-Omics and Cancer Biomarkers: Link to the Biological Truth or Bear the Consequences

Lance A. Liotta and **Emanuel F. Petricoin III**
Center for Applied Proteomics and Molecular Medicine, School of Systems Biology, College of Science, George Mason University, Manassas, Virginia

The search for clinically relevant tissue and body fluid–borne biomarkers has never been more important to the war on cancer. New -omic technologies can measure hundreds to tens of thousands of analytes at once. The field is intoxicated with the prospect of coupling these platforms, and the derived data, with sophisticated bioinformatics. The outcome promises to herald a revolutionary systems biology era for cancer research and clinical implementation. Electronic medical records, national biobanking initiatives, large-scale epidemiologic studies, new rapid sequencing tools, sophisticated gene expression analysis, and advances in protein arrays and mass spectrometry (MS), provide the hope that we can reach a new level of molecular understanding concerning carcinogenesis, cancer progression, and drug-response prediction.

Despite this excitement we cannot rush into the future without addressing 2 burning (questions for the field of biomarkers: (i) How do we prioritize our scarce resources without compromising our sense of clinical urgency? (ii) How do we reduce the failure rate for clinical validation of research findings?

We are met with the sobering reality that the past decade has seen a slow drip of FDA cleared or even CLIA-based new markers reaching routine clinical use for any aspect of cancer detection or treatment. How can we do better? We can identify several overarching critical technical and philosophical barriers (Table 1) that have prevented effective bench-to-bedside translation of the science, and threaten to mute the impact of these aforementioned large-scale efforts in the face of broad enthusiasm. We now discuss each of these overarching issues and propose and highlight some new emerging strategies to overcome these barriers

Bias in the Starting Material

Before cancer tissue and body fluid samples are collected, it is important to recognize sources of bias in the size and stage of the cancer that is sampled for analysis, and the selection of the trial cohorts and controls (Table 1). Many investigators fail to plan for the intended use of the biomarker, and thus omit the appropriate control cohorts. Tumor tissue

Corresponding Author: Emanuel F. Petricoin III, George Mason University, 10900 University Boulevard, MS 4E3, Manassas, VA 20110. Phone: 703-993-8646; Fax: 1-703-993-8606; epetrico@gmu.edu.

Disclosure of Potential Conflicts of Interest

samples often do not control for the grade, stage, and size of the tumor. Too often the pathologic stage, grade, or tumor size is not recorded in a biomarker study. Many published biomarker candidates are never validated in well-controlled independent human clinical study sets, particularly those in which serum/plasma was collected in an asymptomatic group of subjects that later developed cancer. The inadequacies of past body fluid sample handling methodologies and procedures have created anxiety in the community as realization has set in that many of our retrospectively collected study sets with long-term follow up are likely fraught with hardwired biases due to inconsistencies in how samples were collected and stored from site-to-site and study-to-study (1, 2). Many investigators fail to plan for the intended use of the biomarker, and thus do not collect specimens from the appropriate control cohorts. If the intended use of a biomarker is to uncover early recurrence of a cancer, then using a biobank of serum collected from patients with early-stage disease is not appropriate. Healthy patient plasma or sera is not a sufficient control for modern cancer biomarker research because it does not account for the overarching inflammatory and benign cellular processes that are coincident within the tumor microenvironment Consequently, the proper controls must include patients who are sick with noncancer illnesses or harbor benign tumors and must include patients with inflammatory illnesses The aggregate of known inflammatory disorders arc much more highly prevalent in most population compared to any single cancer type, thus discovery and verification efforts must be designed to maximize the chances that a given candidate biomarker has the necessary specificity in the intended population for a given intended use. Failure to do so often has mortal consequences for the candidate marker as because it would have to be revalidated in population cohorts that take into account the intended use of the biomarker, adding tremendous cost and time.

Biobanking efforts are only as good as the design of the tissue banking itself and the intended use of the candidate biomarkers that arc generated from the samples. Biomarker discovery efforts focused on uncovering mechanisms of aggressive tumor behavior or premalignant progression require tissue that contain/reflect that phenotype with adequate follow-up. Indeed, hard-wired bias can creep into tumor study sets simply by the analytic/ technical requirements of the assays that will be used to study the tissue. Efforts such as The Cancer Genome Atlas (TCGA; ref. 3) are seeking to generate genomic, transcriptomic, and functional proteomic analysis on hundreds of rumor specimens with the hope that we can link together mutational data to functional molecular determinants and elucidate the "tumor circuitry." In this time of tremendously constrained budgets and tight funding lines, these large-scale efforts require a proportionately huge amount of taxpayer money. Consequently, although these efforts are extremely exciting, we should be cautious and circumspect about maximizing the quality of science supported by every dollar. The TCGA effort uses broad-scale multiplatform molecular profiling efforts that require substantial tissue, and thus TCGA tumors are nearly all large tumors. This predetermination then hard-wires a tumor microecology bias into a high-profile molecular profiling effort. Of course, one of the best predictors of outcome is tumor size, regardless of treatment, and a biobank comprised solely of large tumors and biomarker candidates identified from such a set are then constrained in their potential impact. Although large tumor samples provide a high number of cells for analysis, this may bias the study because large tumors may have a different prognosis associated with their size or growth rate and may not reflect the most common clinical presentation for the cancer being studied. We now know that the molecular architecture of the primary rumor is much different than metastasis (4,5), and because metastasis is the lethal aspect of the disease and cancer evolves from microscopic premalignant lesions, study large primary tumors will likely tell us little about either end of the tumorigenic spectrum. It is imperative that effective biobanking initiatives take into consideration and are matched with the intended biomarker discovery/validation effort.

Sample collection, handling, storage, and processing can significantly introduce bias into biomarker discovery and validation. With regard to tissue, the effects of pre-analytic variables on the tissue microenvironment are often ignored, On the basis of recent publications (6–9) we now know that gene transcription, protein, and phosphoprotein levels change during the tissue collection and processing workflow before fixation and cell death. During the time period, the tissue is alive and many of the very biomarkers that we may use for early detection, prognosis, and prediction can change as a consequence of cellular hypoxia, acidosis, and so on, which occurs as the tissue is sitting on the pathologist's cutting table or as the surgical procedure is taking place. We need to now incorporate new workflows for rapid tissue collection so that prospective biobanking can take into consideration what the recent data is revealing while at the same time identify surrogate markers of tissue fidelity so that we can adequately assess the retrospective banks we currently have. A key component of these workflows will depend on rapid fixation and preservation of the molecular architecture of the tumor microenvironment. Formalin fixation of tissue is a century-old solution, which is incompatible with the current molecular profiling opportunities. Many retrospectively collected materials with attractive long-term clinical follow up should not be used for cancer biomarker work because of the methods used to collect the material. The slow (mm/hr) penetration time of formalin, well-known cross linking, and associated antigen retrieval issues greatly limit the molecular analysis and produce inaccurate data and are simply not acceptable. Just because we can generate data from these sets, docs not mean we should and it can take years to realize that the data is not accurate after extensive validation. We urge caution in this regard.

Fortunately, there are new molecular fixatives (10, 11) and tissue-processing methods (12) that are being developed to eliminate or greatly minimize these sources of preanalytic variables. These new tissue fixatives are being incorporated and evaluated in large national biobanking efforts and specifically provide the molecular preservation equivalence of snap-frozen tissue so that labile biomarkers can be accurately measured concomitant with formalin-like histomorphology and detail (10). Incorporation of these fixatives into prospective rapid tissue procurement-based biobanking could greatly minimize the effects of hard-wired bias for tissue biomarker research.

Even if the tissue is collected and preserved properly, and the biobank designed to take into consideration all of the aforementioned issues, tissue and cellular heterogeneity is a major source of false positives and false negatives in the field of biomarker discovery. Recent publications (13) have revealed that a different mutational landscape is found depending on what part of the tumor piece you sample and points to significant inter-and intratumoral heterogeneity. At this time, from a biomarker standpoint it is not clear what the clinical impact of tumor heterogeneity is, or even if the DNA mutation differences seen because of sampling are simply due to the lack of knowledge of which mutations are the causal driving mutations and which are passenger mutations. The need to eliminate bias caused by tissue cellular heterogeneity has certainly been recognized for over 15 years (14), and this problem has become more acute recently because of the surge of interest in the role of the tumor microenvironment (15–17) and potential for stromal therapy (18, 19). Any given piece of tumor often contains a hodgepodge of tumor epithelium, normal appearing epithelium, nerve cells, immune cells, extracellular matrix, stromal cells, and vascular cells. We now know that all of these cell types contribute to the growth, invasion, and metastasis of the tumor mass. Importantly, all of these nontumor cells can produce biomarkers at a level similar to, or higher than, the tumor cells. When bulk tumor tissue is extracted, the cellular source of the biomarker, and the proportion of cellular subpopulalions are unknown. Consequently, a marker elevated in the tissue extract may reflect the contribution of non-tumor cells. Conversely, biomarkers derived from the unknown proportion of tumor cells can be obscured by dilution. Silvestri and colleagues (20) and Wulfuhle and colleagues (21) have

clearly shown that tumors with an equivalent high proportion of carcinoma cells can have a marked difference in total biomarker levels because of the contribution of the cellular stroma. "Grind-and-bind" type of tissue analysis may reveal some tumor architecture but its likely more akin to understanding the topography of a land-mass by studying the shape and detail of the highest mountaintops. Given the recent findings that simply having tumors with differing stromal content have different outcomes, this issue is of great clinical importance. To truly understand the manifestation of genomic mutations in tumorigenic progression and metastasis, and map the aberrant and druggable protein signaling networks and then ultimately personalize therapy for a given patient, we will need to reorient our approaches to embrace the tissue microecology not ignore it, or pretend it is not that important. Many of the new molecular targeted therapies are directed against hyper-activated protein signaling networks, and the many of the drug targets within the implicated networks such as PI3K-AKT, RAS-ERK, JAK-STAT, and so on are ubiquitously expressed both in tumor epithelium and stromal compartments (endothelial cells, immune cells, fibroblasts, etc.). As we identify means of stratifying patients based on the underpinning molecular signature of their tumor and measure these drug target activities—the absolute onus is on us to get the measurement right. Fortunately bias because of tissue and cellular heterogeneity can be minimized by upfront tissue sample preparation, such as the routine use of Laser Capture Microdissection (LCM; refs. 14, 22, and 23). LCM, already well-established as a reliable and commercially available tool, is now becoming even more important as a means to study the biomarker content of individual subpopulations within the tumor microecology. Armed with LCM, we can now meet the challenge of the tumor ecology head-on. It is now routine to microdissect individual subpopulations of tumor epithelium, premalignant lesions, normal appearing epithelium, different stromal compartments, immune cells, and so on—all at once, from the same specimen, and study the biomarker content of each. Functional marker analysis of tumor microenvironment subpopulations by microdissection will be crucial for developing the next generations of immunotherapy and stromal therapy. This that can bind proteins, peptides, nucleic acids, hormones, and so on for concomitant harvesting, concentration, and preservation of cancer biomarkers in body fluids. The porous nature of the hydrogel particle shell has an effective pore Size that acts as a molecular sieve with a cutoff determined by the cross-link density. Biomarkers small enough to enter the particle are captured by the high-affinity bait. The high affinity permits very rapid sequestration of target analytes and dissociation from carrier proteins. These nanoparticles effectively protected highly labile proteins such as interleukins and growth factors from enzymatic degradation in blood, sweat, and urine, and massively increased the effective detection sensitivity, while improving the precision, of multiple reaction monitoring analysis (37). Used in whole blood as a one step, in-solution preprocessing step, the nanoparticles greatly enriched the concentration of low molecular weight proteins and peptides while excluding unwanted albumin and immunoglobulins; this achieved a 10,000-fold effective amplification of the analyte concentration, enabling MS discovery of hundreds candidate biomarkers that were previously undetectable in the ng/mL and pg/mL range (37).

Lack of Causal Association and Direct Linkage of the Biomarker(s) with the Underpinning Tumor Biology

Assuming that analytic variables are minimized by the method of sample collection and the correct study design, if the biology is ignored, then the conclusions of the biomarker analysis may be difficult to validate, or may not have any clinical impact. Investigators should strive to mechanistically tie the presence of a candidate marker to the biology of the tumor itself. Reliance on correlation without causality diminishes confidence in the candidate marker(s) and makes it extremely difficult to understand its true clinical use. Linking the biomarker to a functional role in cancer premalignant progression, growth,

invasion, and so on, and have the capability to study the biologic/biochemical effect of overexpressing or underexpressing the analyte in experimental models can provide greatly increased confidence in the use of the marker for early detection, high-risk screening, recurrence monitoring, or individualized therapy. The reliance on correlative linkage alone impacts on downstream aspects of biomarker development and implementation. For example, most -omic-based discovery efforts lead to the identification of dozens to hundreds of candidate genes, proteins, metabolites, and so an without a firm biologic basis for ranking and prioritization, a rational approach to verification and validation is stymied and often the effort burns out at that step leaving the field with yet another paper on a promising signature/multi-omic fingerprint, without proper follow-up validation and the finding never reaches the bedside. Recent publications (38) have revealed the utility of linking the biology to biomarker discovery, which provide a good roadmap for these types of endeavors.

Tying the biology to tissue marker analysis can be enabled by focused approaches that are based on known biology and biochemistry. For example, an attractive attribute for functional biomarkers, such as phosphoprotein/pathway biomarkers, or isoforms-specific products of enzymatic activity such as cleavage products (e.g., cleaved PARP), is that the biomarker is very often a direct read-out for a specified biologic process that is being targeted, or is the drug target itself. Monitoring the effect of a kinase inhibitor, say for the mTOR proteins, may be accomplished by a simple assay that measures the phosphorylation of a known mTOR kinase substrate such as p70S6 kinase. In this example, stratification for an mTOR inhibitor trial might be accomplished by measuring the level of phosphorylated p70S6 instead of a multigene signature that is many degrees of separation removed from the underpinning biologic/biochemical process. Pathway-driven biomarker discovery efforts, focused on upfront posttranslational modification enrichment techniques such as phosphoprotein capture followed by high-resolution MS can be an effective way of rationally exploring a biomarker discovery effort based on the known importance of deranged cell signaling pathway activation in tumorigenic processes (39). As discussed, broad-scale pathway activation mapping using techniques such as the RPPM provide a means to quantify the activation/phosphorylation state of hundreds of key signaling proteins and pathways at once from clinical material. Thus, both de novo discovery efforts and focused profiling efforts can be rationally focused, a priori, around biochemical processes that are directly tied to the underpinning biology of the tumor which can greatly assist in translational research efforts and speed validation and clinical implementation of the biomarker(s).

Although the field of -omic-based characterization and analysis of tumorigenesis and progression is set to unlock the mysteries of cancer like never before, there are considerable barriers that must be overcome to truly realize the promise set before us. We have a growing suite of powerful technologies that give us an unmatched view of the molecular landscape of cancer and we can generate huge databases of -omic data in hours. Cancer biomarkers hold the key to effective managed health care and are the gatekeepers of personalized medicine. All of these efforts, however, rest on the shoulders of the weakest links in the entire process. Often times these weakest links are simply ignoring the biologic reality of the tumor microenvironment, which is then manifest in the way the biobanks were designed in the first place, the lack of tumor cell enrichment, the way the tumor and/or body fluid was procured and processed, and missing evidence supporting a link between the biomarker and the cancer. Ignoring the biology will jeopardize and squander the opportunity given to us.

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Table 1

Strategies to overcome barriers to biomarker clinical implementation

Barrier to cancer biomarker progress		Emerging successful strategies to break the barrier	
1	Bias and poor biospecimen design	**a.**	Uniform protocols for collection of tissues and body fluids
	Creation of bias by protocol variation across clinical sample sets	**b.**	Preservation technologies for tissue and body fluid sample collection
	Creation of bias by use of tissue and body fluid study sets that are not representative of the clinical problem	**c.**	Use of tissue study sets that represent the clinical problem
	Generation of false-positives and false-negatives by tissue heterogeneity, improper sample handling and tracking, inadequate or improper fixation and storage	**d.**	Microdissection of tissue cell subpopulations to generate accurate and precise concentrations of the biomarker and cutpoints for clinical implementation
	Failure of blinded clinical validation	**e.**	Inclusion of independent epidemiologically credentialed and matched cohorts with benign tumors, inflammatory disease, and hormonal differences during discovery and verification phases
2	Correlation without causality	**a.**	Validation of the same biomarker across a series of experimental animal tumor models and human xenografts
	Failure to functionally link a blood biomarker to the tumor itself	**b.**	Mechanistically associating the biomarker to tumorigenesis
	Failure to functionally link a tissue biomarker with the biochemical process or mechanism of action of a drug	**c.**	Linking the biomarker with the mechanism of action of a drug
		d.	Showing a change in the biomarker after successful therapy or recurrence
3	Low abundance (<ng/nL) of biomarkers emanating from early-stage cancer and premalignant lesions, minute cancer stem cell populations, and low tumor cell content in needle biopsy specimens		Nanotechnology-based methods for biomarker capture, preservation, and exclusion of unwanted high-abundance proteins such as albumin can amplify mass spectrometry sensitivity 1,000-fold
			Development of new multiplexed assay technologies that have analytic abilities to Quantitatively measure hundreds of analytes at once from tiny input specimens