

Discovery of Protein Biomarkers for Renal Diseases

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Abstract. Animal models and human studies have been useful in dissecting the molecular mechanisms of renal disease and finding new disease targets; however, translation of these findings to new clinical therapeutics remains challenging. Difficulties with detecting early disease, measuring drug effectiveness, and the daunting cost of clinical trials hampers the development of new therapeutics for renal diseases. Many existing laboratory tests were discovered because of inspired recognition that a particular protein might prove useful in clinical practice. New unbiased genomic and proteomic techniques

identify many constituents present in biologic samples and thus may greatly accelerate biomarker research. This review focuses on the steps needed to develop new biomarkers that are useful in laboratory and clinical investigations, with particular focus on new proteomic screening technologies. New biomarkers will speed the laboratory and clinical development of new treatments for renal diseases through mechanistic insights, diagnoses that are more refined, early detection, and enhanced proof of concept testing.

Basic science has made a great deal of progress in dissecting the molecular mechanisms of renal disease; however, translation of these findings to therapeutics used in clinical practice remains challenging (1). Renal diseases garner less interest as a potential area for therapeutic development because they are often poorly characterized, differentiated often only by subtle histopathologic changes on renal biopsy, difficult to diagnose early, follow progression, and determine response to therapy, all of which add complexity and risk to a clinical trial.

Chronic kidney disease (CKD) illustrates the complexity of the problem. Early diagnosis is usually based on either the detection of proteinuria or elevation of serum creatinine. Neither test can accurately diagnose the type of renal injury. As every nephrologist knows, serum creatinine is a poor marker of early CKD because the serum concentration is greatly influenced by changes in muscle mass and tubular secretion (2). Hence, the normal reference interval must be relatively wide, and use of serum creatinine alone to follow disease progression is fraught with difficulty. Testing a therapy for CKD using a clinical end point takes a long time, and intermediate surrogate end points that can be evaluated in a shorter time frame are needed. Furthermore, significant renal disease (*e.g.*, fibrosis) can exist with minimal or no change in creatinine because of renal reserve, enhanced tubular secretion of creatinine, or other factors (2,3). Sensitive markers of early injury, especially those that correlate with early fibrosis and progression, are desper-

ately needed. In acute renal failure (ARF), serum creatinine has an even poorer sensitivity and specificity, because the patients are not in steady state; hence, serum creatinine lags behind renal injury (4). Issues such as these increase the noise and hence size and cost of clinical studies, because either the correct patients are not enrolled or the outcome measures are inaccurate or slowly track the disease. They also increase the risk of failure in drug development. A troponin-like marker of renal dysfunction that would enhance early detection or follow progression would be extremely helpful. Finally, disease markers are also useful in laboratory investigations because they can detect early injury before histologic changes are appreciated (5).

Definitions

Biomarkers and surrogate markers are important tools that can supply some of the needed information, especially when used in conjunction with traditional clinical and laboratory data (6–9). A biomarker is a biologic characteristic that is measured and evaluated objectively as an indicator of normal biologic processes, pathogenic processes, or pharmacologic response to therapeutic intervention (Table 1) (9,10). Biomarkers may be any parameter of a patient that can be measured, for example, mRNA expression profiles, proteins, proteomic patterns, lipids, imaging methods, or electrical signals. The best biomarkers are accurate, relatively noninvasive and easy-to-perform tests that can be done at the bedside or in the outpatient setting. These tests involve a blood or spot urine specimen, can be measured serially, and have a fast turnaround. In the past, most efforts had focused on discovering tissue and urinary biomarkers. However, there has been a recent shift to finding serum biomarkers (11), with new methods and technologies making this more practical. In contrast, a surrogate end point marker is the rare biomarker that can substitute (or be a surrogate) for a

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1046-6673/1507-1677

Journal of the American Society of Nephrology

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DOI: 10.1097/01.ASN.0000129114.92265.32

Table 1. Definitions

Biomarker. A biomarker is a biological characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic response to therapeutic intervention.
Surrogate end-point marker. A biomarker that is used in substitution for other clinical end points, such as survival.
Tissue marker. A biomarker that is detected in tissue. Typically these are immunohistochemical stains but, more recently, mRNA or protein.
Discovery phase. The initial investigations to identify potential biomarkers worthy of further study.

From reference 10.

clinical end point, such as survival, stroke, fracture, or cancer recurrence.

Biomarkers and surrogate end point markers have many uses in laboratory and clinical investigations and in drug discovery (Table 2, Figure 1) (6,12). Biomarkers are useful for diagnosing, classifying, or grading the severity of disease in both laboratory and clinical settings. They may be able to supply efficacy, toxicity, and mechanistic information for the preclinical and clinical phases of drug discovery and be applied with therapeutics to produce commercial tests that aid patient selection or drug dosing (personalized medicine). Because biomarkers and surrogate end point markers can accelerate the speed and decrease the risk of drug discovery, they are highly sought after. The development process is complex. Investigators need a complete development plan and, most important, access to sufficient, well-characterized samples. Unfortunately, many promising biomarkers never make it into clinical practice or even broad application in clinical or laboratory research. Understanding of the entire complex biomarker development process and using a team approach are required for a successful biomarker development project (Figure 2, Table 3). Every step requires validation, of both assay performance and diagnostic

Table 2. Role of biomarkers

Research/preclinical phase
end point marker in animal studies
proof of concept testing
screening tool for leads
rank compounds in portfolio
pharmacodynamic evaluation
toxicity profile
Clinical phase
early detection
differential diagnosis
identify subpopulations for clinical study
type/location of injury
mechanism of disease, mechanism of action
predict severity and prognosis, regression, etc.
Surrogate end point
drug effect, dose ranging studies
focused hypothesis may shorten and decrease size of trial
speed agents through testing and approval process
Commercial phase
test to aid drug dosing

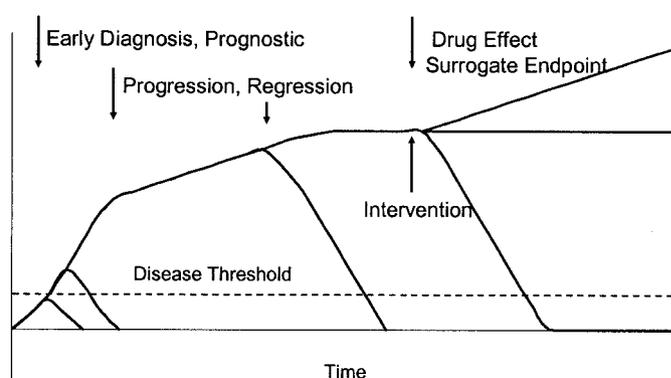


Figure 1. Types of biomarkers and their utility during different time points along the development and progression of a disease. A biomarker can be developed to target one of many different critical decision points during the natural history of a disease. The same biomarker may function for different purposes.

utility, as the biomarker moves toward the clinic (Figure 2, Table 3).

Biomarker Development Pathway

We review the early phases of the biomarker discovery pathway. It is impossible to encompass all details of biomarker development; however, we discuss key issues, new approaches, and compromises that confront an investigator. Because of space limitations, we do not discuss regulatory or intellectual property issues (see ref. 13).

Step 1: Understand and Define the Disease

Proper biomarker development requires detailed knowledge about the disease, including the definition, differential diagnosis, disease subsets, and the local or systemic responses. A reliable case definition is very valuable for proper biomarker development. The definition may be extremely specific (acute myocardial infarction defined as chest pain, EKG, and troponin) or based on a constellation of symptoms and signs (septic shock using fever, tachycardia, tachypnea, organ failure, and hypotension). Ideally, the definition should include a temporal element, for example, as the consensus definition and staging criteria for CKD (14), or the Acute Dialysis Quality Initiative consensus definition of ARF (15,16). If the disease is poorly defined, then the newly discovered biomarker may ultimately change the definition of the disease.

It is also helpful to understand and define diseases that are

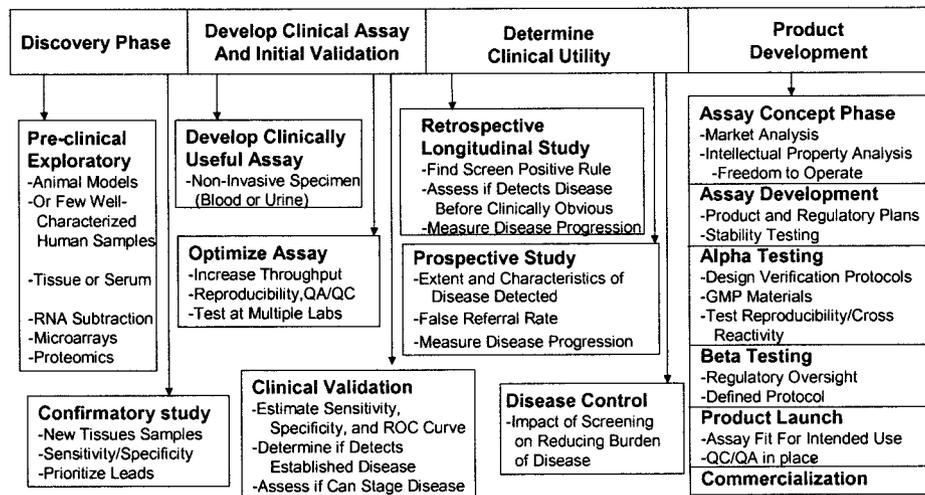


Figure 2. Biomarker and diagnostic assay development pathways. Critical steps in the discovery, clinical assay development and validation, clinical utility determination, and commercial development phases of biomarker development are shown. The discovery phase needs high-quality, well-characterized samples that may be human or from animal models. Once a promising lead is found, the presence of the biomarker should be confirmed in different samples. The next stage is to develop a clinically useful assay (often in serum or urine) and validate if it can detect established disease. The clinical utility of the biomarker is established in a retrospective longitudinal study and a prospective study and finally to determine whether the biomarker screening strategy can reduce the burden of disease. The final stage, often not appreciated, is the commercial development of the assay by industry.

Table 3. Phases of biomarker development

- 1a. Initial preclinical discovery
discovery biomarker on tissue or serum samples
- 1b. Confirmation of preclinical discovery
Validate biomarker on same type of samples
Promising direction identified and prioritized
2. Clinical assay development and validation
set up clinical assay and test on existing samples
clinical assay detects established disease
3. Retrospective longitudinal
test biomarker in completed clinical trial
detects disease early before it becomes clinically obvious
“screen positive” rule is determined; evaluate sensitivity/specificity
4. Prospective screening
Use biomarker to screen population
extent and characteristics of disease detected by test
false referral rate identified
5. Disease control
impact of screening on reducing the burden of disease

Adapted from reference 7.

commonly confused with (differential diagnosis) or are etiologic subgroups of the disease of interest, because this will have an impact on the goal of the biomarker search (step 3) and the strategy used (step 4). Nearby diseases may have similar clinical presentations but different pathophysiologic mechanisms. For example, volume depletion and urinary obstruction are commonly confused with ARF. These diseases may also be the focus of the biomarker search. For diseases for which

important subsets likely exist (e.g., ischemic, septic, toxic ARF) but are difficult to distinguish clinically, microarray or proteomic technologies may detect subclasses using unsupervised clustering, principal component analysis, and other, more statistically rigorous techniques.

Step 2: Frame the Question. What Critical Information Will the Biomarker Provide?

The best biomarkers serve a basic science, translational, or clinical need that advances the diagnosis, prevention, or treatment of the disease. For example, it is important to consider how the biomarker will be incorporated into the care of a patient (diagnostic algorithm or therapeutic management) to complement the clinical history and current laboratory examination of the patient. This process requires ongoing consultation with clinicians and scientists who understand the epidemiology, natural history, pathophysiology, and treatment of the disease to determine the remaining critical unanswered questions. A biomarker may be targeted at early detection of disease or to monitor the stage, severity, progression, or regression of disease after diagnosis (Figure 1, Table 2) or to predict drug response or follow the effect of an intervention. Examples of clinically useful renal biomarkers might include, for example, detection and staging renal fibrosis, prediction or tracking the response of fibrosis to an antifibrotic therapy, differentiation of reversible from nonreversible damage in a patient with systemic lupus erythematosus, early detection of ARF, or early detection of drug response in ARF. The best question may not be the obvious question. For example, if two drugs act synergistically to slow the progression of a disease but one has more side effects, then an obvious question is, “Which patient will progress rapidly without therapy and needs

to be treated?” However, if all patients will get the safer drug, then the critical question might be, “Which patients will not respond to addition of the second drug?” This step should not be overlooked. Biomarkers are often missing at many steps; however, it is critical to prioritize which questions to attack first.

Step 3: Desired Site of Clinical Measurement

A biomarker is usually derived from or modified by a diseased tissue but may be detected in some other fluid. Alternatively, a biomarker might arise from a distant organ or systemic reaction to the disease process (stress proteins, C-reactive protein). Upregulated genes may themselves be poor circulating biomarkers, but their metabolic fingerprints might be detected systemically, for example, pheochromocytoma (17). These last two examples highlight the advantage of starting with serum when developing a biomarker.

Biomarkers can be assayed in easily obtainable fluids, such as serum, plasma, or urine, or other sites, such as saliva, sweat, hair, and kidney biopsy material. Urinary biomarkers might also include shed cells (podocytes), casts, mRNA, or endosomal vesicles (18–21). For the sake of simplicity, they are considered as urine in this article, although the assays may be considerably more cumbersome. Serum or urine biomarkers are preferred because they are easily obtainable. The choice is driven by a balance between clinical relevance, ease of collection, and stability (serum better than urine) *versus* specificity to kidney disease and analytical simplicity of the discovery step (urine better than serum). Urine is more likely to contain biomarkers from the kidney, although, for example, urinary nitrate/nitrite reflects systemic rather than renal activation of nitric oxide system (22). Urine biomarkers may be useful for patient self-testing applications such as detection of infection, kidney stones, or monitoring of diabetic nephropathy or nephrotic syndrome. Proteomic techniques work best on urine because it is less complex fluid than serum; however, urine markers may degrade in the bladder or while sitting for a variable time in a collection vessel. Urine biomarker excretion rate cannot be determined easily because flow rate is not measured easily. Urinary biomarker concentrations are typically adjusted by urinary concentration of creatinine. This is a reasonable approximation in CKD but may not be as accurate in ARF and after renal transplantation.

Serum is often preferred for the final biomarker because of the ease of collection. However, serum markers may measure the systemic response to a disease, although there are organ-specific biomarkers (*e.g.*, troponins (23)). Also, it is difficult to find biomarkers in serum using conventional proteomic approaches because of the wide range of protein concentrations (spanning 10 orders of magnitude), complexity (large number of peptides), and predominance of 10 to 20 proteins (albumin, Ig, etc.) that overwhelm the less abundant signals. Methods to remove these abundant proteins have been developed; however, recent studies have found that many peptide fragments (potential biomarkers) circulate bound to albumin (24). Albumin acts as a sink or reservoir for these molecules and greatly prolongs their half-life from minutes to days (25). Hence,

newer assays that rely on the detection of multiple (uncharacterized) peptide peaks are being developed to probe this peptide space (26); however, it is not clear whether these current peptide fingerprint assays are sufficiently reproducible and robust for clinical use (27–29).

Could a molecular diagnosis of renal disease using novel tissue biomarkers improve renal diagnosis and therapy? Renal biopsy material can be used in the discovery phase, although, ultimately, for a biomarker that would be assayed in serum or urine. Renal biopsies are performed to obtain diagnostic, prognostic, and drug response information only when the disease process is severe enough to warrant the procedure. The kidney is an anatomically complex organ; hence, the biopsy must be evaluated histologically to confirm that the sample contains the correct part of the kidney and that the tissue contains the disease process. The tissue biomarker must provide additional information beyond the histopathologic information obtained from the biopsy.

Step 4: Devise a Strategy for the Discovery Process

The biomarker discovery strategy is influenced by the diagnostic difficulty, disease variability, subclasses, biomarker goals, ultimate site of measurement, and discovery platform (Figure 3). The first critical decision is whether to start the discovery process on the diseased tissue or the material that will make up the final clinical assay (*e.g.*, serum, urine) (Figure 3, a and b, *versus* 3, c and d). This decision involves careful balancing of competing issues. Biomarker discovery from diseased tissue is relatively easy and is very likely to yield many leads, or “hits,” but there are significant drawbacks. First, detecting tissue biomarkers in serum or urine is difficult. Many promising leads may be transcription factors or other low-abundance intracellular proteins that can be detected in tissue but not in serum. Secreted proteins and cleaved receptors typically make the best targets, although a recent study found that fragments of “intracellular proteins” may circulate bound to serum albumin (25). Second, systemic responses to the disease can be missed if the primary organ tissue is used for the discovery effort. Third, it may be difficult to obtain appropriate clinical tissue samples that match the clinical question, especially for early disease biomarkers. Newer proteomic techniques (serum purification followed by two-dimensional gels [2-D], SELDI fingerprinting) bypass the tissue step and allow for a direct search for serum biomarkers (Figure 3, c and d) (26,30). However, it is still difficult to identify an individual serum biomarkers. SELDI methods are particularly good for finding subgroups within a patient population (Figure 3d).

The second decision is whether to use human samples or samples from animal models. In general, human samples should be used when available because many animal model systems do not sufficiently replicate human pathophysiology accurately. For example, endotoxin infusion models commonly used to study sepsis do not accurately predict drug effectiveness in humans (31). Antibodies that work in a rodent system may not translate to human models. However, judicious selection and usage of appropriate animal models can be extremely beneficial, especially when human serum or tissue samples are

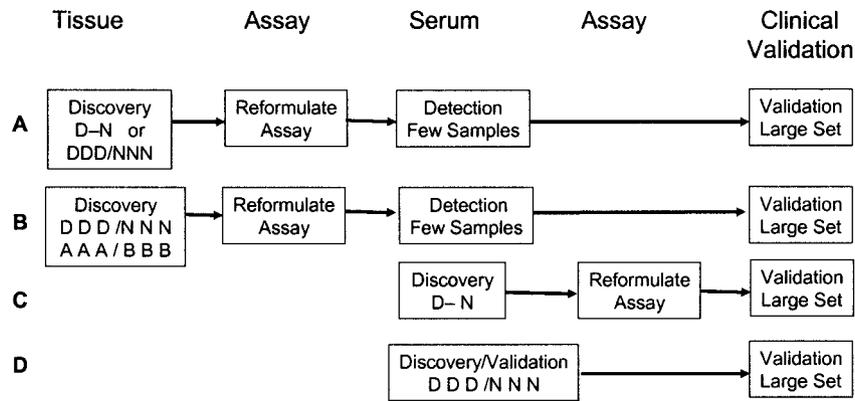


Figure 3. Strategies for biomarker development. Four typical but conceptually different biomarker development schemes are shown. (A) Biomarker for simple disease found in diseased tissue by subtractive method, then clinical assay developed (reformulated) to detect protein product in serum. Clinical assay validated initially on few samples and then on an independent larger set. (B) Biomarker for complicated disease with subgroups or near neighbors found in diseased tissue using multiple-group microarray or proteomic method. Clinical assay developed and validated as in case 1. (C) Biomarker for simple disease detected in serum by subtraction method, then assay reformulated to measure biomarker in serum. (D) Biomarkers for simple or complex disease found using surface-enhanced laser desorption ionization approach and initially validated on same sample set. D, disease; N, normal; D-N, disease minus normal; DDD/NNN, simultaneous measurement of several diseased and normal samples; A, B, closely related diseases that must be differentiated from disease D; DDD/NNN/AAA/BBB, simultaneous measurement of samples from disease, normal, and two closely related diseases.

not available. This is especially true when disease definitions are in flux (ARF), for the development of an early diagnostic biomarker (ARF, CKD), or when it is not practical to find patients early in the disease process (in part because biomarkers are lacking). It is also easier to obtain the proper controls when using animal models. In these situations, use of appropriate animal models that replicate human disease can be catalytic.

The third decision is whether to subtract two samples (disease *versus* normal) or compare several groups of samples in the discovery step (Figure 3, a *versus* b). The most common strategy (Figure 3a) is to subtract normal from diseased tissue, either using representative difference analysis (32) or by microarrays (33). One then hopes that protein will follow RNA levels, which is not always the case (34–37), and that the protein will be detectable in serum or urine. Alternatively, one may use differential proteomic approaches (2-D differential in-gel electrophoresis, isotope-coded affinity tags [ICAT]) on proteins obtained from normal and diseased tissue samples (37–39). These subtractive strategies can work well if the question is presence or absence of disease, the disease itself is uniform, and the differential diagnosis is short (*e.g.*, pregnant *versus* not pregnant). More often, subtractive strategies are used because clinical samples are extremely scarce (*e.g.*, kidney biopsy in a patient with unexpected ARF). The number of samples used may be extremely small; nevertheless, it is often desirable to perform several subtractions of normal *versus* diseased to check the reproducibility of the method and uniformity of the samples.

Alternatively, if patient heterogeneity, complicated differential diagnoses, or multiple heterogeneous disease subgroups are present and must be considered simultaneously, then it often is preferable to analyze multiple groups of samples (Figure 3b),

then use ANOVA and significance testing to find either common or subgroup markers. For example, ARF is caused by ischemia, toxins, and sepsis and is often confused with volume depletion. Each cause has a different renal response pattern and, perhaps, a specific therapy. If a general ARF biomarker is desired, then samples from all of these subclasses should be included in the initial biomarker discovery phase. The analysis, for example, could look for proteins that are upregulated in ischemic, toxin, and sepsis patients but not elevated in volume depletion. This use of complicated AND/OR/NOT logic strategies, although requiring additional clinical samples, often yields a smaller but more focused initial “hit list.”

Innovative strategies should be evaluated. Rather than compare diseased with normal tissue, one can compare samples from the same patient before and after disease. For example, a benign prostatic hypertrophy (BPH) biomarker might be found by comparing serum before and 5 wk after radical prostatectomy (at the risk of identifying a prostate cancer marker; Figure 3c). A renal cell carcinoma marker might be found by comparing serum before and after nephrectomy. Although this approach is harder in renal diseases, it could be applied before and after successful treatment for minimal-change disease.

If the analytic method is extremely expensive (*e.g.*, SAGE, ICAT), then it may be advantageous to pool samples (pool five normal control subjects, and pool five patients with disease). If the disease is heterogeneous, then this method may detect a common disease marker present in all subgroups.

Step 5: Which Samples Should Be Used for the Discovery Phase?

The scientific/clinical question (step 2) and strategy (step 4) drive the choice of samples for the initial discovery effort. These samples are the soil from which the biomarker will be

nurtured and must be as fertile as possible. Too often, the choice is guided by locally available “convenience samples” rather than using samples that are needed; this generally alters the clinical question addressed (step 2). Finding an early disease marker using tissue removed from patients with end-stage disease is unlikely to be fruitful. Thus, it may be easier to obtain serum or urine samples that closely match the clinical question; alternatively, samples from animal models may be used for the initial discovery phase (step 4). This is less of a problem for biomarkers developed to support laboratory and early translational research when the question is very focused and the samples should mirror the disease process being investigated.

It is essential the samples be from carefully defined sources, of high quality, and carefully preserved, because misclassification or degradation will rapidly doom the effort. Clinical samples should be from carefully phenotyped patients, with attention to age, gender, race, ethnicity, and concurrent medications. Sufficient sample volume is essential, as these samples may be used for multiple assays. A discovery set that is constantly changing can bewilder an investigator who is looking at multiple markers.

Tissue may be homogenized to prepare sufficient material for quantitative discovery and initial validation efforts. Alternatively, the diseased portion of the tissue can be isolated for genomic or proteomic analysis using manual or laser capture microdissection (LCM) (40–42). Although involving more effort, this enriches the source tissue and, hence, increases rare signals. Microdissection may be extremely helpful for early markers of glomerulonephritis, where contamination by tubular epithelium would overwhelm the contribution from the glomeruli.

Step 6: Determine Which Discovery Method to Use

The discovery method should be matched carefully to the scientific question, source and number samples, and strategy (Figure 3) and not just limited to methods of convenience or familiarity. These methods were reviewed recently (43–46). The advantages and disadvantages of commonly used methods are summarized in Table 4 and Appendix 1.

Step 7: Review the Significance and Feasibility

After the initial biomarker development plan has been completed, it should be reviewed to determine whether the biomarker is still needed and the plan is feasible. Will the biomarker add value to currently obtained information? Are all of the pieces in place? Are there sufficient discovery samples that match the desired question? A rigorous statistical analysis should be performed to determine the number of patients needed for the validation steps (7). Is there sufficient clinical information and are there enough clinical samples to carry out the development process?

Step 8: Perform Experiments and Prioritize the Hit List

After the initial screen (Table 3, Pepe stage 1a), the investigator should be left with 50 to 500 hits and must narrow down and prioritize the list. The best candidates for serum and urine

biomarkers are often secreted proteins, shed portions of extracellular receptors, or highly abundant intracellular proteins. Some of this information can be gleaned via simple searches (Locus Link, OMIM), comparison with published sets of secreted proteins, or more sophisticated bioinformatics tools (47). Bioinformatic tools can also be used to forecast whether a particular protein is widely expressed or restricted to a particular organ (48) (e.g., Cancer Genome Anatomy Project). Often, it is helpful to use what is known about the disease and the clinical differential diagnosis to narrow down the hit list, a “rational design approach” (49). For example, one can look for genes that are upregulated in all forms of the disease (ischemia, sepsis, toxins), then subtract genes that are upregulated in diagnostically close diseases (volume depletion). A second approach, useful in animal models, is to prevent the disease from occurring, perhaps by preconditioning (50) or drug treatment. Each additional criterion reduces the number of hits, although this must be done carefully, else true positives will also be lost.

Candidates can be confirmed and prioritized on the basis of how well they distinguish normal from disease (Table 3, Pepe stage 1b). A confirmatory study should be carried out on an independent sample set that contains a modest number of normal and diseased samples, with calculation of the true-positive rate and false-positive rate for binary biomarkers (yes/no) or the receiver operating characteristic (ROC) curve for continuous biomarkers. Candidates can then be ranked on the basis of the area under the ROC curve, or false-positive rate (for early disease screening biomarkers). At this early stage, the samples may be from animal models, but ultimately one must evaluate the markers on clinical samples. For tissue-based discovery efforts, this initial screening can be performed on tissue-derived material (mRNA, protein samples) or directly on tissue using tissue microarrays (if available).

Hits can be evaluated using immunohistochemistry and *in situ* hybridization methods to ensure that the putative biomarkers are arising from the diseased tissue, but quantification remains challenging. Some investigators have turned to the use of tissue microarrays (TMA) for target validation to extend the utility of their samples and validate against a larger sample size (51). A well-designed TMA can include the disease and off-axis diseases in the differential diagnosis. However, there are limitations, as the TMA must accurately represent the process being studied. For applications in oncology, this can be done easily; however, for renal disease, it is much harder unless the process is global and diffuse (52).

Step 9: Develop a Robust Clinical Assay and Initial Clinical Evaluation to Detect Existing Disease

To be clinically useful, the biomarker must detect disease when measured in clinically relevant serum or urine samples (Table 3, Pepe stage 2). If tissue samples were used for the discovery effort, then a clinical assay must be developed to measure the biomarker in serum or urine. The clinical assay must be optimized, or “hardened,” so that it can be performed reproducibly at multiple sites. A set of standard operating procedures, quality control, and quality-assurance procedures

Table 4. Advantages and disadvantages of platforms^a

Platform	Advantages for Biomarker Discovery	Disadvantages for Biomarker Discovery
Measurement of mRNA expression (<i>e.g.</i> , differential display, SAGE, microarray)	Able to screen large number of “genes” Commercially available	RNA levels may not directly relate to protein levels Provide no information about posttranslational protein modifications
2-D DIGE ^b	Assay of the actual biomarker not mRNA Allows identification of previously unknown biomarkers Can quantify amplitude of change in biomarker Well established technique Well suited to generating a pattern of peptide peaks corresponding to a disease biomarker High throughput, less labor intensive, and cheaper than 2-D electrophoresis	Difficult to handle large volume of data Poor technique for difficult-to-solubilize proteins (<i>e.g.</i> , membrane proteins), low-abundance proteins, and low-molecular-weight proteins Not high throughput, <i>i.e.</i> , labor intensive Difficult to identify proteins Difficult to measure protein abundance Specimen handling can have large impact on quality
SELDI ^c	Can focus on certain subsets of proteins Higher throughput than 2D DIGE Can identify protein by amino acid sequencing Increased yield of membrane proteins and low-abundance proteins	Need to use ICAT [®] to measure biomarker abundance
LC/MS/MS ^d	High-throughput validation and prioritization of tissue biomarkers (Pepe stage 1b) Obtain protein location by immunohistochemistry	Immunohistochemistry: need antibody; cannot detect “unknown” proteins <i>In situ</i> hybridization—detects mRNA only Quantitation issues Specimen quality issues
Tissue microarray	May produce unexpected new leads about pathogenesis of and biomarkers for disease	Only gives information about an individual’s risk of disease, not presence of disease <i>per se</i> Provides no information about expression of protein
SNP detection		

^a 2-D DIGE, two-dimensional difference gel electrophoresis; TOF, time of flight mass spectrometry; SELDI, surface-enhanced laser desorption ionization; ICAT, isotope-coded affinity tags; SNP, single nucleotide polymorphism.
^b Proteins from normal and diseased samples are labelled with different fluorescent dyes and then separated by two dimensional electrophoresis. Size of peptide (mass to charge ratio) is calculated based on the length of time for the peptide to travel through a vacuum.
^c Proteins from a sample(s) bind to a chip if the coating of the chip allows an adequate protein-surface affinity. For example, hydrophobic proteins bind to a hydrophobic chip surface. Then the proteins are identified by a TOF mass spectrometer.
^d Complex peptide mixtures are separated by chromatography (*e.g.*, reverse phase, cation exchange), then the chromatography fractions are analysed by TOF mass spectrometry. When two TOF mass spectrometers are used in “series,” this is referred to as MS/MS. This allows actual peptide sequencing.
^e Proteins from two different sources (*e.g.*, disease *versus* normal) can be labelled with “light” and “heavy” tags. After LC/MS/MS, the relative abundances of different peptides in the two samples can be calculated.

should be generated. The minimum analytical volume, minimal detectable concentration, and potential cross-reactants should be determined. The stability of the analyte in body fluid and during storage should be established to ensure that the assay will work on stored clinical samples (needed for next step). A good example of assay optimization for a potential renal marker was published and discussed recently (53,54).

Once the assay optimization is complete, the assay should be tested on an independent set of carefully vetted samples to determine whether it can correctly distinguish patients with established disease from normal control subjects (patients with early disease will be tested in the next step). The set needs to be of high quality and of sufficient number to measure accurately the sensitivity, specificity, and area under the ROC curve. The samples can be obtained from the baseline samples of a clinical trial but ideally should be taken from the same type of population as those for whom the test is designed. The reproducibility and portability among multiple laboratories and sites is critical; the assay should be replicated at several sites, with similar results obtained on the same sample at all sites, to ensure universality and portability of the biomarker for widespread usage. The sample set should be large enough to determine whether the biomarker level is influenced by patient factors such as age, gender, and comorbidities (hypertension, diabetes); if so, then the biomarker disease threshold may need to be defined separately for specific subpopulations. Finally, samples can be analyzed to determine whether the marker detects different stages of the disease, or disease severity, or off-axis diseases on the differential diagnosis list.

Biomarkers that are developed for bench science purposes undergo a similar process: Optimization of the assay, followed by validation on an independent sample set. As with clinical biomarkers, it is important that the sample set include normal, disease, and off-axis samples and that the reproducibility and portability be determined.

Step 10: Evaluate the Clinical Utility

The clinical utility of the biomarker needs to be determined preferably under “real-world” conditions. For screening or early

detection biomarkers, Pepe *et al.* (7,8) organized this process into a series of sequential phases that generate progressively stronger evidence. Early detection biomarkers must be able to detect disease before it is clinically apparent. This usually requires measuring the biomarker in banked repository samples from a retrospective longitudinal cohort of apparently healthy subjects who were monitored for the development of the disease (Table 3, Pepe stage 3). By comparing data from patients who developed the disease with age-matched control subjects, a screen-positive rule is established and then used to determine whether the biomarker detects early disease before it is clinically obvious. The study will indicate how the biomarker changes over time in healthy individuals and those with disease, the lead time by which the biomarker predates the clinical diagnosis, whether the biomarker tracks the natural history of the disease, and the sensitivity of the test. Next, a prospective screening study is performed to screen apparently normal individuals and rigorously applying diagnostic procedures to those who screen positive (Table 3, Pepe stage 4). These large, costly studies allow one to determine at which stage the disease is detected (early intervention opportunities), the prevalence of disease in the population, the specificity of the test, and the false referral rate. A low false-positive (or false referral) rate is critical if the early diagnosis marker will be used widely to screen a population. Finally, one must determine whether screening reduces the burden of disease (mortality, morbidity) and is cost-effective in a real-world setting (Table 3, Pepe stage 5). This typically requires a parallel-arm, randomized, clinical trial in which half of the population is randomly screened, although other approaches are possible (7,8). The goal of this ultimate test is to determine whether the disease is detected early enough to make a clinical difference. These studies are extremely expensive, time consuming, and vulnerable to changes in testing method or community adoption that limit enrollment. Testing the clinical utility of other types of biomarkers has not been as rigorously organized but includes similar retrospective and prospective studies to determine whether the biomarker tracks the natural history of the disease or response to treatment (Figure 1).

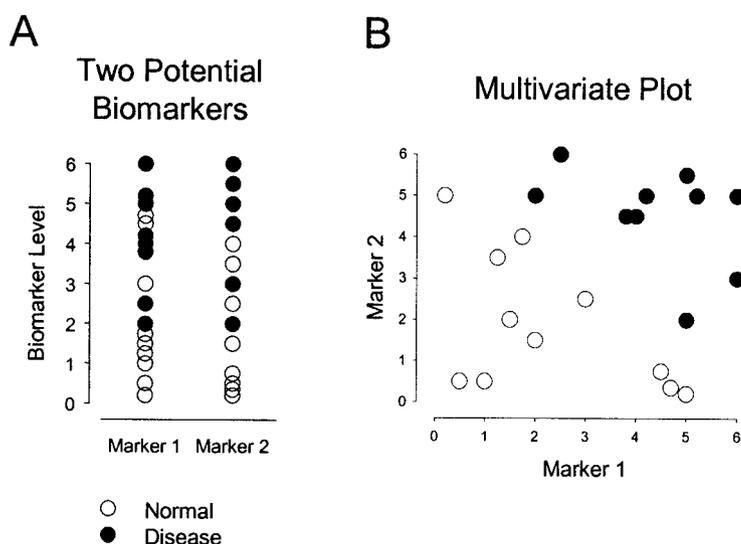


Figure 4. The potential power of multivariate analysis. (A) Two individual biomarkers that cannot discriminate between disease and normal. (B) Simple addition of the two biomarkers allows easy segregation of normal from disease.

Step 11: Combining Biomarker with Clinical Data and Other Biomarkers

Occasionally, a single biomarker will have significant value as a stand-alone test (e.g., human chorionic gonadotropin). Given the complexity and multiple overlapping pathophysiologic mechanisms of clinical diseases, finding a single biomarker with sufficiently high sensitivity and specificity is difficult. Single biomarkers often fail if the disease is heterogeneous or the biomarker level is influenced by several diseases (e.g., prostate-specific antigen is elevated in both prostate cancer and BPH). Thus, investigators are beginning to search for a panel of biomarkers and are combining biomarker data with clinical data. The critical question is not, “Does the biomarker operate alone well?” but rather, “What value/information does the biomarker add to the existing clinical data?” Even “stand-alone” biomarkers such as prostate-specific antigen or troponin are more accurate when combined with clinical data such as age (55) or combined with other biomarkers (56). Biomarker combinations may enhance the sensitivity and specificity over each individual biomarker (Figure 4). These combinations may be found using unbiased techniques or by knowledge of the different pathogenic mechanisms of the disease (56). The algorithm can be “tuned” to the specific goals of the clinical question, using multivariate techniques such as logistic regression or COX modeling to identify the independent clinical factors to include those that enhance the predictive ability. The algorithm can be displayed as a nomogram, or the multivariate equation can be downloaded onto a personal digital assistant (PDA).

Conclusions

The biomarker field is rapidly expanding and provides many opportunities to improve patient health. Table 5 provides a partial list of biomarkers for ARF that are currently in development. This review has illustrated how the sophisticated methods of molecular medicine can be melded with current tools to provide biomarkers for an increasingly complex care environment. As proteomics methods improve, it will be easier to move an idea forward through discovery phase to the validation and commercialization phases. Compared with the cost and risk of drug development, biomarkers offer the opportunity to have an impact on patient health in a more economical manner and may provide an opportunity to speed up the drug development process. Biomarkers represent a catalytic event in the interplay between academia and industry. The result is the development of biomarkers that detect disease earlier and predict which patients will respond to which therapies. In an era of aging population, greater economic constraints, and a goal of providing more targeted care (personalized medicine), biomarkers are certain to have a great presence in patient care.

Acknowledgments

We thank Dr. John Connaughton (National Institutes of Health) for the commercialization section of Figure 2 and Drs. Josephine Briggs, Roz Mannon, Terry Phillips, and Peter Yuen (National Institutes of Health) for careful review of a previous version of this manuscript.

Table 5. Acute renal failure serum and urinary biomarkers under development^a

Biomarker	Question	Discovery Method	Discovery Species, Source	Preclinical Validation	Clinical Validation	Pepe Discovery Stage	Reference
Urinary Kim-1	Detection	Kim-1 antibody	Rodent Kidney	Rodent Urine	Human urine	2	66, 67
Urinary Cyr61	Detection	mRNA then Western blot	Rodent Kidney	Rodent urine		1b	32
Urinary lipocalin	Detection	Microarray	Kidney, Rodents	Rodent urine		1b	57
Urinary NHE3 membranes	Detection	Immunoblot	Human Urine	—	Human urine	2	18
Urinary actin	Detection	Physiology	Human Urine	—	Human urine	2	68
Urinary α-GST	Detection	Physiology	Rat Urine	—			69
Urinary cystatin C and α1-microglobulin	Outcome	CKD analogy	Human Urine	—	Human urine	2	70
Urinary cytokines (IL-6, IL-8, IL-18)	Detection	Physiology	Rodent Urine	Rodent Urine	Human urine	2	68, 71
Urinary mass spec protein profile	Detection	SELDI	Human urine		Human urine	2	72
Serum cystin C	Detection	Analogy from CKD	Human Serum	—	Human serum	3	73
Serum TNF-α receptor levels	Prognosis	Physiology	Human Serum	—	Human placebo arm of CT	3	74
Plasma S100B	Detection	Analogy from brain injury	Rat plasma			1b	75
Plasma fumarylacetoacetate hydrolase	Detection	2-D/MALDI	Rodent plasma			1b	11

^a CKD, chronic kidney disease; MALDI, matrix-assisted laser desorption ionization.

Appendix 1: Individual Discovery Platforms

It cannot be over emphasized that the quality of the starting material is critical. It is critical that the quality of the analyte be examined, including confirmation of the diagnosis or classification of the material.

Microarrays

Microarray technology can be used to perform an unbiased, large-scale screen for changes in mRNA abundance in multiple samples. The method can easily deal with multiple subgroups and replicates. Replicate experiments are essential to limit the false discovery rate. Bioinformatics tools can highlight secreted proteins or shed receptors that might be good for urine biomarkers (57). The weaknesses are that it measures mRNA, not protein abundance; a large amount of high-quality mRNA is required; the deluge of resulting data; the difficulty of predicting which proteins will leak out of cells because of the disease process; and the difficulty of translating a hit to a serum biomarker. With the possible exception of circulating leukocytes, the microarray profile itself is not a biomarker. Signals may be missed if the tissue has several compartments (proximal tubules and thick ascending limbs) that respond differently to the injury. Some investigators have used manual dissection, sieving, or LCM to isolate rare cells or compartments (glomeruli) (40). Choosing the appropriate time point for analysis is essential. Changes in transcription are very rapid and transient, especially compared with changes in the proteome. The most common challenge is obtaining sufficient high-quality samples from an early time point for the development of an early detection biomarker. Although microarrays can be performed on paraffin-embedded material (with or without LCM selection), fresh or frozen tissue is superior for biomarker discovery.

2-D Electrophoresis/Mass Spectrometry

2-D gels are notoriously difficult to process and compare across samples. However, differential in-gel electrophoresis has been developed whereby protein samples are tagged with different (but similarly sized) fluorescent dyes and run simultaneously on a single gel. Two-color imaging and software analysis allow any differences between the two samples to be spotted easily. The spots can be robotically removed, trypsinized, and subjected to matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (TOF) or TOF-TOF for identification (reviewed in ref. 43). This method is good for high-abundant, moderate-pI, moderate-molecular-weight soluble proteins but is not good for membrane, low-molecular-weight, or very acidic or basic proteins. Typically, several thousand spots can be imaged and differences between samples readily appreciated. The advantage is that the compartment that will be assayed for the BM can be assayed directly, skipping steps of data filtering. The major disadvantages are that only two samples can be compared at once, serum samples must be prepurified to remove albumin else it will distort the remainder of the proteome image, and the continuing challenge of identifying the protein sequence. Superior detergents and other solubilization techniques are being

developed to extend its use to membrane proteins, high-pI proteins, and serum.

Surface-Enhanced Laser Desorption/Ionization

This new mass spectroscopy–based instrument can be used either as a discovery tool (58) or as a final clinical assay (59). Samples are selectively adsorbed to a hydrophobic metal surface, and the unbound sample is washed off. After adding an energy absorber, a portion of the sample is vaporized by a laser, and the desorbed material is transferred to a mass spectrometer. Other surfaces (hydrophilic, specific antibody, etc.) can be used. The mass spectrograph provides a low-resolution mass fingerprint of the sample. The strengths of this approach are its ability to use multiple samples, it can detect low-molecular-weight proteins and peptides that cannot be seen by 2-D approaches, and it is serum friendly. Recent studies suggest that it can detect peptides and proteins bound to serum albumin (25). The disadvantages are that the instruments are difficult to calibrated, lot-to-lot variability of reagents, and the peaks are extremely difficult to identify, although a number of sophisticated approaches can be used for identification. Also, instrument reproducibility and porting of results across laboratories and machines has not been determined.

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This new gel-free approach allows the detection of differences in two samples without many of the limitations of 2-D gels (60). Two samples are differentially labeled at free cysteines with oxygen isotopes that are 16 mass units different. The samples are trypsin-digested and mixed together, then the resulting peptides are separated by liquid chromatography and identified by mass spectrometry–mass spectrometry. The advantage of this technique is that it can detect a wider range of molecular weight and pI proteins than 2-D gels. The disadvantage is the cost, technical difficulty, and relative scarcity of this method. Several newer versions allow preferential capture of glycosylated or phosphorylated proteins. This is important, because many circulating biomarkers are glycosylated.

Protein Arrays

Several protein array platforms have garnered a great deal of interest in the biomarker development community (61). Antibody arrays, in which 10 to 500 antibodies are printed on the array, function much like a multiplex ELISA. A single sample is hybridized to a slide that contains 10 to 500 antibodies. In some versions, the samples are first labeled with a fluorescence label. Other versions function more like a multiplex sandwich ELISA; the secondary antibodies are added and then detected. Antibody arrays are tricky to set up because it is difficult to ensure specificity (i.e., lack of cross-reactivity across antibodies) and to keep the assays in the linear range of detection. Because antibody-antigen affinity is so variable and hard to determine, assembling a panel of related antibodies with similar affinities so that their linear ranges of detection are similar is very challenging and is usually tested empirically. Because most antibody arrays are not sandwich arrays, detection methods must be very sensitive. A second approach is reverse-

phase protein arrays, whereby many samples are spotted on the array and a single antibody is used to probe the samples (62,63). This is very similar in concept to a tissue microarray. Dilution curves of the samples are frequently spotted into the array so that linear detection ranges can be determined. The major challenge in this platform is specimen handling. Some methods require SDS and boiling (62), whereas others are more gentle (64). Protein arrays are not widely used because the instrumentation is expensive and the stability of the arrays is uncertain and because of difficulties with labeling methods. Frequently, protein array data cannot be analyzed using the same tools as microarrays because antibodies printed on the arrays are not independent (unsupervised). Novel analysis methods, including linear modeling, show promise; however, classical statistical methods are currently the most accepted means.

Tissue Microarrays

Although not a primary discovery platform, tissue microarrays are commonly used in biomarker discovery. A tissue microarray consists of a microscope slide that contains 50 to 1000 cores of different tissues, which can be used typically for immunohistochemistry or in situ hybridization. These arrays can be constructed from frozen tissue; however, paraffin-embedded tissue microarrays predominate. Typically, a tissue microarray is used to verify and expand on results from microarray experiments or protein array findings. Tissue microarrays are very useful to examine the tissue expression of a biomarker in the disease and can provide crucial information about expression in normal tissue and other disease processes in a rapid manner. Commercial vendors and academic centers are frequent sources of tissue microarrays, although some laboratories will construct their own arrays.

Single Nucleotide Polymorphism–Based Approaches

The newest trend in biomarker development uses a pharmacogenomic approach to identify biomarkers. Single nucleotide polymorphism (SNP) analysis seeks genomic markers (SNP) that co-segregate with a phenotype (propensity for disease, etc.). This approach is widely used to predict the metabolism of drugs and genotype/phenotype relationships in cancer (65). Because this technique analyzes germline DNA, this technique determines only a patient's predisposition, not the presence of the disease. However, if the SNP is in the promoter or coding region of a gene, then either the protein product of the gene or the catalytic product (if an enzyme) might be altered by the disease and, hence, be assayed as a biomarker. Because of the large number of "ifs," SNPs have a great deal of potential in BM development, but there may be many dead ends in the pathway.

Appendix 2

Collecting and Storing Samples

Optimal samples are essential for success, but, unfortunately, many archival samples are compromised in manners in which the investigator is unaware. Stored samples may be very fragile and degrade over time, unless stored in liquid nitrogen. The type of material and the temperature and constancy of the

temperatures are key factors. Both proteins and nucleic acids will degrade in improperly stored samples. Even storage at -80°C for long periods of time will result in degradation. Reliable guidelines are lacking, although efforts are under way to generate industry standards (<http://www.isber.org> and <http://www.tubafrost.org>). Eighteen months is considered a reasonable estimate for storage of serum or tissue in a -80°C freezer for optimal quality. Freeze-thaw cycles are particularly dangerous, so sample aliquoting and sample evaluations (including obtaining a sample of frozen tissue for pathologic evaluation) should be planned carefully to avoid freeze-thaw cycles, optimally at the time of collection. These issues should be discussed initially with clinical chemists and pathologists to improve significantly the quality of material, and this frequently will provide the benefit of additional material. Processing and storage issues of paraffin-embedded tissues are frequently overlooked. Choice of fixative, processing, and storage conditions of paraffin-embedded blocks are essential factors to consider.

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