Molecular and Functional Diagnostic Tools in Precision Oncology for Urological Malignancies

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Abstract Urological malignancies, represented mainly by prostate, bladder, and renal cancers, are some of the leading causes of cancer-related mortalities worldwide. Despite various efforts over decades to develop early detection tests and effective therapeutic paradigms, the response rate to the existing treatments remains low for both primary and late stage/recurrent phases of these cancers. The evolving landscape of molecular diagnostics, aiming to make the diagnosis and treatment more patient-driven, underpins precision oncology and particularly intends to rationally profile individual tumors and highlight the mechanistic insight and complexity of tumor microenvironment in order to develop biomarkers of toxicity risks and response prediction in a clinically oriented dynamical setting. The present review is an effort to capture some of the recent developments in the area of molecular diagnostics and functional testing platforms and their potential application in clinical decision making in the premises of precision oncology of urological malignancies.

Keywords Urological cancer · Precision therapy · Prediction biomarkers · Ex vivo models

Introduction

Our latest understanding of the definition of cancer points towards the fact that rather than considering it as a single

disease, it altogether represents more than 250 genetic diseases. Due to inherent diversity, in recent years, tumor microenvironment has become the field of intense research. Recent studies illustrating the intratumoral diversity within the same patient tumor substantiate the uncertainty of a successful treatment outcome as new resistant clones emerge at the time of one particular therapy. Prostate cancer is the second leading cause of cancer-related death among males. GLOBOCAN 2012 and other recent findings forecast a surprising rise of this cancer in India [1]. Bladder cancer, like prostate carcinoma, is also a major cause of all cancer-specific mortality among adult men and women and one of the most expensive malignancies to treat [2].

Diagnostic Biomarkers

Biomarkers of urological cancers range from screening, diagnostic to predictive/companion diagnostic, therapeutic and even efficacy/toxicity risk. Although molecular diagnostics like detection of prostate-specific antigen at different stages of the disease serve as a tool to understand disease progression, recent recommendations by the US Preventive Services Task Force (USPSTF) against prostate cancer (PCa) screening highlight the pressing need to replace or at least complement it with more specific approaches [3]. Despite these controversies, prostate-specific antigen (PSA) continues to play a critical role in the diagnosis and management of prostate cancer. Some emerging markers of diagnosis and surveillance like hK2 and TMPRSS2-ERG raised hope of augmenting present diagnostics. However, none of them so far are considered as possible replacement of PSA (see Table 1) [4–6]. Similarly in bladder cancer, a number of approved diagnostic, monitoring, and point of care test/markers are available and have been reviewed elsewhere [7].

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Table 1 Biomarkers, devices, and assays of potential applications in urological and other malignancies

Biomarker category	Sample	Cancer type	Application	References
Diagnostics/prognostic				
PSA ^b	Protein	CaP	Diagnosis	5
TMPRSS2-ERG		CaP	Diagnosis	6
BTA ^b	Protein	Bladder	Diagnosis and monitoring	18
NMP22 ^b	Protein	Bladder	Diagnosis and monitoring	17
ImmunoCyte/uCytex ^b	Protein	Bladder	Monitoring	19
UroVyson ^b	DNA	Bladder	Diagnosis and monitoring	21
Decipher ^b	Multigenes	CaP	Metastasis risk prediction	14
Prolaris ^b	Multigenes	CaP	Mortality risk prediction	15
Oncotype DX ^b	Multigenes	CaP	Risk prediction/aggressiveness	16
Predictive/theranostic/toxgnostic				
CAIX	Gene	RCC	IL2 therapy	26
DEFA1, A1B	Gene	CRPC	Docetaxel response	27
PTEN, P13K		CaP	Predicting targeted drug	29
CTC/ctDNA	DNA, RNA	CaP, bladder, RCC	Resistance, recurrence	40–45
Class III β -tubulin	Protein	CRPC	Docetaxel failure	31
CYP3A5*1 ^a	19 gene	RCC	Predictor of toxicity risk	36
Devices and assays				
Unnamed device	PDX	Multiple	Drug delivery and response	73
CIVO	PDX, patient	Lymphoma	Multiple drug response	74
DBP/mitochondrial	Cells	Lymphoma	Cytotoxic and targeted agents	76
MiCK/apoptosis	Patient tumor	mBC	Short term clinical response	77
Functional platforms				
3D organoid	Tissue, CTC	CaP	Recapitulation of TME	71
Organotypic	Tumor tissue	CaP, and multiple	Invasion, pathway inhibitors	64,63
CANScript	Tumor tissue	CRC, HNSCC	Short-term clinical response	48

CaP prostate cancer, RCC renal cell carcinoma, CRPC castration-resistant prostate cancer, CTC circulating tumor cells, CRC colorectal cancer, HNSCC head and neck cancer squamous cell carcinoma, mBC metastatic breast cancer, PDX patient tumor-derived xenograft

^a Single nucleotide polymorphism, (*) represents allele encoding functional CYP3A5

^b FDA-approved test

Molecular Biomarkers in the Era of Precision Therapeutics

Research in precision therapeutics as next-generation patient-driven medicine is gaining momentum, and policy makers are favoring for its rapid implementation. While recent advances showed limited success of biomarkers at various stages of clinical decision making, in the context of urology and other similar malignancies, prognostic and predictive markers of molecular nature are two different aspects having different impacts in treatment. Their applications are also contingent upon extensive clinical validation and deserve thorough critical revisit. In the present article, we will restrict our focus on the developments mainly pertaining to the predictive biomarkers, recent progress in developing ex vivo functional models as complementary

tools and how their coordinated applications may guide to uncover the puzzles of treatment response and failure.

Next-generation sequencing technology is constantly redefining molecular diagnostic approaches in oncology. The Cancer Genome Atlas (TCGA) and International Cancer Genome Consortium (ICGC) projects have been undertaken to complete the sequencing of all major cancers [8] and accelerated molecular subclassification of malignancies; for example, triple negative breast cancers, prostate cancer, and renal cancers [9, 10]. New data that emerged from TCGA's pan cancer analysis shed light on the heterogeneous nature of genes for some cancers including bladder carcinoma which shows similarity with lung and head and neck cancer, while others showed more homogenous cell distributions based on genetic profile analysis. Interestingly, the study also showed

that among all cancers, the most diverse subtyping was seen in the case of bladder carcinomas. Indeed, genetic profiles indicated that out of a total of seven initial subtypes, three major subtypes exist, i.e., LUAD-enriched, squamous-like, and BLCA [9]. In renal cell carcinoma (RCC), microarray profiling led to new molecular subtypes that closely matched with standard histological classification [10]. In the case of androgen deprivation metastatic prostate cancer, Gundem et al. showed that whole-genomic sequencing (WGS) analysis enabled to underpin multiple mutational defects (driver mutations) in androgen signaling which consequently triggered resistance to androgen deprivation therapy [11]. A signature of three genes (FGFR1, PMP22, and CDKN1A) has been identified that together can predict accurately the outcome of low Gleason score prostate tumors [12]. Similarly, a 17-gene signature has been reported to predict the aggressiveness of prostate cancer spread [13]. Irrespective of these findings, it is largely agreed that cancer genome analysis has revolutionized the conceptual progress of new tumor subtypes for a number of cancers and has therefore improved our understanding of the molecular diversity of the diseases.

Prognostic Biomarkers in Urological Cancers

Despite efforts by researchers, the availability of a widely acceptable prognostic biomarker in prostate cancer (CaP) is still elusive. However, three commercial tools are currently available to predict various risks/recurrence of prostate cancer. These include: decipher for recurrence/metastatic risk [14], prolaris for low and high risk patient surveillance [15], and oncotype DX that predicts adverse pathology at radical prostatectomy for low- and intermediate-risk CaP [16]. Similar to prostate cancer, for bladder cancer, a number of FDA-approved tests are available for specific purposes. Bladder tumor-associated antigen or BTA (human complement factor H-related protein); nuclear mitotic apparatus protein (NMP22) [17]; the high molecular weight form of glycosylated CEA; MUCIN-like antigens for monitoring (ImmunoCyt) and detection of aneuploidy for chromosomes 3, 7, and 17; and loss of the 9p21 locus (UroVysion) are some of the promising markers for diagnosis and monitoring (shown in Table 1) [18–21]. The study has showed that the model that combines gene signature and critically involved clinical variables could benefit patients by predicting the chance of recurrence [22]. For clear cell renal carcinoma (ccRCC), a number of biomarkers of potential prognostic and predictive applications have been identified by different investigators and encompass diverse aspects of tumor physiology [23], but have limited scope to elaborate in this review.

Response Prediction Biomarkers

Like many other malignancies, the low success rate of a standard treatment in prostate and bladder cancers is mainly

attributable to a number of critical variables. Tumor as a complex heterogeneous system poses tremendous challenges to pinpoint the most optimal drugs at individualized levels at a particular state of the diseases. Identifying patients who are most likely to respond to a specific treatment plan (patient-drug match) is an important prerequisite for improved survival. The avoidance of overtreatment and drug-related toxicity resulting from non-responsive regimens can also avert unwarranted exposure and side effects. This is the main concern of using PSA and necessitates the search for a number of reliable markers of response prediction in urological cancers, as there is a paucity of validated sensitivity and resistance or non-response biomarkers. The power of sensitivity of biomarkers like prostate cancer antigen 3 (PCA3) and the TMPRSS2-ERG fusion gene is under evaluation in a 6-month extended release formulation of leuprorelin acetate for detecting therapeutic response after hormonal therapy [24]. Earlier studies indicated that enhanced bcl-2/bax ratio could increase the risk of failure to radiotherapy [25]. In RCC, the expression of carbonic anhydrase IX (CAIX) has shown predictive value for sensitivity of treatment with interleukin-2 therapy [26]. Multiple transcripts of defensin genes, i.e., DEFA1, A1B, and A3, showed higher level of detection prior to therapy in blood of castration-resistant prostate cancer (CRPC) patients responsive to docetaxel therapy as compared to the blood of non-responder patients (Table 1) [27]. Loss of chromosome region 10q that encodes PTEN, FAS, and PAPSS2 has potential to predict recurrence after radical prostatectomy [28]. The PTEN-PI3K pathway would also emerge as one of the critical drivers of predicting targeted drugs in the future [29].

Theranostic Markers in Urological Cancers

It covers all major molecular facets like genome, miRNA, proteome, and metabolome and represents predictive biomarkers for therapy response. Recent interest in identifying and validating theranostic biomarkers of clinical utility indicate their potential in redefining treatment strategies. While few theranostic biomarkers in other cancers, like in the case of cancer of gastroesophageal junction, overexpression of HER2/Neu acts as a predictor of improved overall survival when patients receive trastuzumab as first-line therapy compared to the other treatments [30] generated big hope, integration of reliable theranostic biomarkers in prostate and bladder cancer treatment management is still elusive despite a number of candidate molecules are showing translational potential. Elevated expression of class III beta tubulin is a predictor of failure to docetaxel-based therapy for CRPC and therefore a potential theranostic candidate as shown in Table 1 [31]. Similar paucity is observed in the case of bladder cancer. The preclinical model exploring the possibility of using combinatorial theranostic biomarker of p53 deficiency and oncogenic activation of RTK/*HRAS* pathway is one interesting

approach, but further validation in relevant system is required before their application at clinic [32].

Integration of Toxgnostic Biomarkers Can Make the Prediction Multidimensional

As defined by Church et al., toxgnostics is “the systematic, agnostic study of genetic predictors of toxicity from anticancer therapy” [33]. Since toxicity resulting from chemotherapy and radiotherapy is a major health risk for urological cancer and like many other cancers is linked to the underlying genetic background of individual patients, developing drug-specific toxicity prediction at a personalized setting would improve the therapy outcome and quality of life. So far, no such clinically validated testing is available for CaP and bladder cancer. However, studies with a small sample size showed that specific gene sets can differentiate patients that develop late-stage toxicity [34]. Another study showed that 19 DNA repair-related genes highly altered their expression levels in patients that lacked sensitivity to radiation in CaP [35]. In the case of RCC, a single nucleotide polymorphism analysis identified the association of CYP3A5*1 allele with decreased sunitinib dose tolerance [36] (Table 1). However, it is noteworthy that identifying a validated pharmacogenomic predictor of toxicity risk for chemo-drugs and targeted drugs for CRPC would help in changing the treatment paradigm for cancers of endocrine etiology [37–39]. New paradigms combining toxicity risk biomarkers and predictive tools aiming to stratify patients based on dose and response benefit assessment would overcome toxicity-related problems while ensuring maximum therapeutic benefits at individual level.

Circulating Tumor Cells as Surrogate Markers for Predicting Treatment Recurrence

In the recent years, due to the advent of high-precision technologies for detecting tumor DNA, like next-generation sequencing (NSG) at single cell level, there is an increasing interest in exploring more challenging and innovative approaches pertaining to our understanding of response and resistance to therapy and tracking invasive tumor cells in circulation. Popularly known as “liquid biopsies,” this non-invasive technique captures circulating tumor DNA (ctDNA) to monitor tumor recurrence and treatment-driven changes. Recent findings delineated the potential of using ctDNA to design effective therapy options for prostate cancer, and more intriguingly, CRPC. Changes in androgen receptor (AR), specifically emergence of the V7 form (lacking ligand binding domain), and few other mutations potentially known for loss of androgen dependency as a part of ongoing treatment have been evaluated in circulating tumor cells. Multiple point mutations of AR are linked to therapy resistance for a range of anticancer drugs in CaP [40–43]. In more than 90% cases, positive predictive

values of androgen deprivation therapy (ADT) response and resistance based on the AR status in ctDNA were observed, suggesting the application of ctDNA as an effective tool to guide personalized therapy switch depending on the types of modifications prevalent [44]. Despite the big hope it shows for active disease surveillance, there are a number of challenges in implementing these techniques for all cancers. The retention of the cells in circulation and the viability of ctDNA would be major hurdles. Secondly, in addition to ctDNA, mRNA and miRNA can also provide critical clues and their integration with ctDNA can make the prediction strategically more comprehensive (see Table 1) [45].

Functional Testing Tools for Prime Time Precision Medicines

Recent advances in our understanding of tumor microenvironment as a complex heterogeneous system, its clonal heterogeneity, phenotypic plasticity of transient nature, and epigenetically driven adaptation to drugs popularly known as “drug holiday” [46, 47] and conceptual progress in deciphering the critical function of cancer stem cells, immune status, reprogrammed energy metabolism, and perturbation of oncogenic signaling network showed that the heterogeneity of multiple driver molecules in a critical milieu very often paradoxically can limit the scope of biomarker to faithfully predict clinical response of anticancer drugs in diverse personalized contexts [48]. It is understandable that, for a static biomarker to show its predictive power, many active components of tumor microenvironment are required to be in a particular spatiotemporal context as multiple interacting players and their dynamic nature increase the variability of the negative and positive response prediction. This is thought to be the key reason why the same mutation, when contextually delineated in different cancers, resulted in completely opposite predictions [49–52]. Tumor heterogeneity is also a common property of urological cancers. Multiregion exome sequencing identified major subclonal driver aberrations in ccRCC, and this heterogeneity was noted to be intensified with increasing number of biopsies. Like RCC, primary biopsied prostate cancers also display the properties of spatially intratumoral heterogeneity at localized multifocal tissues in which highly heterogeneous single nucleotide variants have been found occasionally with divergent evolution or tumors of independent clonal origin [53]. Similar heterogeneity has been reported in metastatic lesion [54]. Junttila et al. recently described how, depending on the entire tumor-stromal context, same genetic alteration results in different functions. This study and others that described intratumoral heterogeneity and stromal diversity indicated limited utility of genetic profiles, when applied alone, in selecting right drugs for different patients sharing common genetic defects in their tumors [55]. It forms the basis

of exploring the avenue where functional ex vivo tumor models mimicking native tumor state hold key promises.

In recent years, there have been serious efforts to develop more direct and context-dependent personalized approaches underpinning the evolution of a number of next-generation functional diagnostic tools. Functional in vitro testing models have achieved few important critical milestones. The 2D monolayer culture of a panel of cancer cell lines, due to lack of stromal context and multiclonality, shows poor clinical relevance and predictive value. The development of organ culture models accelerated the engineering of clinically relevant 3D technologies [56–58]. Earlier versions of these 3D technologies such as 3D spheroids were critically lacking multiple key components of tumor microenvironment mainly the stromal milieu, including personalized extracellular matrix supports. Indeed, the high throughput nature of these spheroids, the uniformity of their structure, and their ability to preserve certain morphological features of native tumors, for example, basal-luminal polarity in the case of breast cancer and other malignancies have made them the champions in oncology drug screening [59, 56]. A number of studies showed good correlations of these models with in vivo platforms for oncology drug testing [48, 60, 61]. While majority of the recent findings were restricted to spheroids derived from cell lines or patient-derived primary cancer cells with heterologous stromal supports, they showed differential response to anticancer drugs in the presence of these components together compared to the single cell composition [62]. Using patient-derived extracellular matrix scaffold from foreskin, Ridky et al. recreated the tumor architecture (from normal oropharynx, esophagus, and cervix), preserving key features of invasiveness when cells were layered on a collagen matrix network and transduced with mutant *KRAS* gene [63]. The model subsequently allowed testing of the drugs known to have anti-invasive potential. One model demonstrated the stability of the phenotypic and pharmacogenomic profile of primary tumor tissues maintained on commercial organotypic support in the presence of specific CO₂-enriched environment. This study highlighted the preservation of many molecular features including critical tumor microarchitecture, PI3K-AKT signaling axis and the spatiotemporal nature of their integrity in the culture system up to 6 days. Further testing of pathway-specific inhibitors suggested the pharmacodynamic modulation of targeted pathway as a mean to study pathway perturbation [64]. Ex vivo slice culture model developed by other investigators was also able to pinpoint drug-related changes in short-term culture [65]. The latest model of ex vivo functional testing, called “CANScripT”, integrated to a large extent multiple critical growth-promoting and survival factors that are able to maintain the diverse nature of a tumor ecosystem, including multiple autocrine-paracrine growth factor signaling axis heterogeneity, active balance of epithelial-mesenchymal transition markers, cancer stem cell phenotypes, immune cell types

such as tumor-associated macrophages, intratumoral CD8+ T cell infiltrates, and key cytokine-chemokine and angiogenesis profiles. The study showed capturing of heterogeneity of the tumor ecosystem by providing autologous growth factors and personalized cancer-specific matrix support and reported significant predictive values to correlate in vivo testing results of standard-of-care drugs for more than one cancer types and finally using artificial intelligence, predicted short-term drug response at clinic [48]. Recent findings on the fabrication of functional blood vessels in an engineered organ culture model and the generation of tumor spheroids incorporating immune cells, like CD8, at the interface raised hope to understand the crosstalk of multiple phenotypic components in a complex ex vivo model system [48, 66]. On a similar note, latest research described a culture device that showed the ability to dynamically recreate circulating tumor cells and track them in microfluidic culture vessels in real time which may shed new light on the drug response and an impending recurrence [67]. Contextually, preserving intratumoral infection status where it has known pathoetiology (e.g., *H pylori* in CRC, HPV for HNSCC, and cervical cancer) would make the treatment evaluation and prediction more reliable and clinicopathologically oriented. Indeed, proper biosafety precautions and training is required to handle these type of tissues despite the fact that in reality, all tissues in culture are being treated as potentially infectious. A number of these studies prominently focused on developing prostate cancer-specific models. Recently interest has been shifted to utilizing the cells and materials directly originating from clinical tissue samples. Such tissue-derived tumor spheres (TDTS), following partial dissociation of tumor tissues, have been reported in a number of cancer indications including prostate cancer [57, 68, 66, 69, 70]. Another recent study successfully demonstrated the engineering of a long-term 3D organoid system from advanced metastatic prostate cancer samples. This unique study used both patient biopsy samples and autologous circulating tumor cells (see Table 1) [71]. The authors modeled recapitulation of histological and molecular heterogeneity of subtypes including fusion, mutation, amplification, and deletion of specific genes known to have driver roles in the pathogenesis of prostatic neoplasia wherein the loss of p53 and RB tumor-suppressor pathways were commonly retained. The increasing number of such personalized and clinically relevant ex vivo testing platforms for urological cancers will help to develop rationally driven novel therapeutics. On the top of this, tumor gene sequencing projects will be productively more translational oriented if technologies like 3D spheroids and organotypic or personalized platforms replace the traditional 2D cell lines. More intriguingly, projects like PREDICT consortium and recent focus on extraordinary drug responders showed the pressing need to develop patient-derived ex vivo models in order to accelerate development of predictive biomarkers for specific drugs in renal cancer and other similar

malignancies following initial genomic profiling/hit identification on functional siRNA screen [72].

Novel Devices and Assays to Predict Drug Sensitivity

There are a number of innovative devices engineered in recent time that showed promise to precisely target drugs into tumor tissues while it is within the original site and could provide rapid printout of possible response to a drug regimen. Two such products developed by two independent laboratories have been recently appreciated for future clinical applications. Both these implantable microdevices, one unnamed and one called “CIVO,” are based on the release of multiple chemotherapy drugs at microdoses at the tumor sites in controlled manners and positioned to provide new opportunity to test their sensitivity at the same time [73, 74] (Table 1). Initially tested in human xenograft mice model and human and dog lymphomas for pathway drugs and chemotherapy, these devices displayed equivalence of systemic delivery of drugs and their subsequent exertion of antitumor effects. These devices, despite generating great enthusiasm, also have a few limitations. For example, the efficiency of drug diffusion depends on many factors in tumor microenvironment. Tumor stromal stiffness impacts variable diffusion from drug to drug and patient to patient. Secondly, tumor as a heterogeneous entity, due to clonal heterogeneity, would give diverse results in a dynamic spatiotemporal context, and lastly, the device would not support drug delivery in organs where implantation would be anatomically challenging. Sometimes, recovery of exposed tumors needs rebiopsy and proper pathological evaluation. Irrespective of these limitations, proper applications of these devices upon validation may help to accelerate oncology drug testing in diverse patient-friendly settings. An analogous strategy in clinical setting has been described in the ADAPT trial where anticancer agents in breast cancer patients were evaluated by assessing the reduction of Ki67-positive cells in rebiopsied tumors following short-term (15 days) systemic exposure of drugs [75]. Another important aspect of ex vivo functional model is that most of the assays are not independently approved as part of the laboratory-developed process. A large number of anticancer drugs target the mitochondrial pathway and recent studies showed the useful inputs generated from rapid tracking of mitochondrial killing by measuring Bcl2 homology domain3 or BH3 profiling of tumor cells and its improved version called dynamic BH3 profiling (DBP). In a number of liquid and solid tumors, this assay showed good clinical predictive value for specific cytotoxic and targeted agents [76]. Other similar methods such as MicK assay showed strength of predicting short-term response at clinic for metastatic breast cancer [77]. Since in individual tumors more than one anticancer phenotype would be genetically/epigenetically impaired, reliance on a single assay would result in limited predictive utility. Apoptosis marker caspase3(c)

provokes tumor cell repopulation following radiotherapy in specific cancer [78]. In other cases, deletion of cascade genes would affect the interpretation. Therefore, careful integration of mechanism-driven specific assay panels and their proper validation and interpretation in the context of anticancer therapeutics would help in making new 3D ex vivo technologies and other functional tools reliable for forecasting anticancer drug responses. Proper validation of these assays is warranted before considering their acceptance as a companion assay guide in cataloging responder drug panels.

Conclusion and Future Perspectives

Predictive biomarkers warrant thorough assessment of their actual clinical utility by analyzing metrics such as specificity, sensitivity, negative and positive predictive value, and a closer review of their cost-benefit balance. Studies including standard practices compared to the biomarker-guided interventional treatments with respect to the clinical outcome needs to be undertaken where individual level variability within the convincing limits requires further substantiation by population based studies as elaborately hypothesized in recent literature [79–81]. Additionally, consideration of the chance of toxicity in conjunction with quantifiable treatment efficacy would make these strategies multidimensional. Regardless of these issues, any biomarker of predictive value or functional ex vivo testing device to impact clinical decision making would require controlled, double-blinded randomized interventional trials [82]. Collectively, the conceptual progress reviewed in this article, while revolutionizing our knowledge of the molecular variability of individual cancers, can be explored as new avenues for stratification of patients deserving optimal selection of rational therapeutics. The new exciting directions that next-generation functional testing devices showed offer an excellent opportunity to test drug efficacy where complementary roles of biomarkers and individual tumor contexts, guided by function tools in an integrative framework, further highlights the urgent need to combine multiple evolving strategies of precision oncology.

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