REVIEW

Breast cancer biomarker measurements and standards

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Cancer is a heterogeneous disease characterized by changes in the levels and activities of important cellular proteins, including oncogenes and tumor suppressors. Genetic mutations cause changes in protein activity and protein expression levels that result in the altered metabolism, proliferation, and metastasis seen in cancer cells. The identification of the critical biochemical changes in cancer has led to advances in its detection and treatment. An important example of this is the measurement of human epidermal growth factor receptor 2 (HER2), where increased expression occurs in approximately 20–30% of breast cancer tumors. HER2 is a member of the epidermal growth factor receptor family and is an important biomarker expressed on the cell surface. Measurement of the HER2 levels in tumor cells provides diagnostic, prognostic, and treatment information, because a targeted therapeutic is available. The most common methods to measure HER2 levels are immunohistochemistry and in situ hybridization assays. The accurate and reliable measurements of the specific changes in protein biomarkers for detection and treatment of cancer are important challenges. This review is focused on efforts to improve the quantitation and reliability of cancer biomarkers by using standards and reference materials.

Keywords:
Breast cancer / Epidermal growth factor receptor / HER2 / Reference materials / Standards

1 Introduction

This review focuses on breast cancer biomarkers and how the applications of standards and reference materials can be used to improve the quality and reliability of the measurements. Improved biomarkers are needed for the multiple cellular pathways that can go wrong in cancer cells and many new technical approaches are being used. The goals of this review are to review the classical biomarkers for breast cancer, highlight the approaches for the development of new biomarkers, and focus on the opportunities for standards and reference materials.

We start with the current methods to measure the classic biomarkers of breast cancer, followed by the emerging methods to discover new biomarkers based on nucleic acid analysis and protein measurements. Table 1 summarizes the existing methods for biomarker measurement and the emerging methods that are close to routine implementation in clinical laboratories. Human epidermal growth factor receptor 2 (HER2) is used as an example of the progress on biomarker measurements and standards. Throughout this review, the importance of standards and reference materials is stressed as an essential element to improve the reliability and accuracy of the discovery, validation, and applications of new biomarkers in clinical laboratories.

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Abbreviations: ASCO, American Society Clinical Oncology; **ATCC**, American Type Culture Collection; **CAP**, College of American Pathologists; **EGFR**, epidermal growth factor receptor; **FDA**, Federal Drug Admiration; **FFPE**, formalin-fixed paraffin embedded; **IHC**, immunohistochemistry; **HER**, human epidermal growth factor receptor; **ISH**, in situ hybridization; **NCI**, National Cancer Institute; **NIST**, National Institute of Standards and Technology; **PR**, progesterone receptor; **QPCR**, quantitative PCR; **STR**, short tandem repeat

2 Classic biomarkers of breast cancer: Measurements and targeted therapeutics

Measurements of the classic breast cancer biomarkers HER2, estrogen receptor (ER), and progesterone receptor (PR) are

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Certain commercial equipment, instruments, or materials are identified in this paper to foster understanding. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Table 1. Summary of clinical measurements and standards for breast cancer biomarkers using HER2 examples

Target	Technology	Pros and cons	Standards and reference materials (RM) needed
DNA/RNA	FISH (fluorescence in situ hybridization)	FDA approved kits: FISH Inform™ (Ventana Medical Systems, Inc.: http://www.ventana.com), FISH PathVysion™ (Abbott Molecular Inc.: http://www.abbottmolecular.com), FISH Her2 PharmDX™ (Dako Denmark A/S: http://www.dako.com). Pros: partial automation, control for chromosome 17. Cons: increased expense and time compared to IHC, fluorescence microscope, and special training required	Cell/tissue RM, probe validation
	CISH (chromogenic in situ hybridization)	FDA approved: Spot-Light® HER2 CISH™ (Life Technologies, Inc.: http://www.lifetechnologies.com), HER2 CISH PharmDX™ kit (Dako Denmark A/S: http://www.dako.com), Inform™ Dual ISH (Ventana Medical Systems, Inc.: http://www.ventana.com). Pros: standard microscope; built-in internal control, more robust DNA target. Cons: subjective scoring	Cell/tissue RM, probe validation
	SISH (silver in situ hybridization)	FDA pending: SISH EnzMet™ kit (Ventana Medical Systems, Inc.: http://www.her2sish.com/main.php). Pros: HER-2 and chromosome 17 centromere probes, standard brightfield microscope, fully automated. Cons: subjective scoring	Cell/tissue RM, probe validation
	RT-PCR (reverse transcriptase)	OncotypeDX™ (expression of a panel of 21 genes, including HER2) from Genomic Health, Inc.: http://www.genomichealth.com Pros: uses FFPE samples, multiplex gene assay. Cons: preanalytical variables, mRNA degradation, PCR efficiency and dilution artifacts	Cell/tissue/RNA RM
	cDNA microarray	MammaPrint™ is the FDA-cleared breast cancer recurrence assay (Agendia: http://www.agendia.com). The array interrogates 70 genes of the critical molecular pathways involved in the breast cancer metastatic cascade. Cons: dilution artifacts and mRNA stability are critical issues	Cell/tissue/RNA RM
Protein	IHC (Immunohisto-chemistry)	FDA approved several kits: IHC BOND™ ORACLE™ (Leica Biosystems: http://www.leicabiosystems.com), IHC InSite™(BioGenex Laboratories, Inc.: http://www.biogenex.com), IHC HerecepTEST™ (Dako Denmark A/S: http://www.dako.com), and IHC Pathway™ (Ventana Medical Systems, Inc.: http://www.ventana.com). Pros: easy to use, widely available and inexpensive. Cons: effect of tissue pre-analytical variables, and subjective test interpretation	Cell/tissue RM, antibody validation
	ELISA/antibody array (enzyme-linked immunosorbent assay)	FDA approved Siemens ELISA serum HER-2 Advia Centaur™ kit (Siemens: http://www.medical.siemens.com). Pros: existing clinical platform. Cons: sensitivity and dilution artifacts issue, attempts to use this serum-based test have not been widely accepted. Lack of clinical evidence to provide utility for prognostic value or comparison to other methods	Cell/tissue/protein RM, antibody validation
	Circulating tumor cells	Cell Search™ (Veridex, LLC: http://www.veridex.com) cleared by FDA for monitoring metastatic cancer patients. Pros: blood test. Cons: cells present at low concentrations, lack of clinical data to support prognostic value or comparison to other methods for patient management	Cell/tissue RM, antibody validation

routinely done in clinical laboratories to classify tumor samples to determine treatment. The accurate measurements of these biomarkers are essential to determine the correct course of treatment with targeted therapeutics. Unfortunately, the clinical measurements of these biomarkers are not standardized and there are concerns about the accuracy of these measurements [1–3]. These classic biomarkers do not reliably predict the response of all of the patients to therapeutics and it is clear that they do not fully represent the heterogeneity of breast cancer [4].

2.1 HER2 and the EGFR family of cancer biomarkers and therapeutics

Epidermal growth factor receptor (EGFR) was the first member of a family of tyrosine kinase receptor proteins that stimulate cell growth to be discovered. Since the initial discovery of EGFR, three additional members have been added. The family, also referred to as HER (for human) or ErbB, includes EGFR (ErbB1 or HER1), along with HER2 (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). These proteins

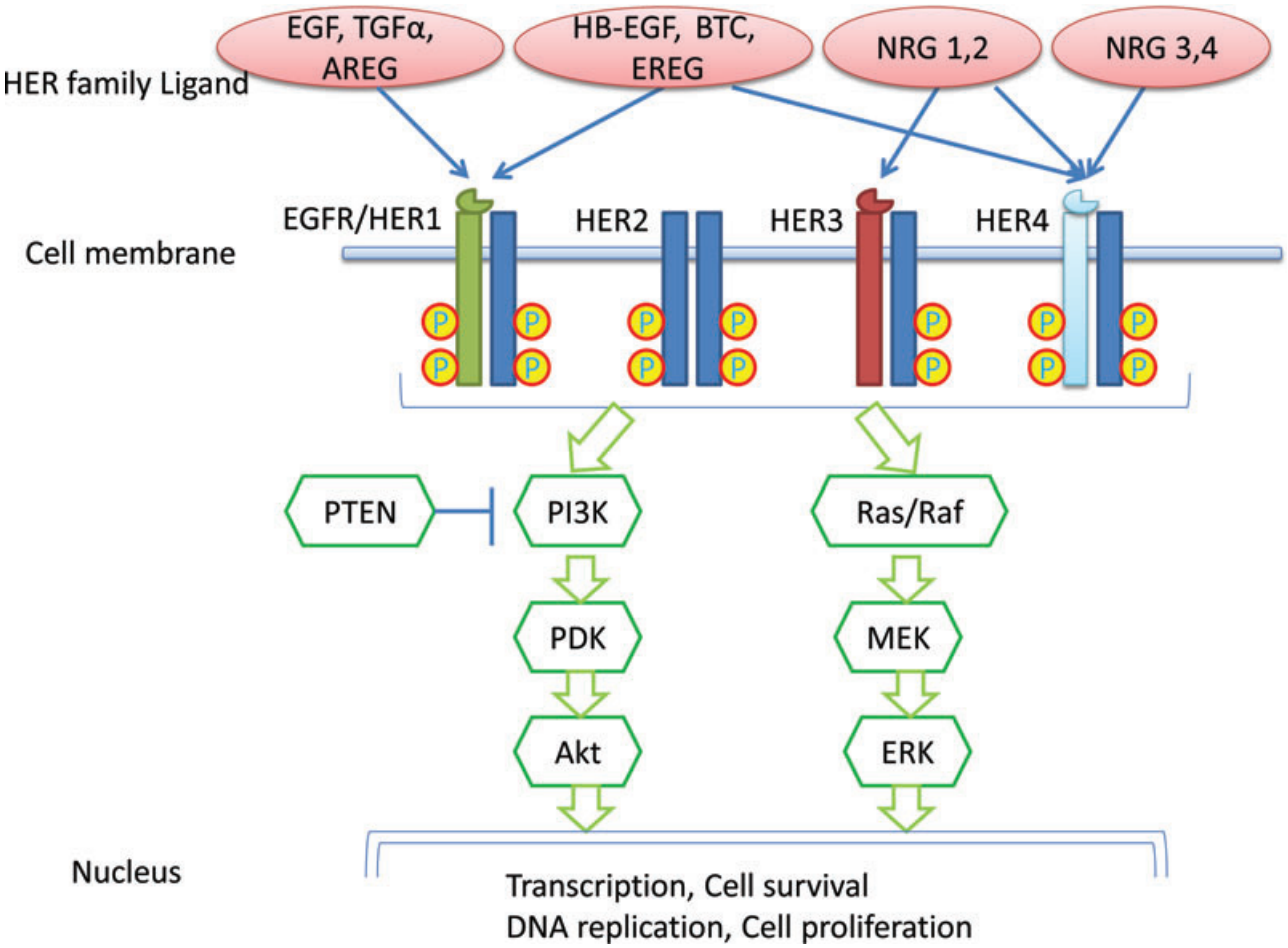

Figure 1. A simplified scheme of the human epidermal growth factor receptor 2 (HER2) key signal transduction pathways. HER2 forms homodimers or heterodimers with the other members of the family. The binding of epidermal growth factor receptor (EGFR) family ligands to the other HERs results in the formation of HER2 heterodimers (AREG, amphiregulin; BTC, betacellulin; EREG, epiregulin; HB-EGF, heparin-binding EGF [where EGF is epidermal growth factor]; NRGs, neuregulins; TGF-α, transforming growth factor-α). Homodimerization and heterodimerization of HER2 leads to tyrosine kinase activation and downstream signaling via the phosphatidylinositol 3-kinase/Akt pathway and the Ras/Raf/mitogen-activated protein kinase pathway to stimulate processes involved in cell survival and proliferation.

are all transmembrane growth factor receptors that share similar structures, but have different biological functions. The HER2 gene is found on chromosome 17q12 and codes for a 185 kDa protein [5]. The EGFR proteins are single chain glycoproteins [6]. The EGFR structure has a large extracellular domain, a transmembrane domain, and the cytoplasmic domain containing the protein kinase activity along with the C-terminal region that contains the tyrosine autophosphorylation sites (Fig. 1) [6, 7].

There are a number of potential ligands that bind to the EGFR proteins triggering their activation and diverse biological effects. The ligands include epidermal growth factor (EGF), transforming growth factor α, amphiregulin, heparin-binding EGF, epiregulin, β-cellulin, and the neuroregulins [8, 9]. The general model of activation for the EGFR family is ligand binding, followed by dimer formation with either the same protein forming a homodimer or with one of the other members of the EGFR family forming a heterodimer. Dimer formation results in the autophosphorylation of the cytoplasmic domain by the tyrosine kinase activity (Fig. 1). The nature of the bound ligand and the protein composition of the dimer determine the biological response [10, 11]. Interactions of other signaling proteins with phosphotyrosine sites on the EGFR dimer trigger the downstream signaling pathways. The downstream signal transduction pathways include the Ras/Raf/ERK kinase pathway, the PI3K/AKT/mTOR (phosphatidylinositol 3-kinase) pathway, resulting in changes in RNA transcription, cell division, apoptosis, cell migration, adhesion, and differentiation [9, 11, 12].

HER3 on its own does not have kinase activity [13] and ligands for HER2 have not been described [14]. HER2 also differs from the other EGFR members in that the 3D structure indicates HER2 may be in an active state even before it forms dimers [6]. HER2 is active even in the absence

of ligands resulting in tumor formation when overexpressed in cells [9, 15]. The biological activities of the EGFR family are determined by both the nature of the dimer formation and the presence of the ligands resulting in a large number of possible combinations and subsequent biological outcomes [9].

Increased expression of HER2 in NIH 3T3 cells results in transformation and tumorigenesis in mice [15, 16]. A major milestone in the establishment of HER2 as a cancer biomarker was a study of 189 primary breast cancers that found HER2 gene amplification in approximately 30% of the cancers [8]. Salomon et al. established that HER2 amplification was a significant prognostic indicator of reduced overall survival and increased relapse in breast cancers [8]. As knowledge of the molecular events occurring in cancer is gained, this knowledge is being used to develop targeted therapeutics [17]. The phosphatidylinositol 3-kinase (PI3K) pathway is frequently activated in cancer. The activation can occur through mutations in *PIK3CA* (the catalytic subunit of PI3K) or through the loss of function of the tumor suppressor PTEN, a phosphatase that inhibits the PI3K pathway, that includes AKT in the signaling pathway. Mutations in PIK3CA were measured in 26% of breast cancer tumors [18]. Mutations in *AKT1* and *PTEN* were found in 1.4% and 2.3% of tumors, respectively, and were detected only in hormone receptor positive tumors [19].

Trastuzumab (Herceptin) is a humanized monoclonal antibody used for the treatment of HER2 overexpressing breast cancers. The mechanism by which trastuzumab acts to treat HER2+ breast cancer patients is not completely known and multiple effects have been observed. The effectiveness of antibody treatment may be due to activation of antibody-dependent cellular cytotoxicity resulting in cell lysis of HER+ cells, reduction of the proteolytic release of the extracellular domain of HER2 into blood, inhibition of PI3K pathway, and inhibition of angiogenesis [20]. It has been found from X-ray crystal structure that trastuzumab (Herceptin) binds HER2 on the C-terminal region of domain IV that includes the binding pocket for the extended domain II loop of inactive forms of HER3 and HER1 [21]. The Herceptin binding close to the membrane region could possibly facilitate endocytosis and avoid kinase activation of HER2 by preventing interaction of the transmembrane regions of the receptors [21].

2.2 Estrogen and progesterone receptor biomarkers and therapeutics

The ER and PR are important biomarkers for the diagnostic classification and treatment of breast cancer. ER and PR are frequently overexpressed in breast cancer tumors. The American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) have developed guidelines to improve immunohistochemistry (IHC) testing of ER and PR in breast cancer samples [22]. The ASCO/CAP recommended that a tumor sample be considered positive if at least 1% of the tumor nuclei stain positive. Their recommendations also include the conditions for the treatment of tissue prior to testing, validation of the assay, the use of external controls, and mandatory proficiency testing each year.

Tamoxifen can be considered the first targeted therapeutic for breast cancer. Tamoxifen is metabolized in the liver and then binds to ER in cells, but it does not activate the receptor and thus partially blocks the action of estrogen produced in the body [23]. A breast cancer tumor that is ER+ is a good candidate for hormonal therapy treatment with tamoxifen. A study of two large databases showed that the PR status was independently associated with breast cancer recurrence and survival, providing useful prognostic value [24]. There are two ER isoforms (α and β) coded by separate genes, and both are nuclear receptors that bind estradiol. ERα is the main isoform found in breast tissue. After binding 17β-estradiol, the activated ER can increase nuclear transcription of genes with estrogen responsive elements and also by interaction with transcription factors such as Fos/Jun [25–27]. ER can also exert effects at the cell membrane. A fraction of ERα (5–10%) is localized to the cell membrane and it can activate growth factor pathways by interacting with surface receptors including HER2 and EGFR or G protein-coupled receptors [26, 28].

AIB1 (also known as SRC-3) is an ER coactivator that is also activated in the HER2 receptor pathway and has been found to be increased in breast and ovarian cancer [29]. The levels of AIB1 and HER2 were measured in 316 patients with breast cancer [30]. In the patients that were receiving tamoxifen therapy, high AIB1 levels were associated with lower disease free survival, indicating AIB1 reduced the antagonistic effects of tamoxifen. Patients whose tumors had high levels of both AIB1 and HER2 had the worst clinical outcomes [30]. Gene expression studies in ER+ breast cancer patients treated with tamoxifen indicated that the measurement of the ER biomarker alone was inadequate to predict response to tamoxifen [31]. Loi et al. found that expression of 181 genes grouped in 13 biological clusters (six of the clusters were pathways related to cell cycle and proliferation) allowed them to place patients into two groups that predicted the clinical outcome response to tamoxifen [31].

2.3 Measurement of the classic breast cancer biomarkers in clinical laboratories

There are two major methods used for HER2 measurements routinely used in clinical laboratories, IHC techniques that use antibodies to measure the protein levels and in situ hybridization (ISH) methods that use nucleic acid probes to measure the amplification of the HER2 gene (Table 1). Both techniques utilize tissue slices from formalin-fixed paraffin embedded (FFPE) tumor samples and both require microscopic examination by skilled professionals. IHC tests use antibodies to localize the HER2 protein on the cell surface and most commonly use an enzyme-linked chromogenic

substrate for detection. FISH (fluorescent in situ hybridization) techniques utilize fluorescently labeled probes to detect the HER2 gene in the nucleus. CISH (chromogenic in situ hybridization) techniques utilize a chromogenic substrate to detect the number of nucleic acid probes to the cell nucleus [32]. The Federal Drug Administration (FDA) has approved commercial kits for IHC, FISH, and CISH tests for the measurement of HER2 (Table 1). The commercially available FISH tests use a fluorescent probe for HER2 and can also incorporate a second probe for the centromere region of chromosome 17 as a control. Methods that incorporate a control probe for chromosome 17 permit the detection of polysomy 17 that can also lead to higher levels of HER2 gene copy [32]. The in situ hybridization tests detect changes in HER2 gene copy numbers, but are not able to detect increased HER2 protein levels that are not due to gene amplification.

A joint publication by the ASCO/CAP gave their recommendations for testing HER2 using IHC and ISH in clinical labs [33]. The recommendations in this 2007 publication have been reconciled with the 2010 ASCO/CAP guideline for ER and PR measurements, in order to ensure samples are handled in a consistent manner. The most recent clinical notice and clarification for the ASCO/CAP guidelines for HER2 testing guidelines and resources are available from the CAP website (www.cap.org). The recommendations are focused on the importance of cold ischemic time, handling of specimens, fixation time in neutral buffered formalin, and the optimal sample for testing. The recommended cold ischemic time (between a tissue sample removal and the time the tissue is placed in fixative) should be 1 h or less to preserve HER2 signals when using FISH measurements [34]. The recommendations for fixation time for HER2 testing is from 6 to 48 h, although needle biopsy samples may be fixed for shorter time periods [33].

IHC tests have the advantages of speed and lower cost compared to the more sophisticated ISH tests. A number of studies have reported problems with the accuracy and the concordance of the results obtained from different laboratories using IHC and FISH (reviewed in [1, 2]). The ASCO/CAP recommends that laboratories include an initial 95% concordance testing of HER2 samples with another validated test method, a quality assurance program, standard operating procedures, external proficiency testing, and current accreditation by a valid laboratory accrediting agency [33]. An interlaboratory comparison (94 laboratories in 21 mainly European countries) of commercial IHC tests for HER2 used cell lines as control reference materials, cell lines BT-20 and MCF-7 as nonamplified controls and cell lines SKOV-3 and MDA-MB-453 as amplified controls [35]. The laboratories had improved results when using the cell line reference materials. Additional studies have also shown the utility of breast cancer cell lines (SKBR-3 and MCF-7, high HER2 expression and normal levels, respectively) for use as reference materials for IHC, FISH, and quantitative PCR (QPCR) assays [36].

3 Emerging methods to discover and measure new breast cancer biomarkers

3.1 Gene expression and gene copy number measurements

Measurement of the gene expression patterns, DNA mutations, and gene copy number abnormalities in tumor tissues from cancer patients and in breast cancer cell lines allow the classification into cancer subtypes [37–43]. Patterns of gene expression could be determined allowing classification into subtypes. The gene expression patterns in normal breast luminal epithelial cells and basal epithelial cells were compared to the expression in different tumors and cell lines. Gene expression measurements of 534 genes in 115 breast cancer tumors allowed classification into a basal-like subtype, a HER2 overexpression subtype, two luminal epithelial subtypes (A or B), and a normal breast tissue expression subtype [38]. Gene expression patterns, genomic structure, and biological activities were measured in 51 breast cancer cell lines and 145 breast cancer samples [41]. Neve et al. [41] found that the genomic heterogeneity, gene copy number variations, and gene expression variations seen in the cancer cell lines mirror the changes seen in the tumor samples, but the cell lines on average contain more genomic aberrations and high-level gene amplifications compared with the cancer tumor samples. Neve et al. felt that the differences could be due to the way that cell lines are frequently derived from late-stage tumors or that high-level amplifications may be an advantage for cell survival in culture conditions [41].

Whole-genome microarrays were used to measure gene expression and gene copy number variations in a set of 52 commonly used breast cancer cell lines [42]. The gene expression results of the breast cancer cell lines indicated, one basal and two luminal (A and B) subtypes. They found in general that the cell lines had higher levels and more complex copy number variations when compared to those found in the tumors and the genetically simpler luminal A subtype (found in tumors) was not seen in the cell lines [42]. Distinctive patterns of copy number variations were seen in the subtypes of breast cancers, indicating that the genetic changes may be correlated with the development of specific cancer subtypes [39].

A large-scale integrated analysis of 510 breast cancer tumor samples combined DNA modifications, gene expression, and protein measurements that classified the samples into four major breast cancer subtypes (luminal A, luminal B, basal-like, and HER2E) [43]. Gene expression patterns and protein levels showed that only approximately one-half of the HER+ tumors were in the HER2E subtype and most of the rest were in the luminal subtypes [43].

A 21-gene multiplex RT-PCR assay was developed to provide prognostic information on tamoxifen-treated, node negative breast cancer expression [44]. This assay is available commercially (Oncotype DX^{TM}) and was designed to work on

FFPE tissues and used to determine the likelihood of cancer recurrence for treatment (Table 1). The genes selected for this assay originated from studies using 250 candidate genes and 447 patients [44].

A method for the prediction of metastases in breast cancer was tested using microarrays to screen gene expression of approximately 25 000 human genes [45]. Starting from an initial set of approximately 5000 genes (regulated in the breast cancer tumors), the authors identified a set of 70 that could be used to differentiate those patients with a good prognosis (low risk of metastases) from those patients with a poor prognosis. The predictive value of the 70-gene set was subsequently confirmed in clinical trials [46]. The test was commercially developed as MammaPrint and cleared by the FDA as the first in vitro diagnostic multivariate index assay for breast cancer recurrence (Table 1). A recent study of the gene expression and gene copy number variations in 355 breast cancer tumors identified six subgroups based on the analysis of the data [47].

QPCR methods for the measurement of HER2 amplification have been developed. A high sensitivity PCR method was used to detect HER2 amplification in as few as 50 cells in archival FFPE tissue sections [48]. Low levels of amplification required laser microdissection of tumor cells from the background of normal cells.

Standards such as the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were developed to encourage the complete description of the assays and samples [49]. The standard recommends that the assay performance be characterized by measuring specificity, PCR efficiency, linear dynamic range, limit of detection, and precision. The Functional Genomics Data Society has established the Minimal Information About a Microarray Experiment (MIAME, www.mged.org) that provides a uniform basis for the deposition of microarray data in public databases, increasing the value of the data and encouraging data standardization. The National Institute of Standards and Technology (NIST) is developing a set of 96 synthetic DNAs of varying lengths and GC contents that can be converted to RNA using in vitro transcription. These standards can be used for DNA or RNA sequencing and microarray spike in experiments [50, 51].

3.2 Proteomic approaches for biomarker discovery and measurements

Proteomic approaches are used to independently identify changes in protein functions or concentrations that occur in cancer and to validate the change predicted by genomic and gene expression experiments. Fractionation techniques can be used to reduce the complexity of the total protein complement for proteomic studies to focus on classes or pathways of proteins [52].

Antibodies are the workhorse affinity reagents. They have a long history, with many applications and the infrastructure for their production and incorporation into assay platforms. Antibodies can be used for detection, as well as for the purification of their target antigens. Instruments and techniques are available for utilization of antibodies in large arrays for discovery of biomarkers [53]. Antibodies can be used for high throughput IHC on tissue microarrays to study the specificity of protein expression, used to probe arrays of tissues or cells (reverse phase protein arrays) for simultaneous detection of many proteins in patient samples, and detection of specific biomarkers in serum using antibody arrays [54]. Reverse phase protein arrays utilize large numbers of individual samples immobilized on a membrane surface and each sample array is probed with an individual antibody. Reverse phase protein assays have proven to be sensitive, reliable, and useful for obtaining data from large numbers of cancer patient samples for biomarker studies [55].

Multiple reaction monitoring (MRM) MS using stable isotope-labeled peptides as standards was used for the quantitation of hundreds of phosphorylation sites in the cell signaling networks [56]. The phosphorylation of 222 tyrosine phosphopeptides was measured using MRM mass spectroscopy after EGF stimulation of 184A1HMEC cells [56]. Most studies of the activation of EGFR focus on phosphoproteins, the most likely targets in cell signaling disrupted in cancer. The transient nature of phosphoproteins makes their analysis difficult. A shotgun analysis of the proteome from dividing A431 cells, EGF-stimulated cells and cells inhibited using the cancer drugs cetuximab or gefitinib revealed differential expression of 12 proteins as candidate markers for the EGFR inhibition signature [57].

A multiplexed immune selective reaction monitoring (SRM) MS assay was developed for the quantification of ER and HER2 levels in cell lines and tumor samples [58]. Schoenherr et al. determined the sensitivity (low fmol/mL) and coefficient of variation (approximately 10%) of the assay. A good correlation with ER/HER2 status measured by traditional clinical assays was demonstrated. The application of MRM for precise detection of cancer biomarkers (including HER2) in FFPE tissues was shown by comparing results to frozen tissues of renal cell carcinoma [59]. The signal intensities of the peptides from FFPE tissues were lower (on average 66%) compared to the frozen samples, but the coefficients of variation of the measurements were the same for both tissues [59]. SRM with stable isotope-labeled standards was also used for the quantitation of EGFR in FFPE cell lines and cancer tissues [60].

SILAC allows the quantitative measurement of proteins by comparison of different cell types or cells that have been differentially treated [61]. SILAC was used to compare the biomarkers associated with the PI3K pathway in the presence and absence of drugs that inhibit the pathway in cancer cells [62]. Andersen et al. measured 375 phosphopeptides in the PI3K pathway, and the expression of 71 of the phosphopeptides changed that were linked to the drug inhibitors [62]. A phosphopeptide biomarker for the PI3K pathway was identified that had improved stability compared to other phosphopeptide markers. The stability was determined by comparing

the recovery at different times during tissue processing [62]. SILAC was also used to identify 23 differentially expressed proteins in a tumor virus-HER2 transgenic mouse model compared to the noncancerous mouse line [63].

The National Cancer Institute (NCI) Office of Cancer Clinical Proteomic Research is dedicated to improving the understanding of the molecular basis of cancer through the use of proteomic technologies (http://proteomics.cancer.gov/). The International Cancer Genome Consortium (http://www.icgc.org/icgc) and the Cancer Genome Atlas (http://cancergenome.nih.gov/) are additional major programs that aim to elucidate the genome, gene expression, and epi-genomic changes that occur in cancer. The NCI Clinical Proteomic Technology Assessment for Cancer program sponsored a number of interlaboratory studies to determine the repeatability and reproducibility of LC-MS/MS proteomic studies [64–66]. Mixtures of purified human proteins and the yeast protein extract (NIST reference material RM 8323) were used as standards for detection of intact protein and tryptic digests in these studies [64–66]. The reference datasets produced from the complex mixture of the yeast proteins and human proteins can be used by others with the same reference samples for confirmation of their results. The consortium sponsored an interlaboratory study of MRM coupled with stable isotope dilution for the quantitation of proteins in human plasma [67]. This study showed that MRM with isotope dilution was a highly reproducible and sensitive technique for analysis of plasma proteins [67]. A peptide standard for MS is available as NIST RM 8327 that consists of three synthetic peptide sequences designed in consensus with the Peptide Standards Committee of the Association of Biomolecular Resources Facilities (ABRF). Information on the sequence, mass, and charge of the peptides is available from NIST (http://www.nist.gov/srm/index.cfm).

3.3 Impact of clinical tissue samples on biomarker measurements

High-quality clinical samples are essential for the discovery of new biomarkers and achieving reliable measurements in clinical laboratories. The utility of FFPE samples for applications utilizing antibodies, measurements of DNA or RNA, and proteomic analysis needs to be validated using standards and reference materials. The preanalytical variables associated with FFPE samples include those associated with the sample collection, the fixation step, processing, and storage prior to analysis, and some of the variables are discussed in the ASCO/CAP guidelines described in Section 2.3. A review article identified 62 preanalytical variables and the analysis revealed that 15 variables had a significant effect on IHC analysis and 12 variables did not have a reported effect [68]. The major variables with an influence on IHC include the following: the time before fixation, fixative type, time in fixation, dehydration conditions, paraffin clearing and impregnation, conditions of slide drying, and storage conditions [68]. A study showed that inadequate fixation of tissues resulted in retention of water in tissue sections causing antigen degradation, which demonstrated the importance of uniform standards for fixation and processing steps [69]. Formalin results in extensive cross-linking of primary amines causing extensive modification of proteins and linking proteins to nucleic acids. It is therefore surprising that IHC and proteomic studies can be done with FFPE tissues. A major step in the use of FFPE tissues for protein analysis was the development of a process called antigen retrieval, done by boiling the FFPE tissue in aqueous solutions resulting in improved detection of proteins by antibodies for IHC techniques [70].

A number of proteomic studies of cancer tissues and other diseases using archival FFPE samples have been published (reviewed in [71]). The quality of nucleic acids extracted from FFPE tissues can be limited by the reduced size of the DNA and RNA extracted. The quality of the RNA and DNA can be low as judged by the low efficiency of the nucleic acids as templates for polymerases. A study compared the effect of different fixation conditions using human tissues on the yield and quality of the DNA extracted [72]. Some treatments improved yield or quality, while others had no improvement and some cases reduced it [72].

The effect of tissue storage time before freezing was measured on the recovery of 53 tissue phosphoproteins and signal pathway proteins [73]. The results revealed that the kinase pathway remained active after tissue collection. The presence of uncontrolled kinase activity and phosphatase activity will change the amounts of phosphorylated proteins preventing reliable measurements of biomarkers. In a busy clinical environment, it can be difficult to freeze tissues in a rapid and uniform manner. Alternative methods to preserve the state of tissues should be developed to stop enzymatic activity that are compatible with histological preparations [73]. An instrument has been developed that can process tissues using a combination of heat and pressure under a vacuum to inactivate enzymes that cause the degradation of proteins or change the posttranslational modifications [74]. This heat and pressure inactivation process was combined with LC-MS/MS to investigate 31 480 phosphorylation sites on 7280 proteins in rat tissues [75]. The time a tissue sits at room temperature before freezing (or fixation) is termed the warm ischemia time and it is an important preanalytical variable. A review of the effects of the time before a tissue is frozen observed that metabolic activity had an effect on gene expression (upregulated genes observed more often than downregulated genes) that was mainly due to the warm ischemic time and that considerable RNA degradation can occur during the thawing step of frozen tissues [76].

The detection of circulating cancer cells in the blood is an active area of research with great potential to monitor treatment and determine cancer status. Due to the low levels of circulating tumor cells in blood, an enrichment step must be used, typically using immunomagnetic beads to capture rare cancer cells in a large background of normal blood cells. A commercial assay and instrumentation

(CellSearch™, www.veridex.com) is available for the semiautomated detection of tumor cells in blood samples (Table 1). The CellSearch™ assay uses magnetic beads with antibodies specific for epithelial cells to enrich epithelial cells, followed by fluorescence detection using monoclonal antibodies to distinguish leukocytes from epithelial cells. The assay has been cleared by the FDA for monitoring cancer progression and evaluating treatment therapy in metastatic breast, colon, and prostate cancer patients.

HER2 levels have been measured in both circulating tumor cells and tumors from breast cancer patients [77, 78]. A study of patients with metastatic breast cancer found that the HER2 levels of the circulating tumor cells were correlated with the levels in the tumor, but the levels in the circulating tumor cells had lower levels of amplification for unknown reasons [78]. A review of previous studies found that approximately 20–50% of patients with HER-negative primary breast cancer tumors had HER+ circulating tumor cells [77].

4 Applications of reference materials and standards to improve measurement quality

4.1 Cancer cell lines as renewable reference materials

Established cell lines are a valuable source of materials, essential for basic research, production of biologicals, and development of new cancer diagnostic tests and therapeutic agents. In order to provide reliable results, the cell lines have to be well characterized and produce predictable results when grown in culture.

The origin or age of cells grown in culture cannot be established by the routine microscopic inspection used to monitor cell growth. The misidentification and contamination of cell lines has been identified as a widespread and serious problem. Stanley Gartler was an early pioneer in the development of methods for the identification of cells in culture using isoenzyme analysis and he used those methods to show that HeLa cells were found to be a frequent contaminant in many cells lines believed to be from other sources [79]. HeLa was found to be a frequent contaminant, but other human cell lines were also found to be contaminants in misidentified cell lines [80]. In 1999, a study at the German Cell Bank showed that 18% of the cell lines submitted were either misidentified or contaminated by other cells [81]. A human breast cancer cell line MCF-7/AdrR was the subject of a large set of publications on drug resistance in cancer cells was shown not be derived from MCF-7, one of the most studied breast cancer cell lines, but was identical to OVCAR-8, an ovarian cancer cell line [82]. The MDA-MB-435 cell line, originally identified as a breast cancer, was shown to be essentially identical to M14 a melanoma cell line [83]. Additional examples of other misidentified high-profile cancer cell lines have also been documented [84].

Fortunately, rapid and low cost methods have been developed to conclusively authenticate the identity of human cell culture lines. STRs are polymorphic repeat elements found in genomic DNA. The STR markers used in human identity testing have been selected with high degrees of heterozygosity, providing high levels of specificity and reliability [85]. A commercial multiplex STR assay was used to screen 253 human cell line samples submitted by the major international cell banks and the unique profiles obtained proved the utility of the approach for identifying closely related cell lines (varieties of HeLa) and independent cell lines [86]. The American Type Culture Collection (ATCC) Standards Development Organization formed a working group to develop the consensus standard "ASN-0002: Authentication of Human Cell Lines: Standardization of STR" [87]. The standard provides users with the recommended methods for genotyping human cell lines, analysis of the data, and recommendations to share the genotyping profiles with public databases to make data accessible to everyone. STR repeats have also been developed for cell lines derived from African Green Monkeys (Vero and COS cells) [88] and markers for additional species are currently under development at NIST for other species, such as mouse and Chinese hamster ovary cells used in research and production of biological therapeutics.

The length of time a cell line is in culture increases the selective pressure on the cells and increases the possibility that changes in the phenotype and genotype can occur. Changes in cell transepithelial electrical resistance, cell density, proliferation rates, para-cellular permeability, trans-cellular permeability, carrier mediated transport, and alkaline phosphatase expression have been found to change with the cell passage number [84]. Changes in genomic structure have been observed in embryonic stem cells while in culture [89, 90]. It is always recommended that authenticated cell lines should be obtained from biological repositories and that cells used with the lowest possible passage numbers be used to obtain the most consistent results.

Osborne et al. obtained samples of MCF-7 cell lines from three different laboratories and a biological repository (ATCC), and although appearing morphologically similar, the cell lines had different amounts of ER and PR receptors and different levels of tumorigenicity when injected into mice [91]. Karyotype analysis by Osborne et al. showed that the cell lines from the three different laboratories had similar chromosome alterations and marker chromosomes, but the cell line from the biological repository did not have the same chromosome alternations compared with the other samples from different laboratories, indicating the different origin of the cell lines [91]. A more recent study by Bahia et al. studied four samples of MCF-7 cell lines, one maintained in their lab for approximately 5 years, one maintained for approximately six and half years, and two samples from different laboratories [92]. Analysis of the metaphase chromosomes of the cell lines using multicolor FISH showed common chromosomal translocations, but each cell line also had unique chromosomal abnormalities [92]. These results highlight the

dangers in using cell lines obtained from other laboratories and culturing cell lines for long periods of time.

Cell culture is an artificial environment that does not adequately simulate the complex environment of a tumor. Most tumors are heterogeneous based on the histological observations, expression of the classical IHC markers, and more recently at the levels of genomic structure and gene expression [4]. The tumor microenvironment provided by the stromal cells has a large effect on the development of cancer [93]. Efforts to investigate the microenvironment of tumors and simulate these factors in cell culture will increase the value of cell culture models of cancer.

Coculture of canine breast cancer cells with canine macrophages or carcinoma-associated fibroblast cells changed the gene expression status as well as the migration and invasiveness of the breast cancer cells [94, 95]. These efforts show that controlling the environment of cell culture lines can increase their value as models to study cancer.

Many of the established breast cancer cell lines were established from metastatic sources, not the primary tumor. These established cell lines may overrepresent the more aggressive types of cancer [96]. Recently progress has been made to establish new cell lines from primary tumors [97] that may be more representative of the initial changes in cancer that occur in tumors.

4.2 Cell and tissue repositories

Sources of high-quality samples from patients with cancer and relevant control tissues are essential for the discovery and validation of biomarkers that can be used for diagnosis and prognostic applications. Commercial and government sponsored cell repositories for microorganisms and cell lines have been highly successful in proving uniform access to high-quality samples. The Coriell Institute for Biomedical Research has established the Coriell Biobanks in order to maintain large collections of cell lines, tissues, biological fluids, and DNA samples for both public and private sources. Examples of other international biological repositories providing validated cell lines include the ATCC, Cell Bank Australia, Leibniz-Institute DSMZ, and the Japanese Collection of Research Bioresources.

The NCI has established an Office of Biorepositories and Biospecimen Research (OBBR) with the purpose of improving the quality and accessibility of high-quality biological materials (http://biospecimens.cancer.gov/default.asp). The OBBR supports scientific research, establishes policies, provides education materials, and partners with stakeholders. A workshop was held by the NCI/OBBR in collaboration with NIST and the FDA Office of Personalized Medicine entitled "Biospecimen Quality Assessment & Standards Development" (http://biospecimens.cancer.gov/global/pdfs/Preliminary_Summary_2125F7-508.pdf). Participants from the workshop identified four major areas to focus on to improve the quality of samples used for cancer research. The four areas were to study the quality of RNA in tissues, DNA in tissues, formalin-fixed tissues, and proteins in serum. The quality of the samples is essential for accurate and reliable measurements of gene expression, genetic mutations, and protein biomarkers.

4.3 Antibody validation

A number of proteomic technologies utilize the selection power of antibodies for studying cancer. Given the importance of antibodies (and other affinity reagents) for the measurement of biomarkers, their validation is vital. There are generally accepted methods to measure the specificity and sensitivity of antibodies, and additional characterization may be necessary [98]. An algorithm suitable for the validation of antibodies used for IHC or quantitative immunofluorescence has been developed [99]. The algorithm is based on measuring specificity using the results of Western blotting, immunoprecipitation, and IHC.

The Human Protein Atlas (http://www.proteinatlas.org/) is an extensive project to develop validated antibodies for every human protein. The goals of the project are to provide high-quality validated antibodies, and uniform information about the expression of the proteins in normal human cells and tissues and cancer tissues [100]. The current version (9.0) contains data on more than 15 500 antibodies from more than 12 200 genes (approximately 61% of the protein coding genes in the human). The antibodies are produced from the recombinant expression of peptides (50–150 amino acids long) obtained from the gene sequences. The peptides are used to produce polyclonal antiserum that is then affinity purified. The goal is to provide at least two antibodies (nonoverlapping) that are highly specific for each human protein.

5 Opportunities for standards to improve discovery and measurement of cancer biomarkers

Table 1 contains examples of the current and emerging tests for breast cancer biomarker measurements, along with the opportunities for standards and reference materials. Standard methods and reference materials are essential as the new methods and instruments are adapted for use in the clinical environment. The necessary reference materials range from tissues to purified biomolecules.

Well-characterized tissue samples would be an ideal standard reference material for many of the tests, but many practical considerations limit their use. The heterogeneity and the limited (nonrenewable) supply of tissues currently limit the use of tissues to research studies. Future development in tissue engineering of surrogate tissues produced in bioreactors may provide adequate amounts of tissues as renewable sources that could be used in clinical laboratories for standards. It is clear that well-characterized cell lines will be

an essential resource in cancer research for biomarker discovery and validation, as well and therapeutic testing. New developments include primary cells available from commercial sources for use as normal controls and new cell lines to include additional tumor types to more fully expand the genetic and proteomic diversity found in cancer. Human cell lines that have been engineered with specific cancer mutations will be an important source of standards for clinical laboratories in the future.

Studying the effect of the preanalytical variables and storage conditions on FFPE and frozen tissues is important for the accurate detection of proteins and nucleic acids. Measurement of gene expression, DNA mutations, and copy number from FFPE tissues are now being used for clinical diagnostic and prognostic values. Serum (or plasma) is the most common type of clinical sample and is widely used because it comes in contact with every tissue in the body. Because of its central role in clinical studies, markers for measuring the quality of proteins in serum samples need to be developed that are reliable and sensitive.

Additional support is necessary to provide access to uniform tissue samples that are essential to improve the identification and validation of cancer biomarkers. The development of standard methods for analysis and well-characterized reference materials will improve the reliability of clinical biomarker measurements. As a part of this effort, NIST is developing HER2 reference materials composed of genomic DNA prepared from well-characterized breast cancer cell lines. The genomic DNA will be used to enhance the standardization of DNA-based assays for HER2. Additional measurements of HER2 protein levels on these well-characterized breast cancer cell lines will be implemented using flow cytometry and mass spectrometry-based techniques in the future. The quantified HER2 protein levels will ultimately assist proteomic based clinical tests. The standards developed at NIST will help increase the confidence and accuracy of cancer biomarker measurements in clinical and research laboratories.

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