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ORIGINAL ARTICLE

The molecular limitations of biomarker research in bladder cancer

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Abstract

Purpose Urothelial carcinoma of the bladder (UCB) is a common malignancy with limited systemic treatment options in advanced stages. Despite recent advances in immunotherapy, the majority of patients do not respond to these treatments. There is an unmet need for developing robust biomarkers to inform treatment decisions and identify patients who are likely to respond.

Methods A MEDLINE/PubMed literature search was performed, focusing on tissue-based and circulating biomarkers, and their potential in muscle-invasive UCB.

Results UCB is a heterogeneous disease that consists of several clonal and subclonal populations, each with a mix of truncal and private genomic alterations. This inter- and intra-tumoral heterogeneous landscape results in the development of treatment resistance. Tumor heterogeneity also constitutes a barrier to the development of robust markers of response and resistance to chemotherapy and immunotherapy. Defects in DNA repair genes and a high tumor mutational burden independently confer sensitivity to cisplatin-based chemotherapy and checkpoint inhibitors. Oncogenic alterations such as FGFR3 mutations and fusions are associated with response to FGFR3 inhibitors. Several emerging potential biomarkers, including gene expression-based molecular subtypes, T-cell receptor clonality, and tissue- or blood-based immune-gene profiling, require prospective testing and validation. Tissue-based biomarkers such as PD-L1 immunohistochemistry have several limitations due to discordance in assay methodology and trial designs. Novel liquid-biopsy techniques are promising as potential biomarkers.

Conclusions Validated biomarkers that capture the complexity of the biology of both the tumor and the tumor microenvironment are needed in muscle-invasive UCB. Standardization of methods is critical to developing reliable biomarkers to guide clinical management.

Keywords Bladder cancer · Biomarker · Molecular biology · Predictive · Prognostic · Chemotherapy · Immunotherapy · Checkpoint inhibitor

Introduction

Bladder cancer is the fifth most common cancer in USA, with 81,190 estimated new cases in 2018 [1–3]. Muscle-invasive and metastatic urothelial cancer of the bladder (UCB) is potentially lethal [3, 4]. Despite systemic therapies with platinum-based chemotherapy and checkpoint inhibitors, the mortality rate remains high, with 17,240 projected deaths in USA in 2018 [2, 3].

The enormous progress in the development of high-throughput technologies has enabled a deeper understanding of the molecular mechanisms that govern the development and progression of UCB. The expanding use of genomics and transcriptomics in the clinical setting raises the prospect of precision medicine approaches. Effective precision medicine requires the development of accurate biomarkers for assessing patients' response to therapy, toxicity, and drug resistance [5]. The World Health Organization (WHO) defines a biomarker as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease" [6]. A more recent definition by the FDA describes a biomarker as "a defined characteristic that is measured as an indicator of normal biologic processes, or responses to an exposure or intervention, including therapeutic interventions"

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[7]. Biomarkers can be classified according to their clinical utility in several distinct categories, including disease-risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamic, and safety [7]. An ideal biomarker should be specific, sensitive, and robust. The clinical utility of a given biomarker with respect to cancer therapeutics essentially lies in its ability to distinguish the subgroups of patients with the highest or lowest likelihood of response to a particular treatment, the longest or shortest progression-free survival (PFS), and/or overall survival (OS), respectively (Fig. 1). However, due to several obstacles hindering biomarker research and validation, out of thousands of proposed biomarkers in the current literature, only a few are clinically useful [8].

In this review, we focus on the current biomarker research in muscle-invasive UCB in relation to tumor heterogeneity of the tumor and the microenvironment's role in the development of drug resistance and metastasis [9–12]. We review different tumor markers investigated in UCB as clinical outcome predictors in the context of chemotherapy and immunotherapy [13]. We also review the concept of using oncogenic addiction to predict whether specific patients could respond to targeted agents [14]. Finally, we discuss the expression-based molecular classifiers of bladder cancer, including the University of North Carolina (UNC), MD Anderson (MDA), The Cancer Genome Atlas (TCGA), and Lund classifications. We discuss the challenges involved in applying these classifiers to clinical practice [15–18].

Methods

We performed MEDLINE/PubMed literature search with different combinations of the terms "bladder cancer", "biomarker", "molecular biology", "predictive", "prognostic", "chemotherapy", "immunotherapy", and "checkpoint inhibitor". We set no time period restriction. We selected original articles, reviews, and editorials based on their clinical relevance. Cited references from selected articles were analyzed to find and include significant papers missed by our initial search. We did not address urine-based biomarkers in muscle-invasive UCB but focused only on tumor- and blood-based biomarkers.

Results

Tumor and microenvironment heterogeneity as a barrier to muscle-invasive UCB biomarker research

Bladder cancer is not one disease. Even within the same patient, muscle-invasive UCB is composed of multiple clones with heterogeneous biological attributes, evolutionary trajectories, and resistance potential. Several genomic studies of spatially and temporally distinct UCBs have analyzed the somatic mutations, copy-number alterations and transcriptional profiles of these tumors [19]. The absence of significant genetic differences between pairs of the early stage non-invasive and late-stage invasive UCB tumors from the same patients supports a common clonal origin [20–22]. However, the total number of mutations generally increases from superficial tumors to invasive tumors [21, 22]. Progressing clones accumulate mutations in tumor suppressor genes (including TP53, KMT2C, FBXW7, and SETD2) as opposed to mutations in FGFR3 KDM6A, and PIK3CA genes which most commonly characterize non-invasive clones [21]. Beyond this temporal, subclonal heterogeneity, a small degree of spatial intra-tumoral heterogeneity can be observed with multi-regional exome sequencing of a single tumor [22]. A second level of heterogeneity emanates from the "field cancer effect" itself, as revealed by multi-regional sequencing of tumor regions and adjacent normal tissue in patients with advanced UCB [23]. The field cancer effect was initially proposed as "field-first-tumor-later" model, whereby aberrant stem cells spread in the urothelium by cellular displacement, creating fields of pre-malignant cells which then transformed into tumors after the accumulation of critical genetic events in individual cells within these fields [24]. Tumors from the same patient harbor both shared and private mutations in driver genes, and likewise, not all mutations are shared among cells within the normal adjacent urothelium [23]. This led to the suggestion of three different models of field disease development through intra-epithelial migration, luminal seeding and implantation, or accumulation of private mutations in an intermixed pool of healthy stem cells and clonal stem cells that initially emerged from the early shared alterations (loss of 9p or 9q or mutations in

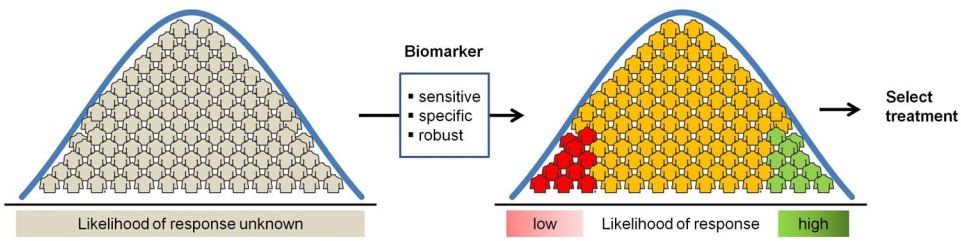

Fig. 1 Normal distribution of UCB patients, whereby an ideal biomarker is able to identify patients with low or high likelihood of response, and guide selection of treatment

TP53) [23]. A third layer of heterogeneity can emerge as an effect of systemic therapy. In our study of matched untreated and chemotherapy-resistant UCB tumors, we found that the majority of mutations between pre- and post-treated tumors were not shared [25]. In addition, chemotherapy-resistant tumors had increased clonality and were enriched in mutations in the integrin-signaling pathway, a known mechanism of drug resistance which promotes the interaction of tumor cells with extracellular matrix proteins [25].

The tumor microenvironment itself is an important source of heterogeneity, as it contains stromal cells, extracellular matrix components, tumor vessels, and infiltrating immune cells [26]. The composition of the immune contexture is variable and includes tumor-infiltrating lymphocytes (TILs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and tumor-associated neutrophils (TANs) [27]. TILs are host lymphocytes (activated T cells, natural killer cells, and non-T or non-B lymphocytes) that migrate to tumor sites to counter cancer cells; however, their abundance and activation is heterogeneous and controlled by tumor-specific immune-escape mechanisms. For instance, regressing and stable metastases are often infiltrated by CD8+ and CD4+ T cells, whereas progressing metastatic lesions are T-cell-depleted [28]. One well-described mechanism of immune evasion and tumor growth in UCB involves the induction of an inflammatory response driven by CD14-expressing cancer cells through IL-6 signaling [29].

In this complex heterogeneous landscape and with continuous tumor evolution, genomic alterations are not constant. Consequently, the identification of "stable" genomic (e.g., driver mutations and copy-number alterations) or transcriptomic (e.g., outliers in gene expression and molecular subtypes) biomarkers extremely difficult. Collectively, the different layers of molecular heterogeneity could result in discordance in the results of molecular testing across sampled lesions or even within a given tumor. Molecular changes accumulating over time can limit the reproducibility and the clinical utility of several biomarkers across different muscle-invasive UCB patient cohorts. Overall, there is a paucity of validated, robust biomarkers that could be used in the clinical setting.

Biomarkers for chemotherapy response and resistance

Cisplatin-based chemotherapy with accelerated MVAC or gemcitabine-cisplatin (GC) remains the standard of care for fit patients in the neoadjuvant and first-line treatment of advanced UCB [30, 31]. Although the mechanistic spectrum of cisplatin's antitumor activity is not fully elucidated, a major cytotoxic mechanism involves the induction of intrastrand and interstrand DNA adducts. Specific DNA damage response (DDR) pathways are responsible for repairing cisplatin-induced DNA damage [32].

Defects in various DDR pathways were tested as potential biomarkers of the improved outcomes in UCB patients that received chemotherapy. Low ERCC1 mRNA expression level was described as an independent predictor of improved overall survival (25.4 versus 15.4 months) in a small cohort of patients ($n=57$) with locally advanced or metastatic UCBs [33]. The gene expression level was normalized to β-actin and seven-fold change was used as a cut-off value. Despite a trend towards longer time to progression in patients with low ERCC1-expressing tumors, there was no clear association with response to cisplatin-based chemotherapy [33]. A similar study evaluated the utility of BRCA1 mRNA expression in transurethral resection (TUR) specimens of patients ($n=57$) with locally advanced UCB who subsequently received neoadjuvant chemotherapy [34]. Low- and intermediate-tercile levels of BRCA1 mRNA expression predicted a greater pathologic response (66% versus 22%) and longer median OS (168 versus 34 months) [34]. A more comprehensive analysis tested the impact of DDR gene mutations on pathologic response (pT0/pTis versus pT2+) to neoadjuvant cisplatin-based chemotherapy by performing whole-exome sequencing (WES) and enrichment analysis in two separate groups of responders ($n=25$) and non-responders ($n=25$) [35]. Out of 3277 genes with at least one possibly damaging somatic alteration, ERCC2 was the only gene significantly enriched in the responder group (36% versus 0% in non-responders) [35]. Importantly, these results from two different institutions were validated in an external cohort of 48 patients with similar characteristics [36]. The presence of ERCC2 non-synonymous mutations was associated with a better response to chemotherapy (40% in responders versus 7% in non-responders) as well as with prolonged median OS [36]. A subsequent study used targeted exome sequencing of 287 genes by the Foundation Medicine platform to analyze pre- and post-treatment tumors from a discovery cohort of UCB patients enrolled in a neoadjuvant accelerated MVAC trial and a validation cohort of patients treated with dose-dense GC [37]. After comparing the rates of pathologic responses (pT0pN0cM0 and ≤pT1pN0cM0), PFS and OS, they found that the majority of responders in either group (87% of responders in accelerated MVAC cohort and 64% in the dose-dense GC group) had an alteration (mutation or/and copy-number change) in one or more of the ATM, RB1, and FANCC genes, compared to non-responders [37]. Defects in this 3-gene panel were also predictive of better PFS and OS in the accelerated MVAC discovery set, with a trend towards significance for OS in the dose-dense GC validation set [37]. A different study group performed exon sequencing in a broader set of 34 DDR genes in a larger cohort ($n=100$) of platinum-treated patients (56% cisplatin-based, 44% carboplatin-based) with advanced or metastatic

UCB [38]. Interestingly, the patients whose tumors harbored DDR alterations (48%) had improved PFS (9.3 versus 6 months) and OS (23.7 versus 13 months) compared to those without [38].

Overall, it is currently unknown which DDR gene combinations provide the best positive predictive value. Several differences including sample size, retrospective versus prospective analyses, lack of uniformity in methodologies used (WES versus Foundation Medicine versus MSK-IMPACT targeted sequencing), and differences in platinum-based regimens exist (Table 1) [35–37]. These differences in study design and conduction limit our ability to draw general conclusions on the clinical utility of DDR-related biomarkers for muscle-invasive UCB. In the neoadjuvant setting, the clinical utility of a biomarker could enhance clinical decision-making with respect to bladder preservation. Patients with alterations in these genes are more likely to achieve a complete response to chemotherapy and thus has a reasonable likelihood of bladder preservation. The evaluation of limited (ATM, RB1, FANCC, and ERCC2) or more extensive DDR panels (ERCC2,3/BRCA1,2/RAD51C/ATR/RECQL4/ATM/FANCC) is ongoing as part of prospective neoadjuvant chemotherapy trials with post-treatment randomization to bladder-sparing versus cystectomy approaches (NCT02710734, NCT03558087, and Alliance A0311701).

Transcriptomic analyses of muscle-invasive UCB tumors have yielded gene expression profiles that were organized into distinct molecular subtypes by different research groups [15–18]. A common theme across different classifications is the emergence of basal-like and luminal subtypes, reflecting different types of urothelial differentiation, in resemblance with the basal-like and luminal subtypes of breast cancer [15]. The discovery of additional classifiers and clusters, including the claudin-low cluster within the updated UNC taxonomy, the p53-like and double-negative subtypes within the MDA classification, the luminal-papillary, luminal-infiltrated and neuronal subtypes within the new TCGA classification, and the refined taxonomy of Lund University validated in the TCGA UCB cohort (urothelial-like, genomically unstable, epithelial infiltrated, SCC-like/Mes-like, SCC-like/UroB, and Sc/NE-like), have provided a gateway to better understanding the biology of UCB (Fig. 2) [17, 18, 39–42]. Simultaneously, because the majority of these tumors across different cohorts were untreated, little can be concluded about the potential of these subtypes as biomarkers of response, other than that patients with basal tumors have a poor prognosis [39, 40]. Analyses of the gene expression profile and subtypes in the MDA discovery and validation cohorts were performed in matched samples before and after neoadjuvant cisplatin-based chemotherapy [16, 41]. The patients whose tumors clustered with the p53-like subtype were resistant to chemotherapy, and those with basal tumors experienced the shortest 5-year OS [16, 42]. These results supported a role for the p53-like subtype as a predictor of chemo-resistance. They also suggested that chemotherapy can transform the natural course of basal tumors [16]. In line with this, four consensus subtypes (claudin-low, basal, luminal-infiltrated, and luminal) predicted by a composite genomic subtyping classifier (GSC) demonstrated distinct prognostic values in a large multicenter patient cohort ($n = 343$) of invasive UCBs [43]. Patients with basal tumors received the greatest benefit in OS with neoadjuvant chemotherapy compared with surgery alone. Patients with claudin-low tumors had the shortest OS, whereas those with luminal tumors had the longest OS irrespective of neoadjuvant chemotherapy [43].

Despite these interesting findings, the diversity and complexity of the existing taxonomies for muscle-invasive UCB hinder the use of transcriptional biomarkers such as the p53-like signature or the GSC in clinical trials and practice. It is also unknown whether these molecular subtypes are truly intrinsic or stable over the course of the disease. Concurrent immunohistochemical (IHC) assessment together with global mRNA expression has revealed that tumor cell phenotypes can diverge or converge with respect to gene expression clusters. This may be explained by intra-tumoral

Table 1 Characteristics and performance of DNA damage repair (DDR) biomarkers for prediction of response to cisplatin-based chemotherapy

Cohort	Biomarker	Setting	*n*	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	ERCC2	NAC					
Discovery	Van Allen et al. [35]		50	36	100	100	61
Validation	Liu et al. [36]		48	80	93	80	93
	ATM, RB1, FANCC	NAC					
Discovery	Plimack et al. [37]		34	87	100	100	90
Validation	Plimack et al. [37]		24	64	85	78	73

NAC neoadjuvant cisplatin-based chemotherapy, *PPV* positive predictive value, *NPV* negative predictive value

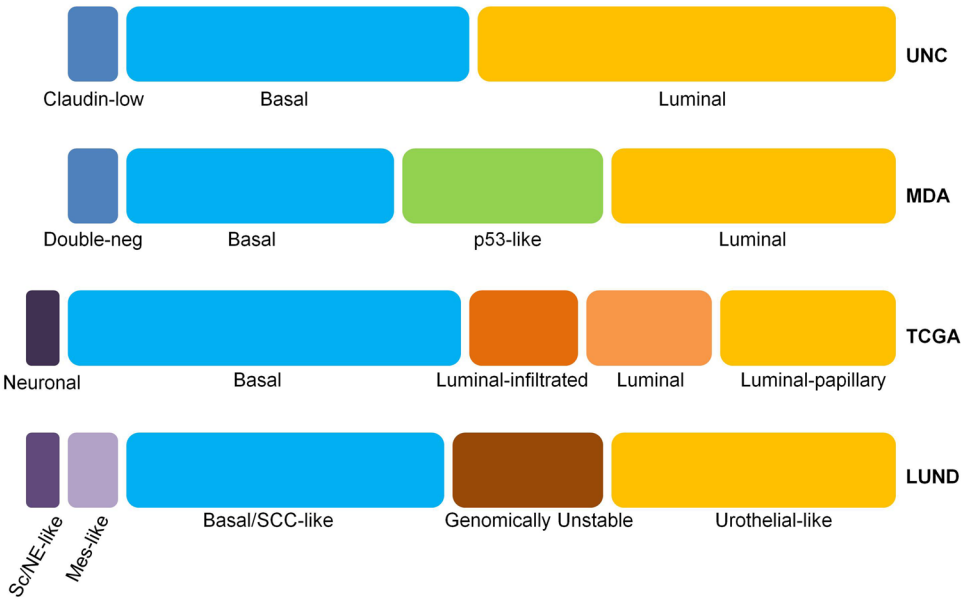

Fig. 2 Schematic representation of updated molecular classification of urothelial carcinoma. *UNC* University of North Carolina, *MDA* MD Anderson Cancer Center, *TCGA* The Cancer Genome Atlas, *LUND* Lund University, *Double-neg* double-negative, *Sc/NE-like* small-cell/neuroendocrine-like, *Mes-like* mesenchymal-like, *Basal/SCC-like* basal/squamous cell carcinoma-like

heterogeneity (infiltrating non-tumors cells) that contributes to these expression signatures [44]. For example, only the amplification of CCND1 gene but not Cyclin D1 IHC protein expression was concordant between primary UCB tumors and lymph-node metastases in one study [45]. With respect to adjuvant chemotherapy response, high nuclear Cyclin D1 expression and CCND1 amplification in the metastases, but not in primary tumors predicted a favorable response [45]. In another cohort of paired muscle-invasive bladder tumors and synchronous lymph-node metastases, discordance in subtype classification was found in approximately one-fifth of tumors and predominantly involved basal/squamous-like tumors in more than half of these cases [46]. Collectively, the small number of studies and the limited number of subtypes explored hamper drawing definitive conclusions regarding gene expression-based biomarkers for chemotherapy response.

The use of two active chemotherapy regimens for invasive UCB, accelerated MVAC and GC, gave rise to the question whether pathologic responses in the neoadjuvant setting are different between these two regimens. To address this question, a phase II biomarker discovery and validation prospective SWOG study was designed (S1314, NCT02177695). The trial is testing the ability of the CO-eXpression ExtrapolatioN (COXEN) algorithm to derive candidate biomarkers by comparing gene expression data between sensitive and resistant cell lines from the NCI-60 (biomarker discovery) [47]. This process is followed by triaging the biomarkers with use of COXEN to ensure concordant expression between data sets and subsequently deriving gene expression models (GEMs) to predict the sensitivity to individual drugs or combinations [47]. The last step involves the use of GEMs to classify patients' tumors based to provide prediction scores based on empirical (in vitro) and clinical (pathologic response pT0) outcomes after four cycles of GC or accelerated MVAC [47] (S1314, NCT02177695).

Biomarkers of response and resistance to immune checkpoint inhibitors

The approval of five immune checkpoint inhibitors (CPIs) targeting the programmed death 1 (PD-1)/PD-ligand 1 (PD-L1) axis is a major therapeutic advance in the treatment of advanced UCB [48–52]. The modest range of objective response rates (ORR 13.4–21.1%) across five phase II or III trials of CPIs (pembrolizumab, atezolizumab, nivolumab, durvalumab, and avelumab) highlights an unmet need to identify biomarkers of response and resistance that could potentially maximize therapeutic effects in a more limited patient subpopulation [48–52]. The induction of an effective antitumor immune response by CPIs is complex and involves numerous stimulatory and inhibitory mechanisms. These include the release of cancer antigens, cancer antigen presentation, priming, and activation of T cells and antigen-presenting cells, trafficking and infiltration of T cells into tumors, recognition, and killing of cancer cells by T cells [53].

The identification of PD-L1 as a distal immune modulator expressed in cancer cells was key to the development of CPIs. Clinical studies in different tumor types, including UCB, evaluated the activity of these drugs in conjunction with IHC assessment of PD-L1 status [48–52]. KEYNOTE-045 phase III study used a combined positive score (CPS) from both tumor cells (TC) and immune cells (IC) with a cut-off set at 10% [48]. PD-L1 positive patients had a significant OS benefit (8 versus 5.2 months) compared

to chemotherapy suggesting a prognostic value of PD-L1 in this setting [48]. The IMvigor211 phase III trial was designed to measure PD-L1 differently and only focused on IC positivity to stratify three levels of expression: IC0 (< 1%), IC1 (1–5%), and IC2/3 (≥ 5%) [49]. However, no significant differences in ORR (23 versus 22%) and median OS (11.1 months versus 10.6 months) were detected between the IC2/3 patients in the atezolizumab group and those in the chemotherapy arm [49]. The other three single-arm phase II CPI trials also used distinct antibodies and definitions for PD-L1 positivity [50–52]. The lack of uniformity of standards and definitions for PD-L1 testing limits the utility of PD-L1 positivity as a predictive or prognostic biomarker, with the major concern being the lack of a common methodology for assessment, cut-offs, and interpretation of IHC staining (Table 2). A study assessing the concordance of 3/5 PD-L1 immunohistochemical assays (22C3 for pembrolizumab, 28-8 for nivolumab, and SP142 for atezolizumab) revealed that pair-wise concordance correlation coefficients between the antibodies in 624 matched primary and metastatic UCB tissue cores ranged from 0.76 to 0.9 for tumor cells, but a wider range was noted for immune cells (0.30–0.85) [54]. Concomitant RNA and protein expression levels showed moderate-to-high agreement (0.72–0.87) [54]. Recently, low PD-L1 expression in two ongoing trials (KEYNOTE-361 CPS < 10%, IMvigor130 IC < 5%) was associated with worse OS in patients who received CPI compared to those that were treated with cisplatin- or carboplatin-based chemotherapy [55, 56]. This led to an FDA alert against the use of these agents in cisplatin-ineligible patients with low PD-L1 expression and discontinuation of enrollment of PD-L1-low patients in the monotherapy CPI arms of these trials [57]. The labels of both drugs were revised to reflect this. Pembrolizumab is now indicated for the treatment of patients with locally advanced or metastatic UC who are not eligible for cisplatin-containing therapy and whose tumors express PD-L1 (CPS ≥ 10), or in patients who are not eligible for any platinum-containing chemotherapy regardless of PD-L1 status. Atezolizumab is now indicated for the treatment of patients with locally advanced or metastatic UC who are not eligible for cisplatin-containing therapy, and whose tumors express PD-L1 (PD-L1 stained IC covering ≥ 5% of the tumor area), or who are not eligible for any platinum-containing therapy regardless of level of tumor PD-L1 expression [57]. The FDA has not changed the indications of these two CPIs for the treatment of patients with locally advanced or metastatic UC who have disease progression during or following any platinum-containing chemotherapy, or within 12 months of neoadjuvant or adjuvant treatment [57].

These findings suggest that low PD-L1 status may predict low response rates (RR) to immunotherapy in locally advanced or metastatic UCB. This was also previously seen in melanoma, RCC and NSCLC tumors, whereby lack of PD-L1 expression correlated with lack of response to PD-1 blockade [58]. It remains to be seen how these recommendations will be implemented in the community as PD-L1 testing has not been routinely performed before treating UCB patients with immune CPIs.

Beyond PD-L1, the ability to mount an effective antitumor immune response is linked to the generation of neoantigens, which are altered peptides that result from somatic mutations [59]. Muscle-invasive UCB has a high total mutation burden (TMB) providing a rationale for testing TMB as a surrogate for response to CPIs [60]. In the exploratory biomarker analysis of the phase II trial of atezolizumab monotherapy in patients with advanced or metastatic UCB (Imvigor210), the median TMB in responders was significantly higher (12.4 mutations per megabase) compared to non-responders (6.4 mutations per megabase) [61]. TMB was assessed in 150 patients with the Foundation Medicine NGS assay, which includes a representative panel of 315 cancer-related genes [61]. Interestingly, this finding was independent of the PD-L1 IC subgroup, implying that assessment of TMB might have a predictive value complementary to PD-L1 status [61]. A similar association between TMB and response to atezolizumab was observed in the first-line setting for cisplatin-ineligible patients [62]. Beyond the potential value of TMB as a positive biomarker of response in Imvigor210, further exploratory analyses revealed the utility of TGFβ-pathway signaling, reflected by a distinct gene expression signature, as a predictor of poor response to atezolizumab [63]. This was particularly evident in patients with tumors which showed exclusion of CD8+ T cells from the tumor parenchyma that were instead found in the fibroblast- and

Table 2 Characteristics of different PD-L1 immunohistochemical staining assays

CPI	Study	Antibody	Platform	Cell stained	PD-L1 cut-off (%)	ORR (%)
Pembrolizumab	Bellmunt et al. [48]	22C3	Dako	TC + IC	≥ 10	26
Atezolizumab	Powles et al. [49]	SP142	Ventana	IC	≥ 10 (IHC 2/3)	23
Nivolumab	Sharma et al. [50]	28-8	Dako	TC	≥ 1	28
Durvalumab	Powles et al. [51]	SP263	Ventana	IC + TC	≥ 25	31
Avelumab	Apolo et al. [52]	73-10	Dako	IC + TC	≥ 25 TC or ≥ 10 IC	24

CPI checkpoint inhibitor, *PD-L1* programmed death ligand 1, *TC* tumor cells, *IC* immune cells, *ORR* objective response rate

collagen-rich peritumoral stroma [63]. The complex interplay between these factors may explain the difficulty in predicting responses to PD-1/PD-L1 blockade. Moreover, similar to PD-L1 testing, there are several methods for measuring TMB, including the use of targeted panels (Foundation Medicine 315, MSK-IMPACT 468 cancer-related genes) versus WES (22,000 gene coding regions). The optimal cut-off number of mutations per megabase can vary between the various bioinformatic pipelines [64, 65].

A hypermutated somatic mutational profile can result from germline or somatic deficiency of the DNA mismatch repair pathway (MMR). A proof-of-concept study of pembrolizumab in tumors of different histologies stratified by genomic analysis of microsatellite instability (a surrogate of defective MMR repair) demonstrated that MMR-deficient patients respond better to CPI compared to MMR-proficient tumors [66, 67]. This led to a tissue-of-origin agnostic approval of pembrolizumab for such patients. This also raises the possibility that other defects in other DDR pathways could also increase sensitivity to CPIs. In fact, a retrospective NGS analysis of 60 metastatic UC patients that were previously enrolled in three different trials of atezolizumab and nivolumab, revealed that the presence of any deleterious DDR alteration (mutation or/and copy-number change) from a panel of 34 DDR genes was associated with higher RR (68 versus 19%), and longer PFS and OS [68]. From a mechanistic perspective, it remains unknown whether there is a particular combination of defective DDR genes which consistently is consistently associated with high RR to CPIs and whether this effect is mediated solely via increased mutagenesis or other pathways.

The systemic nature of the immune response to CPI entails both local and circulating immune cells and cytokines, some of which represent potential biomarkers. The clonality of the T-cell receptor, measured by sequencing from peripheral T cells and TILs is a potential biomarker. In 29 patients with advanced UCB treated with atezolizumab, a low pretreatment clonality combined with expansion of tumor-associated T-cell clones 3 weeks post-treatment was associated with a PFS greater than 6 months [69]. Another immune parameter of potential clinical interest is the abundance of myeloid-derived suppressor cells (MDSCs) in peripheral blood measured by flow cytometry [70]. The presence of MDSCs has been shown to eliminate CD8 T cells in UCB through upregulation of PD-L1 via the COX2/PGE2 pathway [71]. In the CheckMate 275 phase II study of nivolumab in advanced UCB patients, a low baseline level of circulating MDSCs (defined as the lowest tertile) was associated with longer OS compared to medium and high tertiles, particularly when combined with a high tumoral interferon-gamma gene expression signature [70]. These proposed circulating biomarkers are of interest if the results are reproduced prospectively across different cohorts and with multiple CPIs before consideration for clinical use.

Concurrent assessment of composite biomarkers instead of a single metric will potentially provide a more accurate reflection of the underlying biological complexity of the antitumor immune response. Ongoing neoadjuvant CPI trials in UCB (ABACUS atezolizumab, PURE-01 pembrolizumab) have adopted this approach. Preliminary results support a correlation of the biomarkers with pathologic responses (ABACUS: PD-L1 and CD8, PURE-01: DDR or/and RB1 and PD-L1, and 22-gene T-cell-inflamed gene signature) [72, 73]. However, there is currently no clear model capable of incorporating various biomarkers into a unified composite predictive biomarker.

The four expression-based clusters of treatment-naïve invasive UCB from the initial TCGA bladder cohort ($n = 124$) were also tested as tentative biomarkers of response to CPI in exploratory analyses of two separate phase II trials (atezolizumab and nivolumab) [50, 61, 74]. However, results were different between the two studies as patients with cluster II tumors (luminal 2) had the highest response to atezolizumab, whereas patients with cluster III tumors (basal 1) showed the best response to nivolumab [50, 61, 74]. It is unclear how the newer five-cluster TCGA molecular classification correlates with clinical responses in patients treated with CPIs [17].

Oncogenes as potential biomarkers in UCB

Alterations of the RTK/RAS/PI3K pathway are common (71%) in invasive UCB [17]. Given the presence of several other genomic aberrations involving tumor suppressor and cell cycle genes (89%), epigenetic regulation genes (histones and SWI/SNF), DNA damage, oxidative stress, and alternative splicing genes, it is unclear whether the growth of UCB could rely substantially on one single dominant oncogene, according to the oncogene addiction model [17, 75]. However, there is rationale for the design of targeted therapeutics against an oncoprotein and the use of its expression as a biomarker. Biomarkers have the potential to make targeted drug development more rational, optimize clinical efforts, and ultimately help choose a therapeutic intervention for a selected patient population based on the likelihood of response, resistance, or toxicity [75].

Muscle-invasive UCB has high rates of somatic alterations including fibroblast growth factor receptor (FGFR) mutations and fusions (21%). Patients with FGFR3 alterations tend to have a low likelihood of response to chemotherapy [17, 43]. In addition, FGFR3 mutations appear to be enriched in luminal 1 UC subtype, which has a lower ORR to PD-1 and PD-L1 inhibitors (10–19%) compared to infiltrated luminal 2 and basal 3 tumors (ORR 31–34%) [61]. This suggests that FGFR3 mutations occur within a

group of tumors that is less likely to benefit from CPIs. The early results from the development and testing of two pan-FGFR inhibitors, erdafitinib and BGJ398 in cancer patients of different primaries, including UCB, selected for FGFR genomic alterations showed activity [76, 77]. Erdafitinib was further studied in a phase 2 trial that enrolled CPI-resistant patients with FGFR2/3 alterations (mutations or fusions). Based on an ORR of 42% in 59 patients with tumors that harbored actionable FGFR mutations, erdafitinib was granted breakthrough therapy designation by FDA in relapsed/refractory metastatic urothelial cancer [78]. In view of these positive results, erdafitinib development is advanced to the phase III setting in the THOR trial comparing it with chemotherapy or pembrolizumab (NCT03390504). Erdafitinib is also combined in a phase Ib/II trial with the PD-1 inhibitor JNJ-63723283 (NCT03473743).

HER2 mutations (42%) and gene amplification (12%) are common in advanced UCB [17]. This justified the investigation of trastuzumab combined with a platinum-based triplet (paclitaxel, carboplatin, and gemcitabine) in advanced UC patients [79]. Despite tolerability and high RR (70% in 44 patients), a subsequent phase II comparative study reported no ORR-, median PFS-, or median OS benefit from addition of trastuzumab to platinum-based chemotherapy versus chemotherapy alone [80]. A phase III trial of maintenance lapatinib versus placebo after the first-line chemotherapy was also negative (no significant PFS or OS difference between arms) [81]. The presence of sample heterogeneity (biopsies from primary or metastatic sites), variability of assessment methods for HER2 positivity (IHC 2+/3+ and FISH+ versus IHC 3+ only versus serum HER2 extracellular domain ELISA), and the variability in number of cycles and regimens (combination of HER2-targeted treatment with chemotherapy versus maintenance after chemotherapy, cisplatin-based versus carboplatin-based) are factors that limit definitive conclusions about the potential activity of HER2-targeted agents in selected UC patients. Integrated analyses of HER2 status at the DNA, RNA, and protein level showed variation of the frequency of HER2 alterations between the luminal and basal molecular subtypes [82]. Using the Lund classification system, an enrichment of HER2 amplifications and expression (mRNA and protein) was observed in urothelial-like subtype tumors but not in genomically unstable tumors from an advanced UCB cohort when compared to a reference cohort comprising all the stages and grades [83]. Interestingly, less than half of the basal/SCC-like tumors with HER2 amplification had concomitant HER2 mRNA and protein expression, suggesting that the molecular architecture of tumors should be accounted for in future HER2 targeted trials in UCB [83]. Broadening the assessment of ErbB family may also be important. A phase II study of afatinib with prior evaluation of genomic alterations and IHC staining of EGFR, HER2, and ERBB3 reinvigorated interest in targeting this axis in UCB [84]. The median PFS in six patients with HER2/ERBB3 alterations was significantly longer (6.6 months) compared to that of patients without alterations (versus 1.4 months). Although the small study size precludes definitive conclusions about the predictive utility of HER2/ERBB3 genomic testing, this potentially constitute a more robust approach to determine HER2 status compared to IHC, and larger prospective studies are warranted [84].

Biological and technical challenges for future UCB biomarkers

For any biomarker to be clinically useful, it is key to determine the biological compartment where the critical biological activity occurs (e.g., dendritic cells and macrophages in immune biomarkers) within a given tissue [85]. IHC variables including choice of antibody, fixation technique, selection of tumor areas to measure, staining methods, signal detection methods, criteria for positive staining, data interpretation guidelines, and stratification criteria are major challenges for the clinical application of tissue-based biomarkers in UCB management [86]. Standardization and adopting a uniform approach are keys, and prospective comparison of different assays in clinical trial settings is necessary. The recently presented PROPHECY trial, a large multi-assay biomarker-blinded prospective validation study of CTC-ARV7 as a negative predictive biomarker of potent AR inhibition therapy in metastatic castration-resistant prostate cancer (CRPC), could serve as a paradigm for future similar studies in UC [87]. This study is unique within the spectrum of biomarker research trials as it prospectively compared and validated two different assays with different methodologies: the Johns Hopkins RNA-based CTC-ARV7 assay (RT-PCR of EpCAM-selected AR/PSA/PSMA-positive CTCs) and the EPIC IHC-based CTC-ARV7 assay (nuclear ARV7 protein of CK-positive or CK-negative CTCs) [87]. ARV7 positivity in both tests was predictive of poor outcomes (PSA decline, radiographic PFS, and OS) independent of the line of therapy (first-line abiraterone or enzalutamide; second-line taxane) and of the established prognostic factors [87].

The complexity of the tumor–immune interactions remains a major challenge for biomarker development. Several factors including the degree and quality of neoantigen presentation, chemokine expression, stromal density, T cell penetration and MDSCs, Tregs, and non-inflamed versus inflamed tumor microenvironment are involved. Clonal neoantigens tend to be more immunogenic, and conversely, the strongest neoantigens are derived from highly clonal mutations [88]. The contribution of the "host" to the success of treatment is also critical. A patient's HLA germline genotype strongly influences the efficacy of immunotherapy [88]. The

more diverse the HLA alleles, the better the patient responds to immunotherapy. Patients with both diverse HLAs and high mutational load experience the highest responses [89]. Developing biomarkers that identify host-related factors, as well as tumor-related factors, is needed.

Another major step for improving patient selection for current and future therapies in muscle-invasive UCB is to move beyond tissue-based metrics to minimally-invasive circulating biomarkers. Obtaining tumor tissue can be challenging, due to either inaccessibility or limited size, potentially resulting in significant delays in treatment. In contrast, peripheral blood is readily available for biomarker analysis and is also amenable to serial monitoring [90]. In metastatic UCB, this approach is feasible and circulating tumor DNA NGS enabled the identification of a similar profile of genomic alterations for biomarker-driven clinical trials compared with tumor tissues [91]. Blood TMB was associated with improved efficacy in metastatic NSCLC patients treated with atezolizumab [92]. Such circulating biomarkers offer the opportunity of dynamic monitoring during treatment with the potential to inform therapeutic decision-making. For example, the early post-treatment decrease in serum IL-8 can predict response to CPIs in melanoma and NSCLC patients [93]. Likewise, assessment of circulating "exhausted-phenotype" CD8 T cells by flow cytometry could serve as a dynamic, on-treatment biomarker of response to CPIs in melanoma [94].

Conclusions

Several novel biomarkers capturing the salient tumor and immune features are rapidly emerging. The main technical limitation of biomarker research is methodological variability, which poses a challenge for the prospective validation of different biomarker tests. An important future goal is to achieve cost-efficient and robust biomarkers with rapid result turnaround time, which could be introduced for disease-monitoring using real-world clinical samples in clinical settings. Parallel analyses of multiple analytes from a single sample, e.g., DNA, mRNA, and protein could improve the utility of future biomarkers. The optimization of any biomarker requires cooperative networks extending across several scientific and clinical fields.

Several aspects of the biology of muscle-invasive UCB pose additional challenges for biomarker development. Tumor heterogeneity and the clonal evolution of the disease limit the utility of any biomarker. The complexity of the host and cancer-cell autonomous mechanisms that shape the antitumor immune response requires the development of biomarkers that capture these variables. Future biomarker development should be based on a deep biological understanding of invasive UCB.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest with the contents of this work.

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