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Validation of Analytical Methods for Biomarkers Employed in Drug Development

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Abstract

The role of biomarkers in drug discovery and development has gained precedence over the years. As biomarkers become integrated into drug development and clinical trials, quality assurance and in particular assay validation becomes essential with the need to establish standardized guidelines for analytical methods used in biomarker measurements. New biomarkers can revolutionize both the development and use of therapeutics, but is contingent upon the establishment of a concrete validation process that addresses technology integration and method validation as well as regulatory pathways for efficient biomarker development. This perspective focuses on the general principles of the biomarker validation process with an emphasis on assay validation and the collaborative efforts undertaken by various sectors to promote the standardization of this procedure for efficient biomarker development.

Introduction

Biomarkers are playing an increasingly important role in drug discovery and development from target identification and validation to clinical application, thereby making the overall process a more rational approach. The potential use of biomarkers in each phase of the drug development process is summarized in Table 1 (1). The incorporation of biomarkers in drug development has clinical benefits that lie in the screening, diagnosing, or monitoring of the activity of diseases or in assessing therapeutic response. The development and validation of these mechanism-based biomarkers serve as novel surrogate endpoints in early phase drug trials. This has created a much appreciated environment for protein biomarker discovery efforts and the development of a biomarker pipeline which resembles the various phases of drug development. The components of the biomarker development process include discovery, qualification, verification, research assay optimization, clinical validation and commercialization (2).

The role of biomarkers in rational drug development has been a major focus of the Food and Drug Administration (FDA) critical path initiative and the National Institutes of Health (NIH) roadmap (3). While the overwhelming majority of biomarkers are proteins used as surrogate end points for drug development, diagnostic biomarkers may also prove useful for understanding the biology of the disease. Successful biomarker development depends on a series of pathway approach that originates from the discovery phase and culminates in the clinical validation of an appropriately targeted biomarker. Much emphasis has been placed on

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the paradigm of biomarker translation specifically on the principles of biomarker validation in clinical trials and the articles in this edition of *CCR Focus* will address various issues along the validation pathway including the analysis of microarray datasets (4), the validation of predictive models (5), the design of clinical trials using genomics (6), and the overall statistical challenges that exist (7). New biomarkers can revolutionize both the development and use of therapeutics, but is contingent upon the establishment of a concrete validation process that addresses technology integration and method validation as well as regulatory pathways for efficient biomarker development. This perspective will feature highlights on the biomarker validation process and includes a discussion on analytical method validation.

Biomarker Definitions

Numerous publications have described the application of biomarkers in drug development utilizing various nomenclatures to describe distinct aspects of this process. We begin with the standardization of terminology for ease of understanding the biomarker literature. A consensus definition of a *biomarker* is a factor that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (8). A *clinical endpoint* is defined as a variable that measures how patients feel, function, or survive whereas a *surrogate endpoint* is a biomarker that is intended to substitute for a clinical endpoint. In this case, a surrogate endpoint is expected to predict clinical benefit. Examples of surrogate endpoints and clinical endpoints are provided in Table 2.

Another critical distinction should be made when a biomarker undergoes analytical method validation versus clinical qualification. Analytical method *validation* is the process of assessing the assay, its performance characteristics, and the optimal conditions that will generate the reproducibility and accuracy of the assay. Clinical *qualification* is the evidentiary process of linking a biomarker with biological processes and clinical endpoints (9). While “validation” and “qualification” or “evaluation” have been used interchangeably in the literature, the distinction should be made to properly describe the particular phase the biomarker is transitioning through in the drug development process. As such, the term “validation” is reserved for analytical methods, and “qualification” for biomarker clinical evaluation to determine surrogate endpoint candidacy (8,9). Both validation and qualification processes are intertwined and hence their integration guides biomarker development with the principle of linking the biomarker with its intended use (see section on Fit-for-Purpose Method Validation) (10).

Biomarker Qualification Process Map

The FDA has issued guidance for industry on pharmacogenomic data submissions and in classifying the various types of genomic biomarkers and their degree of validity: exploratory biomarkers, probable valid biomarkers and known valid biomarkers.[1] Exploratory biomarkers lay the groundwork for probable or known valid biomarkers and can be used to fill in gaps of uncertainty about disease targets or variability in drug response, bridge the results of animal model studies to clinical expectation, or used for the selection of new compounds (11). Examples of exploratory biomarkers include the use of gene panels used for preclinical safety evaluation or the evaluation of vascular endothelial growth factor as a target to assess the efficacy of angiogenesis inhibitors. For an exploratory biomarker to achieve the status of probable valid biomarker it needs to be “measured in an analytical test system with well-established performance characteristics and for which there is an established scientific

[1]US Food and Drug Administration. Guidance for industry: Pharmacogenomic data submissions, 2005. Available from: http://www.fda.gov/cder/guidance/6400fnl.pdf

framework or body of evidence that elucidates the physiologic, toxicologic, pharmacologic, or clinical significance of the test results." A probable valid biomarker appears to have predictive value for clinical outcomes but has not been independently replicated or widely accepted. The advancement from probable valid to known valid lies in the achievement of a broad consensus in cross-validation experiments which include the independent validation of the biomarker by replicating the outcome at different sites. Thus, a known valid biomarker is defined as "a biomarker that is measured in an analytical test system with well-established performance characteristics and for which there is wide spread agreement in the medical or scientific community about the physiologic, toxicologic, pharmacologic, or clinical significance of the test results." That is, the scientific community at-large accepts these known valid biomarkers to predict clinical or preclinical outcomes. Examples of such valid genomic biomarkers that are listed on the labels of FDA-approved drug products are presented in Table 3.

The qualification process is introduced to bridge the gap from an exploratory biomarker to a known valid biomarker status keeping in mind that a validation process requires a consensus on an efficient and transparent process map for genomic biomarker validation. A qualification process map has been proposed by the FDA that evaluates exploratory genomic biomarkers of preclinical drug safety to assess the potential of genomic technologies in mock submission (12,13) and identify key parameters that can be used to determine the success of these biomarkers in voluntary genomic data submission (14). The proposal transitions an exploratory biomarker to a known valid genomic biomarker through a series of phases from discovery to method development to validation studies and cross-validation consortium (11). In the case of a process map that involves the validation of genomic biomarkers in clinical trials, the regulatory agency will review the biomarker validation package in terms of the usefulness of the biomarker in predicting clinical benefit (11). While this particular qualification process addresses genomic biomarkers, its application can be further extended to other types of biomarkers (e.g., protein or diagnostic biomarkers) granted the qualification approach remains intact. Figure 1 shows the integration of the FDA biomarker qualification process along the phases of the drug development process.

The inclusion of biomarkers in drug development and regulatory review will improve the efficiency of the biomarker development process. Biomarker qualification is also observed in the co-development of biomarkers (in the form of diagnostic tests) and drugs with the use of these biomarkers limited to the drug's application (11).[2] Co-development imposes the necessity to generate specific guidelines describing analytical test validation (sensitivity and specificity of the assays), clinical test validation (ability of the assays to detect and predict diseases), and clinical utility.[3] Furthermore, biomarkers play a significant role in adaptive trial designs in which patient population stratification and efficacy determination is based on biomarker readouts (15). Adaptive trials attempt to maximize the statistical power of the study with a small sample size. The integration of biomarkers into drug development is further discussed in the section below.

Fit-for-Purpose Method Validation

It is important to point out that biomarker method validation is distinct from pharmacokinetic (PK) validation and routine laboratory validation. The FDA has issued guidance for industry on bioanalytical method validation for assays that support PK studies that are specific for small-

[2]US Department of Health and Human Services, Food and Drug Administration. Table of Valid Genomic Biomarkers in the Context of Approved Drug Labels, 2007. Available from: http://www.fda.gov/cder/genomics/genomic_biomarkers_table.htm

[3]US Department of Health and Human Services, Food and Drug Administration. Drug-Diagnostic Co-Development Concept Paper, 2005. Available from: http://www.fda.gov/cder/genomics/pharmacoconceptfn.pdf

molecule drugs and which are not directly related to the validation of biomarker assays.[4] Whereas routine laboratory validation refers to laboratories that perform testing on human specimens for diagnosis, prevention, or treatment of any disease and falls under the jurisdiction of the Clinical Laboratory Improvement Amendments of 1988, there is little regulatory guidance on biomarker assay validation. In October 2003, the American Association of Pharmaceutical Scientists (AAPS) and the US Clinical Ligand Society (CLAS) co-sponsored a Biomarker Method Validation Workshop to address the validation challenges of biomarker assays in support of drug development (16). At this meeting it was concluded that biomarker methods should not be validated by the same guiding principles developed for drug analysis used in bioanalytical method validation. Hence, a “fit-for-purpose” approach for biomarker method development and validation is derived with the idea that assay validation should be tailored to meet the intended purpose of the biomarker study.

Method validation should demonstrate the reliability of the assay for the intended application with the rigor of the validation process increasing from the initial validation proposed for exploratory purposes to the more advanced validation dependent on the evidentiary status of the biomarker (10). The fit-for-purpose method validation is an umbrella terminology that is used to describe distinct stages of the validation process including pre-validation, exploratory and advanced method validation, and in-study method validation (Figure 1). Method validation is thus a continuous and iterative process of assay refinement with validation criteria that is driven by the application of the biomarkers with increasing rigor at each successive validation step and focusing on method robustness, cross-validation, and documentation control.

Technology Integration in Biomarker Validation: Choice of Assays

Before addressing the elements of biomarker assay development and method validation, it is important to recognize that the biomarker method validation process begins with choosing the right assay, followed by developing this assay into a validated method. Indeed, the integration of various technologies proves pivotal to not only biomarker identification and characterization but also validation. In fact, the platform applied in biomarker discovery can also be further developed and used as an analytical platform. Biomarker measurement can be assessed at different biological levels with different technologies; thus, the appropriate choice of assay depends on the application of the biomarker and the limitations of the respective technology. Various types of assays can be used in the biomarker method validation process and range from the relatively low technology end such as immunohistochemistry (IHC) to immunoassays to the high technology end including platforms for genomics, proteomics, and multiplex ligand-binding assays.

A genomics approach consists of various methods that measure gene expression analysis, such as in microarrays, which has become the standard technology used for target identification and validation. Reverse transcription-polymerase chain reaction is a very sensitive, reproducible technology and often time used to validate microarray-generated data. Comparative genomic hybridization can be used to detect chromosomal alterations associated with certain cancers. Proteomics involves global protein profiling to provide information about protein abundance, location, modification, and protein-protein interactions. While proteomics is a discovery technology, immunoassays are routinely used for protein biomarker assessments due to its straightforward clinical application and translation into a potential diagnostic assay. The multiplexing of protein assays can increase the throughput for simultaneous analysis of several proteins; however, it is limited by the need to standardize assay conditions, the lost of sensitivity

[4]US Food and Drug Administration. Guidance for industry: Bioanalytical method validation, 2001. Available from: http://www.fda.gov/cder/guidance/4252fnl.pdf

over single assays, and the quality control of each analyte in the complete multiplex panel (17).

Metabonomics (or metabolomics) is the profiling of endogenous metabolites in biofluids or tissue for characterization of the metabolic phenotype. The analytical platforms used are based on nuclear magnetic resonance spectroscopy and the combination of liquid chromatography with mass spectroscopy. It is principally used in biomarker discovery although by definition it is the ultimate end-point measurement of biological events. Yet the technology is limited by the lack of comprehensive metabolite databases and throughput both of which affects data analysis and interpretation. The integration of these technologies lends to the field of bioinformatics where linking expression data derived from genomic/proteomic approaches to target biological pathways can provide a comprehensive understanding of the disease biology and further validating the application of the biomarker (18).

Furthermore, advances in novel imaging approaches also have profound implications for biomarker development. Molecular and functional imaging technologies are used to assess cell proliferation and apoptosis (e.g., ^{18}F-fluoro-L-thymidine and ^{99m}Tcannexin imaging), cellular metabolism (e.g., ^{18}F-fluorodeoxyglucose positron emission tomography), and angiogenesis and vascular dynamics (e.g., dynamic contrast-enhanced computed tomography and magnetic resonance imaging). Therefore, making the right choice of assay is an important first step to successful biomarker method validation. Validating the developed method or assay to reliably measure the biomarker depends on a series of parameters that is addressed below.

Biomarker Analytical Method Validation

The key parameter assay elements of biomarker method validation are more complicated than for the typical bioanalytical assay that follows good laboratory practice (GLP) guidelines. Table 4 compares these two validation paradigms and highlights some of the validation challenges encountered with biomarker assays. Biomarker assay development and method validation is a complex process that depends on a number of parameters from the choice of the matrix to maintaining sample integrity to assay standardization and accuracy.

Specifications for biological matrices need to be determined taking into consideration the site of biomarker production and the physiology and distribution of the biomarkers. The first challenge is to identify and select a meaningful sample matrix that can be readily accessible, such as whole blood, plasma, serum, or urine. Sources of analytes can influence the validation process as evidenced by feasibility in the acquisition of biological material during the study such as in the collection of non-invasive (sputum, urine, feces, and saliva) versus minimally invasive (blood or plasma) samples. If the method has sufficient sensitivity, then the preferred matrix choice is based on ease of sample collection and analysis. However, if sensitivity is a factor and measurement of the biomarker in the specified matrix poses as a challenge, then the preferred matrix is chosen based on sample concentration even if this presents as a greater challenge for sample collection and preparation.

In addition to the influence of sample sources, material collection and processing should be examined in order to maintain sample integrity. It is important for researchers to realize that biospecimen collection varies across populations; how they are handled and differences in sample processing parameters can dramatically impact a trial's results. Thus, appropriate conditions for collecting, handling, and storing study samples need to be standardized along with adequate training of the clinical trial management personnel to preserve the analyte's stability and integrity. Sample integrity can be affected by repeated cycles of freeze-thawing specimens or by long-term storage and hence the stability of the sample becomes compromised. Depending on the type of samples (biological fluids or tissues), minimization in variability at each step of this procedure from collection to processing is critical to ensure consistent and

valid analyte measurement at subsequent biomarker assays. For biological fluids, differences in the handling of urine versus whole blood samples can affect the analytical assay and thus optimization of a processing protocol is necessary and should be based on the specific biomarker in addition to the source of analyte. Thus, standardization of sample collection procedures and appreciation of the associated limitations allow this variability to be minimized. Moreover, the integrity of reagents is another parameter that can also affect biomarker analysis. Reagents such as antibodies are subject to their own problems of supply, stability, and quality control as they themselves are derived from biologic sources.

Quality control (QC) measures should be undertaken to document analytical performance during clinical studies and to determine the acceptance or rejection of an analytical run during sample analysis (19). Similar to bioanalytical method validation, biomarker analysis requires a systematic review of the analyte stability in calibration standards, QCs and study samples (20). In general, QCs are prepared to evaluate the lower, middle, and upper limits of standard curve ranges. While QC samples are used during study sample analysis to judge the acceptability of assay runs, validation samples (VS) are used in assay validation experiments to estimate intra- and inter-run accuracy/precision and stability. Wherease only three VS concentrations are required in GLP bioanalytical assays (21,22), at least five different concentrations of VS should be analyzed in duplicate on at least six different runs during the pre-study validation because quantitative biomarker assays often exhibit nonlinear calibration curves thus more VS are required (10,23).

Since biomarkers are endogenous substances, difficulty may arise in obtaining biomarker/analyte-free matrices either to perform specificity studies on or to prepare for the calibration curve. Most of the time, the target biomarker molecule is not available to act as a certified calibration standard (19). Researchers then may rely on the use of a noncertified standard, a recombinant protein, or a surrogate matrix in order to construct the calibration curve (10). If assay standards are prepared in a non-authentic matrix, QC samples should be prepared and tested in the same matrix as the study samples to demonstrate that the assay performance is similar between authentic and non-authentic matrices (24). Parallelism studies should be conducted when surrogate standards and matrices are used for calibration purposes. Dilution linearity can also be problematic, as antibody and ligand-binding affinities can vary significantly in different media. Other important components of biomarker assay validation include the reference materials, precision and accuracy, dynamic range, sample recovery, sample volumes, and instrument validation. Additionally, the variability in method validation can also be affected by assay results at different locations and the correct calibration of the assay at different test sites.

The technical validation of biomarkers depends on all aspects of the analytical method including assay sensitivity, specificity, reliability and reproducibility (19,25-27). Specificity refers to the assay's ability to clearly distinguish the analyte of interest from structurally similar substances. Selectivity measures the degree to which unrelated matrix components cause analytical interference. Precision is determined by the assay's repeatability and reproducibility, which are factors used to quantitatively express the closeness of agreement among results of measurements performed under specific conditions (28). Repeatability describes the measurements that are performed under the same conditions, while reproducibility addresses measurements performed under different conditions. The reproducibility of the assay relies on its variability, levels of technical/instrumental and biological noise as well as different validation phases of the method (pre-study and in-study validation of the method).

The acceptance criteria of the assay performance are established based on the study objectives and the known assay variability (29). The nature of the assay methodology and the data generated using that particular assay can influence the establishment of assay acceptance

criteria. The categories of biomarker data that reflect the type of assay employed have been defined at the AAPS/CLAS workshop (16). To aid in the formulation of a method validation plan, a biomarker assay can be placed into various functional categories, each requiring a distinct level of validation. A *definitive quantitative* assay makes uses of calibrators and a regression model to calculate absolute quantitative values for unknown samples. The reference standard must be well defined and fully representative of the biomarker. This type of assay can be validated to be accurate and precise. A *relative quantitative* assay uses a response-concentration calibration with reference standards that are not fully representative of the biomarker. Because the calibration curve may utilize either a noncertified standard or surrogate matrix or both, studies on parallelism and dilution linearity are necessary. Precision can be validated but accuracy can only be estimated. A *quasi-quantitative* assay (possesses certain attributes) does not use a calibration standard, but has a continuous response that is expressed in terms of a characteristic of the test sample. Precision can be validated, but not accuracy (16).

A *qualitative* assay generates categorical data that lack proportionality to the amount of analyte in a sample. The data may be ordinal in that the assay relies on discrete scoring scales like those often used for IHC or nominal such as the presence or absence of a gene product (10, 16). Qualitative assays are only required to show that they are sufficiently sensitive and specific to detect the target analyte. In addition to assay functionality, ensuring that the degree of validation performed reflects the level of importance of the biomarker itself is equally important. Table 5 summarizes the validation parameters for each category of biomarker assay as recommended by the AAPS/CLAS workshop.

What should be the acceptance criteria of the assay performance? Rather then setting the acceptance criteria for precision and accuracy at a fixed value, as in GLP assays, biomarker assays should but evaluated on a case per case basis, with ± 25% acting as default value (± 30% at the lower limit of quantitation, LLOQ) (30). In determining acceptance limits for QCs during sample analysis, either a 4-6-X rule or establishing confidence intervals should be considered (10,30). Such is the case for bioanalytical assays of small molecules where the analytical run is accepted as valid when at least 67% (4/6) of the QCs fall within 15% of their nominal values (the 4:6:15 rule; (21,22,31)). Since the target molecule is often present in pre-dose samples or in the QC matrix, limitations are often placed on LLOQ.

In summary, there numerous factors that impact the biomarker method validation process including the sources of variability in measurements, the intended application of a biomarker, patient selection, sample collection and processing, and analytical validation. As such proper method validation should be carried out in early clinical studies so that these analytical results can be used to assess whether the method affords the sensitivity, precision, and robustness of the assay. During early exploratory phases of drug development, it is not necessary to perform full validation of biomarkers as long as the methods provide reliable data, information, and knowledge (24). As drug development progresses, validation should keep pace with the required precision and reliability needed to achieve the study objectives (24,26). Pre-study validation should be completed before clinical studies are begun and should set the foundation for establishing method acceptance criteria. In-study sample analyses and validation must use QCs to document analytical performance during clinical studies (19). As we advance towards the later stages of drug development, the impact of the biomarker data on decisions around critical safety, efficacy, pharmacodynamic, or surrogate information increases. Thus, an increased rigor in advanced method validation is undertaken as described in the scaled, fit-for-purpose approach (Figure 1).

Standardization and Validation through Collaboration

Recognizing the importance and impact of biomarkers coupled together with the complexity that exists in the drug development process, researchers realized that there is much needed standardization for biomarker development and have therefore joined forces in an effort to integrate biomarkers into drug development. The most recent of these alliances is a consortium called the Cancer Biomarkers Collaborative (CBC) comprised of the American Association for Cancer Research, the FDA, and the National Cancer Institute with an initiative focused on facilitating the use of validated biomarkers in clinical trials (32). The goal of the CBC is to develop guidelines in the areas of biospecimens, assay validation, bioinformatics, and information sharing. The CBC will recommend standards and specifications on how to collect biospecimens and integrate them into drug trials such that the desired endpoint of the biomarker measurement is reached and these endpoints can then be compared among clinical trials. Effort in terms of validation is aimed at identifying and defining how to validate a biomarker assay and make it eligible for inclusion into clinical trials. Hence, the need for a well-defined process with consensus standards and guidelines for biomarker development, validation, qualification, and use is apparent and an important priority of the collaboration. The CBC intends to pave a regulatory pathway for biomarkers, as they transition from the development phase through the FDA approval process and then on to clinical utility.

In addition to the CBC, other alliances in existence include partnerships with government, industry, patient advocacy groups, and other non-profit private sector organizations. The Biomarkers Consortium is a public-private biomedical research partnership formed by the Foundation for the National Institutes of Health, the NIH, FDA, and the Pharmaceutical Research and Manufacturers of America. The Biomarkers Consortium aims to rapidly identify and qualify biomarkers, verify their individual value, and formalize their use in research and regulatory approval to guide clinical practice. Therefore, these collaborative efforts among various sectors have arisen to address the lack of standardized guidelines in biomarker validation, particularly for method validation, and in doing so hope to promote an efficient biomarker development process.

Integrating Biomarkers into Drug Development

Up to this point we have discussed the importance of biomarker method standardization and validation. However, another challenge that remains to be addressed is in understanding how to effectively and efficiently integrate biomarkers into the drug development process. Biomarkers can play a pivotal role in facilitating drug development (Table 1), particularly in oncology drug development where tumor markers appear to correlate with prognosis and potentially be a valuable measure of treatment outcome. The incorporation of biomarkers as surrogate endpoints of clinical efficacy and safety assessment are being intensely evaluated and pursued in rational drug development, especially in the case of biomarker and drug co-developments such as HER2 (also called ErbB2 and Neu) and the development of trastuzumab.

HER2 is a proto-oncogene that became a potential biomarker when studies showed that its overexpression in breast cancer is associated with poor prognosis (33). The role of HER2 as a clinically relevant biomarker led directly to the development of trastuzumab, a specific targeted therapy of a recombinant monoclonal antibody directed against the extracellular domain of HER2. HER2 as a biomarker was used for the selection of the appropriate patient populations (that express or overexpress the oncogenic protein) in clinical trials and to evaluate potential efficacy after therapeutic intervention. Indeed several clinical studies have confirmed that patients with high levels of HER2 receptor overexpression (via IHC staining) are likely to receive clinical benefit from therapy and that HER2 gene amplification (by fluorescence *in situ* hybridization) is most predictive (34). As such, trastuzumab was subsequently approved

in 1998 by the FDA as second-/third-line monotherapy or first-line therapy in combination with paclitaxel for the treatment of HER2-overexpressing metastatic breast cancer (35-38). Thus, biomarker-based patient selection in the early stages of the clinical trial process proves to be critical to the evaluation of a targeted agent as demonstrated by the successful development of trastuzumab.

Another example of the successful use of biomarkers in cancer drug development is the development of imatinib mesylate. This molecular targeted drug is highly efficacious in chronic myeloid leukemia (CML) (39) and gastrointestinal stromal tumor (GIST) (40,41). The BCR-ABL fusion protein translocation in CML provided a biomarker and a therapeutic target for this rationally designed small molecule. In clinical trials of imatinib, assessment of biomarker-based responses facilitated proof of its clinical benefit in CML. Clinical benefit was determined from evaluation of the molecular target in trials using conventional cytogenetics, fluorescence *in situ* hybridization assays of the *BCR-ABL* translocation, and RT-PCR detection of *BCR-ABL* transcripts, which are now used to guide treatment decisions (42). Treatment response and dose optimization can be based on measuring the level of BCR-ABL kinase inhibition achieved *in vivo* as determined by calculating the reduction in protein levels of phospho-CRKL in mononuclear blood cells taken from CML patients (43).

Imatinib also inhibited the receptor tyrosine kinase encoded by the oncogene c-KIT and expression of this oncogenic biomarker provided a rationale for its use in patients with GIST. In addition, the response to imatinib is closely correlated with the presence and type of c-KIT mutation, creating another role for these mutations to serve as biomarkers in treatment selection for individuals with GIST (44-46). GISTs with the most common c-KIT exon 11 mutations are associated with increased imatinib sensitivity, whereas the less common c-KIT exon 9 mutation are less sensitive to imatinib (47,48). GISTs lacking mutations in c-KIT or the alternative receptor tyrosine kinase PDGFRA show much lower rates of response to imatinib (47,48). Thus, the example of imatinib illustrates how biomarkers derived from BCR-ABL can both stimulate initial drug discovery efforts and serve as a useful endpoint assessment of treatment effect in biomarker-based patient and dose selection for imatinib therapy.

With these examples of biomarkers providing key rationale and endpoints in the development of molecular targeted agents, biomarker-based drug development can be regarded as a proven, successful strategy for developing novel anticancer drugs. The scope of evaluation and validation of the biomarker will depend on its intended use as a marker of toxicity, safety, or efficacy. These biomarkers may serve as surrogate endpoints that predict clinical outcomes. As biomarkers are incorporated in drug development, regulatory approaches will be developed and will be more stringent than that needed to guide early drug development to ensure that the development, validation, and implementation of biomarkers into clinical trials is a safe, effective, and efficient process.

Conclusions

The need for a standardized pathway approach towards the biomarker validation process is becoming increasingly important given the recent surge in the biomarker development pipeline. As biomarker research progresses towards establishing the fundamentals of personalized medicine, the future of drug and biomarker co-development resides in identifying the right population that would benefit from that drug. The significant effort and resources that are invested in the development of these biomarkers will expand their roles as surrogate endpoints and diagnostic indicators for disease screening, monitoring disease progression and treatment efficacy, and in assessing patient outcome or identifying potential side effects such as in toxicity. The emphasis now would be placed on biomarker assay development and method validation to eliminate the failure of biomarkers that occur in the clinic as a result of poor assay

choice and the lack of robust validation. Future advancements in biomarker research will be heavily focused on transitioning biomarkers from the development and validation phases to their clinical applications incorporated into drug trials.

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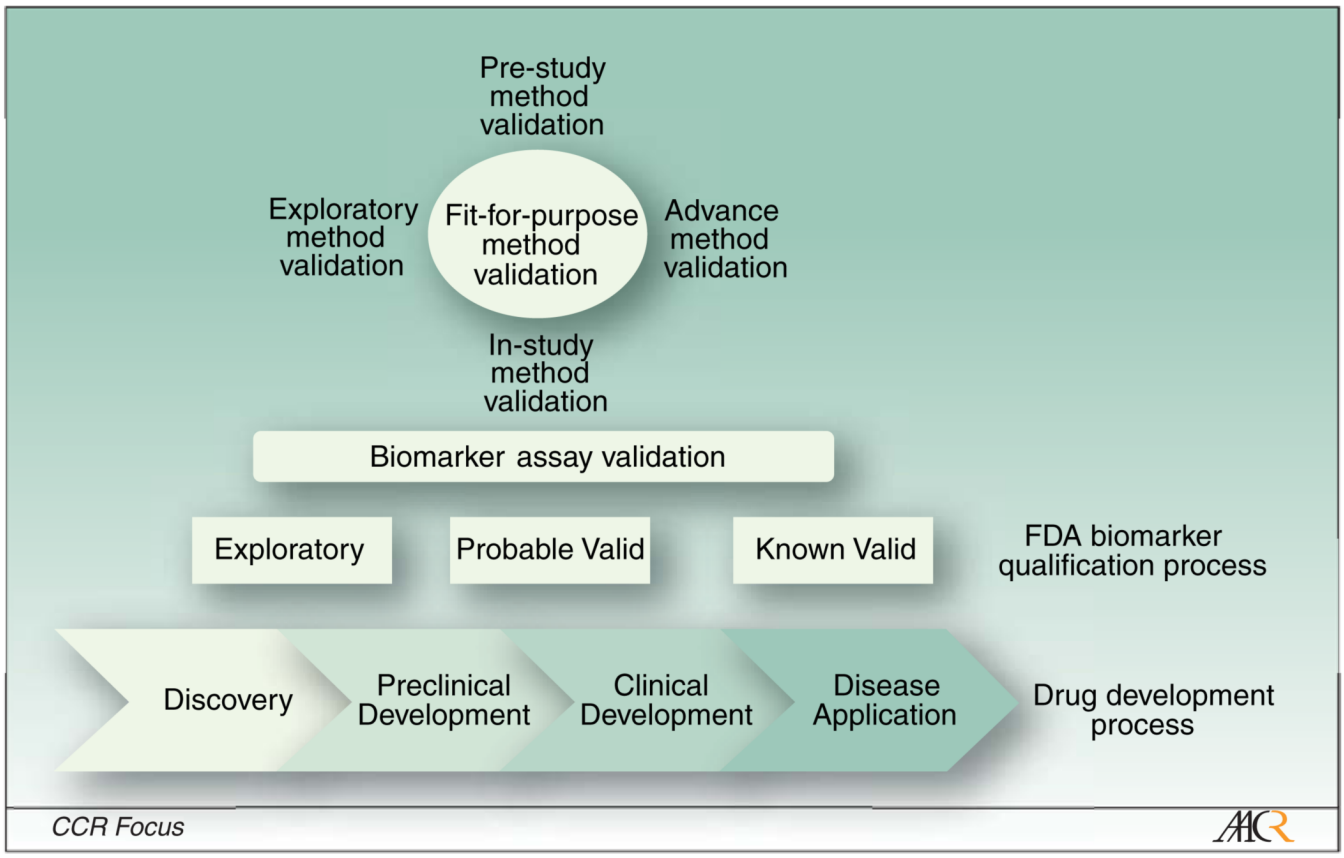

Figure 1.
Integration of the biomarker assay validation and the qualification process with drug development (see text for a description of each process).

Table 1

Potential uses of biomarkers to facilitate the drug development process.

Phases of drug development process	Potential uses of biomarkers during drug development
Target discovery & validation	• Biomarkers used to identify and justify targets for therapy such cellular growth factor receptors, signaling molecules, etc. (e.g. HER2 proto-oncogene frequently amplified in breast cancer and associated with poor prognosis → this correlation provided the rationale for anti-HER2 therapeutic strategies leading to the development of trastuzumab).
Lead discovery and optimization	• Biomarkers used to determine target effects with target-associated assays to identify leads and evaluate the effects of molecular targeted drugs in preclinical development. • Lead agents developed against given target further optimized based on biomarker endpoints in model systems or animal studies.
Preclinical studies	• Development of appropriate animal models of cancer that feature biomarker properties comparable with those seen in patient populations to enhance their utility as predictive models. • Biomarkers can play an essential role in the validation of new disease models (e.g. transgenic mouse models of breast cancer that overexpress HER2). • Biomarkers are used to assess toxicity and safety of the drug.
Clinical trials	• Biomarker-based studies can provide early evaluations of whether the drug is hitting the target (success or failure). • Mechanism-based biomarkers can help guide rational selection of effective drug combinations. • Optimization of dose and schedule can be based on pharmacological effects on biomarker-based end points rather than on maximum tolerated dose. • Biomarkers can serve as tools for the selection of appropriate patient populations or used to stratify patients based on differential clinical response or to identify responders in a subpopulation. • Development and validation of mechanism-based biomarkers that reflect disease activity or the interactions between disease targets and targeted therapy may lead to new surrogate endpoints of clinical benefit. • Selected biomarkers may have the potential to predict clinical outcome.

Table 2

Examples of surrogate endpoints and clinical endpoints.

Disease	**Surrogate Endpoints**	**Clinical Endpoints**
Hypertension	Blood pressure	Stroke
Dyslipidemia	Cholesterol, LDL	Coronary artery disease
Diabetes	Glycosylated hemoglobin (HbA1c)	Retinopathy, nephropathy, neuropathy, heart disease
Glaucoma	Intraocular pressure	Loss of vision
Cancer	Biomarkers Tumor shrinkage, Response rate	Progression-Free Survival Overall Survival

Table 3

Valid genomic biomarkers in the context of FDA-approved drug labels.

Genomic biomarker	Context in label for which biomarker is valid			Other drugs associated with this biomarker
	Representative label	Test*	Drug	
c-KIT mutations+	Presence & type of GIST c-KIT mutations (exon 11 vs. exon 9) predicts sensitivity to imatinib	3	Imatinib mesylate	
CCR5 - Chemokine C-C motif receptor	Drug blocks CCR5 receptor on T-cell that HIV binds to for entry (use only in patients with CCR5-tropic HIV-1 detectable)	1	Maraviroc	
CYP2C9 variants	CYP2C9 PM variants ↑ and EM variants ↓ drug exposure and risk	3	Celecoxib	
CYP2C9 mutations	CYP2C9 mutations ↑ bleeding risk thus requiring lower drug dose	2	Warfarin	
CYP2C19 variants	CYP2C19 variants with genetic defect leads to change in drug exposure (PM ↑ drug exposure and toxicity)	3	Voriconazole	Omeprazole, Pantoprazole, Esomeprazole, Diazepam, Nelfinavir, Rabeprazole
CYP2D6 variants	CYP2D6 PM variants ↑ and EM variants ↓ drug exposure and toxicity	3	Fluoxetine hydrochloride (HCl)	Fluoxetine HCl & Olanzapine, Cevimeline HCl, Tolterodine, Terbinafine, Tramadol & Acetamophen, Clozapine, Aripipraxole, Metoprolol, Propanolol, Carvedilol, Propafenone, Thioridazine, Protriptyline HCl, Atomoxetine, Venlafaxine, Risperidone, Tiotropium bromide inhalation, Tamoxifen, Timolol maleate
Deletion of Chromosome 5q	Cytogenetic abnormality in management of low- or intermediate-1 risk myelodysplastic syndromes	3	Lenalidomide	
DPD deficiency	DPD deficiency ↑ risk of toxicity	3	Fluorouracil++	Capecitabine, Fluorouracil cream, Fluorouracil Topical Solution & Cream
EGFR mutations+++	EGFR mutations ↑ response in NSCLC	3	Gefitinib	Cetuximab
EGFR expression	EGFR (+) expression required for CRC	1**	Cetuximab	Panitumab, Gefitinib
Her2/neu overexpression or amplification	Detection of Her2/neu overexpression or amplification required to select patients for therapy in breast cancer	1	Trastuzumab	Lapatinib
K-RAS mutations++++	K-RAS mutations confer resistance to cetuximab in colorectal cancer	3	Cetuximab	
NAT (N-Acetyltransferase) variants	NAT variants slow and fast acetylators (slow acetylation ↑ drug exposure and toxicity)	3	Rifampin, isoniazid, Pyrazinamide	Isosorbide dinitrate and hydralazine hydrochloride
Philadelphia (Ph1) chromosome-positive responders	Ph1 presence predict response-Busulfan less effective in patients with (Ph1-) chronic myelogenous leukemia	3	Busulfan	

Genomic biomarker	Context in label for which biomarker is valid			Other drugs associated with this biomarker
	Representative label	Test*	Drug	
Philadelphia (Ph1) chromosome-positive responders	Ph1 presence predict response-Dasatinib is indicated for adults with (Ph1+) acute lymphoblastic leukemia	1	Dasatinib	
PML/RAR alpha gene expression	Presence of PML/RAR (alpha) fusion gene predicts response to drug	3	Tretinoin	Arsenic oxide
Protein C deficiencies	Hereditary or acquired deficiencies of protein C may ↑ risk of tissue necrosis	2	Warfarin	
TPMT variants	TMPT deficiency or mutation ↑ risk of myelotoxicity	2	Azathioprine	
UCD deficiency	Contraindicated in UCD patients; evaluation for UCD prior to start of therapy	2	Valproic acid	Sodium phenylacetate and sodium benzoate, sodium phenyl buterate
UGT1A1 mutations	UGT1A1 mutation ↑ drug exposure and toxicity	2	Irinotecan	
UGT1A1 mutations	UGT1A1 mutation ↑ bilirubin levels	3	Nilotinib	
VKORC1 variants	VKORC1 variants confer sensitivity to warfarin thus ↓ dose of warfarin	2	Warfarin	

CRC: Colorectal cancer; CYP: Cytochrome P450; DPD: Dihydropyrimidine dehydrogenase; EGFR: Epidermal Growth Factor; EM: Extensive Metabolizer; G6PD: Glucose-6-phosphate dehydrogenase; GIST: Gastrointestinal stromal tumor; HIV: Human immunodeficiency virus; NADH: Nicotinamide adenine dinucleotide; NSCLC: Non-small cell lung cancer; PM: Poor Metabolizer; PML/RAR: Promyelocytic leukemia/Retinoic acid receptor; TMPT: Thiopurine methyltransferase; UCD: Urea cycle disorders; UGT: UGD glucuronosyltransferase; VKORC1: Vitamin K epoxide reductase complex.

This table is adapted from the following website: http://www.fda.gov/cder/genomics/genomic_biomarkers_table.htm.

CYP: Cytochrome P450; DPD: Dihydropyrimidine dehydrogenase; EGFR: Epidermal Growth Factor; EM: Extensive Metabolizer; G6PD: Glucose-6-phosphate dehydrogenase; GIST: Gastrointestinal stromal tumor; HIV: Human immunodeficiency virus; NADH: Nicotinamide adenine dinucleotide; NSCLC: Non-small cell lung cancer; PM: Poor Metabolizer; PML/RAR: Promyelocytic leukemia/Retinoic acid receptor; TMPT: Thiopurine methyltransferase; UCD: Urea cycle disorders; UGT: UGD glucuronosyltransferase; VKORC1: Vitamin K epoxide reductase complex. This table is adapted from the following website: http://www.fda.gov/cder/genomics/genomic_biomarkers table.htm.

* Reference is made to the requirement of testing for the biomarker (1 = test required, 2 = test recommended, 3 = information only). The test recommendation listed above is current and up-to-date at the time this article is written.

+ Recent studies have shown that c-KIT exon 11 mutations are most common for gastric GISTs and these mutants respond well to imatinib. The less common c-KIT exon 9 mutations occur in intestinal GISTs and are less sensitive to imatinib (47,48). The current FDA-approved drug label for imatinib does not contain this information.

++ Recent studies have shown that higher fluorouracil plasma levels correlated with acute grade 3 toxicity (49).

+++ Recent studies have shown that EGFR mutations and/or amplifications correlate with tyrosine kinase inhibitor activity (50,51). The current FDA-approved drug label for gefitinib and cetuximab does not contain this information.

** Although this is a required test, it is not a predictive marker of activity.

++++ Reference (52). The current FDA-approved drug label for cetuximab does not contain this information.

Table 4

Comparison of bioanalytical assay and biomarker assay validation parameters.

Parameter	Bioanalytical (GLP) assay	Biomarker Assay
Assay method category	Most are definitive quantitative	Most are relative or quasi-quantitative
Regulatory requirement	GLP	No specific guidelines
Nature of analyte	Exogenous	Endogenous
Stability	Drug standards, QCs, sample analyte stability often good	Stability of standards & matrix analytes often poor
Stability testing	Freeze/thaw, bench top, long-term measured by spiking biological matrix with drug	Freeze/thaw, bench top, storage stability with study samples
Standards/Calibrators	Standards prepared in study matrix; certified standard readily available	Standards/calibrators made in matrix different than study samples; certified standards not available
Calibration model	Mostly linear	Choose appropriate calibration model fitting method and tools
Quality controls	Certified standard & blank patient sample matrix available	Certified standard or blank matrix usually not available; substitute with surrogate matrices
Validation sample & Quality control measurements	Made in study matrix. 4-5 VS levels & 3 QC levels	Made in study matrix. At least 5 VS levels & 3 QC levels. If study matrix is limited may use surrogate matrix
Assay acceptance criteria	4-6-15 rule (for small molecules)	4-6-X rule or establish confidence interval
Precision/Accuracy	Robust technology with acceptance criteria	Variable; no acceptance criteria
Specificity/Selectivity	Drugs not present in sample matrix; samples are subject to clean-up & analyte recovery	Specificity issues: biomarkers present in sample matrix; samples not subject to clean-up; assess matrix effects & minimize; investigate sources of interference
Sensitivity	LLOQ defined by acceptance criteria	Limited sensitivity & dynamic range; LLOQ & LOD defined based on working criteria

GLP: Good laboratory practices; QC: Quality controls; VS: Validation sample; LLOQ: Lower limit of Quantitation; LOD: Limit of Detection

Table 5

Summary of validation parameters applicable to each category of biomarker assay.

	Definitive quantitative	**Relative quantitative**	**Quasi-quantitative**	**Qualitative**
Validation Parameters				
Sample stability	X	X	X	X
Reagent stability	X	X		
Assay range	X	X	X	
Parallelism	X	X		
Dilution linearity	X	X		
Accuracy	X	X		
Precision	X	X	X	
Sensitivity	X	X	X	X
Specificity	X	X	X	X
Example of assay	Mass spectrometry	ELISAs	Immunogenicity immunoassays	Immunohisto-chemistry