumoral. Urine serves as a reflection of this tumor heterogeneity, allowing for the detection of mutations that may not be identified through biopsy procedures [99] [100].

The integration of multi-omics data—encompassing genomic, epigenomic, transcriptomic, proteomic, and metabolomic profiles—with advanced machine learning (ML) algorithms holds transformative potential for improving the detection and monitoring of bladder cancer recurrence. By leveraging high-throughput technologies, such as next-generation sequencing and mass spectrometry, researchers can now simultaneously analyze diverse molecular features from urine samples, capturing the complex heterogeneity of bladder tumors more comprehensively than single-marker approaches.

Machine learning models, including deep neural networks and ensemble methods, can be trained on multi-omic datasets to identify subtle patterns associated with recurrence, therapeutic resistance, or aggressive disease phenotypes. These models may enhance predictive accuracy by integrating features such as: Gene mutations (e.g., FGFR3, TP53),Methylation signatures (e.g., TWIST1, SOX1), Non-coding RNA expression (e.g. lncRNA PCAT-1,miRNAs),Protein biomarkers (e.g., NMP22, PD-L1),Metabolic alterations.Such integrated approaches could address key limitations of current biomarkers, including low specificity and an inability to predict treatment responses—particularly for emerging immunotherapies targeting PD-1/PD-L1. For example, ML-driven urine liquid biopsies might stratify patients based on recurrence risk, guide personalized surveillance intervals reducing reliance on cystoscopy, and predict responders to immune checkpoint inhibitors.

Therefore, it is imperative to develop urinary tests that address specific clinical requirements. The clinical validation of these tests must prioritize their potential to influence clinical decision-making processes, particularly regarding enhancements in patient survival and quality of life. Urine biopsy could emerge as a non-invasive testing method with the capacity to significantly improve the diagnostic and therapeutic continuum for BLCA patients. Ideally, a multiplex assay would facilitate the identification of patients who necessitate invasive, costly, and time-intensive procedures such as cystoscopy, while also predicting disease recurrence and guiding optimal treatment options for individual patients. With advancements in technology, urine biomarkers are anticipated to hold promising prospects for future applications.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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