Emerging biomarkers and targeted therapies in urothelial carcinoma

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Contributions: (I) Conception and design: All authors; (II) Administrative support: None; (III) Provision of study materials or patients: None; (IV) Collection and assembly of data: All authors; (V) Data analysis and interpretation: All authors; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abstract: The use of immunotherapy has revolutionized the management of patients with locally advanced, unresectable, and metastatic urothelial carcinoma (UC); however, platinum-based chemotherapy remains a therapeutic cornerstone both in localized muscle-invasive and advanced UC. There is still no predictive molecular biomarker with clinical utility to help guide treatment and select patients most likely to derive benefit from a particular therapeutic modality or regimen. However, recent research has further characterized the inherent biology and immunology landscapes of UC leading to the development of potential biomarkers and therapeutic targets that could be used upon further validation. Emerging interrogation of The Cancer Genome Atlas (TCGA) and other molecular profiling datasets has led to the identification of distinct molecular subtypes with diverse clinical behaviors with potential sensitivity to various therapies. It has also led to the discovery of multiple frequently altered genes and proteins that could lead to perturbation of intracellular signaling pathways and of the dynamic interactions between tumor cells, their "microenvironment", and the host "macro-environment". The advent of molecular profiling and deeper next-generation sequencing has the potential to change biomarker and "real time" drug sensitivity assessment, introducing and testing the premise of "precision oncology" and personalized medicine. Within this review, we summarize emerging biomarkers that may predict response to cisplatin-based chemotherapy, immunotherapy, emerging targeted therapies, and promising combination strategies. We also highlight a few examples of 'precision medicine' trials aiming to improve outcomes in UC. Since our review is not exhaustive we strongly recommend the readers to follow the continuously changing literature in the very interesting and dynamic field of UC.

Keywords: Urothelial carcinoma (UC); bladder cancer; biomarkers; targeted therapies; precision oncology

Submitted May 14, 2018. Accepted for publication May 24, 2018.
doi: 10.21037/atm.2018.05.49
View this article at: http://dx.doi.org/10.21037/atm.2018.05.49

Introduction

Bladder cancer is the fourth most common type of cancer in men and can also affect women, representing about 5% of all new cancers with estimated 17,240 deaths expected in United States alone in 2018 (1,2). Urothelial carcinoma (UC) is the most common histologic type and can arise from the entire urothelial tract, e.g., renal pelvis, ureter, bladder (most common site), urethra. Most patients present with non-muscle invasive disease, which is managed by transurethral bladder tumor resection (TURBT) with or without intravesical therapy usually with favorable

prognosis; however, about 25% of patients present with muscle-invasive bladder (MIBC) with less favorable prognosis, while a proportion progress to metastatic disease that has an estimated 10–15% overall survival rate at 5 years (2,3). The standard management of MIBC remains the use of either neoadjuvant cisplatin-based chemotherapy (for patients who can tolerate cisplatin) followed by radical cystectomy and pelvic lymph node dissection, or concurrent chemoradiation as bladder preservation approach (4-6). For locally advanced, unresectable, and metastatic (advanced) disease, platinum-based chemotherapy and immune checkpoint inhibitors (CPIs) are routinely used, however, there is an urgent unmet need for novel therapies, esp. for those who progress on prior therapies, since advanced UC is usually a fatal condition with major impact on quality of life (7-9).

Unfortunately, only a proportion of patients respond to initial systemic therapy and most, if not all, patients eventually develop resistance with limited responses to chemotherapy in the second-line setting and beyond (10-12). Also, the subset of patients who are cisplatin-ineligible and treated with carboplatin-based regimens in the first-line setting seem to have inferior outcomes compared to those treated with cisplatin-based regimens (9). Further understanding of the key features of the immune system and its ability to attack cancer cells has ushered in an unprecedented era of immunotherapy regimens in both solid and hematological malignancies. Antibodies directed against the checkpoints 'programmed cell death 1' (PD-1) and 'programmed death ligand 1' (PD-L1) were shown to induce rapid and durable responses in advanced UC in the salvage setting and for patients in the first line-setting who are cisplatin-ineligible (13-19). These remarkable findings altered the treatment landscape and led to the FDA approval of five CPI (atezolizumab, avelumab, durvalumab, nivolumab, pembrolizumab) in the platinum-resistant setting, and two agents (atezolizumab, pembrolizumab) in the first-line setting for cisplatin-ineligible patients (advanced UC).

Although significant improvement has been made, there remains no predictive molecular biomarker with clinical utility to determine which patients are most likely to benefit from a specific regimen. There are no curative therapies for patients with advanced disease and most patients eventually progress on systemic therapy highlighting the need for the development of novel therapeutics with high efficacy and good tolerability. Molecular profiling through the TCGA (20,21) and other molecular datasets (22) has further dissected the molecular underpinnings of advanced UC showing that there is major tumor heterogeneity and molecular redundancy. However, key recurrent genetic alterations have been discovered with potential promise of targeted therapeutic approaches for well selected patients. In this non-exhaustive review, we discuss differences between predictive and prognostic biomarkers, currently used systemic therapies, the emerging role of putative biomarkers in predicting response to platinum-based chemotherapy and/or immunotherapy, novel targeted therapies and immune-oncology combinations, as well as future directions, including molecularly driven clinical trials.

Predictive and prognostic biomarkers

A biomarker is defined as the measure of a substance and/or variable whose presence is indicative/surrogate of a disease outcome. It is critical to differentiate between predictive *vs.* prognostic biomarkers, as highlighted in the literature (23,24). A prognostic biomarker is defined by its ability to inform the natural history of disease or specific cancer endpoint independent of a particular treatment. A predictive biomarker is based on its ability to discriminate and determine differences in treatment-specific responses (experimental *vs.* control), e.g., in biomarker-positive and biomarker-negative patients, respectively (23). An ideal biomarker should be reproducible, accurate, validated in multiple datasets, and easy to use. Other solid tumors, such as breast, colorectal and lung cancer, have established the role of predictive biomarkers in clinical practice (25), but the search of predictive biomarkers has remained elusive in advanced UC. The focus of this review is to discuss the role of putative predictive biomarkers in advanced UC.

Current systemic therapy options for advanced UC

For patients who present with resectable MIBC (clinical T2–T4a/N0–1 stage), consensus guidelines recommend the use of neoadjuvant cisplatin-based chemotherapy (for cisplatin-fit patients; level I evidence) followed by radical cystectomy and pelvic lymph node dissection (26). Patients who are cisplatin-ineligible but surgically resectable proceed directly to cystectomy; while clinical trials are a very important option for both patient subsets. There is data regarding the use of adjuvant cisplatin-based chemotherapy in patients who did not receive neoadjuvant chemotherapy. A trial meta-analysis and an intergroup trial (EORTC

30944) comparing adjuvant *vs.* deferred (at the time of relapse) chemotherapy showed statistically significant improvement in recurrence-free survival (and probably marginal overall survival benefit based on the totality of the data) for those receiving adjuvant (*vs.* deferred) chemotherapy (27-29).

In well selected patients who are not surgical candidates or refuse definite cystectomy, bladder preservation with multimodality therapy remains a viable option. The cornerstones of multimodality management involves optimal debulking TURBT followed by radiation therapy with concurrent chemotherapy (30). There have been no reported completed randomized trials comparing bladder preservation *vs.* radical cystectomy (with or without neoadjuvant cisplatin-based chemotherapy). Ideal candidates for bladder preservation approach include those with maximal TURBT prior to starting therapy, absence of tumor-associated hydronephrosis, absence of extensive carcinoma *in situ*, and unifocal tumor without nodal metastases (30). The addition of chemotherapy (cisplatin or 5-fluorouracil/mitomycin-C or gemcitabine) to concurrent radiation has been shown to improve locoregional disease-free survival with trend towards overall survival improvement, and decrease in first recurrence in pelvis (31-33). There are no trials that have defined the optimal chemotherapy regimen to use with radiation and bladder preservation trials are ongoing.

For patients with advanced UC the recent emergence of CPI has revolutionized the field; however, the optimal therapy sequence and defining the ideal patient likely to benefit from each therapy remains to be determined. Gemcitabine/cisplatin and accelerated (dose dense) MVAC (methotrexate, vinblastine, adriamycin, cisplatin) remain the standard of care for cisplatin-eligible patients, while gemcitabine/carboplatin is frequently used for cisplatin-unfit patients. Based on historical controls, for cisplatin-ineligible patients (due to ≥ grade 2 neuropathy or hearing loss, class III/IV congestive heart failure, estimated glomerular filtration rate <50–60 cc/min, or performance status ≥ ECOG 2) (34), the FDA approved single agent atezolizumab and pembrolizumab based on phase II single arm studies (14,16). After first-line chemotherapy, most patients eventually progress and limited benefit remains with non-platinum systemic chemotherapy. The use of CPI in the salvage setting has allowed patients to derive potential durable clinical benefit with less toxicity. Atezolizumab, durvalumab, nivolumab, and avelumab have been FDA-approved in the post-platinum setting based on improvement of durable responses compared to historical control (13,15,18,19). The only CPI approved in the post-platinum setting with level I evidence based on overall survival improvement when compared to chemotherapy (paclitaxel or docetaxel or vinflunine) in a large phase III trial is pembrolizumab (17). Only a subset of patients may respond to CPI (ORR estimated 15–21%) while progression-free survival is roughly 2 months in the salvage setting, and there is no clinically useful predictive biomarker. For those patients who progress after CPI, treatment options are limited; the optimal option is clinical trial, e.g., with antibody-drug conjugates, targeted therapies/anti-angiogenesis agents, immunotherapies, and/or various combinations; while the use of taxane-based or vinflunine chemotherapy has modest results but still used in clinical practice.

Examples of the promising role of molecular profiling in UC

The recent advances in technology, such as next-generation sequencing and gene expression profiling led to further defining the landscape of the common genetic alterations in advanced UC. The TCGA group initially reported findings in 2014 and subsequently updated them in 2017 (20,21). The group also focused on the identification of frequently occurring genetic alterations that could lead to the development of novel targeted therapies. The report in 2014 included extensive profiling (i.e., genomic sequencing, RNA, protein and microRNA expression, DNA copy number and methylation, etc.) of 131 patients with chemotherapy-naïve muscle invasive tumors and identified many frequently altered genes (20). Most alterations were noted in pathways commonly dysregulated in UC, e.g., cell cycle, chromatin remodeling, and signaling transduction pathways. Based on the 2014 data, up to 69% of patients had genomic alterations matching potential therapeutic targets (~42% in the PI3K/AKT/mammalian target of rapamycin (mTOR) pathway (including 17% activating point mutations in PIK3CA pathway, 10% overexpression of AKT3, and 9% with mutations or deletions of TSC1 or TSC2) (20). Alterations affecting receptor tyrosine kinase/RAS pathway (including 17% activation of FGFR3, 9% amplification of EGFR, 9% mutations of ERBB2, and 6% mutations of ERBB3) were noted in 44% of patients.

An updated analysis in 2017, including 412 bladder cancer specimens, resulted in the identification of numerous additional frequently altered genes (21). UC was noted to

have one of the highest mutation rates compared to other solid tumors (mean of 8.2; median of 5.8 mutations per megabase) (21,35). The mutation rate was associated with a mutation signature for *APOBEC* cytidine deaminase (in up to 70%). Patients with the combination of high mutational burden and APOBEC mutation signature had better outcome (potential prognostic biomarker). Further testing and validation of this signature is needed in clinical trials to assess whether it may induce enhanced immune activation and thus potentially improved CPI response.

Further work has led to the identification of distinct molecular subtypes with key genetic alterations and diverse outcomes which can further guide putative targets and trials within each distinct subtype. Work done through TCGA (20,21), Lund University (36), UNC (37), and MD Anderson (38) have led to varying, yet consistent themes, with a degree of overlap between the groups. Although multiple subsets have been reported by each group the most recent update from the TCGA group identified five relevant subtypes with discerning mRNA expression, biological features and reported outcomes. The neuronal subtype (5%) expressed high levels of *TP53* and *RB1* alterations, increased in proliferation and cell cycle state, and inherently may respond better to platinum/etoposide chemotherapy like other neuroendocrine tumors (21). The basal (claudin-low) subset is usually characterized by a more squamous differentiation with epithelial-mesenchymal transition (EMT) predominantly seen; however also with high immune infiltrates (CTLA4/CD274) (20,21,37). The basal subtype seems to behave more aggressively with higher stage upon presentation and worse outcomes, but with better response to neoadjuvant cisplatin-based chemotherapy (39,40).

The luminal subtype can be further differentiated into luminal, luminal-papillary, and luminal with luminal infiltrated subtypes. Within the luminal-papillary subtype, there may be lower response rate to cisplatin-based neoadjuvant chemotherapy (40); however, these patients may potentially derive more benefit from alternative strategies, such as fibroblast growth factor receptor 3 (FGFR3) inhibitors (which tends to be overexpressed in this subset) (20,21,39). Patients with the luminal-infiltrated subtype tend to have high expression of EMT that may confer resistance to cisplatin-based chemotherapy and may potentially benefit from CPI (21). The luminal subtype is not very well defined and optimal treatment strategies need to be determined. The distinct clinical phenotypes and response rates to both currently available and novel systemic therapies certainly need further validation in molecularly driven clinical trials to test the abovementioned hypotheses. The potential role and clinical utility of the several additional biomarkers described by the molecular datasets also need to be further evaluated in prospective clinical trials.

Emerging putative biomarkers predictive of response to cisplatin-based chemotherapy

One of the key mechanisms via which platinum-containing compounds can kill cancer cells is by inducing DNA damage through intra- and inter-strand DNA cross links. UC tumors can be genomically unstable and unique changes in the DNA damage response (DDR) pathways can regulate tumor progression, evolution and potential treatment response (41). The repair of cisplatin-DNA damage is mediated by DDR pathways, such as nucleotide excision repair and homologous recombination. As previously discussed, cisplatin-based therapy has a role in neoadjuvant, adjuvant, metastatic, and concurrent chemoradiation for bladder preservation, settings. It is essential to develop predictive biomarkers to identify patients most likely to benefit from platinum-based therapies; a number of such putative biomarkers is discussed below.

Excision repair cross complementing 1 and 2 (*ERCC1/ERCC2*) is one of the key enzymes responsible in the nucleoside excision repair (NER) of DNA damage. Higher levels of ERCC1 may be indicative of increased function of the NER pathway and thus has been an attractive biomarker to evaluate as prognostic and as predictive biomarker to cisplatin sensitivity. *ERCC1* protein expression by immunohistochemistry (IHC) failed to serve as biomarker predicting pathologic complete response to neoadjuvant dose-dense MVAC (42); however, in the advanced/metastatic setting, low *ERCC1* mRNA expression level measured by RT-PCR (in tumor) in a retrospective study was associated with longer overall survival (43). Further exploration of ERCC2 was analyzed in a retrospective study focusing on whole-exome sequencing on 50 pre-treatment tumor specimens who subsequently received neoadjuvant cisplatin-based chemotherapy (44). The study focused on key differences in responders (N=25; down staging to ≤ pT1) *vs.* non-responders (N=25, ≥ pT2) and determined that ERCC2 was the only gene that was significantly altered in responders (36% *vs.* 0%), data was validated in a subsequent study (45). Another study identified three DDR genes (*ATM*, *RB1*, *FANCC*) that can predict response to

neoadjuvant cisplatin-based chemotherapy in MIBC (46). Genomic data (base substitutions, copy alterations, and select gene rearrangements) from a CLIA-based lab using pre-treatment tumor was sequenced and analyzed for 287 coding exons of cancer-related genes from patients treated in clinical trials. Findings revealed higher number of alterations in those who achieved pathologic complete response *vs.* those with residual tumor (P=0.024) in the discovery and validation sets. *ATM*, *RB1*, and *FANCC* alterations could predict pathologic response in both the discovery (P<0.001) and validation sets (P=0.033) with trend toward longer overall survival. Ongoing clinical trials are evaluating prospectively the role of DDR inactivating mutations in patients with localized MIBC (A031701; HCRN GU 16-257; NCT02710734). It is worth noting that DDR alterations and homologous recombination deficiency may also predict response to a novel class of agents, PARP inhibitors; this hypothesis is being tested in clinical trials, e.g., NCT02546661; NCT03397394.

In patients treated with bladder preservation strategies using multimodality therapy there is no predictive biomarker to select patients most likely to derive benefit from this approach, e.g., chemoradiation. Investigators have evaluated a DNA strand break repair protein, MRE11, and have shown that lower level of MRE11 protein score (IHC) correlated with shorter 3-year cause-specific survival *vs.* those with higher MRE11 score in patients with pre-treatment tumor tissue treated with radiation (43.1% *vs.* 68.7%, P=0.012) (47). A subsequent analysis among six NRG/RTOG trials of MRE11 expression in patients treated with bladder-sparing protocols confirmed higher disease-specific mortality in those with lower expression (HR =2, P=0.03) (48). Further studies are evaluating the biology and role of MRE11 protein expression in the context of clinical trials.

In the advanced setting, retrospective data focused on the role of DDR genes and their role as biomarkers predicting sensitivity to platinum-based therapy. Patients with locally advanced or metastatic UC treated with cisplatin-based chemotherapy had exon sequencing and were placed in two groups (based on presence or absence of genomic alterations in a panel of pre-specified DDR genes) and their outcomes were compared (49). The study revealed that 47 out of 100 patients harbored alterations in DDR genes, while those with DDR gene alterations had longer progression-free (9.3 *vs.* 6 months, P=0.007) and overall survival (23.7 *vs.* 13 months, P=0.006); DDR alterations were also associated with higher number of mutations and copy-number alterations. A trend toward correlation between DDR status and nodal metastases and inverse correlation with visceral metastases was noted, while different DDR pathways suggested variable effect on clinical outcomes. A recent study reported that *ATM* and *RB1* mutations may be a biomarker of poor prognosis in unselected UC patients and may correlate with higher mutational load (50). Further prospective evaluation is needed to address the predictive and/or prognostic role of DDR gene alterations in advanced UC.

Beyond the role of DDR gene alterations investigators have also evaluated molecular subtypes, as previously discussed, to identify patients more likely to benefit from cisplatin-based therapy. Whole-transcriptome profiling was performed in 343 bladder cancer bio-specimens obtained prior to neoadjuvant chemotherapy and were classified according to previous molecular subtypes (claudin-low, basal, luminal-infiltrated, and luminal) (40). A single-sample genomic subtyping classifier was then trained to predict these subtypes in a single specimen and then survival was analyzed and compared according to subtype for patients treated with and without (476 cases) neoadjuvant chemotherapy. The model was able to identify subtypes with expected ratios with high concordance and accuracy (73%). Key clinical findings revealed that luminal tumors had the best prognosis, basal tumors had the worst prognosis without chemotherapy but also the highest benefit with neoadjuvant chemotherapy. Another analysis showed similar findings with the basal molecular subtype having better response compared to luminal and *p53* molecular subtypes in patients treated on a neoadjuvant phase II trial of dose dense MVAC and bevacizumab (51).

Emerging putative biomarkers predictive of response to CPI

The discovery of the inherent biology of unleashing the immune system to eradicate tumors has led to the use of CPI for advanced UC. These agents have revolutionized our approach and have current indications in the first-line cisplatin-ineligible and post-platinum spaces. Identifying patients most likely to respond to CPI has remained very difficult and the search for the ideal biomarkers has remained elusive. The complex interplay between the immune system and tumors include factors present in tumor cells and microenvironment, peripheral blood, germline variants, among others. In this section, we highlight part of the emerging data on putative biomarkers that may predict respond to CPI.

Tumor mutational burden (TMB)

The variation of TMB and the significant mutational heterogeneity among different tumor types may impact response to CPI. UC has one of the highest mutation rates, probably next to lung cancer and melanoma (35). In lung cancer and melanoma, both TMB (number of mutations per coding area of genome) and predicted neoantigens have been shown (in retrospective analyses) to have potential predictive power in determining response to CPI, higher than clinical variables or PD-L1 staining, respectively (52-54). A recent update among multiple tumor types highlighted significant correlation between TMB and objective response rate with correlation coefficient 0.74, attributing that 55% of the differences in objective response among tumor types could be explained by TMB, and suggested the application of a linear calculation formula to predict response rate based on coding somatic mutations (54).

This question was explored in UC via subgroup analysis in the IMvigor210 and Checkmate 275 clinical trials (13,14,55). In the IMvigor210, cohort I (119 tumor specimens analyzed), TMB was associated with longer overall survival (highest level-quartile 4 *vs.* quartiles 1–3) and was significantly higher in responders regardless of TCGA subtype or PD-L1 subgroup (14). In the IMvigor210, cohort II, TMB was estimated in 150 patients via a pre-selected panel of 315 cancer-related genes, revealing higher TMB in responders (12.4/MB *vs.* 6.4/MB; P value ≤0.001) (13). Analysis of TMB in 139 patients in the Checkmate 275 trial also revealed significant correlation between higher TMB and higher response rate (P=0.0006), progression-free (P=0.0001), and overall survival (P=0.003) (determined by continuous variables) when adjusted for baseline tumor PD-L1 expression and other clinical and laboratory parameters (55). Validation data was shown in the IMvigor211 phase III randomized (atezolizumab *vs.* chemotherapy) clinical trial (56). However, unaddressed issues remain regarding optimal TMB cut-off due to variability of assays/methodologies, and whether categorical *vs.* ordinal *vs.* continuous variables should be further prospectively evaluated.

PD-L1 protein expression

PD-L1 and PD-L2 are major ligands expressed in tumor cells and microenvironment leading to immune suppression and tumor evasion of host surveillance. In UC, PD-L1 expression has been shown to correlate with worse clinical outcomes suggesting its role as putative prognostic biomarker, but also confounding its role as putative predictive biomarker (57). Another major issue is that each clinical trial leading to the approval of each CPI in UC had a separate complementary assay for PD-L1 expression, thus conferring lack of standardization, alignment and applicability to clinical practice. There are variable IHC-based assays/methods, antibodies, cell measured (tumor and/or immune infiltrating), and cut-off used to define positivity (13-19). The variability and concordance of those assays have been examined in lung cancer samples, showing that although some degree of concordance may exist among most assays, there is still distinct variability (e.g., tumor *vs.* immune cells expression) leading to debate and confusion regarding the optimal platform to determine PD-L1 positivity (58-60).

There have been higher overall response rates in UC patients with tumors with high *vs.* low PD-L1 expression among studies. The more significant differences were noted with avelumab (54% *vs.* 4%) (19,61) and durvalumab (28% *vs.* 5%) (18,62); however, these were smaller studies with different antibodies/assays. Notably, responses were also noted in patients with low/null PD-L1 expression across trials. An example of how PD-L1 can be a misleading biomarker is the IMvigor211 trial, which compared atezolizumab to chemotherapy in platinum-resistant advanced UC. Based on the specific assay used in that trial, the biomarker ended up showing possibly prognostic value (better outcome with higher PD-L1 expression) but not predictive value regarding response to atezolizumab, therefore the primary endpoint of overall survival difference in the PD-L1 high patient subset was not met; however, in the intent-to-treat population there was significant overall survival benefit with atezolizumab *vs.* chemotherapy (56). Moreover, PD-L1 protein is a dynamic biomarker with variable temporal (can change over time, esp. with interim therapies) and spatial expression (sampling bias). Based on the above findings, PD-L1 expression is currently not used in clinical practice in UC patients.

Molecular subtypes based on gene expression

Investigators have also tested whether the previously discussed molecular subtypes can identify patients more likely to benefit from CPI. The inherent biology described within each molecular subtype may explain potential correlation with CPI response (21). In the cohort II of IMvigor210 trial (atezolizumab), the basal subtype was found to have higher level of PD-L1 expression *vs.* luminal

subtype; however, this did not translate to increased objective response rate; the highest response rate was actually seen in the luminal cluster II biospecimens (13). This trend was also noted in the cohort I of IMvigor210 trial with increased response rate in the cluster II luminal group (14). These findings were not exactly corroborated in the Checkmate 275 trial with nivolumab, where the highest rate of responders were actually seen in the basal subtype (15). Both these subtypes were identified using the 2014 TCGA analysis, not the updated 2017 TCGA analysis. Prospective validation in future clinical trials will offer insight in whether basal and/or cluster II luminal subtypes could respond better to each CPI.

DDR gene defects

The previously discussed family of DDR genes may also have implications in CPI-sensitivity (due to presumed increase in somatic mutations and generated neoantigens). There remains the need for further studies to clearly define the specific genes that should be used when creating predictive models and determining the ideal testing methods, e.g., specific DDR genes and/or genome-wide loss of heterozygosity. A recent study focused on exon sequencing to determine the presence or absence of pre-selected DDR genes (part of the MSKCC-IMPACT panel) in patients treated in phase II trials with atezolizumab or nivolumab (63); response rate was independently associated with DDR alterations. Investigators also tested the role of DDR gene defects in predicting response to cisplatin-based chemotherapy with ipilimumab in a single arm phase II study (64). The objective response rate was 69% for all comers (but with significant toxicity) and was significantly higher in patients with deleterious mutations in pre-selected DDR genes (two-sided Fisher's test P=0.03). Future validation of these findings will be essential via biomarker-enriched prospective clinical trials in advanced UC.

Mismatch repair defects

Further biological exploration has led to the discovery that a large proportion of cancers that have high TMB may also have evidence of microsatellite instability (MSI-high) (65). MSI-high usually results from inherent DNA mismatch repair due to germline or somatic mutations and can be commonly seen in patients with genetic syndromes, such as Lynch Syndrome (hereditary non-polyposis colorectal cancer). This inherent correlation would lead to presumed increased response to CPI in MSI-high patients. Indeed, such correlation led to the first tissue-agnostic FDA approval of pembrolizumab for patients with tumors with MSI-high or deficient mismatch repair (66). This approval was based on five non-controlled, multi-cohort, multi-center, single arm trials showing overall response rate about 40%, with durable responses greater than six months in 78% of responders (66). In UC, especially upper tract disease, there seems to be a higher incidence of underlying mismatch repair deficiency and correlation with CPI response (67,68). Moreover, germline testing should be paramount and essential in the management of patients with UC (especially with upper tract disease and/or young age of cancer diagnosis and/or relevant personal or family history).

Emerging targeted therapies

Most, if not all, patients treated with either platinum-based chemotherapy and/or CPI for advanced UC eventually develop resistance and there is a need for rational targeted therapeutic options to improve outcomes. In the following section, we highlight only a portion of promising agents being developed and studied in advanced UC.

Angiogenesis inhibitors

The upregulation of the vascular endothelial growth factor (VEGF) in patients with advanced UC has been linked to a more aggressive phenotype (69). Targeting this pathway with multiple agents has been studied with no clear evidence of overall survival benefit, while a randomized phase III trial has completed accrual with anticipated results (NCT00942331). Building on the preliminary findings of a phase II trial, ramucirumab (fully humanized monoclonal antibody targeting the VEGF receptor 2) was tested in a multi-center, randomized trial in patients who progressed on/after platinum therapy (70) (previous treatment with CPI allowed). Patients were randomized to docetaxel/ramucirumab *vs.* docetaxel/placebo with the primary endpoint being progression-free survival. Findings revealed an improvement of median progression-free survival (4.1 *vs.* 2.8 months; HR 0.75, P=0.0118) with docetaxel/ramucirumab. Further follow-up is needed to assess overall survival; however, only a very small proportion of patients had received CPI prior to study therapy.

Additional agents, such as bevacizumab (recombinant humanized monoclonal antibody targeting VEGF-A), sunitinib and pazopanib (anti-VEGF receptor tyrosine

kinase inhibitors) have demonstrated limited clinical benefit as single agents in this setting. A phase II trial included 43 patients treated with combination of bevacizumab with cisplatin/gemcitabine showing an impressive overall response rate of 72% and median overall survival of 19.1 months (95% CI: 12.4–22.7 months); but with three treatment-related deaths (71). A large phase III intergroup trial (NCT00942331) randomized trial in the front-line treatment of advanced UC is testing this combination and has fully accrued; but results are pending. There has been limited improvement with sunitinib in the first- or second-line setting (72); while, investigators studied its potential role as switch maintenance therapy in patients who had not progressed after 4–6 cycles of first line chemotherapy (73). Unfortunately, that trial had difficulty accruing and closed prematurely without difference in median progression-free survival. The experience with pazopanib was similar with no survival improvement in a randomized phase II trial (PLUTO) comparing pazopanib *vs.* weekly paclitaxel (74).

FGFR inhibitors

The FGFR pathway is essential for tissue development, regeneration, and angiogenesis. In multiple datasets, the importance of genomic alterations and expression of a specific ligand within this family, FGFR3, and its downstream pathways in the pathogenesis of patients with early to advanced UC, has been clearly illustrated (20-22, 38,75), priming it as a target for potential inhibition. Broadly, the inhibition of this key pathway has been achieved with both selective and non-selective (multi-kinase) inhibitors and is currently being investigated in multiple solid/hematological tumors including patients with advanced UC. FGFR3 expression remains variable between early and advanced stage, while recent studies highlighted that there may be higher incidence of FGFR3 alterations in upper tract *vs.* bladder UC (76).

A selective pan-FGFR inhibitor, BGJ398, showed initial promise after phase I testing that led to an expanded cohort and phase II study in patients with FGFR3 mutations/fusions who progressed on prior platinum-based therapy with overall response rate 36%, minimal toxicities (mostly Grade 1/2) and a complete response in a patient with bone metastases (77). Another pan-FGFR inhibitor, dovitinib, did not show significant activity in patients with advanced UC who progressed on platinum-based therapy (78). Another pan-FGFR inhibitor, erdafitinib, was evaluated in a phase I study in patients with multiple solid tumors showing partial responses (endometrial, glioblastoma, urothelial, and endometrial cancer) (79). Responders had alterations in the FGFR pathway, highlighting its potential role as a predictive biomarker. Rogaratinib is another anti-FGFR agent with promising activity (response rate 24%, disease control rate 73% in prior trial) that is currently being tested (80). An antibody against FGFR3, B-701, is also being investigated in a phase II randomized, multicenter trial, examining the role of B-701 plus docetaxel *vs.* placebo plus docetaxel in patients who progressed after first line therapy, while another trial is evaluating B-701 and atezolizumab in advanced UC. Ongoing clinical trials are being conducted with several anti-FGFR agents in patients with advanced UC with FGFR alterations.

Human epidermal growth factors receptor (HER; ErbB) inhibitors

The ErbB family of proteins includes predominantly four receptor tyrosine kinases that have been implicated in multiple tumors via signaling cascade leading to increased cell proliferation and resistance to apoptosis (81). Both ErbB-1 (EGFR) and the ErbB-2 (Her-2/neu) transmembrane receptors have been found to be critical in the regulation of proliferation of UC (21,75,82), thus rendering them as potential targets.

A phase II study of selected advanced UC patients with evidence of Her2/neu overexpression/amplification (52.3% of screened population) were treated with conventional chemotherapy (carboplatin, paclitaxel, and gemcitabine) plus trastuzumab in the first-line setting with dramatic response rate 70%, median progression-free survival 9.3 months, and median overall survival 14.1 months (83). Cardiac toxicity rate was higher than expected and the true added benefit of trastuzumab needs to be defined in a randomized trial. However, a phase II trial of cisplatin/gemcitabine with or without trastuzumab in advanced UC did not show major difference in outcomes between the two arms (84). A key finding from this study was that only 13% of screened patients were found to have Her2-neu overexpression; thus, potentially clouding the ability of the trial to detect differences between the groups. There also seems to be limited role of maintenance therapy with anti-HER therapy with a study finding that use of lapatinib (Her1/2 inhibitor) did not improve outcomes in patients who had prior upfront-chemotherapy and target overexpression based on IHC (85).

A unique multi-center, non-randomized, phase II 'basket' study ('MyPathway') was recently reported, consisting of

refractory patients with multiple solid tumors, evaluating the safety and efficacy of selected targeted therapies in those with relevant genetic alterations (outside currently labeled indications) (86). Among patients with Her2 alterations treated with trastuzumab/pertuzumab (dual anti-Her2 therapy) a few patients with UC were treated with noted responses including a complete response (ongoing at 15 months). The appeal of this trial lies in the foundation of using CLIA-approved molecular testing and evaluating targeted agents. Further building on this concept, the phase II NCI-MATCH trial (NCT02465060) is enrolling pretreated patients to targeted therapies directed by next generation sequencing. This trial includes patients with Her2 amplification or mutation treated with anti-Her2 therapies.

Studies have also tested the role of targeting other receptors of the ErbB family, such as EGFR with mixed results. Cetuximab (monoclonal antibody targeting EGFR) was tested in a phase II randomized trial in the first-line setting for advanced UC (gemcitabine/cisplatin with or without cetuximab) (87). The overall response rate (61.4% *vs.* 57.1%), median progression-free survival (7.6 *vs.* 8.5 months), and median overall survival (14.3 *vs.* 17.4 months) showed limited clinical benefit with added cetuximab. The triplet combination also contributed to increased Grade 3/4 toxicities, including but not limited to rash and thromboembolism. The use of afatinib (irreversible pan-HER tyrosine kinase inhibitor) was tested in advanced UC patients with platinum-refractory disease in a phase II trial with prespecified tumor analysis for alterations in ErbB family (88). Reporting on 23 patients (with minimal toxicities or dose reductions required), 21.7% of patients met the primary endpoint of 3-month progression-free survival rate. Further dissection of the genomic alterations revealed that 5 of 6 patients with Her2 and/or Her3 alterations achieved progression-free status ranging from 5 to 10.3 months *vs.* none of 15 patients without alterations, with longer median time to progression/discontinuation in those with genomic alterations (6.6 *vs.* 1.4 months). Based on these findings, a phase II open label single arm trial of afatinib in advanced UC patients who failed platinum-based therapy and have known EGFR/Her2/Her3 amplifications or mutations is being conducted (NCT02780687). The phase II NCI-MATCH trial (NCT02465060) is also enrolling pretreated patients with EGFR alterations. Hopefully, these studies will add more insight into the role of ErbB inhibition and the predictive role of ErbB alterations in advanced UC.

PI3K/AKT/mTOR pathway inhibitors

The role of the phosphoinositide 3 kinase (PI3K)/protein kinase B (AKT)/ mTOR has been clearly demonstrated to be commonly activated and remains a critical pathway involved in tumor growth and potential resistance to conventional therapies (20,89). Unfortunately, there has been limited clinical benefit with targeting this pathway in advanced UC. A phase II, single-arm, non-randomized study with everolimus (inhibitor of mTOR pathway) in patients with refractory UC showed minimal response with median progression-free survival 2.6 months, median overall survival 8.3 months, and only 2 responses seen in 45 patients. However, one of these patients was able to achieve a durable response of 26 months (90). Genomic sequencing was performed to better identify the underlying biology of this patient (91). This analysis identified a loss-of function mutation in tuber sclerosis complex 1 (TSC 1; key regulator of the activation of mTOR), which was confirmed to be relevant in other UC patients treated on the trial and seemed an emerging biomarker of response to everolimus. An ongoing phase II trial (NCT03047213) is building on this foundation, testing a novel mTOR inhibitor (sapanisertib; TAK-228) in patients harboring TSC1/2 mutations after progression on platinum-based chemotherapy.

Antibody-drug conjugates (ADC)

ADC is a novel class of drugs composed of monoclonal antibodies (targeting cancer cells) linked through a specific chemical linker to an active compound that can cause cytotoxic effects on cancer cells, more pronounced than expected with monoclonal antibodies alone. An ideal ADC would target specific cancer antigens without expression on normal cells and lead to more cancer specific cell death, sparing normal cells and limiting systemic exposure (92). We highlight promising results from a few ADC that need further validation in ongoing clinical trials.

Enfortumab-vedotin (EV) is composed of anti-NECTIN-4 monoclonal antibody (commonly expressed in patients with advanced UC) attached by a unique linker to the cytotoxic agent monomethyl auristatin E (microtubule disrupting agent). A phase I study included patients refractory to first-line therapy (including platinum-based and CPI) treated with this ADC and revealed that it was fairly well tolerated, while high level of nectin-4 expression was found and promising response rates were noted,

including three complete responses (93). Ongoing clinical trials, e.g., NCT03219333 (phase II single-arm trial of EV alone in patients post platinum-based therapy and/or CPI) and NCT03288545 (phase I single-arm trial of EV with CPI in cisplatin-ineligible or platinum-refractory patients) will further assess this ADC.

Sacituzumab govitecan is another ADC that consists of anti-TROP-2 monoclonal antibody linked to SN-38 (the active metabolite of irinotecan). This was studied in a phase I/II study in patients with advanced UC who progressed on/after first line therapy (platinum-based or CPI) demonstrating overall response rate 36% with a complete response, median progression-free survival 7.2 months, and good tolerance except for grade 4 neutropenia in 16% of patients (94). This compound is further being investigated in triple negative breast cancer and advanced UC. Future development of those and other ADC will hopefully address this unmet need of rational novel agents in advanced UC.

Epigenetic modulation

Epigenetic changes are commonly described as modifications of gene expression or changes that occur to the DNA of cancer cells with preservation of the inherent DNA nucleotide sequence. Analysis through the TCGA and other datasets of advanced UC has highlighted the importance of mutations found in chromatin remodeling (20,75). Based on these findings and extensive preclinical work, the rationale exists that using epigenetic modulators, such as histone deacetylase (HDAC) inhibitors would have potential benefit in treating patients with advanced UC (95). A phase II study was conducted with results pending using a novel selective HDAC 1,2,3,11 inhibitor, mocetinostat [known to target alterations of CREBBP and/or EP300 that are found commonly in advanced UC (20)] in patients with platinum-refractory advanced UC with known CREBBP and/or EP300 alterations (NCT02236195).

Examples of emerging immunotherapy combinations

Anti-CTLA-4 and/or anti-VEGF or chemotherapy and anti-PD1/PD-L1 combination

There is strong preclinical rationale and clinical studies in advanced melanoma that has led to the FDA approval of combining anti-PD1 with anti-CTLA-4 agents (96). The combination strategy has been also tested in a phase I/II open-label trial in platinum-refractory advanced UC patients with different dosing schedules (N1I3: nivolumab 1 mg/kg plus ipilimumab 3 mg/kg) and N3I1: nivolumab 3 mg/kg plus ipilimumab 1 mg/kg) (97). The anti-tumor activity favored N1I3, however, concern of toxicity remains with this combination regimen with rate of Grade 3/4 toxicities in both arms roughly about 32% and discontinuation rates due to toxicities roughly 7.7–13.5%. As clinicians become more aware of early recognition and proper management of expected immune toxicities more patients may be able to be treated with combination therapies in clinical trials. Ongoing trials are being conducted to assess the role of combination regimens, e.g., anti-PDL1 or anti-PD1 with anti-CTLA4 in UC settings, as well as chemoimmunotherapy regimens.

Cabozantinib is a novel multi-tyrosine kinase receptor inhibitor, mainly targeting c-MET, VEGFR2 and AXL. There has been emerging data about the role of anti-VEGF and enhanced response to CPI by enhancing immune cytotoxic effects and reversing immune suppressive effects (98). This led to the phase I study of the double combination of cabozantinib plus nivolumab and the triple combination of cabozantinib, nivolumab, and ipilimumab in patients with advanced UC and other genitourinary tumors (99). Forty patients with advanced UC were included in addition to other genitourinary tumors; both doublet and triplet combinations were tolerated with expected toxicities and overall response rate 32% with 9 out of 11 responses ongoing. There is also a trial evaluating bevacizumab with atezolizumab in advanced UC.

Indoleamine 2,3-dioxygenase (IDO) inhibitors and anti-PD1/PD-L1 combination

Although a few selected patients treated with CPI can obtain durable responses, the majority eventually progress and develop resistance. This principle led to the development of other novel immune therapies. One example is the development of IDO inhibitors which target IDO expressed on the tumor microenvironment (100). The IDO pathway is involved in the tryptophan metabolism and leads to depletion of this essential amino acid resulting in shutting down the effector T cells in the immune system, and is found to be overexpressed in multiple solid tumors (100). Thus, the use of IDO inhibitors as monotherapy or in combination with CPI can further activate and enhance the immune system. A combination phase I/II study with epacadostat (IDO-inhibitor) and pembrolizumab in platinum-refractory advanced UC patients revealed overall response rate 35% and ≥ grade 3 treatment-related adverse events in 20% of patients (101). These results led

to the design of two phase III randomized clinical trials of pembrolizumab with epacadostat in patients with advanced UC; however, the recently reported 'negative' data from a large trial in melanoma has tempered the enthusiasm, negatively impacting the conduction of the UC trials.

Future directions/conclusions

After many years of 'dormancy' in advanced UC, the therapeutic landscape has recently changed with the approval of CPI. The role of platinum-based chemotherapy remains a critical option and there remains an urgent need to develop clinical trials and novel biomarkers to help identify patients that could derive benefit form immune-based strategies, platinum-based chemotherapy, their combination and/or other novel targets, e.g., DDR defects and PARP inhibitors. Emerging biomarkers have shown promise but need further validation in clinical trials before clinical utility is proven. Further molecular interrogation of tumor biology of patients with advanced UC has led to the discovery of targetable alterations which can be potentially treated with angiogenesis inhibitors, FGFR inhibitors, ErbB inhibitors, epigenetic modifiers, PARP inhibitors, etc. Moreover, the use of ADC and combination regimens has the potential to transform our approach to patients with advanced UC. The future for patients with advanced UC remains promising also with innovative "umbrella" and "basket" type of trials currently evaluating the premise of "precision oncology" using next generation sequencing.

Acknowledgements

None.

Footnote

Conflicts of Interest: Dr. Grivas reports personal fees and other from Genentech, personal fees from Dendreon, personal fees and other from Bayer, personal fees and other from Merck, other from Mirati, other from Oncogenex, other from Pfizer, personal fees and other from Bristol-Myers Squibb, personal fees from Exelixis, personal fees and other from Astra Zeneca, personal fees from Biocept, personal fees from ClovisOncology, personal fees from EMD Serono , personal fees from Seattle Genetics, personal fees from Foundation Medicine, personal fees from Driver Inc., outside the submitted work. Dr. Mendiratta has no conflicts of interest to declare.

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Cite this article as: Mendiratta P, Grivas P. Emerging biomarkers and targeted therapies in urothelial carcinoma. Ann Transl Med 2018;6(12):250. doi: 10.21037/atm.2018.05.49

