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Review

Molecular markers of systemic therapy response in urothelial carcinoma

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Abstract Identification of reliable molecular biomarkers that can complement clinical practice represents a fascinating challenge in any cancer field. Urothelial carcinoma is a very heterogeneous disease and responses to systemic therapies, and outcomes after radical cystectomy are difficult to predict. Advances in molecular biology such as next generation sequencing and whole genome or transcriptomic analysis provide promising platforms to achieve a full understanding of the biology behind the disease and can identify emerging predictive biomarkers. Moreover, the ability to categorize patients' risk of recurrence after curative treatment, or even predict benefit from a conventional or targeted therapies, represents a compelling challenge that may reshape both selection for tailored treatment and disease monitoring. Progress has been made but currently no molecular biomarkers are used in the clinical setting to predict response to systemic agents in either neoadjuvant or adjuvant settings highlighting a relevant unmet need. Here, we aim to present the emerging role of molecular biomarkers in predicting response to systemic agents in urothelial carcinoma.

1. Introduction

Urothelial carcinoma (UC) including bladder cancer (BCa) and upper urinary tract urothelial cell carcinoma (UTUC) represents the sixth most commonly diagnosed cancer in Western countries [1]. The estimated new cases and deaths for BCa among Asian men and women in 2012 were respectively 148 568 and 69 294 [2]. Focusing on BCa as

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the most common, it can present as a very heterogenous disease comprising both non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) disease [3]. Although radical cystectomy (RC) with pelvic lymph node dissection represents a milestone in the treatment of MIBC, over the past 10 years cisplatin-based neoadjuvant chemotherapy (NAC) has evolved as the standard of care for treatment with curative-intent [4] showing a 5%—10% absolute survival benefit [5]. Furthermore, pathological complete response (pCR) and pathologic downstaging (pDS) after NAC (<pT2) at RC specimens are strong predictors of survival [6,7] (Tables 1—3).

Despite this evidence [4,8], NAC is still underutilized. This is likely related to the modest benefits, advanced age at diagnosis, existing comorbidities, and treatment toxicity [9]. Nevertheless, nearly half of patients undergoing RC are cisplatin-ineligible based on impaired renal function [10]. Furthermore, approximately only 40% of patients experienced a pCR following NAC [4] whereas chemotoxicity is a common event that universally impacts these patients. With such prerogatives, many physicians feel that MIBC can be adequately treated with upfront surgery avoiding any delay of potential local curative treatment [11]. As a consequence, adjuvant chemotherapy (AC) after RC is considered in chemotherapy-naive patients with postoperative high-risk factors (≥pT3 and/or pN positive disease) [12]. Despite many advances, RC remains a highly morbid operation with postoperative complications that can impact the administration of AC. With such clinical dilemma, the search for specific predictive biomarkers for patient's response to a conventional or target treatment such as immune checkpoint inhibitors (ICIs) has intensified widely in the recent past. Furthermore, non-responders can be considered for alternative and potentially more effective treatments, avoiding unnecessary toxicity. Not secondary, target therapies can be very expensive and so reserved for responders with important economic implications. Although several clinical and pathological tools have demonstrated predictive efficacy capable of influencing survival outcomes [13—17], currently no molecular biomarkers are used in the clinical setting to predict response to systemic agents in NAC and AC settings highlighting a relevant unmet need.

In this narrative review, we aim to present the emerging role of predictive molecular biomarkers for response to systemic agents in UC. For conventional platinum-based chemotherapy, the predictive role of DNA damage response and repair (DDR) genes, driver mutations, molecular subtyping, liquid biopsy, and novel microRNAs (miRs) were discussed, whereas for immunotherapy the predictive value of PD-L1/PD-1 expression, tumor mutational burden (TMB), and liquid biopsy were reviewed across different therapy lines. Novel expanding systemic targeted therapies including fibroblast growth factor receptor (FGFR) inhibitors and antibody—drug conjugate against Nectin-4 were further explored. We highlighted the impact of different therapies and regimens on *in vitro* or *in vivo* pre-analytical activity, pCR or pDS, and radiological response according to RECIST criteria [18] and clinical endpoints such as overall survival (OS), progression/disease/recurrence-free survival (P/D/RFS), and cancer-specific survival (CSS).

2. Platinum-based chemotherapy

Cisplatin is an antineoplastic in the class of alkylating agents. These agents are so named because of their ability to add alkyl groups to many electronegative groups under conditions that present in cells. One of the key mechanisms via which platinum-based drugs can stop tumor growth is by cross-linking guanine bases in DNA double-helix strands—directly attacking DNA. This makes the strands unable to uncoil and separate. As this is necessary in DNA replication, the cells can no longer divide. Cisplatin enters the cell through several membrane transporters and three different operating mechanisms have been described: First, attachment of alkyl groups to DNA bases, resulting in the DNA being fragmented by repair enzymes in their attempts to replace the alkylated bases, preventing DNA synthesis and RNA transcription from the affected DNA; second, DNA damage via the formation of cross-links which prevents DNA from being separated for synthesis or transcription; and third, the induction of mispairing of the nucleotides leading to mutations [19]. Conversely, four mechanisms of cisplatin resistance have been proposed: First, pre-target resistance that involves steps preceding the binding of cisplatin to DNA; second, on-target resistance that directly relates to DNA-cisplatin adducts; third, post-target resistance concerning the lethal signaling pathways elicited by cisplatin-mediated DNA damage; fourth, off-target resistance affecting molecular circuitries that do not present obvious links with cisplatin-elicited signals [20]. In each of these steps, there are different genes, enzymes and membrane transporters that can play a critical role in the pharmacodynamics of cisplatin and therefore can act as response biomarkers as well as future therapeutic targets.

2.1. DNA DDR genes

DDR genes are regulators of double-helix repair following platin-based damage though processes like nucleotide excision repair (NER) pathway, mismatch repair proteins, or homologous recombination [21]. Therefore, deficiency like non-synonymous mutations in the involved genes has been expected to confer increased sensitivity to platin-based therapy [19].

Focusing on these, excision repair cross complementing 1 and 2 (ERCC1/2) are two of key regulators involved in NER process, whereas studies about *ERCC2* provided robust data and analysis on ERCC1 led to controversial results. ERCC1 protein expression assessed by immunohistochemistry (IHC) failed to predict pCR to NAC dose-dense methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) [22]. Conversely, low *ERCC1* mRNA expression determined by reverse transcriptase-polymerase chain reaction (RT-PCR) in tumor DNA from 57 advanced and metastatic BCa patients treated with either gemcitabine/cisplatin or gemcitabine/cisplatin/paclitaxel was associated with longer OS [23].

ERCC2 has been extensively studied. Isoform 2 in particular has the task of separating the double helix through a 5′—3′ helicase mechanism after recognizing the error. Whole exome sequencing (WES) on pretreatment

Table 1 Emerging predictive molecular biomarkers of response to systemic therapies in urothelial carcinoma.

Biomarker	Cohort	Source	Setting	Systemic therapy	Summary
DDR genes alterations					
ERCC2	50	Tissue	NAC	Cisplatin-based	*ERCC2* mutations correlate with pCR at RC [24].
	48	Tissue	NAC	Cisplatin-based	*ERCC2* alterations confer vulnerability to cisplatin traducing in better OS [25].
	32	Tissue	NAC	Gem-Cis	Deleterious *ERCC2* alterations strongly predicted pDS and superior RFS [26].
ATM	34	Tissue	NAC	MVAC (discovery), Gem-Cis (validation)	*ATM* mutations predicted pCR and better OS/PFS in both sets [27].
	24	Tissue	NAC	MVAC (discovery), Gem-Cis (validation)	*ATM* mutations predicted better OS/DSS in both sets [28].
RB1	34	Tissue	NAC	MVAC (discovery), Gem-Cis (validation)	*RB1* mutations predicted pCR and better OS/PFS in both sets [27].
	24	Tissue	NAC	MVAC (discovery), Gem-Cis (validation)	*RB1* mutations predicted better OS/DSS in both sets [28].
FANCC	34	Tissue	NAC	MVAC (discovery), Gem-Cis (validation)	*FANCC* mutations predicted pCR and better OS/PFS in both sets [27].
	24	Tissue	NAC	MVAC (discovery), Gem-Cis (validation)	*FANCC* mutations predicted better OS/DSS in both sets [28].
Driver mutations					
ERBB2	71	Tissue	NAC	MVAC, Gem-Cis/Car (discovery/validation)	*ERBB2* missense mutations predicted pCR and better CSS [33].
	52	Tissue	NAC	Gem-Cis	*ERBB2* mutations were correlated with pDS [34].
FGFR3	52	Tissue	NAC	Gem-Cis	*FGFR3* mutations were correlated with pDS [34].
	72	Tissue	NAC	Gem-Cis	*FGFR3* alterations were correlated with pNR and with worse RFS [36].
	74	Tissue	AC	Gem-Cis	*FGFR3* alterations were associated with worse RFS [36].
PIK3Ca	52	Tissue	NAC	Gem-Cis	*PIK3Ca* mutations were correlated with pDS [34].
HUS1	23	Tissue	NAC	Gem-Cis	Amplification of *HUS1* predicted pNR and worse RFS [37].
ABCA13	23	Tissue	NAC	Gem-Cis	Amplification of *ABCA13* predicted pNR and worse RFS [37].
EGFR	23	Tissue	NAC	Gem-Cis	Amplification of *EGFR* predicted pNR and worse RFS [37].
FIGNL1	23	Tissue	NAC	Gem-Cis	Amplification of *FIGNL1* predicted pNR and worse RFS [37].
IKZF1	23	Tissue	NAC	Gem-Cis	Amplification of *IKZF1* predicted pNR and worse RFS [37].
Liquid biopsy					
CTCs	20	Blood	NAC	MVAC or Gem-Cis	Patients with medium/high (cut-off 10 CTCs) count showed pNR [58].
CTCs	31	Blood	Metastatic	MVAC	Patients with favorable CTCs trend showed better PFS and OS rates [59].
ctDNA	68	Plasma	NAC/AC	Gem-Cis/Car; Car-Eto; Gem	Dynamics of ctDNA was associated with RFS and OS but not with pDS [70].
ctDNA	17	Plasma	NAC	Cisplatin-based	Persistence of ctDNA detection during NAC predicted disease recurrence [72].
	NA	Urine	NAC	Cisplatin-based	
miRs					
miR-886-3p	30	Tissue	AC/metastatic	MVAC or Gem-Cis	miR-886-3p was correlated with CR (RECIST) and better OS [76].
miR-923	30	Tissue	AC/metastatic	MVAC or Gem-Cis	miR-923 was correlated with CR (RECIST) and better OS [76].
miR-944	30	Tissue	AC/metastatic	MVAC or Gem-Cis	miR-944 was correlated with CR (RECIST) and better OS [76].
miR-138	30	Tissue	AC/metastatic	MVAC or Gem-Cis	Decreasing miR-138 increased the *in vitro* Cis-sensitivity [76].
miR-27a	30	Tissue	AC/metastatic and *in-vitro* analysis	MVAC or Gem-Cis	miR-27a overexpression increased the *in vitro* Cis-sensitivity [76].

	354	Tissue		Cisplatin-based	miR-27a increased the *in vitro* Cis-sensitivity through SLC7A11 axis [77].
miR-642	30	Tissue	AC/metastatic	MVAC or Gem-Cis	miR-642 overexpression increased the *in vitro* Cis-sensitivity [76].
miR-101	NR	Tissue	*In-vitro* analysis	Cisplatin-based	miR-101 downregulation induced Cis-resistance through COX-2 axis [78].
miR-193a-3p	NR	Tissue	*In-vitro* analysis and *in-vivo* analysis	Cis, Pa, Ad, Epi	miR-193a-3p promotes the multi-chemoresistance [114].
miR-203	108	Tissue	AC	MVAC or Gem-Cis	Low miR-203 expression was correlated with worse PFS and OS [115].
miR-372	83	Tissue	Metastatic	MVAC or Gem-Cis	High miR-372 expression was associated with worse PFS [79].
miR-21	83	Tissue	Metastatic	MVAC or Gem-Cis	High miR-21 expression was associated with a shorter PFS [79].
miR-34a	20	Tissue	NAC and *in-vitro* analysis	Cisplatin-based or Epi	Increased miR-34a expression mediated *in-vitro* Cis-sensitivity [116]. miR-34a overexpression resulted in increased *in-vitro* Epi-sensitivity [117].
miR-101-3p	89	Tissue	*In-vitro*	Gem-Cis	miR-101-3p overexpression increased chemosensitivity through EZH2 [80].
Cdr1as	NR	Tissue	*In-vitro*	Cisplatin-based	Cdr1as increased Cis-sensitivity through miR-1270/APAF1 axis [81].

ABCA13, ATP binding cassette subfamily A member 13; AC, adjuvant chemotherapy; Ad, adriamycin; ATM, ataxia telangiectasia mutated 1; APAF1: apoptotic protease-activating factor 1; Car, carboplatin; CR, complete response; CSS, cancer-specific survival; CTCs: circulating tumor cells; DDR, damage response and repair; DSS: disease-specific survival; EGFR, epidermal growth factor receptor; Epi, epirubicin; ERBB2, Erb-B2 receptor tyrosine kinase 2; ERCC2, excision repair cross complementing 2; Eto, etoposide; FANCC, FA complementation Group C; FGFR2−3, fibroblast growth factor receptor 2−3; FIGNL1, Fidgetin-like protein 1; Gem-Cis, gemcitabin−cisplatin; HUS1, HUS1 checkpoint clamp component; ICIs, immune checkpoint inhibitors; IKZF1, IKAROS family zinc finger 1; MVAC, methotrexate, vinblastine, doxorubicin, and cisplatin; NAC, neoadjuvant chemotherapy; OS, overall survival; Pa, paclitaxel; pCR, pathologic complete response; pDS, pathologic downstaging; PFS, progression-free survival; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit *α*; pNR, pathologic non-response; RB1, RB transcriptional corepressor 1; RC: radical cystectomy; RECIST, response evaluation criteria in solid tumors; RFS, recurrence-free survival; VAF, variant allele frequency; Vin, vinflunine; NA, no available; NR, not report.

tumor and germline DNA of 50 patients who received platin-based NAC showed that somatic *ERCC2* mutations were significantly associated with pCR (pT0 or Tis) at time of RC [24]. Furthermore, in a validation cohort of 48 MIBC patients who received three cycles of NAC, *ERCC2* was predictive of response to therapy—40% of responders and 7% of non-responders had a non-synonymous *ERCC2* genetic alteration (odds ratio [OR] 8.3, $p=0.01$) and this translated to a better OS [25]. In a multicenter prospective phase II trial, testing the efficacy and tolerability of six cycles of dose-dense gemcitabine and cisplatin in the NAC setting, Iyer et al. [26] performed a biomarker analysis using next generation sequencing (NGS) assays in pre-treatment tumor tissue within a panel of 29 DDR genes. *ERCC2* mutations were identified as strongly predictive for chemosensitivity, pDS (<pT2), durable response, and better 2-years RFS [26].

In a milestone paper in this field, Plimack et al. [27] suggested that other DDR genes can also affect response to NAC. DNA from pretreatment tumor tissue was sequenced for all coding exons of 287 cancer-related genes and was analyzed for base substitutions, indels, and copy number alterations. Among two independent prospective data sets, the 3-gene signature (*ATM, RB1, and FANCC*) predicted pathologic response and better progression free survival (PFS) and OS. Updated analysis with long-term follow-up (median 74 months) confirmed a significantly greater OS and PFS in patients with *ATM, RB1, or FANCC* genes mutations [28].

WES on 43 MIBC patients who received different AC regimes showed that the presence of somatic mutations in one or more of six DDR genes (*ATM*, *ERCC2*, *FANCD2*, *PALB2*, *BRCA1*, and *BRCA2*) was associated with RFS [29].

Teo et al. [30] analyzed the relationship between DDR alterations and response to PD-1/PD-L1 blockade. Sixty metastatic UC patients treated with atezolizumab or nivolumab who had targeted exon sequencing to determine the presence of preselected DDR mutations were evaluated. A higher response rate was observed in patients whose tumors harbored known or likely deleterious DDR alterations (80%) [30]. In a similarly designed study (combined conventional chemotherapy and ipilimumab), Galsky and colleagues [31] observed a significantly higher response rate in patients that harbored deleterious somatic mutations.

The RETAIN (risk enabled therapy after initiating neoadjuvant chemotherapy for bladder cancer) trial requires a mention here. This is a phase II, parallel arm, multi-institutional clinical trial (NCT02710734) that aims to evaluate a risk-adapted approach to the treatment of MIBC. Patients will receive NAC with accelerated MVAC. Pre-NAC transurethral resections of bladder tumor (TURBT) specimens are submitted for deep sequencing to identify variants in a panel of cancer-relevant genes. Those with an alteration in *ATM, RB1, FANCC* or *ERCC2* and no evidence of residual disease at restaging TURBT and imaging post-NAC will begin a pre-defined active surveillance regimen whereas their counterpart will receive a direct therapy. Primary endpoint is 2-year metastasis-free survival [32]. DDR alterations provided many promising findings, but much more has to be learned and validated. In this context,

Table 2 Predictive molecular biomarkers of response to immunotherapy in urothelial carcinoma.

Biomarker	Cohort	Source	Setting	Systemic therapy	Summary
IHC					
PD-L1/PD-1	1213	Tissue	Metastatic	Atezolizumab+Gem-Cis/Car	- High PD-L1 expression correlated with better PFS [118].
	1837	Tissue	Metastatic	ICIs	- Patients who progressed after first-line Cisplatin-based therapy had better OS and PFS when PD-L1/PD-1 was positive [92].
Liquid Biopsy					
ctDNA	29	Plasma	Metastatic	Durvalumab (discovery/validation)	- On-treatment reduction in ctDNA VAF may be a useful predictor of OS/PFS benefit [105].
ctDNA	50	Plasma	NAC	Gem-Cis	- Patients with metastatic relapse had significantly higher ctDNA levels compared with disease-free patients [71].
	10	Plasma	Metastatic	Atezolizumab, Car-Eto, Gem-Cis, Vin	

IHC, immunohistochemistry; PD-L1, programmed cell death-ligand 1; PD-1, programmed cell death protein 1; NAC, neoadjuvant chemotherapy; Gem-Cis, gemcitabin-cisplatin; ICIs, immune checkpoint inhibitors; PFS, progression-free survival; OS, overall survival; VAF, variant allele frequency.

Table 3 Predictive molecular biomarkers mutations of response to novel expanding systemic targeted therapies in urothelial carcinoma.

Biomarker	Cohort	Source	Setting	Systemic therapy	Summary
FGFR3	67	Tissue	Metastatic	Infigratinib	- Infigratinib is active in patients with *FGFR3* alterations resulting in both reductions in tumor volume and stabilization of disease (RECIST) [107].
FGFR1-3	52	Tissue	Metastatic	Rogaratinib	- Rogaratinib is active in patients selected by overexpression of *FGFR1−3* mRNA achieving an objective response (RECIST) [109].
FGFR2-3	99	Tissue	Metastatic	Erdafitinib	- In patients with prespecified FGFR alterations erdafitinib was associated with an objective tumor response (RECIST) [110].

FGFR, fibroblast growth factor receptor; RECIST, the Response Evaluation Criteria in Solid Tumours.

such designed risk-adapted studies could prompt the integration of specific DDR mutational profile into routine clinical management.

2.2. Driver mutations

Exploring new frontiers, mutations in *ERBB2* [33], *FGFR3* and *PIK3C*a [34] were found predictive in MIBC patients receiving different NAC regimes and were targetable with novel therapeutic agents. For example, afatinib (oral irreversible inhibitor of the *ERBB* family) was found to be active in platinum-refractory metastatic patients harboring *ERBB* alterations meeting the PFS endpoint [35].

FGFR3 alterations occur in almost 15% of UC with particular predisposition to be expressed in UTUC and luminal papillary tumors. Teo et al. [36] retrospectively demonstrated that patients who harbored *FGFR3* mutations had worse RFS following perioperative chemotherapy. Three cohorts consisting of MIBC patients treated with dose-dense gemcitabine-cisplatin NAC ($n=72$), metastatic UC treated with first-line platinum-based chemotherapy ($n=27$), and MIBC from the TCGA who received AC ($n=74$) were analyzed. Among patients who harbored *FGFR3* mutations, no pCR after NAC was observed [36].

A retrospective experience performed a comprehensive WES and genomic variant call format (VCF) to calculate tumor mutational burden (TMB) to predict cisplatin-gemcitabine NAC response in 23 MIBC patients. DDR alterations confirmed a significant and positive correlation with high TMB and were noticed in 38.1% cases with *TP53* (45%), *ARID1A/B* (40%), and *KMT2B/C/D/E* (35%) as the three most frequently mutated genes. Although a trend as higher TMB load was noticed in patients who achieved pDS, this did not meet the significance endpoint. Virtual karyotype analysis observed an amplification of the chromosomal region 7p12 in 71.4% of non-responders. This

location encompasses several genes which are implicated in the acquisition of resistance to cisplatin such as *HUS1*, *ABCA13*, *EGFR*, *FIGNL1*, and *IKZF1* [37].

Furthermore, the preliminary results of SWOG S1314 require a mention. This was a randomized phase II controlled trial of co-expression extrapolation (CoXEN) comparing two types of neoadjuvant chemotherapy: Dose-dense MVAC or cisplatin-gemcitabine-based NAC. CoXEN represents a "correlation of correlations", an algorithm based on gene expression model including 60 cell lines from nine common malignancies types (NCI-60) to predict the individual patient's probability of responding to a specific NAC scheme. Preliminary results demonstrated that CoXEN was not predictive of response in individual arms [38].

2.3. Molecular subtypes

Substantial work by multiple groups has defined key molecular subtypes of UC characterized by distinct gene signatures, varying expression of potential drug targets and differing chemotherapy sensitivity [39—42]. Whole transcriptome mRNA expression profiling has independently been conducted for this purpose by several working groups [39—42]. Although a general agreement about division in luminal, basal/squamous, and neuronal subtypes already exists, recently an international panel of experts reached a consensus on a set of six molecular classes. Luminal papillary, luminal nonspecified, luminal unstable, stroma-rich, basal/squamous, and neuroendocrine-like have been defined as different entities regarding underlying oncogenic mechanisms, infiltration by immune and stromal cells, and histological and clinical characteristics including outcomes [43]. Generally, the basal subtype was strongly characterized by high expression of *KRT5/6*, *KRT14*, indetectable *FOXA1* and *GATA3*, whereas the opposite scenario together with *FGFR3* mutation was peculiar in the luminal subtypes [44].

Choi et al. [41] demonstrated that basal subtype was predictive of response to NAC, whereas the p53-like subtype poorly responded. Luminal papillary tumors harbored the best prognosis irrespective of the treatment received [41]. These results were next validated within the context of a prospective phase II clinical trial of neoadjuvant dose-dense MVAC plus bevacizumab before RC confirming the chemoresistance of p53-like tumors traducing into a significant worse 5-years OS rate: Basal 91%, luminal 73%, and p53-like 36%, respectively. Interestingly, a paradigm shift toward p53-like in tumor that started as either basal or luminal was noticed with the development of resistance to MVAC [45].

Whole transcriptome profiling was performed on a retrospective cohort of 343 MIBC patients treated with NAC at different schemes (mainly cisplatin-gemcitabine). Similar to previous study, basal subtype was predictive of response to NAC showing a relevant improvement in OS compared with surgery alone. Claudin-low subtypes were associated with poor OS irrespective of treatment regimen [46]. Claudin-low tumors were characterized by the highest indication of immune infiltration. They also demonstrated gene expression patterns typical of active immunosuppression likely associated with broad upregulation of cytokine and chemokine levels from low *PPARG* activity. Immune gene signatures included increased rates of *RB1*, *EP300*, and *NCOR1* mutations and decreased rates of *FGFR3*, *ELF3*, and *KDM6A* mutations. Clinical relevance might be represented as an indicator of response to novel ICIs [47].

As newly recognized as an independent molecular subtype of conventional UC, neuroendocrine (NE)-like, Grivas et al. [48] performed a single-sample genomic classifier on a retrospective multicenter cohort of patients who underwent cisplatin-based NAC and subsequent RC. NE-like subtypes robustly expressed neuronal-associated genes including *SYP*, *TUBB2B*, and *PEG10*. The author showed that NE-like tumors had a significant worse CSS if compared to their counterpart but pDS rate was comparable (36.5% *vs.* 40.0%, respectively) [48].

To characterize the value of epithelial—mesenchymal transition (EMT) effectors to predict response to cisplatin-based NAC, a retrospective analysis of tissue microarray by Hensley et al. [49] showed that NAC decreased cofilin phosphorylation and induced EMT phenotype. Increased expression of mesenchymal markers and actin-cytoskeleton regulators in pretreatment specimens was associated with pathological progression on NAC and adverse clinical outcomes [49].

As the effect of conventional and targeted systemic agents is both cytotoxic and genotoxic, the concept of tumor plasticity should be emphasized. This is a dynamic process in which changes in biomarker landscape from matched pretherapy and post-therapy tumor samples reveal the complex interplay between the disease, immune system and microenvironment and how much tumor cells can adapt and respond to therapeutic stress. Molecular subtyping seems attractive and promising as it represents the mirror of biological and clinical interpatient heterogenicity in response to systemic agents potentially allowing a comprehensive pre-treatment counselling.

2.4. Liquid biopsy

Liquid biopsy, such as circulating tumor cells (CTCs) or circulating cell-free/tumor DNA (cf/tDNA), constitutes a promising and less invasive technique that can be a useful tool to overcome the limits related to conventional diagnostic methods [50]. Isolating and analyzing such materials from bodily fluids represents a crucial step to interrogate metastatic disease allowing longitudinal monitoring for progression and response to therapy [51].

2.4.1. CTCs

CTCs are cells that have shed into the vasculature or lymphatics from a primary tumor. CTCs can undergo biological processes such as the EMT or centrosome amplification [52]. The detection and analysis of CTCs can assist on determining patient prognosis, personalized treatments as well as initial diagnostic and monitoring procedures. Moreover, CTCs are particularly suited to interrogate functional heterogeneity by combining genetic and transcriptomic

assessment of single CTC [53] or by transcriptome and epigenome analysis [54] providing information about tumor biology. Several technologies for CTCs isolation and detection are based on negative selection of white blood cell membrane markers such as CD45 and positive selection using magnetic beads coupled to anti-epithelial cell adhesion molecule (EpCAM). Alternatively, employing immunofluorescence proprieties of CTCs to enrich for them, as the CellSearch method, is commonly utilized [55]. The detection rate of CTCs in localized MIBC is still relatively low, and only 30% of patients are CTC-positive [56]. Furthermore, CellSearch system depends on EpCAM expression which can be influenced by changes in the tumor with loss of EpCAM expression, the latter is expected as metastatic cells undergo processes such as EMT.

In a small prospective study, the authors examined the CTCs using CellSearch in 26 patients undergoing cisplatin-based NAC and the response rate according to the RECIST criteria. Four patients had more than five CTCs detected prior to NAC administration. All four patients experienced a decline in CTCs post-NAC (median decline of 17 CTCs). Furthermore, the patient who experienced the largest CTCs decline showed better PFS [57].

Alva et al. [58] correlated CTCs number with pathological stage in BCa patients receiving cisplatin-based NAC at baseline and after one cycle using CellSearch isoflux. Median number of CTCs decreased from 10.5 to 3.75 after the first cycle, and patients with pDS had a more significant decline compared to their counterpart [58].

Among 31 patients (26 BCa and five UTUC) treated with first-line MVAC for metastatic UC, Fina et al. [59] evaluated CTCs mRNA detected by AdnaTest from blood samples at baseline and after two cycles. No association between CTCs count and objective response to MVAC was found. However, CTCs dynamic changes better predicted 3-year PFS and OS compared to CTCs status assessed at single time points. This suggests that early CTCs changes during treatment could serve as a predictive tool [59].

Several studies have failed to prove the predictive role of CTCs regarding systemic therapy administration. Among the subset of patients who received NAC, no differences were found in terms of CTCs' number compared to those who didn't suggest similar clinicopahological characteristics in two cohorts [60]. Moreover, considering a subset of 50 BCa patients who received platinum-based AC for $\geq$pT3 or pN+ after RC, presence of CTCs did not influence outcomes [61]. Cirguidance (NTR4120) is currently recruiting MIBC RC candidates aiming to assess the patients without detectable CTCs (as a marker of good prognosis), in whom NAC administration can be omitted. The selected cut-off of CTCs is 1 with the primary endpoint of 2-years OS [55].

2.4.2. Circulating cfDNA and circulating tumor DNA (ctDNA)

Most of cfDNA is double-stranded molecules which circulate as nucleoprotein complexes in blood. In human plasma, cfDNA circulates predominantly as nucleosomes [62] in which histone modifications may also be tumor-specific [63]. Studies on different malignancies have shown that cfDNA from patients with advanced cancer contains ctDNA, and that its presence in plasma correlates with disease recurrence and poor outcomes [64]. DNA released from tumor cells by different molecular processes such as cell apoptosis, immune response-related, necrosis, micrometastasis, and secretion has been considered to be an important cancer biomarker [65]. Limited data suggested that in BCa, the level of ctDNA in plasma or in urine could be useful diagnostic and prognostic marker [66,67].

Deep sequencing of ctDNA could provide a window into the tumor genome potentially predicting response to specific treatments [68]. Agarwal and coworkers [69] confirmed that among 369 metastatic UC patients, cfDNA NGS was able to identify a similar profile of genomic alterations for biomarker-driven clinical trials compared with tumor tissue. Thus, blood-based genomic screening tests will be increasingly attractive as a non-invasive technique to characterize eligibility for targeted therapies.

In a comprehensive study of ctDNA in patients with BCa, Christensen et al. [70] addressed the prognostic and predictive value of ultra-deep sequencing of ctDNA in 68 patients who received NAC (including cisplatin/carboplatin, gemcitabine or etoposide) and RC for MIBC (median follow-up of 12 months). They first performed WES of tumor tissue to identify specific somatic variants and next interrogated cfDNA using multiplex RT-PCR NCG based on 16 specific mutations. A total of 656 plasma samples were procured at time of diagnosis, during NAC, before RC and during surveillance. The authors found that the presence of ctDNA before NAC was predictor of worse RFS and OS. Of note, after NAC and before RC in ctDNA-positive patients, a significantly higher overall 12-month recurrence rate was observed (75% *vs.* 11%). No pCR was observed in ctDNA-positive patients and all pT0 patients were ctDNA-negative. After RC, ctDNA analysis correctly identified all patients who developed metastatic relapse during disease monitoring (100% sensitivity and 98% specificity). Furthermore, expression profiling for tumor subtype and immune signature analyses found a high contribution of mutational signature associated with ERCC2 status in patients who responded to NAC [70].

Performing digital droplet PCR (ddPCR)-based assays targeting single nucleotide variants or small insertions or deletions (INDELs) located in cancer driver genes to monitor BCa after treatment, Birkenkamp-Demtröder et al. [71] evaluated 60 patients. Of these, 50 patients were scheduled for NAC (mainly cisplatin-gemcitabin scheme) and 10 patients for salvage therapy due to metastatic progression (including vinflunine, carboplatin-etoposide and atezolizumab). This approach first analyzed the primary tumor mutations and next cfDNA was interrogated using ddPCR. The detection of ctDNA after RC predicted clinical relapse significantly earlier compared to conventional radiology (137 *vs.* 275 days). In addition, there was a significant difference in the plasma ctDNA level after RC between disease-free patients and patients with clinical relapse. Notably, ctDNA was also detectable in half of the disease-free patients at scheduled control visits, however, at low mean copies amount (2.6 copies/mL). Considering only the

subset of patients with metastatic disease, overall decrease of ctDNA after two to five cycles of treatment was observed ($p=0.002$) [71].

Sequencing the primary tumor on each patient with the intent to identify specific mutations and then using ctDNA analysis for surveillance could be time consuming and expensive. In addition, as ctDNA may harbor *de novo* mutations, novel methods to analyze ctDNA are needed. As a consequence, Patel and colleagues [72] studied both plasma and urine cfDNA in 17 MIBC patients before and during cisplatin-based NAC. They used a targeted NGS panel including eight commonly mutated BCa genes and assessed single nucleotide variants (SNVs) and copy number alterations (CNAs) in cfDNA and shallow whole genome sequencing (sWGS). During NAC, five out of six patients with detectable ctDNA (plasma or urine) experienced disease relapse which did not occur in their counterpart ($p=0.003$) [72].

Liquid biopsy has shown the potential to guide perioperative decision making in patients undergoing systemic therapies. Data about ctDNA especially with patient-specific NGS assays or ddPCR seem hopeful but far from ready for prime time and incorporation to clinical daily practice. It should be integrated in currently planned UC trials to shed more light on molecular circulating biomarkers field. Such circulating biomarkers offer the opportunity of dynamic and non-invasive monitoring during treatment with the potential to inform therapeutic decision-making.

2.5. miRs

miRs are endogenous small noncoding RNA molecules (19—22 nucleotides in length) that bind to the 3′-untranslated region (3′-UTR) of mRNA and can modulate gene expression. They are implicated in the regulation of different process like proliferation, migration, invasion, and apoptosis and many of them can be easily found in tissues, serum, and urine and evaluated with the real-time quantitative reverse transcription PCR (RT-qPCR) [73]. Several, miRs have confirmed their oncogenic or oncosuppressor role in various human carcinomas [74]. Differences in different miRs signatures have been described, and some data support their diagnostic and prognostic value [75]. The use of NGS approach has revolutionized the analysis of miRs allowing a more detailed and comprehensive evaluation compared to the micro-array and RT-qPCR methods that have historically been the preferred techniques [51]. Although for many other tumors robust data are already available, only sparse data exist about the predictive role of these molecule exists in UC.

In a prospective study of 30 patients with locally advanced ($\geq$T4 and/or N2-3) and/or metastatic (M1) UC who were treated with first line platinum-based chemotherapy, Nordentoft and colleagues [76] profiled the expression of 671 miRs in formalin fixed paraffin embedded urothelial tumors. Authors observed miRs profiling correlated with radiological response with reported effects of regulation of key miRs on the cisplatin sensitivity. Moreover, they validated the top five ranked molecules by Taqman RT-PCR. Three (miR-886-3p, miR-923, and miR-944) of these were associated with both endpoints. In an *in vitro* sub-analysis including eight BCa cell lines with different cisplatin sensitivity, they found that decreasing miR-138 increased the cisplatin sensitivity and increased level of miR-27a and miR-642 [76].

Drayton et al. [77] measured miRs expression in paired cisplatin-resistant and -sensitive cell lines studying the expression of a panel composed by 357 miRs with microfluidic cards. The authors evaluated tissue samples from 354 patients, of which 215 were treated with cisplatin-based AC in a phase III study. miR-27a was found to target the cystine/glutamate exchanger SLC7A11 playing a critical role in cisplatin resistance through modulation of glutathione (GSH) biosynthesis. Furthermore, clinical relevance has been highlighted identifying that cisplatin resistance could be reversed by either reintroduction of the specified miR and small interfering RNA (siRNA)-induced knockdown of SLC7A11, or by inhibition of this exchanger with a target molecule [77].

As an integration between these miRs and key regulators of inflammation-producing prostaglandins such as COX-2, Bu et al. [78] showed that overexpression of miR-101 significantly increased the anti-proliferative effects and apoptosis induced by cisplatin. Furthermore, downregulation of miR-101 induced cell survival and cisplatin-resistance through the upregulation of *COX-2* expression. A luciferase reporter vector demonstrated that *COX-2* was a direct target gene of miR-101. As a consequence, inhibition of COX-2 using COX-2 siRNA canceled the drug resistance mechanism induced by miR-101 downregulation [78].

Studying RNA extracted from tumor tissues of 83 patients with advanced BCa receiving first-line platinum-based or MVAC AC (39% *vs.* 54%, respectively), Bellmunt et al. [79] evaluated a miR panel by RT-qPCR. Though the *in vitro* analysis could not confirm miR-21 as modulator of platinum sensitivity at multivariate level, higher levels of E2F1, miR-21, and miR-372 were independently associated with a shorter PFS [79].

Including tissue specimens from 89 cases of BCa who received three courses of cisplatin-based NAC, Li and coworkers [80] evaluated the expression of miR-101-3p and *EZH2* gene signature. The authors found that the expression of miR-101-3p is positively related to cisplatin treatment sensitivity through targeted silencing *EZH2* gene pathways.

A recent experience conducted by Yuan et al. [81] considered the role of circular RNA (circRNA). circRNA is a covalently closed RNA molecule without a 5′ terminal cap structure and 3’ terminal polyadenylated tail that can act as a competitive endogenous RNA to regulate the biological activity of different miRs influencing their inhibitor action in a target gene region [82]. The authors found that circRNA had a cisplatin-chemosensitization effect on BCa cells through the Cdr1as/miR-1270/*APAF1* axis [81].

Lastly, long noncoding RNAs (lncRNAs) are RNA transcripts longer than 200 nucleotides which are not transcribed into a protein. LncRNAs outnumber the protein-

coding genes, and their expression is often lineage-specific and in malignancies even cancer-specific. Many of these are able to modify gene expression at different levels, either through interactions with chromatin modifiers or through the regulation of mRNA stability and transcription [83] and even were shown able to modify chemotherapy response in BCa [84]. Dudek et al. [85] analyzed lncRNA expression associated with response to platinum-based chemotherapy in metastatic UC patients using data from the MiTranscriptome lncRNA expression database. LINC00857 was found to be upregulated in tumors from patients who did not respond to therapy. Moreover, high expression of LINC00857 is predictive of shorter RFS and OS. Knockdown of LINC00857 using selective siRNAs sensitizes BCa cells to cisplatin [85].

As an emerging field of investigation in BCa, miRs appear to be far away from having a definitive role as predictive tool. Nevertheless, even if embryonic, the results seem promising and could form the basis for the search for new therapeutic targets.

3. Immunotherapy

While ICIs have demonstrated an OS benefit in metastatic UC, only the minority of the patients gain that benefit, and ongoing research is aimed at identifying biomarkers of response to PD-1 and other checkpoint inhibitors. Initial efforts on grouping responders by PD-L1 expression, the Cancer Genome Atlas Program (TGCA) subtype, and interferon-gamma signatures have not yielded consistent associations, and have failed to identify clinically relevant predictive factors of benefit [86,87]. Focusing on molecular subtypes, in the IMvigor210 trial TCGA cluster II benefited most from treatment with atezolizumab [88]. Conversely, in the CheckMate275 patients grouped into TCGA cluster III showed a better response to nivolumab compared to the other subgroups [89].

The predictive value of PD-L1/PD-1 expression has been extensively studied. In the first-line setting, phase II trial of pembrolizumab or atezolizumab in cisplatin-unfit patients demonstrated responses across all PD-L1/PD-1 groups [90]. Conversely, interim analysis of phase III trials Keynote-361 and IMvigor-130 showed inferior survival among patients with low biomarker expression [91]. Thus, the Food and Drug Administration (FDA) has restricted ICIs (pembrolizumab or atezolizumab) in this setting in patients who harbored high PD-L1 expression based on companion assay [91]. Recently, a systematic review and meta-analysis considering patients who progressed after first-line platinum-based therapy found higher overall response rate, OS, and PFS in patients who were PD-L1/PD-1 positive compared to those who were negative [92]. Common weakness of PD-L1/PD-1 biomarker evaluation included high heterogenicity in employed assays and cut-off across studies.

3.1. Tumor mutational burden (TMB) and ICIs

UC carry a high TMB providing a rationale for testing TMB as a surrogate for response to ICIs [93,94]. Such instability is one of the leading cause of tumorigenesis and contributes to the expression of neoantigens that can activate CD8+ effector cytotoxic T lymphocytes (CTL) to act against tumors [95]. TMB is generally defined as the number of somatic, coding, indels mutations, and base substitution per megabase of genome examined [96].

In the exploratory biomarker analysis of the IMvigor210 trial testing atezolizumab monotherapy in patients with advanced or metastatic BCa, high TMB was predictive of better OS with higher median TMB in responders. IMvigor210 findings were independent from the PD-L1 expression implying that assessment of TMB might have a predictive value to complement the PD-L1 status [88]. These results were next validated in IMvigor211 [97].

Extended follow-up and biomarker analysis from CheckMate275 that explored nivolumab in patients with advanced platinum-resistant UC, showed that higher TMB correlated with objective response rate, better PFS, and OS. Furthermore, when TMB was combined with PD-L1, it better predicted these endpoints than PD-L1 expression alone [89].

A recent Chinese experience conducted by Zhu et al. [99] first identified somatic mutations using TCGA dataset and International Cancer Genome Consortium (ICGC) dataset finding eleven frequently mutated genes (*FGFR3*, *TTN*, *XIRP2*, *CREBBP*, *PIK3CA*, *TP53*, *MUC16*, *EP300*, *ARID1A*, *ERBB2*, and *KDM6A*). *EP300*, known to encode an adenoviral E1A-binding proteins functioning as a transcriptional cofactor and histone acetyltransferase (KAT) [98], was associated with higher TMB speculating; its mutation might enhance immune response as a mainstream action in ICIs therapies [99].

Lim et al. [100] systematically identified TMB within the germline genetic polymorphisms associated with variable tumor tissue gene expression across 24 human cancer types, which showed that *ERAP2* gene stratified OS in a subset of patients with BCa receiving atezolizumab [100].

As a cornerstone paper in this field, PURE-01 (NCT02736266), a phase II trial investigating pembrolizumab at NAC regimen, showed a pCR rate of 42% [101]. The biomarker analyses included a comprehensive genomic profiling and PD-L1 combined positive score (CPS, Dako 22C3 antibody) assessment of pre- and post-therapy tissue samples (cut-off 10%). Revealing the complex interplay between TMB and CPS, Necchi et al. [101] showed that increasing TMB and CPS values featured a linear association with logistic pT0N0 and for patients harboring high TMB values, complete response was significantly associated with higher CPS. Based on these data, a composite biomarker-based pT0N0 probability calculator was proposed.

Disappointingly, these results are in disagreement with those reported in ABACUS trial (NCT02662309): A phase II study, testing atezolizumab before RC in 95 cisplatin-ineligible patients with MIBC. According to this experience, preexisting T cell immunity seems to be the driving factor to achieve the pCR endpoint whereas TMB in association with DDR genes signatures failed to show any predictive value [102]. A partial agreement between ABACUS [102] and PURE-01 [101] results is represented by the correlation with pathologic response and the level of

pre-existing immunity, documented by CD8+ T-cell infiltration or by immune-related gene signatures.

3.2. Liquid biopsy

Anantharaman et al. [103] assessed PD-L1 expression on CTCs in the nucleated cells by immunofluorescence staining being able to determine its presence in both keratin-positive and keratin-negative CTCs. Results were still premature but may suggest PD-L1 expression on CTCs as a marker for response to ICIs. However, the roles of PD-1 and PD-L1 expression even on primary immune or tumor cells as a biomarker for response to ICIs remain unclear which are partially due to the use of different methods and cut points between studies [88,90,104].

In a mixed evaluation on two validation cohorts of metastatic UC patients ($n=29$) who progressed after first-line treatment and non-small cell lung cancer (NSCLC) patients ($n=72$) treated with novel anti-PD-L1 ICI durvalumab, Raja et al. [105] explored the utility of ctDNA in an open-label dose-escalation and dose-expansion setting. Using a broad NGS-based mutation panel, they demonstrated a strong relationship between clinical outcome (PFS and OS) in the metastatic setting and early reduction (6 weeks) in ctDNA variant allele frequencies (VAF) after initiation of treatment with durvalumab.

To date, the Treatment of Metastatic Bladder Cancer at the Time of Biochemical reLApse Following Radical Cystectomy (TOMBOLA) study (NCT04138628), enrolls patients who have undergone NAC and RC. The authors aim to monitor cfDNA after surgery using ddPCR as a tool to receive further treatment (atezolizumab) if cfDNA is detectable.

For the time being, in UC setting, no clinically applicable biomarker has succeeded to identify patients that will benefit from PD-L1/PD-1 inhibition therapies. The increasing number of patients treated with ICIs highlights the much needed reliable biomarkers to inform efficacy and help prevent unnecessary treatment toxicity. Ongoing studies in this space are in place.

4. Novel expanding systemic targeted therapies

Novel expanding systemic targeted therapies such as FGFR inhibitors and antibody—drug conjugate against Nectin-4 provide additional salvage options.

The group of FGFRs comprises four structurally related tyrosine kinase receptors (FGFR1-4) whose activation pathway is a common event in UC [106]. Considering data coming from phase I trial, the predictive role of FGFR3 alterations was confirmed among 67 metastatic patients unable to receive platinum-based therapy. This trial tested the efficacy of infigratinib, a potent and selective pan-FGFR antagonist, showing a complete response rate of 25.5% [107]. As complementary information, Pal et al. [108] demonstrated a high concordance (79%) about FGFR3 expression between matched pair tissue samples and plasma cfDNA among metastatic UC patients token infigratinib. Thus, plasma cfDNA could be interrogated serving as non-invasive platform of counselling [108].

A phase I dose-escalation and dose-expansion study of rogaratinib, another oral pan-FGFR inhibitor, including 52 UC patients ineligible for standard therapy demonstrated an objective response rate of 23.1%. Biomarker positivity was defined by *FGFR3* mRNA overexpression and was mandatory for enrollment [109]. Erdafitinib, a pan-FGFR inhibitor, showed an objective response rate of 40% (3% complete and 37% partial) in locally-advanced or metastatic setting. Using a custom RT-PCR assay on RNA from formalin-fixed tumor samples, the authors enrolled patients who harboured at least one *FGFR3* mutation or *FGFR2/3* fusion as oncogenic drivers [110]. Owing to these findings, erdafitinib was approved by FDA. Indications included locally-advanced or metastatic UC in platinum-refractory patients who had selected *FGFR2/3* mutations or fusion [111].

Another solution has entered in the third-line scenario: Enfortumab vedotin has been approved in the post-platinum, post-immunotherapy setting based on Phase II single-arm data [112]. This is an antibody—drug conjugate against Nectin-4, a cell adhesion molecule expressing in more than 90% of UC samples [113]. The response rate achieved was 44% with a median PFS and OS of 5.8 months and 11.7 months, respectively [112]. Since the presence of Nectin-4 and it's mostly widespread, biomarker testing is not needed for treatment with enfortumab vedotin.

Although promising data could come from ongoing trials, given the variable efficacy of these agents, the combined administration (ICIs and other therapy lines) with generated side effects due to pan-activity, makes the validation of predictive biomarkers for novel molecules very challenging.

5. Conclusions

Ongoing trials testing the safety, feasibility, and efficacy of novel targeted agents in any therapy line that incorporate relevant biomarkers provide an ideal setting for prospective evaluation of different platforms of analysis. Main limitations of the above-mentioned studies include small cohorts that varied in composition, gene expression or molecule profiling technologies, retrospective collection of clinical data, and incomplete information regarding patient treatments. Thus, further prospective validations are awaited. As a paradox, opportunities for biomarker development and validation are decreasing. Current and upcoming trials largely focus on combinations of ICIs with different systemic therapies including novel targeted (NCT03288545) or conventional agents (NCT02853305). Such a settings could not allow to identify a solid correlation between a biomarker and a specific treatment.

Predicting response to systemic agents is a fascinating challenge which develops through the evolution of a panel of convincing molecular biomarkers capable of performing despite interpatient and intratumor heterogenicity. Emerging biomarkers have shown promise but need further validations before entering into the clinical daily practice.

Further understanding of molecular tumor biology is a mandatory step to find a predictive signature that can be used to select appropriate treatments towards the convergence of precision oncology with both conventional and novel systemic agents.

Author contributions

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Conflicts of interest

The authors declare no conflict of interest.

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