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CURRENT OPINION

Implementation of a Precision Pathology Program Focused on Oncology-Based Prognostic and Predictive Outcomes

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Abstract Personalized or precision medicine as a diagnostic and therapeutic paradigm was introduced some 10–15 years ago, with the advent of biomarker discovery as a mechanism for identifying prognostic and predictive attributes associated with treatment indication and outcome. While the concept is not new, the successful development and implementation of novel 'companion diagnostics', especially in oncology, continues to represent a significant challenge and is currently at the forefront of smart trial design and therapeutic choice. The ability to determine patient selection for a specific therapy has broad implications including better chances for a positive outcome, limited exposure to potentially toxic drugs and improved health economics. Importantly, a significant step in this paradigm is the role of predictive pathology or the accurate assessment of morphology at the microscopic level. In breast cancer, this has been most useful where histologic attributes such as the classification of tubular and cribriform carcinoma dictates surgery while neoadjuvant studies suggest that patients with lobular carcinoma are not likely to benefit from chemotherapy. The next level of 'personalized pathology' at the tissue-cellular level is the use of 'protein biomarker panels' to classify the disease process and ultimately drive tumor characterization and treatment. The following review article will focus on the evolution of predictive pathology from a subjective, 'opinion-based' approach to a quantitative science. In addition, we will discuss the individual components of the precise pathology platform including advanced image analysis, biomarker quantitation with mathematical modeling and the integration with fluid-based (i.e. blood, urine) analytics as drivers of next generation precise patient phenotyping.

Key Points for Decision Makers

The integration of quantitative diagnostic-predictive and prognostic pathology into clinical genomic analysis should be at the forefront of all current personalized medicine approaches.

The observed challenges in deciphering patient selection and response for immune-modulatory therapies such as the check-point inhibitors has placed a spotlight on the importance of the tumor microenvironment and the need for assessment at a cellular level.

The characterization of intratumoral heterogeneity including the identification of cancer stem cells through morphometry and quantitative biomarker assessment remain critical drivers for treatment selection and ultimately smart trial design.

Early detection and longitudinal disease assessment must incorporate multiple fluid-based analytics including exosomes and CTCs and such efforts should become part of the pathologist's comprehensive precise pathology armamentarium.

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1 Introduction

A simple definition of precise or precision pathology is the ability to more accurately define the architectural and individual components of a disease process at a level which surpasses traditional, subjective microscopic examination. By emphasizing the importance of cell to cell interactions and microanatomic histology we begin to acknowledge the significance of the cellular ecosystem in the context of a disease phenotype. Since genotype drives phenotype one would argue that focusing on the morphological attributes may further refine our basic understanding of pathophysiology at a cellular level [1].

In traditional observational pathology, the lack of cellular differentiation has always been a sign of a more aggressive phenotype. This is well established in prostate cancer with the introduction of the original Gleason grading system some 50 years ago [2], and in breast cancer with the Nottingham modification of the Scarff–Bloom–Richardson score [3] where morphologic attributes are combined to ultimately assign a prognostic risk. The extremes (i.e. low, high) of these grading systems are quite robust; however, where the classification begins to deteriorate is when intermediate architectural-cellular feature profiles dominate the histology. In this setting tumor volume and percentages of one grade or one feature over another are quite subjective and therefore the system becomes less reliable. This is most evident in the recent International Society of Urologic Pathology (ISUP) 2014 guidelines for prostate cancer where an intermediate Gleason score 7 cancer by traditional methods is now subdivided into an ISUP 2 category for Gleason 3 + 4 and an ISUP 3 for Gleason 4 + 3 in an effort to refine risk assignment [4].

1.1 Histology as a Biomarker

It is well established that basic histologic categorization has been shown to be insufficient in guiding treatment as evidenced by the identification of specific DNA mutations and or gene fusions (i.e. translocations) with treatment selection and improved response, especially for lung, colon and melanoma. One of the first examples was the association of epidermal growth factor receptor (*EGFR*) mutations in a specific sub-type (i.e. bronchioloalveolar cell carcinoma) of non-small cell lung cancer (NSCLC) [5]. In these early studies the *EGFR*-positive cases were predominantly associated with the non-mucinous phenotype, which has since evolved into additional morphologically defined categories including in situ, minimally invasive, lepidic and papillary non-small cell adenocarcinomas [6]. There is compelling evidence that the adenocarcinoma phenotype, in particular what was formerly bronchoalveolar subtype (BAC), is a significant and independent predictor of *EGFR* mutation status; however, this group represents some 4–24% of all histologic subtypes of lung cancer and approximately 80% of adenocarcinomas are mixed, limiting the use of morphology alone to assess response to specific tyrosine kinase inhibitors [7].

1.2 Clinical Genomics is not the Whole Story

Obviously improved methods over standard pathology are needed to further advance risk stratification and ultimately guide effective treatment. The advent of tumor DNA profiling has progressed to include several methodologies such as multi-gene genomic ‘hot-spot’ regions that are frequently mutated in human cancer genes, whole exome sequencing of all expressed genes in the genome (i.e. the exome) and finally whole genome analyses which interrogates the entire gene library. For NSCLC, characterizing epidermal growth factor receptor (*EGFR*), Kirsten rat sarcoma viral oncogene homolog (*KRAS*), anaplastic lymphoma receptor tyrosine kinase (*ALK*), Ros proto-oncogene 1 (*ROS1*) and RET proto-oncogene (*RET*) as well as erb-b2 receptor tyrosine kinase 2 (*ERBB2*), B-Raf proto-oncogene, serine/threonine kinase (*BRAF V600E, mutation*) and MET proto-oncogene, receptor tyrosine kinase (*MET*) have provided very useful biomarker-driven data for guiding therapy and are currently part of the 2016 National Comprehensive Cancer Network (NCCN) guidelines for patient management. Unfortunately, this collective treatment group represents a modest number of all patients diagnosed with lung cancer today, does not account for tumor heterogeneity or epigenetic changes and is a static time point in the disease continuum.

Tumor cellular diversity poses both challenges and opportunities for cancer therapy and is often not considered important or necessary given the current emphasis on clinical genomics. Recent DNA mutation-centric ‘basket’ trials, designed to be histology agnostic, have demonstrated that although a common mutation may exist, the therapeutic efficacy and ultimate outcome is not straight forward [8]. The results of these mutation-selective (biomarker) trials even within a specific class of tumors such as NSCLC have provided overwhelming evidence that a more integrated and comprehensive approach that incorporates the genome, transcriptome, and phospho(proteome) along with histology is necessary to overcome the biological complexity of cancer [9]. By excluding the functional effect of a gene and the biological-tissue context including accompanying mutations, epigenetic changes, and gene-protein expression patterns, we are only sampling a very small part of the entire process.

1.3 Interface Between Histology and Genomics

Breast cancer is a very relevant example where current analytic platforms such as the AmpliSeq Cancer Hotspot Panel (Thermo Fisher), whole exome, and whole genome panels have demonstrated the limitations in using these types of approaches to inform prognosis and guide treatment decisions. In addition, although the goal of The Cancer Genome Atlas (TCGA) was to identify all of the known cancer driver genes; what was not included is how these genetic abnormalities would manifest in vivo and whether this information could be useful in deciphering a more complete phenotype. A recent study has proposed that current breast cancer histologic grading systems are closely correlated with specific driver mutations and may actually represent a surrogate phenotypic (bio) marker for understanding these mutations in vivo [10]. Furthermore, the results suggest a biological relationship between tumor grade and known mutations and/or amplifications of well characterized oncogenic genes such as tumor protein p53 (*TP53), v-myc avian myelocytomatosis viral oncogene homolog (MYC), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA)* and ATA binding protein 3 (*GATA3)*. This suggests that understanding driver gene composition in the context of histologic grade may help in treatment selection practices, i.e. high-grade tumors with *PIK3CA* + *TP53* mutation or amplification of *MYC* will most likely not respond to a *PIK3C* inhibitor only.

In addition to the somatic genomic profile there is accumulating evidence supporting more comprehensive tumor profiling, which includes an interrogation of the microenvironment and the 'immune' host response. A recent study which used image analysis to quantify the degree of heterogeneity within the tumor microenvironment including stromal and immune populations demonstrates the importance for including these types of analyses in discriminating disease potential [11]. In this study, tumors with an increased ecosystem diversity index (EDI) or tumor cellular heterogeneity (i.e. composition of stroma, endothelial cells, lymphocytes, epithelial cells, etc.) as determined through H&E stained sections were associated with a more aggressive phenotype of breast cancer. The subsequent successes of various immune check-point inhibitors in a variety of solid tumors and some of the early trials in triple negative breast cancer would further support the necessity for expanding the analysis beyond the characteristics of the epithelial compartment of the tumor [12, 13].

It is clear that the next generation of cancer therapies must include technologies that address a more complete understanding of the malignant microenvironment. Carcinogenesis involves multiple genetic and epigenetic events, yet the organizing principles underlying their choreography are poorly understood. In addition to clinical genomics, epigenetics and the transcriptome, one must also include histologic and cellular composition of the tumor as important attributes when designing truly patient-specific treatment scenarios.

2 Tumor Heterogeneity and Comprehensive Phenotyping

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 once again 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 [14]. Providing this information at diagnosis and prior to treatment should aid in appropriate therapy selection and provide a baseline phenotype as the disease process evolves. However, even with such introduced clinical oversight there continues to be challenges in consistency between pathologists on the ability to accurately assess grade. Interestingly, the recently published MINDACT trial demonstrated that a low-risk 70-gene signature (MammaPrint) may avoid chemotherapy for patents with high-risk clinical characteristics as determined by a modified Adjuvant! on-line tool which includes pathologic grade [15]. Although additional studies are warranted and the stated trial outcome difference may not be meaningful for some patients, the study also reaffirms the importance of pathology in the decision process.

This is also true for genito-urinary (GU) cancers where the recently reported clonal disparity identified within renal-cell carcinoma has further emphasized the challenges facing genomic medicine and how to leverage this information in clinical practice. Unfortunately, even with subclassification we are not routinely able to predict response to classic treatments as with tamoxifen and breast cancer and anti-androgens and prostate cancer.

2.1 Prostate Cancer: Prototype Disease

In Europe and the USA, prostate cancer is the most common solid neoplasm and the second leading cause of death in men [16]. There are approximately 180,890 anticipated new cases in 2016 with 26,000 deaths [16]. Although the biomarker discovery process has been quite adept at identifying increasing and seemingly relevant numbers of potentially interesting markers, only prostate-specific antigen (PSA) is routinely used by urologists. Major

advances have been made in cataloging the genomic alterations in prostate cancer (PCa) and understanding the molecular mechanisms underlying the disease. These findings suggest that prostate cancer 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 transmembrane protease, serine 2 (*TMPRSS2*) gene (21q22.3) and ERG, ETS transcription factor (*ERG*; ets related group, 21q22.2) of the E74-like ETS transcription factor 1 (*ETS*) family [17]. Currently more than 20 different fusions have been reported implicating *ERG* with *TMPRSS2*, which is generally caused by an interstitial deletion at locus 21q22 and a reciprocal translocation [17]. The fusion is present in approximately 50% of all diagnosed prostate cancers with little consensus on association with risk. Noteworthy, the presence of the translocation has been used in RNA expression arrays to identify a subgroup of prostate cancer patients that are at intermediate risk for disease progression and the gene fusion has also been found to be relevant for potential therapy selection using histone deacetylase (HDAC) and Poly(ADP-ribose) polymerases, PARP inhibitors; further illustrating a role for genomics in personalized medicine [18, 19].

2.2 Role of the Cancer Stem Cell and the Immune Phenotype

Even with the above successes including the prevalence of the fusion gene, prostate cancer remains one of the most molecularly diverse and therapeutically challenging of all GU cancers. The published gene expression studies have confirmed signaling pathway diversity and clonal evolution through therapeutic pressure. 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 (CSC) 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. There is strong evidence that the CSC is in fact capable of asymmetric division, and is both cytokeratin and HLA1 negative, i.e. undifferentiated and capable of escaping immune response [20]. Such less differentiated cells are highly resistant to docetaxel as a result of activation of both Notch and Hedgehog pathways and targeting these pathways abrogates resistance through inhibition of AKT and Bcl-2 [21]. 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 [22]. Additional efforts have also implicated the GATA2-IGF2 axis as playing a role in chemoresistance for prostate cancer further emphasizing the need to accurately phenotype post-treatment residual tumor [23]. Further studies are necessary to understand the epigenetic changes associated with fluctuations in CSC activity, number or phenotype(s) and the role they play in overall therapeutic response.

The recent importance of immunotherapy in many solid and liquid tumors including the complex and heterogeneous role of various check-point inhibitors has emphasized the importance of understanding tumor composition at the cellular level. Notably, the PD-1 and PD-L1 inhibitors have shown promise in melanoma, NSCLC, renal cell, head and neck cancer, but thus far play a limited role in prostate cancer, a tumor that by general histology appears to be quite 'immunogenic' [24]. Immunotherapy for prostate cancer still has significant drawbacks and currently several improvements are needed before routine use in clinical practice, including the identification of robust biomarkers for optimal patient selection and for determining response [25]. Reliance on the expression profile of a single biomarker such as PD-L1 as a measure of patient selection will no doubt be insufficient for evaluating attributes of disease progression and only provides a very simplistic view of a very intricate, dynamic and complex process.

3 Systems 'Precise' Pathology

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 or precise pathology [26–28]. 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 which 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 which selects features based on their association with a clinical event. This results in a highly accurate multivariate predictive model which 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 which 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 of prostate cancer (using the patient's own biopsy tissue) or post-surgery [28, 29]. The quantitative and standardized assessment of cell-specific biomarkers such as AR and Ki67 [utilizing quantitative multiplex immunofluorescence (QMIF), 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). The biopsy model was also 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 (HR 3.6, $p < 0.0001$), primarily using dynamic changes of in situ androgen receptor (AR) levels within the primary tumor coupled with morphometry [30]. An example of prostate cancer in a prostate needle biopsy analyzed with the systems pathology platform illustrates the degree of heterogeneity present in by standard H&E analysis is quite homogeneous (Fig. 1).

Furthermore, these multiplex protein-network strategies should prove especially useful for identifying and quantifying (counting) complex rare cell events such as the cancer stem cell (i.e. individual cells which are: CK18−/HLA1−/GLI1–2+/Notch2+) characterized by Domingo-Domenech et al. [21]. 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. Most recently, we were able to demonstrate how such approaches can successfully dissect immune-modulation at the cellular level in patients with head and neck squamous cell carcinoma receiving an intratumoral immunostimulant Poly-ICLC and associate these results with outcome [31].

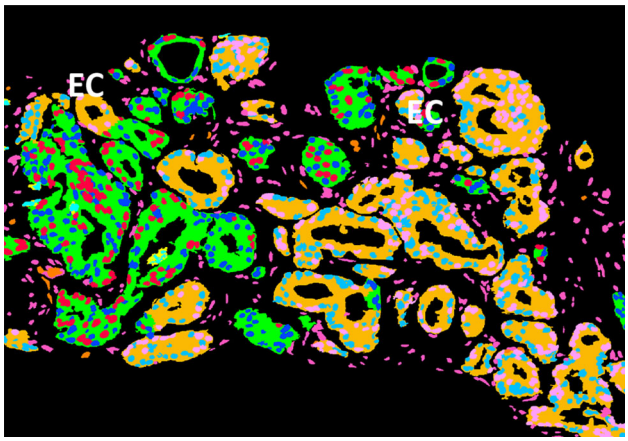

Fig. 1 Formalin-fixed, paraffin-embedded (FFPE) 5 μm section of a prostate needle biopsy with prostate cancer (PCA) stained with a multiplex immunofluorescent assay consisting of cytokeratin 18+ (epithelial cells (EC), *green*), alpha-methylacyl-CoA racemase (AMACR+EC, *orange*), the androgen receptor (AR+/AMACR−/EC, *red nuclei*; AR+/AMACR+/EC, *pink nuclei*) and DAPI (4',6-diamidino-2-phenylindole) stained nuclei. Acquired images were subjected to object-based biomarker morphometric image analysis to illustrate the heterogeneity of AMACR and AR levels across the tumor; quantitative features generated for prognostic model development. X400

Finally, the future of tissue biomarker analysis in prostate cancer and other solid malignancies will undoubtedly involve a combination of the approaches outlined in this review including quantitative biomarker thresholds coupled with morphometry (systems pathology), deep learning feature assessment, RNA expression profiles and genomic changes represented as SNPs or methylation signatures. Novel technologies including next-gen immunohistochemistry and the incorporation or labeled aptamers and mass spec will undoubtedly change the field of in situ protein networking [32, 33]. Their incorporation into systems modeling efforts will certainly address the complexity of the disease and provide the necessary 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 morphometry, network/pathway analyses and model incorporation. A recent study employed multispectral imaging with chromogenic immunohistochemistry on renal-cell carcinoma and identified that levels of Ki67 were associated with increased recurrence [35]. For advanced proteomic technologies using mass spectrometry (MS), MS-imaging (MSI) and MALDI (matrix assisted laser desorption ionization) there have been limited studies; however, a group in Germany was able to phenotype bladder cancers using this methodology in a tissue microarray format and identify potentially relevant prognostic markers [36].

As precise pathology moves towards encompassing the entire disease process from diagnosis through treatment, the ability to monitor patient response with minimal intervention will become even more important. In addition to tissue analytics there has been expanded effort to utilize body fluids such as blood and urine as alternative and

complementary source materials (e.g. genomic, cellular) for interrogation. The following section will focus on some of the more advanced technologies with the premise that phenotypic information from these analyses represents an additional element of the precise pathology program.

Although the current focus today is on body fluid genomics there are recent prognostic assays measuring specific proteins found in saliva (e.g. CD44 and total protein) that hold promise for identifying head and neck squamous cell carcinoma at an earlier stage, specifically for patients who are at higher risk [37]. Collectively, the ability to create a time-line of disease from early diagnosis through treatment and disease progression is the first step towards true precise medicine.

4 Fluid-Based Methods to Evaluate Heterogeneity Including CTCs, Cell-Free/Cell-Tumor DNA and Exosomes

The next section will focus on the evolution of the liquid biopsy from a novel biomarker discovery platform to a clinical (molecular diagnostic) assay. Initially associated with the quantitation of breast cancer circulating tumor cells (CTCs) found in blood as a measure of tumor burden, the field has incorporated the isolation and capture of cell free/tumor DNA (Cf-DNA), exosomal RNA species and even peptide–protein analytes in all body fluids including blood, CSF and urine (Table 1) [38, 39].

4.1 Circulating Tumor Cells (CTCs)

The presence of CTCs in peripheral blood functions as a "liquid (fluid) biopsy", supplementing other more invasive procedures, and over the past few years continues to receive abundant attention in clinical-translational research [40]. CTCs are isolated from blood fairly routinely and used to longitudinally follow patients over time, providing significant information regarding tumor biology and in particular, tumor cell dissemination. In addition, the recent ability to molecularly characterize the CTC has yielded a unique opportunity to improve our understanding of metastasis and resistance to established therapies, for both advanced and possibly early-stage patients. Since enumeration of CTCs has demonstrated utility in the clinical management of patients with breast malignancies, various analytical and versatile platforms for CTC detection, isolation and phenotypic characterization have been developed. New areas of research are directed towards developing novel assays for single CTC analysis (including cancer stem cells) and molecular characterization of discrete CTC phenotypes (e.g. PD-L1+) with the promise of utilizing the genomic profile to guide treatment decisions [41]. 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 FDA almost a decade ago. Notably, a splice variant of the androgen receptor, ARv7, shown to predict resistance to AR axis inhibitors enzalutamide and abiraterone, has been identified in CTCs from patients with castrate-resistant prostate cancer [42]. Even the expression of PD-L1 on CTCs has been shown to play a prognostic and possibly predictive role for check-point inhibitors [43].

4.2 Cell-Free (cf)-DNA

Circulating cell-free DNA (cf-DNA) represents a non-invasive biomarker isolated from human plasma, serum, urine, stool and other body fluids [44]. Cf-DNA offers a unique opportunity for serially monitoring tumor biology and genomics in a non-invasive manner, functioning as a surrogate for the phenotypic characteristics of the tumor. Interestingly, serum has more cf-DNA than plasma but with more false positives due to cf-DNA from normal cells, and for this reason the majority of studies evaluating cf-DNA use plasma as the primary medium. A simple increased level of cf-DNA is not unique to cancer and is present in normal physiologic processes including inflammation, exercise and aging, therefore specificity of cf-DNA as a cancer biomarker becomes highly important. Particularly in cancer patients, a considerable proportion of

Table 1 Liquid (fluid) biopsy methodologies currently utilized in clinical medicine

Liquid-biopsy	Biofluid	Result	Indication
CTCs	Whole blood	Enumeration, mutation detection, sequencing	Metastatic breast, colon, prostate Ca
Cell-free DNA	Plasma/serum	Mutation detection	Lung, prostate, colon Ca
Exosomes	Urine/plasma/serum	RNA levels, RNA sequencing, mutation, gene fusions, copy number	Prostate and lung Ca, melanoma, Glioblastoma

CTCs circulating tumor cells, *Ca* cancer, *RNA* ribonucleic acid

plasma cf-DNA 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, cf-DNA 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 [44]. The predominant source of cf-DNA is however from apoptotic bodies, which are ~180 bp in length as a consequence of programmed digestion of genomic DNA. Furthermore, in solid tumors, cf-DNA can also be released through necrosis, autophagy, necroptosis and other physiological events induced by micro-environmental stress and chemotherapy treatment [45, 46]. The biology behind the generation of cf-DNA is both complex and multidimensional. Interestingly, cf-DNA is also reported to reflect tumor heterogeneity, tumor burden and doubling time of multiple cancer (and normal cells) although the absolute source of cf-DNA is still not entirely certain, with a proportion most likely derived from nucleated blood cells and wild-type DNA. Recent success including the development of the first and only (as of this writing) FDA-approved blood-based (cf-DNA liquid biopsy) companion diagnostic for the drug Tarceva (erlotinib) in patients with NSCLC have further realized the potential [46].

A major limitation of this approach, from a biomarker prospective, is the requirement for prior knowledge of the tumor mutation profile followed by assay design to identify and quantify the mutational lesion. Drug resistance 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 cf-DNA to identify resistant clones prior to treatment [47].

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 a wide screening of multiple mutations. Furthermore, all of these efforts will require an appropriate complement of cf-DNA from non-cancer individuals in the analysis profile.

On the other hand, cf-DNA can potentially identify subclones underrepresented in the tissue biopsy, allowing for a more personalized and comprehensive treatment plan. To truly estimate the clinical utility of cf-DNA 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: (1) will cf-DNA be equally relevant for early stage, small volume and all stages of cancer? and (2) will sample timing and technical practice standards be limitations for clinical adoption?

4.3 Exosomes

Exosomes are extracellular lipid bilayer vesicles (EVs) 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 [48]. 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, and CD9), heat shock proteins, cytoskeletal proteins and even the prostate-specific membrane antigen (PSMA). 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 [48]. 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 [49].

Importantly, exosomes carry proteins, lipids and genetic materials including (DNA, RNA, and 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 demonstrated that prostate-related genes could be successfully detected in urinary EVs, suggesting their potential use in examining prostate cancer-related transcripts [49]. Recent work has validated that exosomes can be routinely and reliably isolated from urine in men presenting to their urologist with an equivocal PSA 2–10 ng/mL for their initial prostate needle biopsy [50, 51]. These approaches have generated the first commercial clinical assay [ExoDx *prostate* (*IntelliScore*)] EPI, to predict high-grade prostate cancer by isolating exosomal RNA in first-catch urine specimen without a prior digital rectal exam. RNA contained within the exosome is of extremely high-quality, and exhibits a series of well-characterized prostate cancer centric genes including: PCA3, ERG, TMPRSS2:ERG, and AMACR, which are reflective of the tumor phenotype [52]. These results, along with the

published biology of the exosome including its role in immune-modulation, suggest that the exosome will play a significant part in sorting out tumor heterogeneity, early diagnosis, treatment choice and monitoring therapeutic response.

5 Summary/Conclusion

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 which need to be accounted for including tumor morphometry, the 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 considering 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.

Compliance with Ethical Standards

Conflict of interest MJD and CCC have no conflicts.

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