EXPERT REVIEWS

Overcoming tumor heterogeneity in the molecular diagnosis of urological cancers

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Our understanding of tumor heterogeneity and impact on treatment response is still in its infancy, presenting significant challenges to the molecular pathologist, treating physician and ultimately for the patient. Given that tumor recurrence due to treatment resistance is the most common cause of cancer death, there remains a critical unmet need to change the current paradigm. The mechanisms which underlie tumor heterogeneity can be broadly divided into genomic instability and non-mutational processes, including stochastic variations in cellular responses, modulation by tumor microenvironment and or phenotypic/ functional plasticity relating to cancer stem cells. We believe that these biological mechanisms are not mutually exclusive and emphasize the need for more suitable methodologies to exploit the spatiotemporal patterns of intratumoral heterogeneity using novel approaches such as quantitative tissue-based biomarker assessment and systemic fluid analytics. Generating a comprehensive patient-centric phenotypic disease profile should generate a 'codex' which can be employed to change the current treatment decision process.

Keywords: biomarker • exosomes • next-gen sequencing • system pathology • tumor heterogeneity

Tumor molecular diagnostics

Tumor heterogeneity exists at the histologic and molecular level, presenting significant challenges for accurate classification, outcome risk assignment and treatment selection. Fortunately, the clinical science of *molecular diagnostic pathology* has been at the forefront of tumor (and disease) characterization, aiding the 'next-gen' pathologist with tools that define phenotypes, elucidate biochemical processes and decipher tumoral heterogeneity. Some of the more clinically relevant and useful molecular assay developments have included quantitative DNA and RNA FISH, quantitative PCR (Q-PCR) and most recently hot-spot (gene) mutation detection, next-generation sequencing (including whole-genome and whole-exome analysis) and RNA expression profiling (RNA sequencing 'seq').

Numerous studies have investigated the genomic evolution of cancers in multiple tissues, demonstrating that by the time of diagnosis, the tumor has already acquired a complex subclonal architecture with distinct mutational profiles [1,2]. Successful 'molecularly' defined therapies are able to debulk a tumor, but at the same time create conditions that promote outgrowth of drug-resistant cancer stem cells (CSCs), thereby increasing tumoral heterogeneity. Whether these resultant clonal cell populations contain inherent mechanisms of resistance or are epigenetically and environmentally modified has yet to be fully elucidated. A recent analysis has identified a dynamic and reversible CSC drug-resistant mechanism which links the integrin AvB3 and NFKB activation as a pro-survival signaling mechanism [3]. In addition, further studies are necessary to understand the epigenetic changes associated with fluctuations in CSC activity or phenotypes and the role they play in overall therapeutic response.

Over the past several years, there have been important discoveries in genitourinary (GU) oncology, including the identification of

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somatic mutations (e.g., EGFR, KRAS, PIK3C) across multiple GU tumors, structural-translocation events including Xp11.2: TFE3 in renal cell carcinoma (RCC) and TMPRSS2:ERG in prostate cancer, disease-organ specific miRNA/lncRNA profiles (e.g., miR145, miR200; SChLAP1 and PCAT-1) in bladder and prostate cancer, respectively, and the over-expression of novel proteins including IMP3 (insulin-like growth factor II mRNA-binding protein) as prognostic signatures in kidney and bladder cancer [4–7]. By analyzing the macromolecular content of the tumor, the 'primary' molecular pathologist is able to identify relevant, potentially 'oncogenic driver' or 'trunk' pathway genes that not only aid in a more accurate diagnosis but also circumvent, to some degree, the impact of tumoral heterogeneity, allowing for more appropriate risk stratification and treatment choice.

Another emerging field in the management of tumor heterogeneity is through the incorporation of a systems or precise pathology program to provide complex models that integrate clinical variables with regional histologic and biologic features derived directly from the intact tissue specimen. By avoiding the 'grind and find' approach, there is a possibility to reproducibly identify and quantify discrete populations of cells for further annotation [8–10]. This strategy becomes critical for understanding and quantifying specific attributes of any given tumor, including the role of novel tumor antigens, host tumor immunologic activity and the CSC in therapeutic response and outcome. Finally, interrogating cell-free/cell-tumor (cf/ct) DNA, circulating tumor cells (CTCs) and exosomes will no doubt allow the investigator/clinician to assess the dynamic physiologic state of the tumor (along with the host), providing another dimension to our understanding of the mechanisms which are responsible for cancer growth and metastasis. Ultimately, this will serve to address the resistance pathways, either in existence at diagnosis or uncovered as a result of treatment.

Urologic tumor heterogeneity

Tumor heterogeneity is one of the major problems limiting the efficacy of targeted therapies and stands to compromise successful trials, new drug approvals and ultimately treatment outcomes. Breast cancer is an excellent example where a combination of efforts including protein immunohistochemistry and gene expression arrays have classified a complex tumor type into three main categories (i.e., luminal, Her2 and basal type) based on the expression patterns of steroid and growth factor receptors coupled with morphologic markers [11]. Similar approaches have been initiated for GU cancers with variable success. Providing this information at diagnosis and prior to treatment should aid in appropriate therapy selection and create a useful baseline phenotype, as the disease process evolves post-therapy.

These methods have led to several treatment strategies for urologic cancers, including sunitinib and sorafenib in RCC, FGFR3 inhibition in bladder cancer and targeting prostate cancer by impacting the AR pathway with abiraterone/enzalutamide and *TMPRSS2-ERG* gene fusions with Poly (ADP-ribose) polymerase (PARP) inhibitors [12–17]. Recent successes with the immune checkpoint inhibitor to PD-L1 in bladder cancer and the importance of drug combinations (i.e., docetaxel with antihormone therapy) in hormone-responsive metastatic prostate cancer) further illustrate the impact of tumor–host heterogeneity on outcome [18,19]. Unfortunately, early therapeutic response for most cases results in disease progression and the majority of patients in all selected therapy trials (with the notable exception of chronic myelogenous leukemia and imatinib) develop resistance and succumb to their disease.

This review will focus on some of the new advances being developed to decipher tumoral heterogeneity both at the tissue and at the fluid-biopsy level and how these approaches will help in understanding tumor composition and ultimately advance pragmatic and future, dynamic clinical trial design. Over the next few sections, we will briefly review examples of tumoral heterogeneity in specific GU tumors with an emphasis on kidney, prostate and bladder and then focus on some novel technologies, which stand to change the treatment paradigm and ideally advance our understanding of disease and impact on outcomes.

Renal cell carcinoma

Approximately 65,000 patients are diagnosed with cancer of the kidney or renal pelvis, and approximately 13,680 will die of their disease with a worldwide prevalence exceeding 200,000 [13,20]. Improved understanding of both histologic and molecular phenotypes over the past three decades has led to the recognition that RCC represents a heterogeneous collection of malignancies arising from the kidney, with many cases diagnosed at locally confined stages (stages I–III) [21]. However, even in the metastatic setting, nephrectomy represents an appropriate step for management. Clear cell RCC (ccRCC) comprises the vast majority of RCC (92%), with other histologic types including papillary 1 and 2, chromophobe and other less common varieties representing a very small percentage [21]. Approximately 17% of patients present with metastatic disease, with tyrosine kinase inhibitors targeting the vascular endothelial growth factor receptor as the mainstay of treatment [21]. Metastasectomy, careful monitoring of indolent disease and high-dose IL-2 therapy remain options in carefully selected cases.

The molecular profile, particularly of ccRCC, seems fairly well established; however, a recent study evaluating somatic genetic heterogeneity using exon sequencing, copy number analysis and ploidy on multiple different regions from several primary and metastatic ccRCC tumors demonstrated a general lack of overall concordance between mutations in primary versus metastatic specimens, with some tumors exhibiting unique mutational profiles, identified as 'private mutations', and metastases exhibited a phenomenon of clonal evolution secondary to selective pressure that resulted in common genetic responses [22]. These results not only confirm the presence of molecular heterogeneity but also stress the necessity for creating a phylogenetic codex to identify key driver events in the biology of RCC. Leveraging this codex with functional activity, that is, protein over-expression or pathway activation, would

have a significant impact on association with outcome. Another interesting point is the role of the immune system in the long-term management of patients with RCC, especially metastatic disease. Immunotherapy is currently making a strong comeback through novel checkpoint agents [23], and still to this day the only agent that can afford a hope for complete response, specifically for patients with clear cell disease, is IL-2 [24]. Importantly, the heterogeneity of RCC including the molecular underpinning and host response must be included in any comprehensive treatment process [25].

Prostate cancer

In Europe and the USA, prostate cancer (PCA) is the most common solid neoplasm and the second leading cause of death in men [20]. There are approximately 229,000 anticipated new cases in 2014 with 28,000K deaths [20]. Although the biomarker discovery process has been quite adept at identifying increasing and seemingly relevant number of potentially interesting markers, only prostate-specific antigen is routinely used by urologists. Major advances have been made in cataloging the genomic alterations in PCA and understanding the molecular mechanisms underlying the disease. These findings suggest that PCA may in fact be a collection of homogenous subtypes identifiable by molecular criteria, associated with distinct risk profiles, and amenable to specific management strategies or targeted therapies. The identification of structural chromosomal aberrations (translocations, deletions or inversions) and mutations in prostate cancer has supported the biological stratification of this disease, notably the identification of a fusion between the *TMPRSS2* gene (21q22.3) and *ERG* (ets-related group, 21q22.2) of the ETS family [26]. Currently, more than 20 different fusions have been reported, implicating *ERG* with *TMPRSS2* that is generally caused by an interstitial deletion at locus 21q22 and a reciprocal translocation [26]. The fusion is present in approximately 50% of all diagnosed PCA with little consensus of its presence to predict risk. The existence of the translocation has been used in RNA expression arrays to identify a subgroup of PCA patients who are at intermediate risk for disease progression [27]. Finally the presence of the gene fusion has also been found to be relevant for potential therapy selection using histone deacetylase and PARP inhibitors illustrating a role for genomics in personalized medicine [15].

Even with the above successes including the prevalence of the fusion gene, PCA remains one of the most molecularly diverse of all GU cancers. Unfortunately, most of this work has been done on limited patient samples and therefore, further studies are necessary to completely understand. The published gene expression studies have confirmed signaling pathway diversity and clonal evolution through therapeutic pressure; however, the majority of this diversity has been demonstrated at the tissue level. A recent identification of a 'well-differentiated' malignant clone in a prostatectomy specimen that exhibited a combined PTEN loss and *TP53* mutation further illustrates both the inherent heterogeneity of the tumor and our current inability to classify based on simple histopathology [28].

Bladder cancer

Bladder cancer is the seventh most common cancer worldwide and the fourth most common cancer among men in the USA, with a threefold higher incidence than women, most likely related to smoking [20]. It is the seventh most common cause of death from cancer in men and the eighth most common cause in women. In most western countries, bladder cancers are transitional cell carcinomas; conventional clinical and pathological indexes are widely used to grade and stage tumors and to eventually predict clinical outcome. However, their predictive value is limited because of low accuracy in bladder cancer patients [29].

Bladder cancer is associated with high recurrence and mortality rates and, in the US, is one of the most costly cancers to treat [29]. These tumors show vast heterogeneity reflected by diverse morphologic manifestations and various molecular alterations associated with very specific disease phenotypes. The majority of incident bladder cancer diagnoses are noninvasive and the mainstay of diagnosis remains cystoscopy and transurethral resection, with enhanced optical techniques potentially improving detection of nascent disease and identification of topographic, gross-level heterogeneity [25]. Intravesical chemotherapeutic and immunotherapeutic agents reduce the likelihood of recurrence and progression, with novel agents showing promise. The identification of variant histology with aggressive phenotypes permits identification of patients unlikely to respond to intravesical agents, in whom early cystectomy is advocated. Risk stratification of patients with non-muscle invasive bladder cancer continues to improve and should be used to inform surveillance and treatment paradigms.

Biomarkers that prospectively evaluate disease aggressiveness, progression risk, probability of recurrence and overall prognosis would improve patient care. Integration of molecular markers with conventional pathologic staging of bladder cancers may refine clinical decision making for the selection of adjuvant and salvage therapy. In the past decade, numerous bladder cancer biomarkers have been identified, including various tumor-suppressor genes, oncogenes, growth factors, growth factor receptors, hormone receptors, proliferation and apoptosis markers, cell adhesion molecules, stromal factors and oncoproteins. Recognition of two distinct pathways for urothelial carcinogenesis (notably FGFR3 and P53) represents a major advance in the understanding and management of this disease [25]. Nomograms for combining results from multiple biomarkers have been proposed to increase the accuracy of clinical predictions; however, even with identification of multiple genomic mutations and pathways, the clinical success rate is minor [26]. A recent review for both renal and urothelial cancers acknowledged that tumoral heterogeneity is the underlying limitation for treatment selection [25].

Disruptive novel technologies address histologic & molecular heterogeneity

A recently described mechanism for helping to bridge these various investigational studies with clinical medicine has been through the introduction of an analytic modeling platform

known as systems pathology [8–10]. The derived systems-based pathology models utilize the patient's own clinical data and intact tissue specimens to construct a baseline phenotype for defining a clinical risk state. These biological–quantitative models provide a biomarker profile that is linked to treatment and outcome. Systems pathology represents a major advance in the standard practice of tissue-based pathology through its integration of molecular and imaging data with the patient's clinical history. These dissimilar data sets are effectively analyzed with machine learning analytics that selects features based on their association with a clinical event. This results in a highly accurate multivariate predictive model that identifies an individual's probability of experiencing a specific outcome over time. The working hypothesis was that by using this approach and expanding the clinical-pathological variables to include standardized and objective morphometric features and molecular biomarkers, one could develop a more robust tool for predicting patient outcome, reducing heterogeneity to parameters that are more generalizable and robust.

The systems pathology program was able to produce clinically effective models to predict outcome both at the time of diagnosis (using the patient's own biopsy tissue) and post-surgery [8–10]. The quantitative and standardized assessment of cell-specific biomarkers such as androgn receptor (AR) and Ki67 (utilizing quantitative multiplex immunofluorescence, image analysis and morphometry) have proven quite successful in stratifying patients and guiding treatment decisions including enrollment in active surveillance (AS) programs, brachytherapy ± androgen ablation, salvage radiotherapy and surgical approach (i.e., incorporation of a lymph node dissection and extent of surgical margin). Most recently, the biopsy model was applied on a large cohort (n = 181) of men enrolled in an AS program and successfully predicted time to treatment (i.e., exit from AS) with an accuracy of CI 0.65 (hazard ratio 3.6, $p < 0.0001$), primarily using dynamic changes of *in situ* AR levels within the primary tumor and morphometry [30]. Furthermore, these multiplex protein-network strategies should prove especially useful for identifying and quantifying (i.e., counting) complex rare cell events such as the CSC (i.e., individual cells which are: CK18-/HLA1-/GLI1-2+/Notch2+) characterized by Domingo-Domenech *et al.* [31]. There are a variety of examples where a mutation identified in one tumor type is not a driver mutation and therefore not responsive to a specific inhibitor in a different cancer due to activation of feedback loops or alternative mechanism for tumor growth, identified only at the protein level. Being able to evaluate this cell population in biopsy tissue sections will further identify those men most likely to have lethal (e.g., *de novo* chemoresistant) disease.

Finally, the future of tissue biomarker analysis in prostate cancer will undoubtedly involve a combination of the approaches outlined in this review including quantitative biomarker thresholds coupled with morphometry (systems pathology), RNA expression profiles and genomic changes represented as single-nucleotide polymorphisms or methylation signatures. Additional novel technologies including next-gen immunohistochemistry and the incorporation of labeled aptamers and mass spectrometry will undoubtedly change the field of *in situ* protein networking [32,33]. Their incorporation into systems modeling efforts will certainly allow for the complexity of the disease and provide the means to understand the process going forward. An additional consideration is the ability to effectively evaluate and quantify phosphorylated proteins in fixed/frozen tissue materials [34]. Careful consideration of appropriate biomarkers and tissue formats (needle biopsy vs resection specimens) must be considered in the overall utility of such markers for network/pathway analyses and model incorporation.

The utility for using quantitative multiplex immunofluorescence as described in the systems pathology platform has not been attempted in other GU-related cancers, notably renal cell and bladder. A recent study did employ multispectral imaging with chromogenic immunohistochemistry on ccRCC and identified that levels of ki67 were associated with increased recurrence [35]. For advanced proteomic technologies using mass spectrometry, mass spectrometry-imaging and matrix-assisted laser desorption ionization, there have been limited studies; however, a group in Germany demonstrated the promise of such an approach to phenotype bladder cancers in a tissue microarray format and subsequently identified potentially relevant prognostic markers [36]. In addition to genomic studies, the most relevant and accessible information for the urologist and uropathologist will come from protein-based assays confined to the tissue sample. Methods that utilize digital imaging and exploit the intact cellular architecture, including identification of rare cell events, will be more easily integrated in the laboratory flow of a next-gen clinical pathology department of the future.

Furthermore, there are several promising fluid-based methodologies that will add a further level of complexity for improving patient risk stratification.

Fluid-based methods to evaluate heterogeneity including CTCs, cell-free/cell-tumor DNA and exosomes

The evaluation of CTCs in peripheral blood can serve as a 'liquid biopsy' approach, supplementing other more invasive procedures, and has the potential to enhance GU clinical-translational research [36,37]. Traditionally, CTCs have been isolated from blood fairly routinely and have been used to follow patients over time as these cells provide significant information for a better understanding of tumor biology and, in particular, tumor cell dissemination. CTC molecular characterization offers the unique potential to elucidate the biology of metastasis and resistance to established therapies, and analysis of these cells presents a promising field for both advanced and possibly early-stage patients [38]. Since detection of CTCs has been shown to be of considerable utility in the clinical management of patients with GU malignancies (e.g., prostate), various analytical and versatile platforms for their detection, isolation and phenotypic characterization have been developed. New areas of research are directed toward developing novel assays for single-CTC analysis (including CSCs) and molecular characterization to predict dynamic response to treatment [38]. As previously

reported, the clinical significance of CTCs has been evaluated in many types of solid cancers, and the CTC enumeration test (i.e., number of CTCs) in metastatic breast, colorectal and prostate cancer was cleared by the US FDA almost a decade ago [36]. Of note, the very nature of how the CTC is likely released would allow a potentially broader assessment of the tumor than by needle biopsy; however, whether these processes are passive or active and truly represent the complete tumor physiology has yet to be fully determined. CTCs have been identified in both RCC and bladder cancer but with considerable variability reported in the biology, origin and malignant significance of these cells, particularly with RCC [39]. Interestingly, CTCs in bladder cancer, however, appear to hold some promise in predicting early systemic disease, and the identification of survivin-expressing CTCs may even prove to be an independent prognostic factor for disease-free survival [37]. Though there have been improvements in the isolation strategies and the use of single-cell genomics, there is still much to learn on how to incorporate the CTC feature in routine GU clinical practice. Of note, a recent publication has demonstrated that presence of the androgen receptor splice variant 7 messenger RNA in CTCs was associated with resistance to enzalutamide and abiraterone [40]. Although this requires prospective validation prior to use in clinical practice, it further illustrates the importance of fluid-based analytics in personalized medicine.

Cell-free-DNA

Circulating cell-free DNA (cfDNA) represents a noninvasive biomarker isolated from human plasma, serum, urine, stool and other body fluids [41] cfDNA offers a unique opportunity for serially monitoring tumor biology and genomics in a noninvasive manner, functioning as a surrogate for the phenotypic characteristics of the tumor. Interestingly, serum has more cfDNA than plasma but with more false-positives due to cfDNA from normal cells, and for this reason, the majority of studies evaluating cfDNA use plasma as the primary medium. A simple increased level of cfDNA is not unique to cancer and is present in normal physiologic process including inflammation, exercise and aging; therefore, specificity of cfDNA as a cancer biomarker becomes highly important. Particularly in cancer patients, a considerable proportion of plasma cfDNA originates from tumor cells, most likely from apoptosis and necrosis of cancer cells (including CTCs) as well as various immune-related cell types, thereby presenting a challenging environment for clinically accurate biomarker selection.

In the blood, cfDNA is detected in different forms such as free-floating DNA, protein-bound DNA (nucleosomes and virtosomes) and vesicle-bound DNA, including apoptotic bodies and microvesicles [42]. The predominant source of cfDNA is, however, from apoptotic bodies, which are approximately 180 bp in length as a consequence of programmed digestion of genomic DNA. Furthermore, in solid tumors, cfDNA can also be released through necrosis, autophagy, necroptosis and other physiological events induced by microenvironmental stress and chemotherapy treatment [42,43]. The biology behind the generation of cfDNA is both complex and multidimensional. Interestingly, cfDNA is also reported to reflect tumor heterogeneity, tumor burden and doubling time of multiple cancer (and normal cells), although the absolute source of cfDNA is still not entirely certain, with a proportion most likely derived from nucleated blood cells and wildtype DNA.

A major limitation of this approach, from a biomarker perspective, is the requirement for prior knowledge of the tumor mutation profile followed by assay design to identify and quantify the mutational lesion. Drug resistance in the GU malignancies may be predetermined by the existence of primary subclones under-represented or not detected due to assay sensitivity in the initial biopsy employed to decide the therapeutic protocol. Certainly, the overwhelming majority of newly diagnosed tumors in this category have a low malignant/metastasis potential, and there is very little information available to identify which tumors will progress. A possible novel application will be to use whole-genome sequencing of cfDNA to identify resistant clones prior to treatment [43].

The most important difference and challenge for the oncology field is that the mutation frequency of specific cancer types is limited and there will be a high false-positive rate without wide screening of multiple mutations. Furthermore, all of these efforts will require an appropriate complement of cfDNA from non-cancer individuals in the analysis profile.

On the other hand, cfDNA can potentially identify subclones under-represented in the tissue biopsy, allowing for a more personalized and comprehensive treatment plan. To truly estimate the clinical utility of cfDNA as a biomarker for cancer, the most conclusive results should be generated by defined clinical trials with well-annotated specimens, involving larger cohorts of patient and controls with multiple centers. Several outstanding questions remain including: Will cfDNA be equally relevant for early stage, small volume and all stages of cancer? and Will sample timing and technical practice standards be limitations for clinical adoption? To date, there have been only limited and inconclusive patient studies across multiples GU cancers evaluating the role of cfDNA in both diagnosis and prognosis with certainly more on the horizon [41]. In clinical practice, the use of cfDNA in blood from patient's with prostate, renal cell and bladder will most likely succeed in establishing prognosis and determination of treatment including monitoring of response but will be limited in utility for early diagnosis [44,46] Signatures and panels of markers, including promoter methylation strategies, have been progressing, but all require further validation in independent studies with larger numbers of patients before advancing to current patient care [44]. Further controlled studies are required to understand the role for cfDNA in the management of GU neoplasms.

Exosomes

Exosomes are extracellular lipid bilayer vesicles, usually 30–200 nm in diameter, that are released by most cell types into various biological fluids such as bronchoalveolar lavage,

blood (serum and plasma), ascites, urine, saliva, CSF and malignant effusions [47]. Because of their endosomal origin, exosomes contain several proteins involved in the endosomal sorting complexes required for transport and fusion. Additional markers expressed on exosomes include tetraspanins (e.g., CD81, CD63, CD9), heat-shock proteins, cytoskeletal proteins and relevant to this review, prostate-specific membrane antigen [48]. Interestingly, these cell-surface proteins are thought to represent a mechanism by which exosomes can specifically target various recipient cells by either interaction with cell-surface adhesion molecules or through interaction with cell-surface heparin sulfate proteoglycans [47]. Alternatively, exosomes can enter another cell via lipid-dependent endocytosis, in which a high content of sphingomyelin/ganglioside GM3 in the exosomal membranes enhances the fusion efficiency with the plasma membrane of target cells [48].

Importantly, exosomes carry proteins, lipids and genetic materials including (DNA, RNA, miRNA) and play a critical role in cell-to-cell communication in response to physiological and or pathological cues. They have been found to be important mediators of intercellular communication and regulators of the cellular niche. Previous studies have demonstrated that prostate-related genes could be successfully detected in urinary extracellular lipid bilayer vesicles, suggesting their potential use in examining prostate cancer-related transcripts [49,50]. Recent work has further shown that exosomes can be routinely and reliably isolated from urine in men presenting to their urologist for a prostate needle biopsy. RNA contained within the exosome is of extremely high quality and represents a series of well-characterized prostate cancer centric genes including *PCA3*, *ERG*, *TMPRSS2:ERG* and *AMACR,* which are reflective of the tumor phenotype [50,51]. These results along with the published biology of the exosome, including its capacity in immune modulation, suggest that the exosomes will play a significant part in sorting out tumor heterogeneity, early diagnosis, treatment choice and monitoring therapeutic response [52]. The extracellular mRNAs/miRNAs found in both the urine and blood exosomal fraction have shown a great potential as diagnostic and prognostic biomarkers in urologic cancers; however, standardization of sample collection, exosome isolation, normalization and quantification are critical [53] The most mature efforts have been identified in urinary exosomes for prostate cancer; and, it is our belief that this approach will continue to improve in accuracy, specifically for bladder and renal cell where the urine may actually be the most relevant substrate for biomarker identification.

Expert commentary

The classification of GU malignancies has historically been limited to their histologic-phenotypic diversity, utilizing expert observation parameters to subtype individual tumors based on specific diagnostic criteria. For GU cancers such as renal, prostate and bladder, such approaches have been quite effective, although there continues to be a clinical need to better define risk of progression at diagnosis and improve treatment regimens for advanced disease. This is particularly relevant for prostate cancer where over-diagnosis and over-treatment is profound, and markers which define aggressive disease have not been validated. It is also true for bladder cancer where 80% of the diagnosed tumors are superficial lesions with a favorable diagnosis by histology, but many will recur and some will eventually develop a more aggressive phenotype.

Importantly, over the past 5–10 years, there has been a focused effort to molecularly define and classify tumors in order to identify prognostic/predictive biomarkers and potential therapeutic targets. Although, for the most part, this has been successful, challenges remain in how best to implement this information in a clinical setting. Given that cancers arise through a complex series of genetic and epigenetic changes that transform somatic cells causing clonal expansion, there are multiple additional factors that need to be accounted for including tumor microenvironment, host response and impact of chemotherapy. The ability to identify, quantify and assimilate these attributes through novel tissue and fluid-based technologies with the incorporation of mathematical clinical models will undoubtedly provide the necessary tools to decipher the impact of heterogeneity on durable cancer control.

One important point worth consideration is that the degree of intratumoral (molecular) heterogeneity as presented in this review may not reflect an enhanced malignant potential. There is considerable evidence to suggest that many of the mutations that have been identified are passenger mutations and therefore not particularly clinically relevant. The challenge will be to identify the significant drivers of the disease process and target these effectively, most likely through the rational design of combinatorial therapy regimens that would match the dynamically changing cellular and molecular composition of the tumor. Although we are in an era of personalized medicine and targeted therapies, it is our view that we have yet to effectively embrace the impact of tumor heterogeneity, either in preclinical models or in clinical trial design. More work is certainly needed to further understand the tumor milieu, using both tissue- and fluid-based system approaches in concert to develop a truly personalized approach to disease management.

Five-year view

The era of next-gen sequencing and clinical genomics is becoming embedded in current medical practice. As technology platforms evolve and become more cost–effective, such approaches will eventually be standard of care for risk stratifying newly diagnosed patients, informing appropriate therapy selection and used for monitoring the course of disease. Continued advances in radiology, molecular imaging and nanotechnology, although not addressed in this current review, will also be necessary to manage this complexity in a thoughtful and beneficial way for the patient.

Over the next 5 years, there will be an emphasis on sequencing all patient tumor specimens, whether it is DNA focused hot-spot/targeted panels or whole-genome/whole-exome/ RNA sequencing efforts, to provide a more comprehensive profile of

the disease. To address the heterogeneity question, we anticipate that there will be an increased emphasis on obtaining multiple samples/biopsies from a patient's tumor at the time of diagnosis to understand tumor molecular content at baseline and, if possible, compare primary and metastasis if available. To monitor the course of the disease (including response to treatment), we believe there will be a shift toward the use of fluid-based approaches including proteomics, CTCs, cf DNA and exosomes as 'liquid biopsy' tools to profile the tumor and host response in a more dynamic fashion.

Efforts will be made to maximize the use of available specimens with strategies surrounding DNA/RNA amplification and single-cell analyses becoming more critical. Improving the preanalytic impact on genomic/protein assays in a clinical genomics environment will also advance during this period as tumor (quantity) content, quality etc. become even more critical in accurate phenotyping. In addition to the genomic activity, there is an evolving pressing need to understand pathway activation at the cellular level using systems/precise medicine approaches. We anticipate that these tissue programs will yield true spatiotemporal patterns of heterogeneity and produce dynamic clinical models where specific attributes can be incorporated to understand risk, guide treatment and so on.

Finally, a key to the success of these approaches is the implementation of a bioinformatics support network with next-gen clinical geneticists and online portals to manage and distribute information reliably and effectively in a virtual or real-time effort. Of course, this generation and management of knowledge will require partnerships between industry and academic centers of excellence with access to large and diverse patient groups in order to be effective.

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Key issues

- Intratumoral heterogeneity will negatively impact the success of targeted therapies in standard clinical trials unless steps are introduced to reduce sampling bias, improve specimen quality/preservation and develop molecular analytic techniques amenable for small-volume specimen characterization.
- Identifying and understanding the observed molecular (DNA/RNA) diversity present within tumor specimens (primary diagnostic, post-treatment or metastasis) will be critical for developing an effective treatment plan, which may include the use of combinatorial drug regimens in a clinical trial setting.
- Biologic–biochemical information contained in the intact tumor specimen must be interrogated and extracted using novel biomarker assessment tools integrated with clinical models as described in the systems/precise pathology platform.
- Employing fluid-based approaches such as exosomes and cell-free DNA represents the future of dynamic disease management from diagnosis through treatment response.

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