

Classification of Molecular Biomarkers

Ankeet Shah, Dominic C. Grimberg, Brant A. Inman✉

Duke Cancer Institute, Division of Urology, Duke University Medical Center, Durham, United States

Abstract

A “biomarker” is any measurable characteristic that indicates the presence or absence of disease or the biological response to a stimulus, typically an exposure or intervention. The FDA-NIH Biomarker Working Group has produced a document called Biomarkers, EndpointS and other Tools (BEST), which defines 7 categories of biomarkers according to their clinical usage: susceptibility and risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamic and treatment response, and safety. We approach the classification of biomarkers in 2 additional ways: their bodily source and their measurement type. In the context of their use in genitourinary malignancy, we also consider factors that influence their use and reliability in clinical and research applications.

Introduction

A “biomarker” is any measurable characteristic that indicates the presence or absence of disease or the biological response to a stimulus, typically an exposure or intervention. The FDA-NIH Biomarker Working Group has defined 7 categories of biomarkers according to their clinical usage: susceptibility and risk, diagnostic, monitoring, prognostic, predictive, pharmacodynamic and treatment response, and safety. We approach the classification of biomarkers in 2 additional ways: their bodily source and their measurement type. In the context of their use in genitourinary malignancy, we also consider factors that influence their use and reliability in clinical and research applications.

Biomarkers by Source

Blood

Blood and its various components represent a valuable source for a wide variety of molecular biomarkers. Although direct sampling of cells in solid tumours of urologic oncology is not accomplished with peripheral blood draws, circulating tumour cells, as well as cell-free circulating DNA, can be used for genomic biomarkers [1,2]. Proteomics, lipidomics, and metabolomics in oncology are growing fields that can also be applied to blood samples for additional biomarker evaluation [3].

The means used to obtain blood are less invasive than those used to obtain tissue and some biofluids, and many patients with urologic malignancies are likely to undergo blood draws for standard care. Blood is largely composed of water but also contains erythrocytes, leukocytes, platelets, fibrinogen and other clotting factors, proteins including albumins and globulins, glucose, and electrolytes. Importantly, these components may limit the assessment of a given analyte if the blood is not processed appropriately [4,5]. It is also challenging to control the variation of individual components that make up blood that can occur in disease states such as dehydration, infection, or malignancy [3,4,6].

To prevent degradation, blood and blood fractions have traditionally been cryopreserved in aliquots to limit the damage to target analytes caused by thawing and re-freezing within the specimen. A major critique of this approach is that the cost associated with cryopreservation can be significant [7,8]. Alternative methods of storage that aim to decrease costs tied to cryopreservation include drying with newer methods such as lyophilization and isothermal vitrification; however, these methods are not yet standardized [9,10]. For low molecular-weight protein, drying on silica

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Abbreviations

DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
FFPE	formalin-fixed, paraffin-embedded
RNA	ribonucleic acid
RNase	ribonuclease

chips is feasible but does not protect specimens at higher temperatures. Dried blood spots using a paper system to evaporate water and contain blood components are useful in settings where access to cooling is limited for initial specimen handling. However, DBS requires controlled storage conditions, and certain analytes are more susceptible to oxidative damage. Novel techniques for safeguarding blood components remain an area of exploration [10].

Serum and plasma

Although whole blood has many uses for biomarker assessment, certain measurement modalities require sample refinement to optimize detection of a particular analyte. To this end, separating the cellular fraction out from the liquid portion of blood facilitates spectroscopy-based analysis with less interference from blood cells. The liquid fraction of blood can be isolated as either serum or plasma. Plasma is stored in a way that prevents coagulation and clot formation. Various clotting factors, fibrinogen, and platelets are maintained in suspension in plasma. Serum, on the other hand, is allowed to clot over 30 minutes before use and can give a cleaner sample when interference from platelets and other contaminants is undesirable. There are trade-offs of the 2 forms [4,11], and the liquid fraction used should be individualized to the analyte of interest [12].

Cellular fractions

Cellular components of blood are also used in a variety of biomarkers. For example, a high neutrophil to lymphocyte ratio has been found to be a poor prognostic marker of systemic inflammation and to correspond to worse outcomes in a variety of malignancies [13,14], while anemia and thrombocytopenia are used in risk stratification for renal cell carcinoma [15] and may broadly correlate with late stage tumours [16]. Isolation of cellular fractions may be achieved by centrifugation and separation by size or using advanced spectroscopy [17,18]. Cellular fractions are less subject to coagulation when blood is stored as plasma. Reassessment of cellular biomarkers from blood samples may be facilitated with such specimens, although the anticoagulant or freezing technique used may affect the viability of cells [19,20]. Flow cytometry and other immunological techniques can be used to characterize

the cellular components of blood to a high degree of precision using fluorescent antibody labelling [21].

Urine

Among the least invasive liquid biomarkers to obtain, urine also has the advantage of a simpler constituent matrix than other biofluids. Urine is more thermodynamically stable than other biofluids and generally requires less processing for preservation. Also, in the case of urinary tract facing malignancies, an opportunity exists to capture tumour cells and their biochemical by-products. Urinary extracellular vesicles containing a wide variety of molecular biomarker classes have also been discovered. A vast majority of the molecular biomarker classes are identifiable in urine. Not all patients are able to supply urine for analysis, depending on their renal function or disease state. When urine can be provided, it is subject to variations in composition and pH, which can have varying effects on any given class of biomarker. Uniquely, urine is also subject to contamination by the urinary microbiome, which can make interpretation of the source of particular analytes challenging [22-25].

Ejaculate and Prostatic Secretions

Of particular relevance to prostate cancer are prostatic biofluids, which capture analytes more effectively than other sources [26]. Of course, an intact prostate and ejaculatory pathway is required for procuring these specimens. The post-prostatic massage urine is a proxy for capturing prostatic secretions, and so this particular biofluid is also subject to the constraints of urinary specimens noted above. There are different social acceptability thresholds for semen and prostatic secretions, compared to other biofluids, making these secretions more procedurally intensive to collect. Recent efforts have shown the ability to collect RNA, DNA, proteins, and other molecular biomarkers from these biofluids [26-30]. Few data exist on storage considerations of prostatic secretions, although cryopreservation of seminal ejaculate is a standard practice in fertility scenarios [2,27,30].

Tissue

Arguably, tissue is the most invasive specimen type to obtain, and using tissue has additional costs for procurement, processing, and storage. In urologic oncology, though, tissue samples are often already obtained during routine clinical practice and may be used to identify biomarkers that guide treatment or provide prognostic information [31,32]. The full range of molecular biomarkers can be obtained from tissue samples, including more direct measurement of immune parameters at the tumour site (eg, tumour-infiltrating leukocytes), which influences endogenous

immune response to tumour as well as chemotherapy and immunotherapy efficacy [33,34].

A major advantage of tissue specimens is the inherent ease with which the signal-to-noise ratio can be optimized in evaluating molecular biomarkers derived from tumours or tumour microenvironments. Depending on the biomarker of interest, a sample may be “enriched” to exclude normal tissue and prioritize tumour tissue for analysis (eg, laser capture microdissection). Recently, efforts have been made to standardize the manner in which tissue samples for various types of tumours, are delineated from surrounding stroma on histopathologic analysis with the intent of decreasing inter-observer variability of certain biomarker assessments [35].

Like other sources of biomarkers, tissue-based biomarkers are subject to degradation and contamination. This is particularly true in fresh frozen tissue samples, in which tissue will be subject to predictable ischemic changes in the ex vivo state, such as apoptosis and in situ coagulation until freezing occurs. The timeliness of such processing would affect the accuracy and quality of biomarker analysis across a range of analytes, including more sensitive proteins [36].

Formalin-fixed, paraffin-embedded (FFPE) samples increase the longevity of the specimen regardless of storage temperature. However, residual paraffin (even after appropriate treatment) can contaminate the analysis of such a preserved sample [36]. There are trade-offs of additional processing considerations for FFPE samples obtained for clinical evaluation. These may be associated with different contaminants or constraints in methodology for evaluation, and are discussed in more detail below [37,38].

Biomarkers by Type

Genomic biomarkers

The European Medicines Agency, in concert with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, has defined a genomic biomarker as “a measurable DNA and/or RNA characteristic that is an indicator of normal biologic processes, pathogenic processes, and/or response to therapeutic or other interventions” [39].

Factors affecting genomic biomarkers

Although DNA and RNA are generally reliable biomarkers, there are some commonly encountered situations in biospecimen collection that occur in clinical medicine that can affect nucleic acid quantity and quality and impact their accuracy as biomarkers. A few of these conditions are described here.

Pre-fixation time: Pre-fixation time is the duration of time between obtaining the biopsy or surgical specimen and its preservation. As the tissue samples removed are ischemic during this interval, several important biologic processes occur in the tissue that can affect nucleic acids. RNA, in particular, is susceptible to the effects of this “cold” (ie, <37 °C) ischemia. Changes that are seen during cold ischemia include increased expression (quantity) of RNA molecules from hypoxia response genes (eg, hypoxia-inducible factor 1 α [HIF-1 α]); digestion and loss of RNA molecules with short half-lives; and broad RNA degradation and reduction in quality, starting at about 5 to 6 hours at room temperature [40]. In general, the shorter the time from patient to preservation (preservative or freezing), the better.

Formalin: Formalin fixation is a common method used to preserve biological tissue samples that have been obtained surgically or by biopsy, and subsequent paraffin-embedding allows for the cutting of thin slices for histological examination. FFPE samples are abundant and represent the standard method of clinical tissue preservation in most hospitals. Formalin has several effects on DNA that affect DNA quality, including DNA denaturation and cross-linking with cytosine residues [41]. As a result of these and other effects on DNA, formalin induces artificial mutations at a rate of approximately 1 mutation per 500 base pairs. RNA shares these formalin effects, but it is also affected by formalin in other ways which impede reverse transcription [41, 42]. Factors that increase the formalin-induced artificial mutation rate include increasing formaldehyde concentration, increasing temperature, increasing duration of fixation, and decreasing pH [41].

Tissue nucleases: Deoxyribonucleases (DNases) and ribonucleases (RNases) are tissue nucleases that digest DNA and RNA, respectively. RNA molecules are particularly susceptible to degradation by RNases, and for this reason, RNase inhibition is part of most RNA extraction protocols. DNase is felt to be an important contributor to DNA degradation in FFPE tissue samples [43].

Storage conditions: The age of the FFPE sample and storage temperature can have an impact on nucleic acid quality [44]. In general, storage at –20°C is better than room temperature, and shorter duration of storage is better.

DNA

DNA has many attributes that make it an excellent biomarker. First, DNA tends to be a very stable molecule—a biological requirement, as it directs the replication of all human cells—and is consequently

affected less by environmental conditions than many other molecules. Second, many characteristics are measurable in DNA, including single-nucleotide variants (formerly single-nucleotide polymorphisms), variability of short repeated segments (eg, microsatellites), epigenetic modifications (eg, methylation), haplotypes, deletion mutations, insertion mutations, copy number variations, and cytogenetic variations (eg, translocations, duplications, deletions, or inversions).

One important distinction with DNA is the difference between germline changes and somatic changes. Germline DNA is the complement of genes that an individual is born with and can pass on to future progeny. Generally, blood leukocytes are used as the source for germline DNA, but there are scenarios (eg, leukemia) where this is not ideal, and buccal swabs, saliva, or other normal tissue are used. Most evidence suggests that buccal swabs and saliva yield similar DNA quality to blood leukocytes, although quantity is usually less [45,46]. Germline DNA alterations can inform the presence of an inherited tumour syndrome (eg, von Hippel-Lindau disease), a susceptibility to exposures (eg, glutathione-S-transferase [GSTM1] null and *N*-acetyltransferase 2 [NAT2] slow acetylator increase the risk for bladder cancer), an ability to metabolize drugs, and a susceptibility to developing certain diseases or adverse events associated with treatment.

Somatic DNA refers to DNA collected from an affected tissue or organ, usually a tumour, and reflects a change that occurred in the DNA after conception. Somatic alterations are not passed on to children. Somatic alterations are useful for predicting responsiveness to treatment (eg, microsatellite instability and programmed death 1 ligand 1 [PD-L1] response), determining prognosis, and diagnosing the presence or absence of disease.

RNA

RNA is the transmitter of genetic information coded in the DNA and is therefore a significantly more dynamic molecule than DNA. RNA quantity and composition change significantly from tissue to tissue under normal physiologic conditions. Characteristics that are measured in RNA include sequences, splicing, expression levels, and subtype (eg, miRNA). As alluded to above, while RNA is a more responsive molecule and, perhaps, a better reflector of genetic activity within a particular tissue, it is also substantially less stable and is affected by a larger number of environmental conditions than DNA.

There are numerous types of RNA molecules and they are generally classified as the following: (a) those

involved in protein synthesis, (b) those involved in RNA modification, and (c) those whose function is mainly regulatory [47]. A non-exhaustive summary of the main types of RNA is shown in [Table 1](#).

Protein

Proteins are the workhorses of the cell and are often highly dysregulated in disease states. Proteins can be isolated from nearly all biofluids but, like all analytes, they are also subject to degradation and alteration. Human blood and urine contain proteases that cleave proteins into smaller peptides, which can be cleaved by peptidases into even smaller pieces [48]. Interestingly, the pattern of cleavage can be used as a signature to identify certain cancers [49]. Adding protease inhibitors to biospecimens can help reduce artifactual changes in proteins caused by enzymatic degradation, although these additions can also affect downstream applications.

Urine can be a particularly challenging source for protein biomarkers because of dramatic changes in pH (ranges from 4 to 8), the influence of hydration status on protein concentration, and proteolysis that occurs during storage in the bladder [50]. About 30% of urinary proteins are derived from glomerular filtration and 70% from the renal tubules and urothelium, so the urine protein pool is a mix of systemic and local-regional sources [51].

Protein-based biomarkers have generally been focused on the quantification of a particular protein or isoform. However, assessment of post-translational modifications is also important. Post-translational modifications that can be important to biomarkers include phosphorylation, methylation, glycosylation, ubiquitination, acetylation, and lipidation [52].

Glycans

The attachment of carbohydrates to molecules, such as proteins and lipids—a process known as glycosylation—is common, occurring in > 50% of human proteins [53]. Several important glycoproteins have been found to be good biomarkers in urology, including α -fetoprotein, prostate-specific antigen, and human chorionic gonadotropin. There are different forms of protein glycosylation, including N-linked (glycan attached to the nitrogen of asparagine) and O-linked (glycan attached to the oxygen of threonine and serine). Tumours may show differences in the amount, size, and type of glycosylation when compared with normal tissue. For example, N-linked glycans tend to become larger and more branched, whereas O-linked glycans tend to be truncated and expose underlying peptide epitopes. Other glycans can be important biomarkers, too. For example, glycolipids (glycans bound to lipid molecules) and glycosaminoglycans (mucopolysaccharides) have been studied as biomarkers.

TABLE 1.
Main Types of RNA

Protein Synthesis	
Type	Function
Messenger (mRNA)	<ul style="list-style-type: none"> • Transcription of the information contained in DNA exons (recipe for a protein) • Subject to alternative splicing, which creates different protein isoforms
Ribosomal (rRNA)	<ul style="list-style-type: none"> • Primary constituent of the ribosomes, where mRNA is translated into protein • Most abundant RNA in cells (about 80%)
Transfer (tRNA)	<ul style="list-style-type: none"> • Carries an amino acid matching the mRNA to the ribosome, required for translation
RNA modification	
Type	Function
Small nuclear (snRNA)	<ul style="list-style-type: none"> • Processing and splicing of mRNA in the nuclear spliceosome
Small nucleolar (snoRNA)	<ul style="list-style-type: none"> • Involved in methylation and pseudouridylation of rRNA and tRNA
Ribonuclease P	<ul style="list-style-type: none"> • Ribozyme (enzyme made of RNA) that cleaves RNA
Ribonuclease MRP	<ul style="list-style-type: none"> • Ribozyme that processes rRNA in the nucleus
Regulatory	
Type	Function
Micro (miRNA)	<ul style="list-style-type: none"> • Single stranded RNA, 22 bp length, interferes with other RNAs
Small interfering (siRNA)	<ul style="list-style-type: none"> • Double stranded RNA, 20–25 bp length, interferes with other RNAs
Long non-coding (lncRNA)	<ul style="list-style-type: none"> • Single stranded RNA, >200 bp length, interferes with other RNAs
Short hairpin (shRNA)	<ul style="list-style-type: none"> • Artificial RNA molecule designed to inhibit other RNAs, has a tight hairpin turn structure
Antisense (asRNA)	<ul style="list-style-type: none"> • Single stranded RNA complementary to a mRNA to which it binds and inhibits

Lipids

Lipids are key molecules in cellular metabolism and are a critical structural component in the biological membranes that wrap all human cells. Lipids are different from other biomolecules in that they are soluble in organic solvents, which is an important processing step in lipid analysis and characterization [54]. Lipids are subdivided into 8 classes, each of which has had some biological role described in cancer biology: fatty acyls, glycerophospholipids, glycerolipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [55]. Mass spectroscopy and related techniques are the main tools used for profiling biological lipids.

Imaging

Although it may not seem intuitive, imaging can also serve as a biomarker [56,57]. Examples of widely

available imaging-based biomarkers include basic radiological lesion characteristics (eg, size, shape, location), lesion density (computed tomography), lesion echogenicity (ultrasound), lesion signal intensity (magnetic resonance imaging), and contrast enhancement. The Response Evaluation Criteria In Solid Tumors (RECIST) criteria for evaluating tumour response to therapy is a radiological biomarker that is commonly used in clinical trials [58,59]. Functional molecular imaging has been further developed, whereby specific molecular features are studied using novel radiological ligands. For example, in positron emission tomography (PET) imaging, functional biomarkers are being explored to improve the detection of cancer, including, 18F-fluorodeoxyglucose (18F-FDG), carbon 11 choline (11C-choline), 68Gallium prostate-specific membrane antigen (68Ga-PSMA), and numerous others.

In other cases, theranostic imaging is being pursued whereby a molecular target is imaged in a patient in vivo before the administration of a targeted agent against that molecular target [60].

Pathology

The histological evaluation of tissue samples (or blood smears) is not only a routine clinical component of cancer care but also an important source of clinical biomarkers. Many standard descriptors of tissue morphology can be quantified and used as biomarkers. Common examples in genitourinary oncology include tumour grade, presence of lymphovascular invasion, presence of mitoses, and histological tumour type and subtype. More recently, digital imaging has allowed for a new era of digital pathology, in which pattern recognition and artificial intelligence software tools can

be used to characterize tissue sections with increasingly precise and reproducible methods [61,62]. It is highly likely that in the future digital pathology tools will form the backbone of the analysis of most tissue sections.

Conclusions

Biomarkers can be obtained and characterized from a highly diverse set of biological sources of measurement. There is no clear optimal biomarker, and each has inherent strengths and flaws. The future will likely consist of a collation of large networks of biomarkers that are merged computationally to provide a consensus picture of the pathological process that is occurring in the patient. This will undoubtedly require new informatic and artificial intelligence tools but will also lead to a new era of precision medicine.

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