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Review

Cancer biomarkers☆

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ABSTRACT

Biomarkers have many potential applications in oncology, including risk assessment, screening, differential diagnosis, determination of prognosis, prediction of response to treatment, and monitoring of progression of disease. Because of the critical role that biomarkers play at all stages of disease, it is important that they undergo rigorous evaluation, including analytical validation, clinical validation, and assessment of clinical utility, prior to incorporation into routine clinical care. In this review we address key steps in the development of biomarkers, including ways to avoid introducing bias and guidelines to follow when reporting results of biomarker studies.

With the tremendous increase in knowledge about the biology of cancer and the rapid changes in molecular technology that have occurred in the past decade, studies of biomarkers in cancer are published almost daily. Because of this overabundance of information, it is necessary for clinicians and scientists to have a thorough understanding of biomarkers and biomarker development so they can critically review the literature, in order to determine whether and in what setting a biomarker can and should be used for patient care, or whether additional evaluation is required before it can be incorporated into routine medical practice.

1. What is a biomarker?

According to the National Cancer Institute, a biomarker is "a biological molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease,"(NCI) such as cancer. Biomarkers typically differentiate an affected patient from a person without the disease. The alterations can be due to a number of factors, including germline or somatic mutations, transcriptional changes, and post-translational modifications. There is tremendous variety of biomarkers, which can include proteins (e.g., an enzyme or receptor), nucleic acids (e.g., a microRNA or other non-coding RNA), antibodies, and peptides, among other categories. A biomarker can also be a collection of alterations, such as gene expression, proteomic, and metabolomic signatures. Biomarkers

Abbreviations: ASCO, American Society of Clinical Oncology; EGFR, epidermal growth factor receptor; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry; LOE, Level of Evidence; NCCN, National Comprehensive Cancer Network; NCI, National Cancer Institute; PSA, prostate specific antigen; TMUGS, Tumor Marker Utility Grading Scale.

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can be detected in the circulation (whole blood, serum, or plasma) or excretions or secretions (stool, urine, sputum, or nipple discharge), and thus easily assessed non-invasively and serially, or can be tissue-derived, and require either biopsy or special imaging for evaluation. Genetic biomarkers can be inherited, and detected as sequence variations in germ line DNA isolated from whole blood, sputum, or buccal cells, or can be somatic, and identified as mutations in DNA derived from tumor tissue.

2. Potential clinical uses of biomarkers

Biomarkers can be used for patient assessment in multiple clinical settings, including estimating risk of disease, screening for occult primary cancers, distinguishing benign from malignant findings or one type of malignancy from another, determining prognosis and prediction for patients who have been diagnosed with cancer, and monitoring status of the disease, either to detect recurrence or determine response or progression to therapy. Examples of each of these settings are given below and in Table 1. Importantly, some biomarkers are only used in a specific setting, whereas others can serve more than one purpose.

Determination of a patient's risk of developing a malignancy is helpful if risk reduction strategies (such as lifestyle changes, prophylactic surgery, or chemoprevention) or screening have been shown to be effective. Applying these strategies to high risk groups is much more efficient than wholesale application to the entire population. Biomarkers have been identified that can be used to determine an individual's risk of developing cancer. For example, a woman with a strong family history of ovarian cancer can undergo genetic testing to determine if she is a carrier of a germline mutation, such as BRCA1, which will increase her risk of developing breast and/or ovarian cancer (Easton et al., 1995). If so, she could opt for more intensive screening, chemoprevention with tamoxifen, or prophylactic bilateral mastectomy and/or bilateral salpingo-oophorectomy in order to decrease her risk of developing a malignancy (Domchek et al., 2010; Fisher et al., 1998; Kauff et al., 2002; Rebbeck et al., 2002).

Biomarkers can be used to screen otherwise healthy patients for malignancy. A commonly used but controversial biomarker for screening is prostate specific antigen (PSA). Following its approval by the Food and Drug Administration in 1986, increased screening of men over age 50 led to an increase in the diagnosis of prostate cancer, but there were concerns raised about overtreatment. The most recent U.S. Preventive Services Task Force analysis found insufficient evidence for routine screening with PSA (Lin et al., 2008).

In a patient with an abnormality, biomarkers can also be used to distinguish between different possibilities that are in the differential diagnosis. For example, if a patient is found to have a lung nodule on chest CT, histologic evaluation of the biopsy specimen can determine whether the tissue is cancer, infection, inflammation, or another benign process. If cancer is detected, further evaluation with specific immunohistochemical markers can be used to try to identify the tissue of origin.

In patients who have been diagnosed with a cancer, biomarkers can help determine prognosis, or likelihood of disease recurrence independent of treatment. Traditionally, the clinicopathologic characteristics of a tumor have been used for determination of prognosis. More recently, newer technologies are being utilized to assess prognosis for individual tumors. For example, in breast cancer, there are a number of gene expression signatures that have been developed that can be used to estimate prognosis for an individual patient based on assessment of the tumor (Paik et al., 2004; van't Veer et al., 2005). In the metastatic breast cancer setting, circulating tumor cells have been shown to be prognostic for overall survival (Cristofanilli et al., 2004). Their utility for prediction of response to palliative therapy has not yet been established, and is the focus of an ongoing cooperative group trial.

Biomarkers can also be used as response modifiers, or "predictive factors," for a specific therapy, or for determining which therapy is likely to be most effective. In colorectal cancer, KRAS is a predictive biomarker, because somatic mutations in KRAS are associated with poor response to anti-epidermal growth factor receptor (EGFR) directed therapies (Allegra et al., 2009). Similarly, overexpression or gene amplification of the *HER2* gene in breast and gastric cancers predicts for response to anti-Her2 agents such as trastuzumab (Bang et al., 2010; Piccart-Gebhart et al., 2005; Romond et al., 2005),

Table 1 – Potential uses for cancer biomarkers.

Use	Example	Reference
Estimate risk of developing cancer	BRCA1 germline mutation (breast and ovarian cancer)	Easton et al., 1995; Hall et al., 1990
Screening	Prostate specific antigen (prostate cancer)	Lin et al., 2008
Differential diagnosis	Immunohistochemistry to determine tissue of origin	
Determine prognosis of disease	21 gene recurrence score (breast cancer)	Paik et al., 2004
Predict response to therapy	KRAS mutation and anti-EGFR antibody (colorectal cancer)	Allegra et al., 2009
	HER2 expression and anti-Her2 therapy (breast and gastric cancer)	Bang et al., 2010; Piccart-Gebhart et al., 2005; Romond et al., 2005
	Estrogen receptor expression (breast cancer)	EBCTCG 2011
Monitor for disease recurrence	CEA (colorectal cancer)	Locker et al., 2006
	AFP, LDH, βHCG (germ cell tumor)	Gilligan et al., 2010
Monitor for response or progression in metastatic disease	CA15-3 and CEA (breast cancer)	Harris et al., 2007

and overexpression of the estrogen receptor in breast cancer predicts for response to anti-endocrine therapies such as tamoxifen (EBCTCG, 2011).

Potential somatic biomarkers for prediction of response to therapy are chemotherapy sensitivity and resistance assays, which have been studied in multiple tumor types. Numerous clinical studies have been published and these assays are commercially available. However, they are not recommended for clinical decision-making by ASCO outside of a clinical trial setting because of lack of sufficient evidence to support their use (Burstein et al., 2011).

Germ line genetic mutations can also be used to predict adverse reactions to a specific therapy. This is the basis of the field of pharmacogenomics. In 2005, the United States Food and Drug Administration changed the labeling for irinotecan because of demonstration of an association between homozygosity for the *UGT1A1*28* mutation and increased risk of developing severe neutropenia and diarrhea with standard doses of the chemotherapy (Innocenti and Ratain, 2006).

In patients who have completed adjuvant therapy, biomarkers can be used to detect early recurrence of disease, before patients become symptomatic. For example, CEA is monitored serially following adjuvant treatment for colon cancer with the goal of detecting liver metastases when they are still resectable and potentially curable (Locker et al., 2006). Similarly, alpha feto-protein, beta-HCG, and lactate dehydrogenase are monitored serially in nonseminomatous germ cell tumors in order to detect early disease recurrence (Gilligan et al., 2010).

Biomarkers can also be used to monitor response to treatment in the metastatic setting. Circulating soluble protein tumor markers such as CEA, PSA, CA125, the MUC-1 antigens CA15-3 and CA27.29, and CA19-9 are recommended for monitoring response to palliative therapy in metastatic colorectal, prostate, ovarian, breast, and pancreatic cancers, respectively (Harris et al., 2007; Locker et al., 2006). The role of monitoring these antigens to detect occult recurrences in patients who are free of disease after surgery and during or after adjuvant therapy is unclear. While many clinicians do so, the only clear indication with high evidence of clinical utility is for CEA in patients with colorectal disease, since several studies have shown a small, but real, cure rate in patients with isolated, resectable liver metastases. PSA and CA125 are commonly monitored in prostate and ovarian cancer patients who are free of disease, but there is little evidence that doing so improves outcomes, and indeed the results of a prospective randomized trial refute any benefit in the latter (Rustin et al., 2010). Widely accepted guidelines by NCCN and ASCO recommend against monthly circulating tumor marker assessment to detect occult recurrence in patients with breast cancer (Carlson et al., 2011; Harris et al., 2007; Khatcheressian et al., 2006).

An important distinction should be made between biomarkers and targets, since in many cases these are not equivalent. For example, as mentioned above, KRAS is an excellent biomarker in colorectal cancer, even though it is not the actual target of therapy. Instead, mutations in KRAS render tumors less responsive to anti-EGFR therapies (Allegra et al., 2009). It is important to remember this distinction when planning clinical studies of potential biomarkers.

3. Identification of a potential biomarker

Potential biomarkers can be identified through multiple approaches. The classic approach has been to identify candidate biomarkers based on the biology of the tumor and surrounding environment, or the metabolism of the pharmaceutical agent. With the explosion of new knowledge about tumors and advent of new technology, biomarker identification is now frequently performed using a "discovery" approach, using techniques such as high-throughput sequencing, gene expression arrays, and mass spectroscopy to quickly identify individual or groups of biomarkers that differ between cohorts. The vast amount of data generated using these techniques means that particular attention needs to be paid to the study design and the data analysis, in order to minimize the chance of identifying associations that are subsequently determined to be false positives. Key aspects of biomarker development that will be discussed in detail include careful study design to avoid bias, comprehensive testing and validation, and accurate reporting of the results.

4. Steps in the development of a candidate biomarker

There are a number of hurdles that a potential biomarker must surpass before it can be applied in the clinic. First, a cohort of samples is analyzed to test a specific potential new biomarker, or to try to discover a new biomarker. Subsequent testing then involves analyzing an independent sample cohort to validate the original hypothesis-generating findings, and additional evaluation to confirm that the new biomarker will provide additional information that is useful for clinical decision-making. These concepts have been termed analytic validity, clinical validity, and clinical utility (Teutsch et al., 2009).

4.1. *Analytic validity*

When a new potential biomarker is being developed, it is important to focus on both pre-analytic and analytic issues of the assay to detect the biomarker. Pre-analytic validity refers to the handling of the sample that will be tested using the new assay (Moore et al., 2011). The results of the assay could be influenced by a number of sample-related factors, including (a) time and storage conditions between sample collection and processing, (b) type and duration of fixation or lack thereof, and (c) storage time and conditions following processing. It is important to account for these pre-analytic issues, since they can influence the outcomes and ability to reproduce the findings for the potential biomarker.

Analytic validity describes evaluation of technical aspects of the biomarker assay itself, which must meet certain criteria. It is important to determine the sensitivity, specificity, and robustness of the assay. In addition, it must be accurate and reproducible, both within an individual laboratory and between laboratories. Problems can arise, for example, when laboratories perform an assay using an antibody whose quality varies from lot to lot. Proficiency testing programs are now being developed for certain key biomarkers, including estrogen receptor and Her2 assessment in breast cancer, in order

to establish consistency for sample handling, sample testing, assay interpretation, and reporting of results across laboratories (Hammond et al., 2010; Wolff et al., 2007). Those laboratories that successfully participate in the programs can become accredited by the College of American Pathologists or other certifying organizations.

4.2. *Clinical validity*

Once a technically valid assay has been developed, the biomarker must be studied to determine if it has clinical, or "biologic", validity. Clinical validity relates to the observation that the biomarker reliably divides the overall population of interest into two distinct groups, such as those more or less likely to suffer an event. Clinical validity does not indicate that the biomarker should be used to direct clinical care. As in all of science, observation of apparent clinical validity needs to be reproduced in a completely independent set of samples in order to confirm validity. Several approaches to reproducibility, or validation, have been proposed. However, if the samples in the test and validate groups are not independent, overfitting can lead to the appearance that a test has excellent discriminatory ability, which cannot be reproduced when independently validated. Some researchers argue that the independent validation should be performed by a completely independent group of researchers, whereas others believe that the initial validation study should be performed by the original researchers using the original method, but with an independent sample cohort (Ransohoff, 2007).

4.3. *Clinical utility*

In order for an assay to be used to direct patient care, it must be shown to have clinical utility with very high levels of evidence (Simon et al., 2009). Clinicians will often measure prognostic biomarkers for their patients. Lack of clear guidance about how to use the information for patient care, however, can lead to confusion and worse, incorrect treatment decisions. Therefore, appropriate rigor to ensure analytic validation and clinical utility of a biomarker is important in order to ensure that new biomarkers that are introduced into clinical practice will appropriately direct patient management, and will provide information in addition to currently used decision-making factors. Assessment of clinical utility includes an assessment of the effectiveness of a biomarker, as well as the benefit-to-harm ratio. Prognostic or predictive biomarkers that have been inappropriately clinically validated have the potential to harm patients directly through inappropriate treatment selection, or indirectly through increased health care costs.

In spite of three decades of research and thousands of reports of circulating biomarkers, very few tumor markers have established clinical utility. One example of a biomarker with established clinical utility is assessment of KRAS mutations in colorectal carcinoma, as described above (Allegra et al., 2009). Assessment of this new biomarker, in addition to traditional clinicopathologic assessment of the tumor, yields information regarding likely benefit from anti-EGFR therapy.

5. Guidelines for reporting and evaluating biomarker studies

Reporting the results of biomarker studies are a key component of evaluating a new biomarker, since this will enable other researchers to critically review the study design and the data, and provide them with sufficient information to independently validate the findings. Therefore, guidelines have been developed for reporting results of biomarker studies in order to ensure that all necessary information is included. The Biospecimen Reporting for Improved Study Quality (BRISQ) and REporting recommendations for tumor MARKer (REMARK) prognostic studies criteria have been developed for reporting the details of pre-analytical and analytical issues related to potential prognostic factor studies in an organized and transparent fashion (McShane et al., 2005; Moore et al., 2011). Other, more specific reporting guidelines that have been developed include the Standards for Reporting of Diagnostic Accuracy (STARD) for publishing diagnostic tests (Bossuyt et al., 2003) and the Minimum Information About a Microarray Experiment (MIAME) guideline for reporting microarray research (Taylor et al., 2007).

In addition to reporting guidelines, efforts have been made to place cancer biomarker results into various levels of evidence to determine clinical utility. In 1996, the ASCO Tumor Marker Guideline Committee proposed TMUGS to facilitate critical evaluation of biomarkers (Hayes et al., 1996). In TMUGS, the highest LOE (level I) required evidence from a prospective clinical study designed specifically to test the biomarker of interest, or evidence from a meta-analysis or systematic overview of well-conducted LOE II studies. Similar to level I studies, level II studies also provide evidence about a biomarker from a prospective clinical trial, but they were not designed to test the biomarker as the primary objective of the trial. These two types of trials provide the strongest evidence to support the clinical utility of a new biomarker.

More recently, a more detailed scale of levels of evidence has been proposed to more clearly define clinical utility for tumor marker studies (Simon et al., 2009). In this revised system, prospective clinical trials in which the biomarker is the primary objective receive the highest level (see Sargent et al., 2005 or Freidlin et al., 2010 for a more detailed description of prospective cancer biomarker clinical trial designs). However, a sufficiently high level of evidence can also be obtained by "prospective retrospective" analyses of archived specimens correlated with therapeutic clinical trials, but similar rigor must be applied in these studies as well (see Simon et al., 2009 for further details).

6. Biomarker effect size

For a marker to have clinical utility, it is important to consider the impact that the biomarker has on the clinical decision being considered. For example, by how much does a biomarker that is associated with increased risk of a disease actually increase that risk? By 10%? Two-fold? Regardless, what level of difference in magnitude is required for a patient, clinician, or third party payer to elect to treat the patient differently than

if the marker results were not known. The answers to these questions vary, depending on the disease, the situation, and perspectives regarding absolute benefits, risks, and economics. There are many highly cited articles in the literature that demonstrate strong effects for individual biomarkers. Ioannadis and Panagiotou recently performed an analysis comparing the effect size noted in the highly cited articles with effect sizes for the same biomarkers in larger studies or in meta-analyses (Ioannidis and Panagiotou, 2011). In the vast majority of cases, the effect size in the highly cited article was much higher than that in either a larger study of the same marker (86% of the time), or in a meta-analysis (83%). Therefore, it is important for researchers to critically evaluate the literature when considering using a biomarker, and not just rely on a reference to a frequently published study in a review article.

7. Study design issues, or how to avoid bias

What can lead to erroneous conclusions about the strength of the effect of a biomarker? Three threats to biomarker validity include play of chance, lack of generalizability, and inadvertent introduction of bias (Ransohoff and Gourlay, 2010). One critical factor that can introduce bias is subject selection. Unlike studies of new pharmaceuticals, which are evaluated in randomized clinical trials, most studies evaluating potential new biomarkers are comprised of samples of convenience. These cohorts frequently represent a heterogeneous population of subjects who all have a specific diagnosis but whose clinicopathologic characteristics and treatments may differ. In order to minimize bias, it is important to select populations that address the clinical question. Various strategies can be applied to subject selection. For example, in order to appropriately address some questions it is important for the cases and the controls to be as similar as possible to each other, except for the disease of interest, so controls are frequently matched to the cases based on age, sex, and other factors. Underlying differences between the cases and controls can lead to bias, which can be subtle and therefore unrecognized, but which can substantially impact the results.

As an example of inadvertent bias due to sample selection, a promising biomarker for identifying prostate cancer was identified that could discriminate between affected patients and healthy controls (Villanueva et al., 2006). However, although the patients were older men, the controls were primarily younger females, and it was therefore unclear whether the detected difference in the biomarker between the two populations was due to the presence of prostate cancer, or simply to sex- or age-related differences in biomarker concentration.

Another factor that can introduce bias is sample handling. Frequently, cases are identified over time and are stored until analysis, whereas controls are often collected at a different time. It is important that the samples be handled similarly in terms of collection and processing methods, storage duration and conditions, and number of freeze/thaw cycles. Samples should be analyzed in a blinded manner using optimized procedures. In addition, if samples cannot all be analyzed together in the same batch then the cases should be intermingled with the controls when analyzed, not run in separate batches. In one study of a proteomic signature for ovarian cancer, it was determined that the discrimination between cases and controls was likely due to variation in the assay over time and differences in sample handling (Petricoin et al., 2002; Baggerly et al., 2004). As these examples show, attention to these pre-analytic, analytic, and post-analytic factors is critical for establishing the clinical validity of an assay and avoiding false positive results.

There are a number of examples of biomarkers that are routinely used for clinical care that have not met these stringent criteria. Although immunohistochemistry for estrogen receptor in breast tumors has been performed for years, it has become apparent that many clinical assays do not accurately assess this critical predictive biomarker. In Canada, it was discovered upon retesting that 40% of women originally diagnosed with estrogen receptor negative breast cancer actually had estrogen receptor positive tumors, and thus were deprived of a potentially life-saving treatment (Allred, 2008). Similarly, Her2 assessment has been very controversial, as a substantial amount of discordance has been detected between laboratories and between assay methodologies (IHC vs FISH). As a result, guidelines for assessment of both Her2 and estrogen receptor have been published within the past few years by the College of American Pathology and ASCO, with the goal of improving assay performance and patient diagnosis (Hammond et al., 2010; Wolff et al., 2007).

8. Summary

Biomarkers factor into the diagnosis and treatment of almost every patient with cancer. When new pharmaceuticals are developed, they are required to pass high levels of scrutiny and be tested in carefully designed, randomized clinical trials prior to governmental approval. Unfortunately, similar requirements are not in place for biomarkers, although they too can significantly influence patient outcomes. Therefore, it is important for clinical, translational, and laboratory-based researchers to be acutely aware of the issues surrounding appropriate biomarker development, in order to facilitate entry of clinically useful biomarkers into the clinic, while avoiding the introduction of biomarkers that have not been sufficiently evaluated and therefore may be useless or even potentially detrimental to patient care.

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