
Cancer Biomarkers Discovery and Validation: State of the Art, Problems and Future Perspectives

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Abstract

Cancer is one of the major public health problems worldwide representing the leading cause of morbidity and mortality in industrialized countries. To reduce cancer morbidity and mortality as well as to facilitate the evolution from the traditional “one size fits all” strategy to a new “personalized” cancer therapy (i.e., the right drug to the right patient at the right time, using the right dose and schedule), there is an urgent need of reliable, robust, accurate and validated cancer biomarker tests.

Unfortunately, despite the impressive advances in tumor biology research as well as in high-powerful “omics” technologies, the translation of candidate cancer biomarkers from bench to bedside is lengthy and challenging and only a few tumor marker tests have been adopted successfully into routine clinical care of oncologic patients.

This chapter provides an updated background on biomarkers research in oncology, including biomarkers clinical uses, and discusses the problems of discovery pipeline, biomarkers failures and future perspectives.

Keywords

Biomarkers • Biomarkers failures • Cancer • Cancer biomarker: discovery and validation • Cancer control studies • Cancer therapy • Clinical assay development • Clinical uses • Discovery • Pharmacodynamic markers • Preclinical exploratory studies • Predictive markers • Prognostic markers • Prospective screening studies • Retrospective longitudinal repository studies • Risk assessment • Validation

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2.1 Background

“The term *cancer* defines over one hundred different diseases that can arise from virtually any tissue or organ in the body and, while sharing common properties of local invasion and distant spread, may have different causative factors, molecular composition, natural history of disease, methods for diagnosis and methods by which they are treated” [1].

Cancer is a major public health problem worldwide, representing the leading cause of morbidity and mortality in industrialized nations, where the lifetime risk of developing cancer is approximately 40 % (about 43 % for males and 38 % for females) [2].

According to GLOBOCAN 2012 [3], the International Agency for Research on Cancer’s online database, the global burden of cancer increased to 14.1 million new cancer cases and 8.2 million cancer-related deaths worldwide in 2012 compared with 12.4 million and 7.6 million in 2008, respectively [4]. Furthermore, GLOBOCAN 2012 predicts that there will be 19.3 million new cancer cases per year by 2025, due to growth and ageing of the global population, environmental exposures, cancer-associated lifestyles (e.g., diet, obesity, smoking and sedentary life) and late diagnosis with low survival rates. More than half of all cancers (56.8 %) and cancer deaths (64.9 %) in 2012 occurred in less developed regions of the world, and these proportions will increase further by 2025.

More than 30 % of cancer deaths could be prevented by modifying or avoiding key risk factors (behavioural and dietary), including high body mass index, low fruit and vegetable intake, lack of physical activity, tobacco and alcohol use.

To reduce cancer morbidity and mortality thereby alleviating both the economic and social costs caused by cancer, there is an urgent need to develop novel tumor biomarker tests which are sensitive and specific enough for early diagnosis, for staging and monitoring disease progression and for predicting and monitoring therapeutic response, paving the way to a “personalized” cancer treatment [5].

Unfortunately, despite the impressive advances in knowledge of tumor biology as well as in “omics” and “lab-on-a-chip” technologies, the translation of candidate cancer biomarkers from bench to bedside is long and challenging and only a few tumor marker tests have been adopted successfully into routine clinical care of oncologic patients.

Here, we review the state of the art on biomarkers research in oncology and discuss the reasons that impede the translation of findings from tumor markers research to standard clinical practice and also the ways in which this is being addressed.

In particular, this review aims to focus on the following questions: what is a cancer biomarker, and which are the potential clinical uses for a tumor biomarker test and the strategies for discovering and validating novel cancer biomarkers; finally, what are the reasons why many cancer biomarkers do not perform well in clinical practice.

2.2 Cancer Biomarkers: Definition, Types and Potential Clinical Uses

2.2.1 Definition of Cancer Biomarker

The Biomarkers Definitions Working Group of the National Institutes of Health defines a biomarker as a cellular, biochemical, and/or molecular (including genetic and epigenetic) characteristic that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [6].

A cancer biomarker, in particular, is a “biological molecule produced either by the tumor cell or by human tissues in response to cancer that is objectively measured and evaluated as an indicator of cancerous processes within the body” [7].

Alternatively, a tumor marker may be defined as a “molecule that indicates the presence of cancer or provide information about the likely future behaviour of a cancer (i.e., likelihood of progression or response to therapy” [8].

Table 2.1 Human specimens for cancer biomarker discovery

Human specimen	Cancer type
Blood (Serum or Plasma)	Broad spectrum
Cerebrospinal fluid	Brain
Nipple aspirate fluid	Breast
Breast cyst fluid	Breast
Ductal lavage	Breast
Cervicovaginal fluid	Cervical and endometrial
Stool	Colorectal
Pleural effusion	Lung
Bronchoalveolar lavage	Lung
Saliva	Oral
Ascites fluid	Ovarian
Pancreatic juice	Pancreatic
Seminal plasma	Prostate and testicular
Urine	Urological

Scientists commonly use the terms “bio-marker”, “marker”, “molecular diagnostic” and “signature molecule”, interchangeably.

Biochemically, cancer biomarkers can be DNA (germline or somatic), RNA, proteins, peptides, hormones, metabolites, and even biological processes such as apoptosis, angiogenesis or proliferation.

Cancer biomarkers can be detected in the circulation (whole blood, serum or plasma) or in secretions (stools, urine, sputum or nipple discharge) or in others human biological fluids (Table 2.1) [9] and thus easily assessed non-invasively and serially, or can be tissue-derived and require either biopsy or surgical resection [10, 11].

An ideal tumor biomarker should be [8, 9]:

- (a) produced only by the tumor cells;
- (b) correlated with tumor burden and endowed with a sufficient lead time (i.e. the time between asymptomatic cancer still localized to the organ of origin and clinical diagnosis; for example, aggressive cancers have shorter lead times than indolent cancers);
- (c) present in measurable quantities (or in concentrations significantly higher than normal) in the blood (or other human biological fluids) of cancer patients at early or preclinical stages (preferably in one cancer type only);

- (d) undetectable (or present at a very low levels) in the blood (or other biological fluids) of healthy individuals or with benign disease;
- (e) easy to measure even in small amounts and with little preparation, by means of a reliable test, cost-effective and associated to high analytical sensitivity (the percentage of individuals with cancer who test positive for the biomarker) and to specificity (the percentage of individuals without cancer who test negative for the biomarker). An ideal biomarker test would have 100 % sensitivity and specificity (i.e. everyone with cancer would have a positive test, while everyone without cancer would present a negative test).

To date, the U.S. Food and Drug Administration (FDA) have just approved 19 protein cancer biomarkers, only 11 of which are detectable in the blood (Table 2.2).

Although these biomarkers are routinely in clinical practice, nevertheless they are far from ideal, for, as the saying goes, “the ideal tumor marker does not actually exist” [8].

2.2.2 Types of Cancer Biomarkers

Based on their clinical use, three major types of cancer biomarkers are currently distinguished: (1) prognostic, (2) predictive, and (3) pharmacodynamic markers [1, 12–15]. Notably, an individual biomarker may serve more than one purpose and thus can fall into more than one of the above categories [16].

2.2.2.1 Prognostic Markers

Prognostic markers are factors that predict “disease outcome in the absence of systemic therapy or portend an outcome different from that of patients without the marker, despite empiric (not targeted to the marker) systemic therapy” [13, 17].

Practically, prognostic markers predict “the natural course of an individual cancer, distinguishing *good outcome* tumours from *poor outcome* tumours, and they guide the decision of whom to treat and/or how aggressively to

Table 2.2 List of FDA-approved protein cancer biomarkers

Biomarker	Specimen	Clinical use	Cancer type	Methodology
α -fetoprotein (AFP)	Serum	Staging	Nonseminomatous testicular	Immunoassay
Human chorionic gonadotropin- β (β-hGC)	Serum	Staging	Testicular	Immunoassay
Carbohydrate antigen 19–9 (CA 19–9)	Serum	Monitoring	Pancreatic	Immunoassay
Carbohydrate antigen 125 (CA 125)	Serum	Monitoring	Ovarian	Immunoassay
Carbohydrate antigen 15.3 (CA 15.3)	Serum	Monitoring	Breast	Immunoassay
Carbohydrate antigen 27.29 (CA 27.29)	Serum	Monitoring	Breast	Immunoassay
Carcinoembryonic antigen (CEA)	Serum	Monitoring	Colorectal	Immunoassay
Fibrin/fibrinogen degradation products (FDP)	Serum	Monitoring	Bladder	Immunoassay
Human epidermidis protein 4 (HE4)	Serum	Monitoring	Ovarian	Immunoassay
Prostate specific antigen (PSA)	Serum	Screening and monitoring	Prostate	Immunoassay
Thyroglobulin (TG)	Serum	Monitoring	Thyroid	Immunoassay
Epidermal growth factor receptor (EGFR)	Tissue	Prediction	Colorectal	Immunohistochemistry
v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT)	Tissue	Prediction	Gastrointestinal	Immunohistochemistry
Estrogen receptor (ER)	Tissue	Prognosis and prediction	Breast	Immunohistochemistry
Progesterone receptor (PR)	Tissue	Prognosis and prediction	Breast	Immunohistochemistry
v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (HER2-neu)	Tissue	Prognosis and prediction	Breast	Immunohistochemistry
Nuclear matrix protein 22 (NMP-22)	Urine	Screening and monitoring	Bladder	Immunoassay
Bladder tumor antigen (BTA)	Urine	Monitoring	Bladder	Immunoassay
High molecular CEA and mucin	Urine	Monitoring	Bladder	Immunofluorescence

treat” [14]. Prognostic markers are therefore particularly important at the time of initial diagnosis of malignancy and in cancers that vary widely in patients’ outcome (e.g. prostate and breast cancer) [13, 18].

However, as emphasized by Duffy and Crown [18], “no prognostic marker can accurately predict outcome for an individual patient; it provides a probability estimate of outcome for a heterogeneous population of patients”.

Importantly, prognostic markers may be crucial to reduce overtreatment of patients with indolent malignancy and so minimizing the side effects of adjuvant systemic therapies, and to avoid under-treatment of patients with aggressive and life-threatening malignancy for which would

be recommended to receive the most appropriate local and systemic therapy [18].

In the last years, hundreds of prognostic biomarkers have been proposed, but few have progressed to clinical use (see Sect. 2.4). Some of the best-validated and/or clinically used prognostic markers as well as other markers proposed but not used routinely in clinical oncology have been recently critically reviewed by Duffy and Crown [18].

2.2.2.2 Predictive Markers

Predictive markers are molecules that “provide upfront information as to whether or not a patient is likely to benefit from a specific therapy” [19]. Predictive biomarkers assess the likelihood that

the tumor will respond to the drug, and thereby allow a level of personalization to be introduced into the treatment regimen [1]. There are a small number of predictive biomarkers that have found clinical utility [20], and others are gaining clinical acceptance as objective measurements that inform on the clinical response to the drug (i.e., only patients expressing the marker will respond to the specific treatment or will respond to a greater degree than those without the marker) [1, 17]. Predictive markers, by prospectively differentiating populations of “responder” from “non-responder” patients, can guide the choice of anticancer therapy [17] thereby saving patients from unnecessary side effects [18]. At the same time, predictive markers might result in considerable cost savings (especially for the new biological therapies), as anticancer drugs would be used only in patients likely to derive benefit. Predictive markers, especially the very few ones that are in clinical use or close to entering clinical use, have been critically reviewed by Duffy et al. [19] and La Thangue and Kerr [1]. Again, putative predictive genomic biomarkers for cancer targeted therapies have been recently reviewed by Simon and Roychowdhury [15].

2.2.2.3 Pharmacodynamic Markers

Pharmacodynamic markers provide information on the effects of the drug on the body (i.e., drug targets and mechanisms of action), including both early effects on its molecular target (i.e. whether a drug engages and inhibits a target, and the degree and timing of the inhibition) and also later effects on downstream events [21–23]. On the other hand, pharmacokinetics evaluates the effect of the body on the drug: that is, the process by which a drug is absorbed, distributed, metabolized and eliminated by the body.

Consequently, endpoints of pharmacodynamic markers include assessments of protein phosphorylation markers, measures of cellular proliferation/apoptosis, cell-cycle regulation biomarkers, and epigenetic changes [21, 24]. In oncology, pharmacodynamic biomarkers are utilized in optimizing doses of chemotherapeutic drugs below their cytotoxicity level and in understanding response/resistance mechanisms [21, 22].

2.2.3 Potential Clinical Uses of Cancer Biomarkers

The different types of cancer biomarkers that can be used in multiple clinical settings depend on the disease stage (and hence on patient status). Biomarkers, indeed, can be accounted for before cancer diagnosis (in risk assessment and screening for premalignant lesions or early invasive disease), at diagnosis (in staging, grading, and selection of initial therapy) and after diagnosis (in monitoring therapy, selecting additional therapy and detecting recurrence) (Fig. 2.1).

“Consequently, the spectrum of cancer patient status can range from unaffected individuals who are concerned about whether they should adopt preventive or screening strategies, to patients with early-stage disease for whom considerations of appropriate primary (surgery and radiation) and adjuvant systemic therapies (chemotherapy, hormone therapy, biological therapy or various combinations of these therapies) are critical, to those who are free of disease but are concerned about recurrence, and finally to patients with established metastatic disease” [11].

Remarkably, some biomarkers are only used in a specific setting, whereas other ones can serve in more than one mode [10].

In this regard, tumor biomarkers might be useful for: (1) risk assessment, (2) screening for early cancer detection, (3) diagnosis, (4) prognosis, (5) selection and monitoring of anticancer therapy [10, 11, 25].

2.2.3.1 Risk Assessment

“Risk assessment is the search for factors that provide the earliest evidence of the impending cancer in persons not yet diagnosed with the disease” [26].

Cancer, traditionally viewed as a series of genetic diseases, is now recognized to involve epigenetic modifications along with genetic mutations [27]. Moreover, genetic and epigenetic alterations are not separate events in cancerogenesis, but the “*crosstalk*” between these two mechanisms ultimately promotes genomic instability and abnormal gene expression contributing to the various phases of neoplastic development

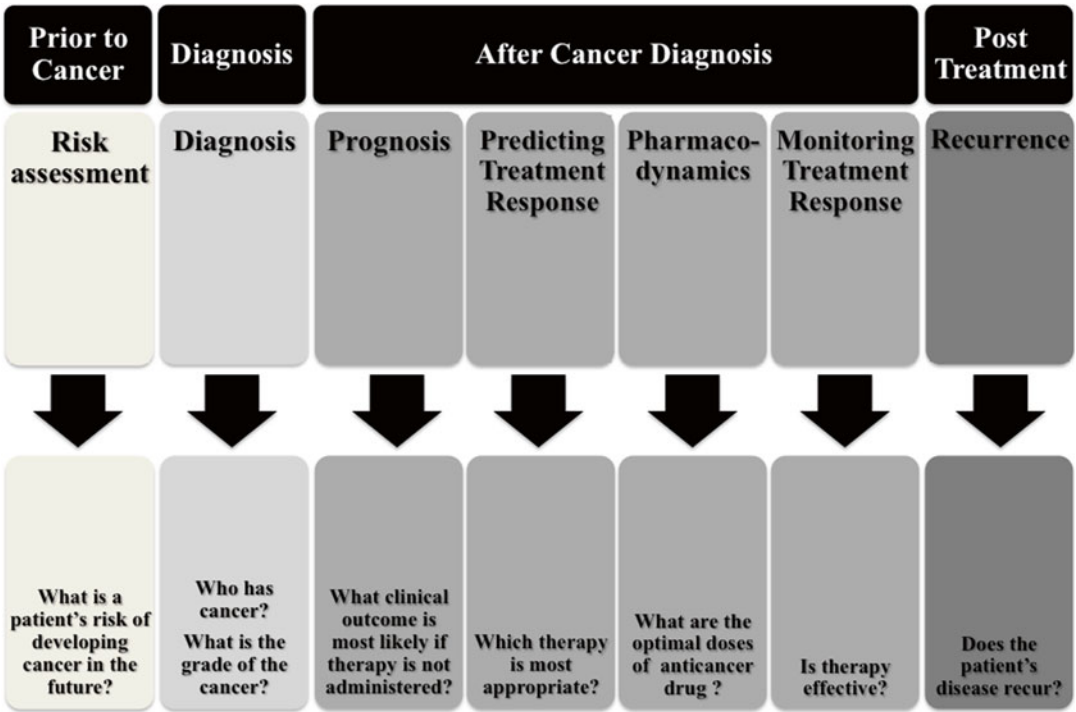


Fig. 2.1 Potential fields of application of a cancer biomarker test

including initiation, promotion, invasion, metastases and chemotherapy resistance [28]. Consequently, genetic (e.g. point mutations, translocations and copy number variations) and, more important, epigenetic materials modifications (e.g. DNA methylation, histone post-translational alterations, chromatin remodeling, and small, noncoding microRNAs expression) may represent an early and promising analytical tool for biomarker discovery, with broad potential applications in risk assessment, screening for early cancer detection, prognosis, and prediction of response to therapy [29].

In particular, because epigenetic modifications may constitute a signature of specific exposure to certain risk factors, they have the potential to serve as highly specific biomarkers for risk assessment [29].

Genetic and epigenetic biomarkers and their clinical implications in risk assessment and early cancer diagnosis are diffusely discussed in recent pivotal reviews [27, 30–33].

2.2.3.2 Screening for Early Cancer Detection

Many cancer types, if diagnosed and treated early, can be cured or, at least, transformed to a chronic disease. Therefore, early cancer detection in asymptomatic patients still remains a priority of cancer research with a high potential of improving both patients’ survival and quality of life [34].

A useful screening test for early detection of cancer must exhibit most, if not all, the characteristics previously described for an ideal tumor marker. “First of all, a screening test should be able to detect malignancy at an early and asymptomatic stage thereby resulting in decreased morbidity or increased survival rates” [35]. In addition, the test must be inexpensive and safe enough to be applied to mass populations. Moreover, a screening test should have a very high sensitivity and an exceptional specificity, to avoid too many false positives in populations with a low cancer prevalence.

Unfortunately, besides some notable exceptions (e.g. human chorionadotropin for germ cell tumors and gestational trophoblastic disease and α -fetoprotein for hepatocellular and testicular carcinoma), none of the biomarker tests currently used in clinical oncology is suitable for population screening or early diagnosis of cancer, that still remains the biggest clinical challenge of all [34, 36, 37].

A list of selected promising molecular markers for early detection of cancer is reported in [38].

2.2.3.3 Diagnosis

Contrary to screening, a diagnostic test would be prescribed to an individual who has already manifested symptoms of cancer. Currently, however, there is no biomarker test recommended in clinical practice guidelines for cancer diagnosis, but many of the well-known markers are widely used as aids in diagnosis and/or sub-classification of a particular malignancy state [8, 35].

2.2.3.4 Prognosis

For prognostic markers see Sect. 2.2.2.1.

2.2.3.5 Selection and Monitoring of Cancer Therapy

For predictive and pharmacodynamic markers see Sects. 2.2.2.2 and 2.2.2.3, respectively.

2.3 Cancer Biomarker: Discovery and Validation

The process of discovering and developing molecular cancer biomarkers “is a work in progress and is evolving” [39], representing an “integral component of contemporary cancer research” [35].

In 2001, the National Cancer Institute’s Early Detection Research Network (EDRN), to promote efficiency and scientific rigor in biomarkers research, introduced guidelines “to guide the process of biomarker development” consisting of five “phases that are generally ordered according to the strength of evidence that each phase provides in favour of the biomarker, from weakest to strongest and the results of earlier phases are generally necessary to design later phases” [40].

These guidelines propose specific aims, subject selection, outcome measures, and evaluation of results for each of the five phases of a biomarker discovery pipeline in the context of progress being made in the field and relevant published studies [40–42]. The phase structure of biomarker development pipeline includes: Phase 1 (Preclinical exploratory studies), Phase 2 (Clinical assay development), Phase 3 (Retrospective longitudinal studies), Phase 4 (Prospective screening studies), and Phase 5 (Cancer control studies).

The phases are not rigorously distinct from each other and to proceed from one phase to another a candidate biomarker needs to overcome pre-analytical, analytical and post-analytical challenges at different levels. Only biomarkers that will reach the last step successfully will be implemented in the clinic [9, 35, 43, 44].

A major implication of this framework is that the time required from the initial discovery to clinical adoption of a biomarker is lengthy, generally a decade or more [25].

2.3.1 Phase 1: Preclinical Exploratory Studies

The beginning of the discovery phase in the biomarker development pipeline involves preclinical semi-quantitative studies to identify one or more promising biological molecules (“candidate biomarkers”) that, exhibiting discriminating potential between cancer patients and healthy subjects, might be useful to develop clinical tests for early detecting and monitoring cancer or for managing cancer therapy.

The discovery process of new putative tumor markers can involve two major complementary approaches: (a) “knowledge-based” or “hypothesis-driven” or “targeted” method; (b) “unbiased” or “discovery-based” or “untargeted” method [7, 45, 46]. The “hypothesis-driven” approach identifies candidate biomarkers by a deductive method that relies on ever-increasing knowledge of the molecular mechanisms underlying cancer biology and therefore only a specified set of molecules supposed to be involved in cancerogenesis is measured (“targeted” method). In contrast, the “discovery-

based” approach identify candidate biomarkers by an inductive method that, exploiting the extraordinary potentiality of new high-throughput-omics technologies (capable of identifying multiple rather than just a single marker by performing parallel rather than serial analyses), select molecular species on the basis of their differential expression between normal (controls) and diseased (cases) states, without an *a priori* target identification (“untargeted” method) [7, 45, 46].

Although high-throughput-omics technologies are frequently used for biomarkers discovery, hypothesis-driven method is now endorsed as the preferred one [7, 35, 47, 48] as the key advantage of this approach is that “defining an intended use for the tumor marker at the early stages of the discovery process allows better control of the variables (other than the cancer itself) that may influence measured levels of the marker during the discovery process” [7].

Regardless of which method is selected, a rigorous and accurate study design is essential to reach the required results. The major topics to be defined when discovery studies are planned include: (a) the number of samples to analyse; (b) inclusion/exclusion criteria for the samples; (c) collection and handling requirements; (d) limitations of the analytical methodology(ies); (e) appropriate statistical analysis of the acquired data; and (f) validation of the findings in independent datasets and by independent investigators. Moreover, complete and transparent reporting of results is also necessary so that other investigators can assess the “soundness of the study” [7, 49].

“Judging by the numerous publications reporting novel candidate biomarkers, the discovery phase seems to be productive” [35], however the majority of cancer biomarkers do not progress beyond this phase. The main reasons for this failure include modest differences in the concentration of the biomarker in cases compared with controls and large variability in the levels of the biomarker in healthy subjects [50].

For an in-depth discussion of these and other issues of the discovery phase of biomarker-development pipeline, the reader is referred to several excellent published reviews [7, 35, 37, 44, 49] and to the references therein.

2.3.2 Phase 2: Clinical Assay Development

Once a promising cancer biomarker is identified, the next crucial step is to develop and validate a robust, accurate and reliable test (at this regard, “it is essential to distinguish a cancer biomarker from a cancer biomarker test, that is a specific assay that measures the marker reliably” [51]) to measure the analyte of interest both in the clinical trials of the biomarker-development pipeline and, more important, in an eventual routine laboratory practice [40].

“Assay development and validation is an iterative process that occurs at every step in the pipeline and may not end even after an assay is marketed” [35]. To progress to clinical practice, a candidate biomarker test, like any other medical technology or intervention, must undergo a rigorous evaluation that involves the assessment of its: (a) analytical validity, (b) clinical validity, and (c) clinical utility (Fig. 2.2). The terms “analytical validity”, “clinical validity” and “clinical utility” have been coined in 2009 by the Evaluation of Genomic Applications in Practice and Prevention Working Group in the development and implementation of a rigorous process able to support the translation of scientific evidence on genomic testing into clinical practice [52].

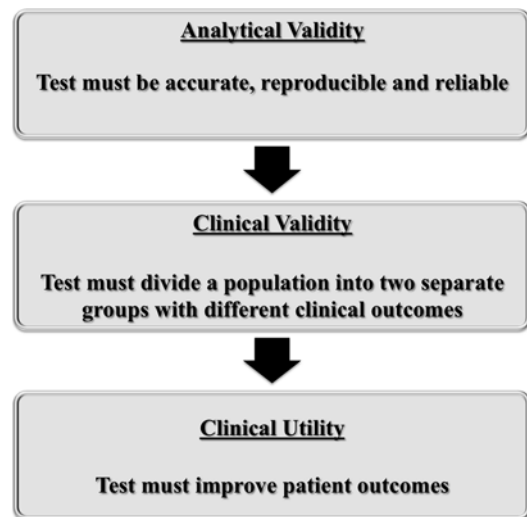


Fig. 2.2 Validation process of a candidate cancer biomarker test

Analytical validity is defined as the “test’s ability to accurately and reliably measure the analyte of interest in the clinical laboratory, and in specimens representative of the population of interest” [10, 52–54].

In other words, analytical validation means establishing that the “test measures what it claims to measure, and does so accurately with adequate sensitivity and specificity” [15]. “Analytical validity refers not just to the hardware platform used for measuring test but to the entire process of treating a sample including sample preparation, performing the assay and the computational pipeline for assembling the sequence readouts and calling variants” namely the three different phases of assay development: pre-analytical, analytical, and post-analytical phase [15, 55].

In order to develop an analytically robust biomarker assay, at least the following parameters should be assessed: accuracy, trueness, precision, reproducibility, robustness, linearity, reportable range, reference range, interfering substances, analytical sensitivity and specificity, and limit of detection [7, 10, 56, 57]. Lastly, a useful biomarker test should be easily performed by routine clinical laboratories.

About the analytical platform used for measuring test, since most of the cancer biomarkers have a plasma or serum concentration in the range of picogram to nanogram per milliliter, immunoassays, such as enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA), remain the method of choice for protein quantification in clinical samples [35, 58–60]. Immunological techniques, indeed, still offer a higher level of sensitivity (two to three orders of magnitude), reproducibility and dynamic range than the more sophisticated nonimmuno-based technologies (e.g. mass spectrometry assay) [58]. At the state of art, mass spectrometry technique, due to their high assay complexity, high cost, and expertise requirements is not yet transferable into routine use in clinical laboratories [35, 60]. It is noteworthy that the majority of FDA-approved protein assays utilize immunoassay (see Table 2.2), and not a single mass spectrometry-based protein assay has been approved for clinical use yet [59, 61].

“Robust analytical performance is an essential but insufficient prerequisite for the successful clinical deployment of a novel tumor marker test” [7], therefore, once verified the analytical (or “technical”) validity, the biomarker assay must be tested for evaluating its clinical (or “biological”) validity.

Clinical validity is defined as the “test’s ability to consistently and accurately identify or predict the intermediate or final clinical outcomes of interest”. Practically, clinical validity implies that the cancer biomarker test separates a population into two or more distinct groups with different biological characteristics or clinical outcomes. Clinical validity encompasses clinical sensitivity and specificity (integrating analytic validity), receiver operating characteristic (ROC) curves analysis, as well as positive predictive value (PPV) (i.e. the chance that a person with a positive test has cancer), and negative predictive value (NPV) (i.e. the chance that a person with a negative test does not have cancer) [7, 52, 54, 57]. Clinical validity studies may be conducted retrospectively or prospectively on samples collected from clinical trials, tissue banks or other sources.

Finally, before a cancer biomarker test is introduced into standard clinical management, it must also demonstrate to have what is commonly called “clinical utility”. Clinical utility is defined as “the test’s ability to significantly improve measurable clinical outcomes, and its usefulness and added value to patient management decision making compared with current management without testing” [52].

Therefore, “whereas analytic and clinical validity transform data into knowledge, demonstration of clinical utility is the critical last step that allows for application of a tumor biomarker test in patient care” [62].

The end point for establishing clinical utility is generally survival or progression-free survival, whereas the end point used for establishing clinical validity is often tumor or clinical response [15]. Key features for evaluations of the clinical utility of diagnostic testing are summarized in [63].

Ideally, the clinical utility of tumor biomarker test is best assessed through prospective ran-

domized controlled trials, as these studies are the least prone to bias. However, such trials are not always feasible because they are often costly, require very large sample sizes, and have ethical challenges [63, 64]. Clinical utility may be alternatively determined either from prospective–retrospective studies using archived specimens from previously conducted prospective clinical trials [64] or, if adequate archived specimens are not available, from prospectively direct clinical trials [17, 65, 66].

At this regard, an experts committee convened by the Institute of Medicine (IOM) of the United States to define the best practices for translation of omics-based tests from the research laboratory into clinical trials, and ultimately to clinical care, has recently proposed a roadmap for development of putative new omics-based tests from initial concept to ultimate clinical utility [67]. Although specifically ideated for omics-based tests, the roadmap is still applicable to any diagnostic test, and in particular to tumor biomarker tests. The roadmap involves two stages: (1) discovery and test validation, and (2) evaluation for clinical utility and use. The first stage can be divided into two separate but linked phases: (a) discovery of a tumor biomarker test of potential biological or clinical interest, and (b) analytical development of a tumor biomarker test with biological/clinical validity.

In the second stage, an analytically-validated tumor biomarker test is further evaluated for clinical utility, either in a prospective-retrospective study using archived specimens or in a prospective clinical trials where the test either is used to direct patient management or is prospectively determined to be the primary objective of the trial [68].

Notably, the potential pathways suggested by the IOM to generate high levels of evidence necessary to demonstrate clinical utility of the biomarker test follow quite closely the recommendations of Simon et al. [64].

Most biomarkers do not progress beyond this phase primarily because the validation study shows that the biomarker test does not have sufficient sensitivity or specificity to be clinically useful (see Sect. 2.4).

2.3.3 Phase 3: Retrospective Longitudinal Repository Studies

With a good clinical assay in hand, a retrospective analysis using stored samples can be employed to determine if the biomarker can truly detect the outcome of interest and define the cut-point for a biomarker with many values [40, 43].

The phase 3 of the biomarker development pipeline is aimed at evaluating the capability of the biomarker test to detect preclinical disease on samples collected and stored longitudinally from research cohorts. Accordingly, the biomarker level is measured in specimens collected from cancer case subjects prior to their clinical diagnosis and compared to that measured from specimens collected from age-matched healthy subjects (i.e., subjects who have not developed cancer). Furthermore, the retrospective longitudinal studies can be useful to compare multiple markers of interest with a view to select those that are most promising and to develop algorithms for combinations of biomarkers. Criteria for ‘positive’ screening defined in this phase are used in the subsequent phase 4.

In contrast to the discovery phase, retrospective longitudinal studies require large numbers of samples to ensure a rigorous statistical analysis, as well as samples that reflect the biological variability of the targeted population.

The retrospective longitudinal studies of phase 3 are not able to establish the stage or nature of the cancer at the time that it can be detected.

2.3.4 Phase 4: Prospective Screening Studies

The phase 4 intends to determine whether a biomarker test can detect a cancer at an early stage of development [40]. In particular, the primary aim of this phase is to determine the “operating characteristics of the biomarker based screening test in a relevant population” by calculating the detection rate or PPV (i.e. the proportion of screened subjects who test positive and have the disease), and the false-referral rate (i.e. the proportion of screened subjects who test positive but do not have the disease).

In other words, in phase 4 “the biomarker is tested to determine if it can do what it is hypothesized to do” [43].

In the prospective studies of phase 4, asymptomatic subjects are screened using the biomarker test, and those with a positive result are followed up to determine if they have cancer and, if so, its stage.

Notably, in contrast to studies in phases 1, 2, and 3, which are conducted on retrospective analysis of stored specimens, studies in phase 4 involve screening people and lead to diagnosis and treatment [40].

2.3.5 Phase 5: Cancer Control Studies

The final phase of the biomarkers development pipeline evaluates how the biomarker test performs in the population [43]. Large-scale population studies are designed to determine whether screening test reduces the burden (in morbidity and mortality) of cancer on the population [40].

2.4 Cancer Biomarkers Failures

Despite the impressive volume of research that has been addressed to identify cancer biomarkers and the increasing number of putative tumor markers reported in literature (and some of them in very prominent journals), “very few, if any, new circulating cancer biomarkers have entered the clinic in the last 30 years” [69].

Paraphrasing the title of a Buchen’s article, it seems right to ask: why is it so hard to find a test to predict cancer? [36] Furthermore, why do most cancer biomarkers fail to reach the clinic?

“The problem of identifying novel cancer biomarkers cannot be attributed to the lack of pathophysiological knowledge, powerful techniques, or investment of funds, but it may reside in difficulties that are associated with biomarker discovery, which have apparently been persistently underestimated” [69, 70].

Although “the high failure rates in the cancer biomarker field are no different from those of therapeutics”, “therapeutics leading to relatively

small improvements in patient survival (weeks to months) are likely to be marketed, diagnostics with relatively small improvements in patient diagnosis or prognosis will likely fall by the wayside. Hence, similar advances in therapeutics and diagnostics can be hailed as *successes* in the former and *failures* in the latter” [69].

Recently, the reasons responsible for cancer biomarker failure to reach the clinic have been widely discussed and classified into distinct categories [69, 71]. The classification is aimed to “help in understanding what goes wrong in each case and offer some lessons on how we could try to avoid similar problems in the future” [71].

According to Diamandis [69], tumor biomarkers failures can be classified into three distinct categories: (a) fraudulent reports, (b) discovery of biomarkers with weak clinical performance, and (c) false discovery or artifactual biomarkers.

The first category involves fraudulent publications that, in spite of the huge attention that they usually receive by scientific community and press, are extremely rare and responsible for a negligible percentage of biomarker failures [72, 73].

The second and largest category of failing biomarkers includes those biomarkers that have been discovered and validated by using robust and reliable techniques (*true discovery biomarkers*), but that never reach the clinic because of poor clinical performance (i.e. low specificity, low sensitivity, low prognostic/predictive value, and information not necessary for clinical decision-making) [35, 69].

Further details regarding this type of failing biomarkers have been discussed elsewhere [35, 58].

The third category includes cancer biomarkers that at first look highly promising (or even “revolutionary”) but later on show several shortcomings, either at the discovery or validation phase (pre-analytical, analytical, post-analytical and statistic/bioinformatic artifacts), which invalidate the original performance claims (*false discovery* or *artifactual biomarkers*). Therefore, these cancer biomarkers do not reach the clinic because

“the original performance claims cannot be independently reproduced in subsequent validation studies” [69].

Examples of such artifactual biomarkers have been summarized elsewhere [74–76].

Ioannidis [71] has recently proposed a classification of tumor biomarker failures, slightly different from that of Diamandis [69], that recognizes four different types: (A) clinical reversal, (B) validation failure, (C) nonoptimized clinical translation, and (D) promotion despite nonpromising evidence.

Type A failure (or clinical reversal) occurs when a “widely used biomarker that has already been implemented in clinical practice is shown to be largely useless or even harmful and therefore needs to be abandoned” [71]. The problem is to eliminate this obsolete test that regularly continues to be used, more frequently for non-scientific reasons, for example because of conflicts of interest of specialist practitioners.

Type B failure (or validation failure) occurs when a “biomarker shows great promise in one or more early studies, the claims are later found to be wrong or exaggerated, and the biomarker is eventually never implemented into clinical practice” [71]. Type B failure of Ioannidis’s classification may be considered analogous to the third category of failing biomarkers (false discovery biomarkers) described by Diamandis [69].

Type C failure (or nonoptimized clinical translation) occurs when a “biomarker shows some genuine promise in one or a few early studies but this result is not followed up systematically toward clinical implementation” [71]. Type C failure of Ioannidis’s classification may be considered analogous to the second category of failing biomarkers (true discovery biomarkers) described by Diamandis [69].

Type D failure (or promotion despite nonpromising evidence) occurs when a “biomarker shows no or little promise, but nevertheless is enthusiastically promoted for widespread clinical or population use” [71].

Reasons for biomarker failures and some solutions to overcome these challenges have been discussed in a seminal review [35].

2.5 Concluding Remarks and Future Perspectives

The traditional approach to treat cancer is commonly defined “trial and error” or “one size fits all”. This therapeutic approach is largely empirical, costly, and frequently ineffective thus resulting in an inappropriate treatment or, worse, in drug-related toxicity [13]. As a result, “some patients with aggressive malignancy may be undertreated, and some with indolent disease may be overtreated” [13].

In the last years, cancer therapy is evolving from the traditional “one size fits all” to a new “personalized” or “individualized” approach based on the molecular characterization of the tumor and on the concept that cancer is a highly heterogeneous disease (both within a tumour and between a primary tumour and metastases) [77, 78] and consequently “each individual solid tumor and hematologic malignancy in each person is unique in cause, rate of progression and responsiveness to therapy” [79–82]. Therefore, the ultimate goal of personalized cancer therapy is to deliver “the right drug to the right patient at the right time, using the right dose and schedule” [83].

To accomplish this ambitious outcome, personalized oncology needs reliable, robust, accurate and validated cancer biomarker tests [13] in order to:

- (a) differentiate patients with indolent malignancy from those with aggressive forms (prognostic markers);
- (b) predict response or resistance to specific therapies so that the right patients receive the right drugs (predictive markers);
- (c) identify patients who are likely to develop severe toxic side effects from specific treatments (pharmacodynamic markers).

Unfortunately, the cancer biomarkers field, just in the case of protein tumor markers, appears to be currently stagnant [36, 58] and most of the newly discovered cancer biomarkers have been either abandoned or not clinically validated because they failed to satisfy the analytical criteria necessary for clinical implementation [58, 84].

Nevertheless, recent advances in genomic technologies (i.e. next-generation sequencing) as well as in diagnostic platform for the detection of circulating tumor cells (CTCs) have allowed to identify and characterize new circulating cancer biomarkers, which are collectively defined by the scientific community with the term of “liquid biopsy” [85, 86]. *Stricto sensu*, the expression “liquid biopsy” should be restricted to CTCs by analogy with the standard definition of “tissue biopsy” [87]. However, the term “liquid biopsy” is used to identify different circulating cancer biomarkers such as CTCs but also cell-free tumor DNA (ctDNA) and microRNA (miRNA) [85, 86, 88, 89].

In this view, the detection of CTCs, ctDNA and/or miRNA could serve as a true “liquid biopsy” for cancer patients, and results much less invasive compared to surgical or endoscopic biopsy permitting also repeated samplings so tracking the current status of tumor characteristics.

“Liquid biopsy” analysis might be potentially useful at different stages of the diagnostic/therapeutic course of cancer patients, namely for: (a) early diagnosis, (b) monitoring tumor dynamics, (c) identification of genetic determinants for targeted therapy and resistance mechanisms (predictive marker), (d) evaluation of early treatment response, (e) stratification and real-time surveillance of therapies, and (f) estimation of the risk for metastatic relapse or metastatic progression (prognostic marker) [81, 89–91].

The first report on the presence of CTCs in the peripheral blood of a cancer patient was attributed to Ashworth [92]. Since then, CTCs have received enormous attention, representing one of the most active areas of translational cancer research, with more than 15,850 publications listed in PubMed in December 2014. CTCs, moreover, are used as biomarkers in more than 280 clinical trials registered at ClinicalTrials.gov.

“CTCs are rare cells that are shed from primary and metastatic tumour deposits into the peripheral circulation, and represent a means of performing noninvasive tumour sampling” [93], thus providing the opportunity to monitor serial changes in tumour biology [94].

Generally, CTCs occur at very low concentrations in the peripheral blood of cancer patients (one CTC in a background of approximately 10 million leukocytes and 5 billion erythrocytes in 1 ml of blood) [93, 95], therefore the accurate detection of CTCs with sufficient sensitivity and specificity is a major technical challenge. Up to date, the only FDA cleared technology for enumeration of CTCs in whole blood is the CellSearch® system (Veridex, Raritan, NJ) [81, 96].

The main clinical evidence for CTCs detection in various types of cancer (e.g., breast, prostate, lung, and colon cancer) is discussed in several reviews [91, 96–98]. Although CTCs are already used in numerous clinical trials as potential cancer biomarker, their clinical utility in oncology is still under investigation [88, 99].

In parallel to the progress in CTCs research, significant advancement has also been made with circulating cell-free DNA (cfDNA), whose clinical utility has been investigated in many disciplines of medicine. Circulating cell-free DNA exists at steady-state levels and increases, sometimes dramatically, with cellular injury or necrosis [100]. Like normal cells, tumor cells also release DNA fragments (ctDNA) into the circulation and significant differences in the amounts of plasma ctDNA are detected in cancer patients as compared to subjects with benign disease or healthy individuals [89]. Notably, ctDNA, which represents a very small fraction (<1.0 % and possibly as little as 0.01 %) of total cfDNA [100, 101], differs from normal cfDNA by the presence of mutations (commonly single base-pair substitutions) and may be therefore used to reconstruct tumor genomes [89, 102, 103]. The techniques for ctDNA analysis as well as the potential role of ctDNA as a diagnostic, prognostic and predictive cancer biomarker have been extensively reviewed by Ignatiadis and Dawson [104] and by Heitzer et al. [89]. Although the analysis of ctDNA constitutes a promising area of investigation, ctDNA is not yet routinely measured in clinical practice may be because is considered “not yet ready for a starring role in the clinic” [101].

Another cancer biomarker that is gaining popularity and might be used as a “liquid biopsy” is

microRNA (miRNA) [105–108]. MicroRNAs are fragments of single-stranded (18–25 nucleotides long) non-coding RNAs that regulate a variety of genes (more than 50 % of all protein-coding genes) by targeting mRNA transcripts and thereby controlling various cell functions such as apoptosis, proliferation and differentiation [105, 109]. Additionally, miRNAs have emerged as critical factors in cancer pathogenesis and progression by modulating many pathological aspects related to tumor initiation, growth, metastasis, and drug resistance.

“Expression patterns of miRNAs are unique to individual tissues and differ between cancer and normal tissues. Some miRNAs are overexpressed or downregulated exclusively or preferentially in certain cancer types” [109]. The high specificity together with the remarkable stability in a wide variety of human biological fluids, including blood, make circulating miRNAs attractive biomarkers in early cancer diagnosis, prognosis and response to therapy [105–107]. The potential roles of circulating miRNAs in various cancer types are summarized in several recent reviews [105, 109, 110]. These initial promising findings notwithstanding, the role of circulating miRNAs is still under investigation and further research is warranted to ascertain the potentiality of these interesting non-coding RNA molecules [108].

Difficult as it may seem, the proposal of “liquid biopsy” looks scientifically sound. The outlook from the bedside will of course remain the same, that is the need to determine when the neoplastic process has effectively started, where is localized, what type of malignancy (aggressive or indolent) is being developed and what therapeutic approach is best for that particular patient.

In conclusion, many important information for early diagnosis, prognosis and treatment of cancer are “*written in one’s blood*” whose script is still not fully decoded and made available for an application to a valid biomarker test by present knowledge and technology.

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