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REVIEW

The Evolving Role of Companion Diagnostics for Breast Cancer in an Era of Next-Generation Omics

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A companion diagnostic is a test for a specific biomarker—approved by the United States Food and Drug Administration—qualifying a patient to receive a specific, associated therapy. As interest has grown in precision medicine over the past decade, the principle of companion diagnostics has gained increasing purchase among laboratory professionals, clinicians, regulators, and even patients. The evolution of the biomarkers used to stratify and treat breast cancer illustrates the history of companion diagnostics and provides a lens through which to examine potential challenges. As new targeted therapies and corresponding biomarkers accumulate, algorithms for diagnosis and treatment necessarily become lengthier and more complex. To accommodate future needs of breast cancer patients, the companion diagnostic model will continue to adapt and evolve. (*Am J Pathol* 2017, 187: 2185–2198; <http://dx.doi.org/10.1016/j.ajpath.2017.04.021>)

Companion Diagnostics

As interest has grown in precision medicine, the concept of companion diagnostics has gained increasing purchase among laboratory professionals, clinicians, regulators, and even patients. The United States Food and Drug Administration (FDA) defines an *in vitro* companion diagnostic device (an IVD companion diagnostic) as a device or test “that provides information that is essential for the safe and effective use of a corresponding therapeutic product” (<https://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM262327.pdf> and <http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-meddev-gen/documents/document/ucm510824.pdf>, last accessed April 4, 2017). This definition is fairly broad and could in principle be applied to a sphygmomanometer used to prescribe a β -blocker; in practice, a companion diagnostic has been more properly understood as a test for a specific finding that rules a patient in or out for a specific therapy based on that finding. The specificity is important; a sphygmomanometer detecting a blood pressure above a certain threshold may trigger the use of β -blockers, but that should not be the only therapy considered.

Conversely, β -blockers may be prescribed for multiple conditions, only some of which are triggered by a specific blood pressure. Thus, the term companion diagnostic is best understood as a dyad between test and therapy.

Molecular genomic findings often carry the most specific association with a targetable disease mechanism. For this reason, companion diagnostics are most often understood and applied (and indeed, evolved as a concept) in the context of specific molecular genomic findings identifying oncology patients likely to respond to targeted therapy. Indeed, the prototype for the companion diagnostic model would almost certainly be translocation between chromosomes 9 and 22 [the Philadelphia chromosome, t(9;22)] as indication for treatment with imatinib (Gleevec; Novartis, Basel, Switzerland). To date, the current list of FDA-approved IVD companion diagnostics remains almost exclusively based on molecular oncology targets; in breast cancer, most are based on protein detection by immunohistochemistry

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This article is part of a review series on next-generation breast cancer omics.

Table 1 IVD Companion Diagnostics in Breast Cancer

Biomarker	Companion diagnostic	Manufacturer	Method	FDA status	Associated therapy
Her-2 (<i>ERBB2</i>)	HercepTest	Dako (Glostrup Municipality, Denmark)	Overexpression (IHC)	Approved	Trastuzumab Pertuzumab Ado-trastuzumab emtansine
	HER-2 IQFISH pharmDx	Dako	Amplification (FISH)	Approved	Trastuzumab Pertuzumab Ado-trastuzumab emtansine
	Inform Her-2/Neu	Ventana Medical Systems, Inc. (Oro Valley, AZ)	Amplification (FISH)	Approved	Trastuzumab
	PathVysion Her-2	Abbott Laboratories (Lake Bluff, IL)	Amplification (FISH)	Approved	Trastuzumab
	Pathway anti—Her-2/Neu	Ventana Medical Systems, Inc.	Overexpression (IHC)	Approved	Trastuzumab
	InSite Her-2/Neu	Biogenex (Fremont, CA)	Overexpression (IHC)	Approved	Trastuzumab
	SPOT-LIGHT Her-2 CISH	Life Technologies (Invitrogen; Carlsbad, CA)	Amplification (CISH)	Approved	Trastuzumab
	Bond Oracle Her-2 IHC	Leica Biosystems (Wetzlar, Germany)	Overexpression (IHC)	Approved	Trastuzumab
	HER-2 CISH pharmDx	Dako	Amplification (CISH)	Approved	Trastuzumab
	Inform Her-2 Dual ISH	Ventana Medical Systems, Inc.	Amplification (2-color CISH)	Approved	Trastuzumab
	HERmark	Monogram Biosciences (South San Francisco, CA)	Overexpression (protein quantification)	NA (CLIA validated)	NA
	FoundationFocus CDxBRCA	Foundation Medicine, Inc. (Cambridge, MA)	Massively parallel sequencing (MPS)	Approved*	Rucaparib
BRCA1/2	BRCAnalysis CDx	Myriad Genetics (Salt Lake City, UT)	PCR & Sanger sequencing	Approved*	Rucaparib
	myChoice HRD	Myriad Genetics	Mutational signature	Investigational	Olaparib Talazoparib Niraparib Veliparib Talazoparib Niraparib Veliparib
	PD-L1 IHC 22C3 pharmDx	Dako	Expression (IHC)	Approved*	Pembrolizumab
PD-L1	PD-L1 IHC 28-8 pharmDx	Dako	Expression (IHC)	Approved [†]	Nivolumab
	PD-L1 (SP142) assay	Roche (Basel, Switzerland)	Expression (IHC)	Approved [†]	Atezolizumab
	PD-L1 (SP263) assay	Roche	Expression (IHC)	Approved [†]	Durvalumab
PIK3CA	cobas 4800 PIK3CA assay	Roche	Mutation (sequencing)	Investigational	Alpelisib (BYL719)

The table is meant to be representative, not exhaustive.

*Approved in another cancer type.

[†]Approved as a complementary diagnostic, rather than a companion diagnostic.

CDx, companion diagnostic; CISH, chromogenic *in situ* hybridization; CLIA, Clinical Laboratory Improvement Amendments; FISH, fluorescent *in situ* hybridization; HRD, homologous recombination deficiency; IHC, immunohistochemistry; NA, not applicable; PD-L1, programmed cell death 1 ligand; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α .

(IHC) (Table 1 and Figure 1) (<https://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm>, last accessed April 4, 2017).

The evolution of biomarkers used to stratify and treat breast cancer illustrates the history of companion diagnostics, and provides a lens through which to examine current and future challenges. Although not all biomarkers considered below are IVD companion

diagnostics according to the FDA definition, many function in a similar manner, and they all provide biological, clinical, and historical context and are therefore useful to consider. Table 1 provides a comparison of several existing and investigational IVD companion diagnostic assays for breast cancer, and Figure 1 places the most commonly used assays in the context of their clinical application.

B

Expression Pattern	Molecular Subtype	Ki67	Grade	Prognosis	Standard Therapy	Selected Genomic Alterations
ER/PR +	Luminal A	Low	Low (Grade I)	Favorable	Endocrine	PIK3CA variants
ER/PR+ & Her2 +	Luminal B	↑	Intermediate (Grade II)	↑	Endocrine / Her2 targeted	ERBB2 amplification
Her2 +	Her2 +	↓	High (Grade III)	↓	Her2 targeted	↓
Triple Negative	Triple Negative	High	High (Grade III)	Poor	Chemotherapy	TP53 variants

Figure 1 Evaluation of invasive breast cancer includes companion diagnostic (CDx) assays. **A:** A schematic representation of the most commonly applied diagnostic assays for invasive breast cancer illustrates the role for CDx assays. Tests that currently include a CDx component are highlighted in bold red text. Mandatory testing for invasive breast cancer per National Comprehensive Cancer Network guidelines (https://www.nccn.org/professionals/physician_gls/pdf/breast_blocks.pdf, last accessed April 4, 2017). Before or concurrent with histopathologic assessment of the tumor, evaluation of personal and family history may necessitate germline testing for hereditary *BRCA1* or *BRCA2* variants (https://www.tri-kobe.org/nccn/guideline/gynecological/english/genetic_familial.pdf, last accessed April 4, 2017). Currently, assays for the *BRCA* genes are approved as CDx for poly-ADP ribose polymerase inhibitors only in the context of ovarian cancer (Table 1). Importantly, germline testing should always be accompanied by appropriate counseling and should not be performed solely on tumor tissue. Narrowly targeted testing for the confirmation of a known familial variant may differ in methodology from assays that are agnostic regarding the presence of a specific variant, such as the CDx assays listed in Table 1. Regardless of whether germline testing is performed, all invasive breast carcinoma is evaluated for estrogen receptor (ER) and progesterone receptor (PR) expression by immunohistochemistry (IHC). Overexpression of Her-2/Neu protein may also be evaluated by IHC or may be inferred from the amplification of the *ERBB2* gene, detected by fluorescent or chromogenic *in situ* hybridization (FISH or CISH). Additional testing (not mandatory by current guidelines) is often applied for predictive (therapeutic) or prognostic purposes. IHC for Ki-67 may be applied concurrently with IHC for other markers, offers a global assessment of the proliferative nature of the cancer, but standardization and reproducibility are of concern. If immune-checkpoint inhibitors are a therapeutic consideration, IHC assays for programmed cell death 1 (PD-L1) expression may also be applied if there is sufficient tissue. Only one IHC assay is approved as a CDx in non-small cell lung cancer, with others as complementary diagnostics or in development. Nucleic acid–based tests (gene expression arrays and multigene sequencing panels) have different tissue processing, equipment, analytical, and interpretive requirements, which can extend turn-around time. Moreover, these assays are often sent to external laboratories. **B:** Gene expression arrays can categorize breast cancer by molecular subtype and may inform prognosis and therapeutic response (particularly to chemotherapy). The reliability and equivalence between different gene expression assays are not well established. Multigene sequencing assays (usually by massively parallel sequencing) vary considerably in target coverage and detectable genomic alterations (eg, whether specific genes are included, copy number changes are detectable, and the maximum size of detectable insertions/deletions), but when properly designed they can produce predictive and prognostic data. Such assays are currently most helpful in the context of disease progression, unexplained therapeutic resistance, and candidacy for clinical trials, but they can also provide information otherwise arrived at through CDx assays (eg, deleterious *BRCA1/2* gene variants and *ERBB2* amplification). The data from these assays are used, along with clinical data and pathologic staging, to stratify patients for treatment.

ER, PR, and Her-2

Estrogen receptor (ER), progesterone receptor (PR), and Her-2 are the protein products of the *ESR1*, *PGR*, and *ERBB2* genes, respectively. The use of IHC to detect expression of ER and/or PR predates the formal adoption of the IVD companion diagnostics model by decades; however, expression of either ER or PR stratifies patients by their presumptive response to therapy targeted at the estrogen-response mechanism.^{1–5} In the case of these hormone receptors, the diagnostic finding is not paired to a single drug (or even class of drugs) in a stereotyped manner. Instead, expression of either hormone receptor corresponds with several treatments and classes of drugs that converge on the same molecular pathway.⁶ Thus, ER/PR testing functions similarly to FDA-approved IVD companion diagnostics. Indeed, some of the challenges posed by ER/PR testing informed later companion diagnostic development, providing contrast and context to more conventional companion diagnostics.

Evaluation of ER and PR began in the 1970s to assess potential response to antiestrogen therapy, including aromatase inhibitors, selective estrogen modulators, and oophorectomy. ER and PR are hormone receptors that bind their respective ligands and translocate to the nucleus, where they bind DNA and activate transcription of downstream targets. The transcriptional targets of activated ER drive a number of cellular processes related to cancer, including proliferation and differentiation. Approximately 70% of patients with breast carcinoma have tumors that express ER and/or PR, and therapy directed at the estrogen pathway, which acts to remove or antagonize the stimulatory effect of estrogen, significantly reduces the recurrence and mortality rates in these patients.^{7,8} Given the prevalence of breast carcinoma (there are an estimated 252,710 new cases of invasive breast cancer per year), ER and PR testing by IHC has improved clinical outcomes for a substantial number of breast cancer patients (<https://www.cancer.org/research/cancer-facts-statistics/all-cancer-facts-figures/cancer-facts-figures-2017.html>, last accessed April 4, 2017). Endocrine therapy to block the ER pathway is highly effective, but its usefulness is limited by common intrinsic and acquired resistance mechanisms, including deregulation of various components, alterations in cell cycle and cell survival signaling molecules, and activation of escape pathways that can provide tumors with alternative proliferative and survival stimuli. Among these, increased expression or signaling of growth factor receptor pathways, especially the epidermal growth factor receptor/Her-2 pathway, is associated with both experimental and clinical endocrine therapy resistance. New treatment combinations targeting ER and growth factor receptor signaling which block the crosstalk between these pathways and eliminate escape routes have proved highly effective in preclinical models. Results of recent clinical studies, although partly supporting this approach, also highlight the need to better identify *a*

priori the appropriate patients whose tumors are most likely to benefit from specific cotargeting strategies.

Although expression of ER and PR is an important marker for breast cancer, genetic changes driving tumor behavior initially remained opaque. That changed with a deepening understanding of the molecular mechanisms driving cancer. In the late 1980s, amplification of the Her-2/Neu genomic locus was identified as an independent prognostic factor in breast cancer.⁹ Her-2/Neu (alias Her-2 or *ERBB2*) encodes a receptor tyrosine kinase member of the epidermal growth factor receptor family. Excess kinase drives downstream oncogenic pathways, which include a number of other known oncogenes. Because the Her-2 protein product is a receptor kinase, it provided an opportunity to develop a comparatively specific, targeted therapy directed at a specific genomic alteration. In the early 1990s, trastuzumab (Herceptin; Roche, Basel, Switzerland) was approved by the FDA along with one of the earliest true IVD companion diagnostics, the immunocytochemical assay HercepTest, designed to detect Her-2 protein in formalin-fixed, paraffin-embedded tissue.^{10–12}

Conventional ER, PR, and Her-2 analyses, including the approved companion diagnostics, rely on IHC or *in situ* hybridization (ISH) techniques, which require visualization of the biomarker in tissue. These techniques and their interpretation carry with them some variability. In the early days of testing, a lack of reproducibility between laboratories led to clinical uncertainty and divergent outcomes. Ultimately, this uncertainty drove considerable effort to standardize processes and techniques. Much of the inconsistency was attributed to variability in specimen handling before analysis, primarily the amount of time between surgical removal of the tissue and fixation (ischemic time), and the amount of time the tissue spent in formalin fixative (fixation time).^{13–17} Analytic variability arose from differences in properties of different specific IHC antibodies¹⁸ and inconsistent interpretation of the IHC signal, which even under precisely controlled conditions varies in both staining intensity and the proportion of cells stained.^{7,19} Recognition of the breadth and scope of confounding factors led to extensive efforts to determine appropriate standards and apply them to practice guidelines.^{6,20–24} Recent work has further suggested that not all IHC antibodies are equivalent in their prognostic or predictive ability, because Her-2 undergoes cleavage into intracellular and extracellular domains as part of its biological mechanism, suggesting that antibodies directed at extracellular epitopes may better predict response to therapy directly targeting Her-2.²⁵ The struggle to standardize assessment of ER/PR and Her-2 directly has affected the development and adoption of later biomarker assays and more generally influenced the overall FDA strategy of IVD companion diagnostics.

A number of other assays using other techniques have since come to market as alternatives to HercepTest, making use of alternative IHC antibodies, fluorescence ISH, chromogenic ISH, or proprietary protein quantification methods

(Table 1). However, the clinical utility of the growing number of alternative Her-2 assays has yet to be borne out in practice. For instance, the use of alternative Her-2 fluorescence ISH probes in cases previously scored as equivocal with the use of standard fluorescence ISH probes may result in the re-classification of up to 40% of such cases as amplified. Nevertheless, the lack of prospective clinical trials to evaluate the response of these patients to targeted therapy renders these results clinically ambiguous.²⁶

Not all variability in therapeutic response is attributable to different assay technologies or their practical application; biology maintains inherent variability as well. Although most tumors expressing ER and/or PR respond to estrogen-modulating therapies, a significant portion do not. Some of this discrepancy can be explained by the intratumoral heterogeneity. Breast cancer, as with most malignancies, evolves over time, accruing new genomic changes. This process can generate intratumoral subclones or distant metastases with distinct genomic profiles (spatial heterogeneity).^{27,28} Differences among subclones can lead to different responses to therapy, including the development of resistance (temporal heterogeneity). Indeed, heterogeneity in Her-2 expression is an independent predictor of poorer outcomes in breast carcinoma.²⁹ IHC and ISH—although capable of identifying heterogeneity of expression—cannot necessarily identify the acquisition of resistance mutations.

Variants in *ESR1* may alter the responsiveness of the receptor to antiestrogen therapy without changing the expression of the protein.^{30–33} *ERBB2* can also acquire therapeutic resistance via a number of genetic mechanisms, many of which are undetectable by current companion diagnostics.³⁴ Moreover, the mRNA transcript is subject to alternative splicing, which can also disrupt ER function without altering ER IHC detection.³⁵ In contrast to the resistance variants in *ESR1*, activating mutations have been described in *ERBB2*.^{36–38} Some of these variants respond to anti-Her-2 therapy, even in the absence of Her-2 overexpression. In the presence of resistance or loss-of-function alterations (because of mutations or alternative splicing) standard IHC or ISH testing will overestimate patients who will respond to targeted therapy, whereas in the presence of driver mutations the same testing will fail to identify patients who may benefit from such therapy. Thus, the more additional information available regarding specific cellular and genetic properties of a tumor, the more precisely therapy can be tailored. The relevant data, though, are not provided by existing FDA-approved IVD companion diagnostic assays.

Sequencing, Clinical Genomics, and Future Companion Diagnostics

Despite the broad use of ER, PR, and Her-2 as biomarkers in treatment, these genes are not among the most frequently mutated in breast cancer. A host of studies mining large data

sets have identified recurrent molecular alterations in breast cancer, including potential driver mutations, molecular signatures, and other diagnostic features,^{27,28,39–46} any of which may potentially lead to a targeted therapy that could be paired with an IVD companion diagnostic. Not every potential therapeutic target easily lends itself to a straightforward diagnostic test, however.

Two of the most prominent genes in breast cancer pathology—the homologous DNA-repair genes breast cancer 1 (*BRCA1*) and breast cancer 2 (*BRCA2*)—are best known for harboring germline variants associated with hereditary breast and ovarian cancer syndrome,⁴⁷ although somatic variants occur as well.⁴⁸ Rucaparib, a poly-ADP ribose polymerase inhibitor, has recently been approved for use in ovarian cancer with a PCR/Sanger sequencing–based companion diagnostic (BRACAnalysis CDX; Myriad Genetic Laboratories, Salt Lake City, UT),^{49–53} and a massively parallel sequencing (MPS, alias next-generation sequencing) assay (FoundationFocus CDxBRCA; Foundation Medicine, Inc., Cambridge, MA) (Table 1) (<https://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm>, last accessed April 4, 2017). Notably, Myriad Genetic Laboratories also offers an alternative diagnostic product identifying the mutational signature of homologous repair deficiency (myChoice HRD). Although many IVD companion diagnostics directly identify the target of therapy, the identification of a mutational signature for *BRCA1/2* deficiency carries some advantages. The *BRCA1* and *BRCA2* genes are large and subject to a number of different kinds of alterations, not all of which are reliably identified or interpreted from direct sequencing (eg, large deletions and noncanonical splice-site variants). Perhaps more importantly, homologous repair deficiency itself is agnostic regarding the specific deficient gene and therefore indicates dysfunction in any gene in the homologous repair complex, which includes multiple genes and interacts with a number of other pathways. Accordingly, assays evaluating the functional status of the homologous repair complex, whether directly assessing its functional capacity⁵⁴ or identifying the telltale mutational signature resulting from its dysfunction,^{46,55} may prove to be more inclusive of rare (or even undescribed) drivers of cancer, potentially identifying more patients who might respond to targeted therapy.

BRCA1 and *BRCA2* are not unique in interacting as components of larger functional complexes or networks. *PIK3CA*, the most commonly mutated gene in breast cancer,²⁸ encodes the catalytic subunit of the phosphoinositol-3 kinase (PI3K) complex, an upstream component of a multiprotein pathway [PI3K/mechanistic target of rapamycin (mTOR)/AKT pathway], which itself interacts with a number of other cellular pathways. *PIK3CA* harbors several variant hotspots (eg, variants at amino acids H1047 and Q546), which drive constitutive activity of the kinase complex. Targeted therapies specific to *PIK3CA*-mutated cancer, including breast cancer, are seeing preliminary

success in preclinical and clinical trials,^{56,57} and a research use only assay for *PIK3CA* variants is available from Roche on the cobas 4800 platform (FDA-approved as a platform in other contexts, such as detection of *EGFR* variants in lung cancer). Other investigational therapies, though, target the PI3K pathway less specifically,⁵⁸ such that patients harboring variants in other pathway components, including *PIK3R1*, *AKT* genes, or *PTEN* (Figure 2), might benefit from treatment. Resistance variants may also develop during therapy anywhere in the pathway. Design of IVD companion diagnostic assays addressing even a majority of relevant genomic alterations to the PI3K pathway may prove challenging. To compound the biological complexity, variants in *PIK3CA* specifically modify prognosis in ER-positive and Her-2—positive breast cancer, independent of *PIK3CA* as a direct therapeutic target.^{28,59} Multiparametric assays, including genomic analysis, proteomic analysis, and expression arrays, could be used to detect relevant genomic variants in multiple genes and direct anti-PI3K therapy, but none of these types of assays are easily paired with a single therapy or class of therapies.

Variants in the tumor protein p53 (*TP53*) gene are also common across cancer, including breast cancer.²⁸ *TP53* encodes a protein that regulates multiple pathways in opposing oncogenesis, including detection of DNA damage and activation of repair mechanisms, cell-cycle arrest, apoptotic activation, and inhibition of angiogenesis, among others. The p53 protein is normally viewed as a tumor suppressor, with loss-of-function variants predominating; however, oncomorphic single-nucleotide variants at hotspot codons are also described.⁶⁰ Like *PIK3CA*, p53 interacts with a network of other proteins (Figure 2); therefore, it carries some of the same difficulties in developing any effective companion diagnostic for a therapy directed specifically at p53 dysfunction. With regard to targeted therapy, the most notable direct protein interaction of p53 is with mouse double minute homolog 2 (*MDM2*), which binds p53, maintaining a quiescent state. In the presence of functional p53, *MDM2* antagonists (such as idasanutlin, currently in clinical trials) release p53 to perform its normal tumor suppressive functions.^{61–64} In a significant proportion of breast cancer, *MDM2* is amplified or *TP53* is mutated (usually in a mutually exclusive manner), suggesting that anti-*MDM2* therapy may be efficacious in the appropriate genomic context. It is straightforward to imagine the development and implementation of a companion diagnostic for *MDM2* amplification; however, successful treatment with *MDM2* inhibitors does require the presence of at least one functional *TP53* allele. *TP53* is one of the most mutable genes in cancer, both before and during treatment. Combinatorial detection of *MDM2* amplification (by ISH or IHC) with p53 expression (by IHC) has potential as a diagnostic strategy, but it cannot account for oncomorphic variants in p53 without additional testing.

Loss of inhibition of the cell cycle is a common finding across breast cancer, although the specific mechanism can

vary (Figure 2). Drivers of the cell cycle can be activated or amplified [eg, cyclin D1 (*CCND1*) or cyclin-dependent kinase-4 (*CDK4*)]. Alternatively, cell-cycle repressors may be dysfunctional or lost [eg, cyclin-dependent kinase inhibitors 1B (*CDKN1B*/p27) or 2A (*CDKN2A*/p16)]. In these situations, therapy directed at inhibiting CDK4 and/or CDK6 (palbociclib; Novartis) may provide particular benefit.^{65,66} As with *BRCA1/BRCA2* testing, designing a companion diagnostic to capture all of the potential targets that might be responsive to anti-CDK4/CDK6 therapy poses challenges, and like *MDM2* inhibition, CDK inhibition can be rendered ineffective by loss-of-function variants in an entirely separate, mutable gene, retinoblastoma 1 (*RBI*).

A number of other genomic biomarkers have been identified with enough frequency in breast carcinoma to warrant research and development toward targeted therapeutics with companion diagnostic assays. Amplifications and activating mutations of the fibroblast growth factor (*FGF*) and fibroblast growth factor receptor (*FGFR*) gene families collectively occur with enough frequency to make them a viable target to the variety of FGF and FGFR inhibitors under investigation.⁶⁷ Variants in lysine methyl-transferase genes (eg, *KMT2C*, *KMT2D*, alias *MLL3* and *MLL2*, respectively) may help specifically target therapy with histone deacetylase inhibitors.⁶⁸ Sequencing-based assays targeted at these genes or indirect assays detecting aberrant methylation profiles can be envisioned in a companion diagnostic strategy for therapy.

Gene Expression Profiling and Ki-67

As molecular data grow more complex, so too do corresponding clinical diagnostic tests. Gene expression profiling does not meet the strict definition of an IVD companion diagnostic: the results are used to stratify patients for expected response to standard chemotherapeutic regimens but do not necessarily indicate a specific targeted therapy. Operationally, though, gene expression profiles serve a theranostic function, predicting patient response to conventional chemotherapy and, in particular, predicting which patients may be spared the toxicity of chemotherapy. For instance, the TAILORx trial recently confirmed the utility of Oncotype Dx (Genomic Health, Redwood City, CA) in identifying the subset of patients with ER-positive, axillary lymph node-negative invasive carcinoma that may be safely treated with adjuvant endocrine therapy alone.⁶⁹

Expression of Ki-67 protein (the product of the *MKI67* gene) is often used in the evaluation of breast carcinoma, but it is not appropriately considered a companion diagnostic. Rather than a targetable biomarker, the Ki-67 antigen is expressed in cells in some phase of the cell cycle. As such, it represents an easily visualizable read-out of a number of different cellular pathways converging on cellular proliferation. Each pathway may contain many components (cyclins, cyclin-dependent kinases, receptor kinases,

Figure 2 Molecular pathways assessed by companion diagnostics (CDx) in breast cancer are complex and interconnected. This diagram provides an overview of the molecular pathways driving breast cancer that are targeted by available therapies, therapies available in other cancer types, or therapeutics under clinical trials. Because immune-checkpoint inhibitors act on immune cells, they are not depicted in this diagram. The diagram is not comprehensive—not every pathway, protein, or potential interaction is included. **Asterisks** indicate markers assessed by immunohistochemistry (IHC); **dagger**, marker assessed by fluorescent *in situ* hybridization (FISH)/chromogenic *in situ* hybridization (CISH); **double dagger**, marker assessed by nucleic acid sequencing assay. **1:** Estrogen binds dimerized membrane-associated estrogen receptor (ER α), resulting in translocation of the complex to the nucleus, where it associates with DNA, transcription factors, and transcription machinery to drive expression of target genes, including those involved in cellular growth and proliferation. ER α also directly activates the cell cycle by antagonizing cell-cycle inhibitors, including p27 (CDKN1B), p15 (CDKN2B), and p16 (CDKN2A). Neoplastic cells expressing ER α and/or progesterone receptor (not shown) are identified by IHC, indicating antiendocrine therapy (eg, selective estrogen receptor modulators, aromatase inhibitors, etc.) may antagonize the neoplastic processes. **2:** Separately, growth factor ligand binds a receptor tyrosine kinase heterodimer consisting of Erb-B2 receptor tyrosine kinase 2 transmembrane protein (ERBB2, alias Her2 or Her2/Neu) and epidermal growth factor receptor, ERBB3, or ERBB4. Binding of ligand activates a signaling cascade involving other kinases, including RAS proteins, RAF proteins, mitogen-activated protein kinase kinases (MAP2Ks or MEKs), and mitogen-activated protein kinases (MAPKs, alias extracellular signal regulated kinases). These ultimately phosphorylate downstream targets, including nuclear ER α and other targets, further promoting transcription. Activation of the Her2 pathway also activates the phosphoinositide-3-kinase complex (PI3K), including the catalytic subunit phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA). PI3K activates a number of downstream pathways by phosphorylation of target proteins, including AKT isoforms 1, 2, and 3. AKTs in turn activate downstream targets, including the mechanistic target of rapamycin (mTOR) complexes 1 and 2, which activate many downstream processes, including ER α -mediated transcription. Neoplastic cells expressing Her2 may be therapeutically targeted in at the extracellular domain (by trastuzumab) or the intracellular domain (by lapatinib) and are identified by several different *in vitro* CDx assays making use of IHC, FISH, or CISH. Notably, different IHC antibodies may preferentially detect either the extracellular or intracellular portion of Her2. **3:** DNA damage is detected by the ataxia telangiectasia mutated (ATM) protein, which activates poly(ADP-ribose) polymerase 1 (PARP-1) and recruits the breast cancer associated (BRCA) complex (including BRCA1 and BRCA2, among others). PARP inhibitors (PARPi) such as rucaparib are approved treatment in the context of ovarian cancer, when a CDx assay (based on direct sequencing of BRCA genes or mutational signature) indicates mutation or dysfunction of the BRCA complex. ATM separately recruits p53, mediating a distinct DNA-repair mechanism and antagonism of the cell-cycle. p53 is normally inhibited by mouse double minute homolog 2 (MDM2), which is itself inhibited by an investigational class of drugs called nutlins (eg, idasanutlin). **4:** The cell cycle is driven by a complex periodic interaction of a number of proteins, including cyclins, cyclin dependent kinases (CDKs), CDK inhibitors (including p15, p16, and p27), checkpoint kinases (CHKs), and regulated by retinoblastoma 1 (Rb1). Palbociclib inhibits CDK4 and CDK6. The proliferative status of a tissue can be assayed using the IHC for expression of the marker of proliferation Ki-67 (MKI67). PTEN, phosphate and tensin homolog; TSC, tuberous sclerosis.

DNA-damage detection, repair proteins, etc.), each component may carry one or more genetic variants, and any or all may be involved in carcinogenesis. Evaluation of Ki-67 by IHC is believed to be an independent prognostic predictor, with greater proliferation corresponding to poorer outcomes. At very high and very low levels of expression, Ki-67 is effectively used to stratify patients into cohorts likely to benefit from standard chemotherapy and unlikely to benefit. For those patients with intermediate expression, however, reproducibility of clinical outcomes been inconsistent.^{70–74} Many of the relevant confounding issues from other IHC assays pertain, including ischemic time and fixation time, but the Ki-67 antigen also demonstrates additional biological heterogeneity, with higher proliferation rates often being observed at the edge of the tumor and frequent hotspots.⁷⁰ Some data suggest more consistency may be achieved by computer-assisted image analysis.⁷⁵ Assessment of Ki-67 provides another example of a pathway-centered approach to diagnostics: a comparatively simple assay evaluating a pathway or global process, rather than a specific targetable protein or genomic alteration.

Immune-Checkpoint Inhibitors

Immune-checkpoint inhibitors (ICIs) have been successfully deployed to treat a number of cancers,^{76–80} and they have shown promise in the treatment of breast cancer.^{81–83} Unlike other targeted therapies discussed above (*ER*, *PR*, and *Her-2* and *Sequencing, Clinical Genomics, and Future Companion Diagnostics*), ICIs do not act to antagonize specific molecular alterations driving cancer (as trastuzumab inhibits overexpressed *Her-2*); rather, ICIs relieve inhibition of the immune system by blocking the T-cell surface molecule, programmed cell death 1 (PD-1, alias CD279); programmed cell death ligand 1 (PDL-1, alias CD274), expressed on the surface of tumor cells; or another T-cell surface molecule, cytotoxic T-lymphocyte associated protein 4 (CTL-4, alias CD152). Relevant clinical biomarkers for ICIs differ from many cancer biomarkers, because they identify properties of the tumor and the tumor environment not directly related to cancer drivers, including expression of the target molecules or their ligands, the presence of tumor infiltrating lymphocytes (TILs), and the antigenicity of the tumor [neoantigen expression or tumor mutational burden (TMB)]. Attempts to implement and integrate these biomarkers clinically illustrate advantages and challenges for the companion diagnostic model.

Only one IHC test (PD-L1 IHC 22C3; pharmDx; Dako, Glostrup Municipality, Denmark) is currently approved by the FDA as an IVD companion diagnostic for an ICI (pembrolizumab) for use in non–small cell lung cancer (<https://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm301431.htm>, last accessed April 4, 2017). At least three other IHC assays [PD-L1 IHC 28-8 pharmDx (Dako); PD-L1 (SP142) (Ventana Medical Systems,

Inc., Oro Valley, AZ); and PD-L1 (SP263) (Ventana)] are FDA approved as complementary diagnostics for nivolumab, atezolizumab, and durvalumab, respectively. The complementary designation remains to be formally defined, but it currently confers a recommendation (rather than a requirement) for use with an associated therapy.⁸⁴ Several other ICIs are approved or in development, each with a potentially different IHC platform or antibody clone associated with the target. The challenges of standardizing the technical and interpretive guidelines for IHC for *ER*, *PR*, and *Her-2* are thus compounded for ICIs, with different drugs associated with different platforms, each with a different scoring algorithm.^{85,86} Moreover, PD-L1 can show significant expression in infiltrating immune cells, potentially confounding the interpretation of a positive IHC signal.⁸⁶ Further complicating the use of these companion diagnostics is the indirect nature of ICIs; it is quickly becoming clear that additional biological information about a given patient can increase the prognostic value of anti–PD-L1 IHC.

Because ICIs act by disinhibition of the immune system, the antigenicity of the neoplastic cells relates to the efficacy of therapy. The degree to which neoplastic cells acquire and present neoantigen peptides to TILs is indirectly assessed by quantification of TMB, with a high TMB correlating with improved outcomes when patients are treated with ICIs. Although in some cases, high TMB can be inferred from other patient data (eg, *BRCA* status), direct assessment of TMB makes use of MPS to estimate the number of mutations per megabase. TMB varies between tumor types and between patients, and it does appear to correlate with patient response to ICIs.^{87–93} Many questions remain regarding the standardization and guidelines for the detection and reporting of TMB, but at least one prominent MPS reference laboratory is reporting estimates of TMB (Foundation Medicine, Cambridge MA). Irrespective of the TMB (or even the expression of the appropriate ICI target), if T cells are not present in the tumor tissue, treatment with ICIs may be less effective.^{79,87} The presence of TILs has itself been demonstrated to be an independent prognostic factor in breast cancer, particularly in tumors that are *ER/PR/Her-2* negative,^{94–98} but TILs have yet to be specifically associated with response to ICIs in breast cancer. In non–small cell lung cancer, the nature of TIL subpopulations (determined by cell-surface marker expression) carry prognostic associations as well.⁹⁹ As more data accumulate, the assessment of both TMB and TILs may need to be integrated into treatment algorithms for ICIs.

Targeted genetic sequencing will also likely play a role in monitoring and possibly selecting ICI therapy. As discussed above in the context of *ESR1* and *ERBB2* resistance variants, most cancer treatments will place cancer cells under pressure to evolve, generating escape mutations over time. Loss-of-function variants leading to ICI escape have been reported in Janus kinase genes (*JAK-1* and *JAK-2*), β -2 microglobulin, and *PTEN* in melanoma and colon cancer.^{100,101} More such variants are likely to be identified as the indications for ICI therapy expand to include other

diagnoses, and longer-term outcomes can be assessed. It would not be inappropriate to recommend sequencing these genes in the context of ICI therapy, particularly when there is reason to suspect therapeutic resistance. Moreover, because ICIs make use of the patient's own immune system, it is quite likely that the patient's germline genomic context influences the effectiveness of treatment, although this is an area that has yet to be explored with much rigor.¹⁰²

ICI therapy is a promising new tool in the armamentarium against breast cancer. As a companion diagnostic, IHC for PD-L1 maintains some of the advantages associated with other IVD companion diagnostics, particularly that it reduces the complexity of the immune system to a comparatively straightforward clinical assay. The reductive nature of the test, though, also illustrates many of the challenges in using an IVD companion diagnostic to assay a complex system.

Challenges for Companion Diagnostics

The companion diagnostic model is attractive in its simplicity; the detection of a clearly defined molecular abnormality or abnormalities indicates the use of a specific therapy, and the test and the drug are approved and regulated by a single authority (the FDA). As new targets are identified and new therapies produced, the simplicity of the model starts to pose challenges for patients, the health care system, and manufacturers (<http://www.fda.gov/MedicalDevices/NewsEvents/WorkshopsConferences/ucm436716.htm>, last accessed April 4, 2017).^{81,103,104} When the spectrum of clinically relevant molecular or genomic changes characterizing a specific neoplasm is narrow and the number of potential therapies for any given target is low, a companion diagnostic model functions well, as with Her-2 amplification/overexpression acting as the principle predictive biomarker for treatment by trastuzumab in breast carcinoma. However, neither biology nor industry naturally operate according to strict one-to-one relationships. Multiple genetic variants might alter response to a single therapy (eg, *MDM2* amplification and *TP53* variants affecting *MDM2* antagonists), and multiple therapies might be indicated by a single biomarker (eg, estrogen ablation or antagonism based on ER expression). Our understanding of biology and our ability to intervene continually advance, altering the therapeutic landscape, sometimes drastically.

Indeed, as illustrated above and elsewhere in this issue, the generation of massive amounts of genomic, expression, and proteomic data regularly introduces novel biomarkers that inform and modify each other. If even a subset of these biomarkers result in targeted therapies, the number of individual assays necessary for a given patient will increase exponentially. All breast cancer patients are already assessed for ER, PR, and Her-2; depending on the clinical context, a significant proportion will have *BRCA1* and *BRCA2* testing as well (Figure 1). Increasingly, patients will

go on to have expression array or genomic sequencing-based testing, particularly those patients with advanced disease. New therapies and biomarkers will continue to accumulate, and algorithms for diagnosis and treatment will necessarily become lengthier and more complex. Any individual biomarker may also be confounded by driver or resistance variants (as with Her-2, among others) which may also need to be identified to guide therapy.

This is not a trivial issue for patients, the laboratory, or clinicians. Tissue requirements, particularly from small biopsy specimens, may limit the number of independent tests that can be reasonably performed. To support multiple tests, laboratories could adopt multiple testing platforms, requiring expertise, training, capital investment, and space. Others might opt to send out some or all of their testing. Either strategy potentially increases cost and time to diagnosis or treatment for patients. Maintaining quality control for each test can be challenging as well. As described for ER, PR, and Her-2, above, a significant challenge in breast cancer has been identification and standardization of variables affecting biomarker testing. Integration of the resultant diagnostic data, arriving from different sources at different times, poses an interpretive and informatics challenge for pathologists and clinicians. Compounding the problem is the possibility that different therapeutics might target the same biomarker, but do not necessarily use the same companion diagnostic. One has only to look at the landscape of ICIs to begin to see this type of burgeoning complexity.¹⁰⁵

For research, the introduction of multiplex, multiparametric, and high-throughput approaches has been an unequivocal boon, driving a rapid expansion in the ability to interrogate cancer genomes, transcriptomes, and proteomes to identify novel drivers and biomarkers of cancer (Figure 2). For clinical care, the increased understanding offered by the omics techniques may be something of a mixed blessing; greater insight into cancer biology brings the potential to develop an abundance of targeted therapeutics, but also a potential web of independent diagnostic tests. Despite this, as methods such as expression arrays, DNA-MPS, RNA-sequencing, and mass cytometry continue to evolve from research tools to a robust diagnostic techniques, they might provide the path forward in the clinical realm. For example, MPS-based diagnostic assays (when appropriately designed) are capable of detecting thousands of different variants from thousands of different genes, all on the same assay. Importantly, a single assay may simultaneously identify different types of variant: single-nucleotide variants, insertions, deletions, splice-variants, promoter alterations, copy number variants (including loss of heterozygosity), gene fusions, and other structural rearrangements are all identifiable in the same assay.¹⁰⁶ Moreover, such multiplex assays can also identify biological patterns in a patient specimen, including tumor heterogeneity,^{27,28} TMB,⁸⁹ and specific mutational signatures.^{46,55} If RNA-sequencing is included, either as an integrated assay or in parallel, expression data and improved detection of splice variants and novel structural variants may

be incorporated. Patient classification into clinically relevant subtypes may be accomplished on the same assays as identification of driver mutations, resistance mutations, therapeutic modifiers, and germline risk variants.^{28,107} Although much more limited in current clinical adoption, proteomic approaches can also detect differential expression profiles that can elude genomic techniques,¹⁰⁸ including post-translational modifications¹⁰⁹ and extracellular matrix proteins.¹¹⁰ Mass spectrometry can be coupled with immunohistochemical methods to permit *in situ* multiplexed identification at the cellular and even subcellular level.¹¹¹ Whether the substrate is nucleic acid or protein, these multiplexed, multiparametric techniques maximize the identification of clinically relevant information (including many biomarkers currently assessed by IVD companion diagnostics) with the potential for reducing overall tissue requirements, standardizing quality control, and consolidating interpretation and reporting. These are all developing techniques, though, with challenges of their own because of their high technical and interpretive complexity. Importantly, they are techniques rather than specific assays and do not currently fit cleanly into the IVD companion diagnostic model.

Companion diagnostics provide unequivocal therapeutic and prognostic benefit to patients with breast cancer diagnoses. In the near-term, the generation of massive data sets from genomic studies will continue to identify new biological targets, generate new therapies, and yield novel uses for existing therapies. The more successful the strategy becomes, the more the model must adapt to accommodate patients who may harbor multiple actionable targets, requiring multiple therapies and multiple diagnostic modalities. The FDA has already intimated that another class of tests, termed complementary diagnostics, might provide an alternative in the near-future, but details and guidance in this area are limited thus far.⁸⁴ Massively parallel assays, capable of identifying large numbers of biomarkers of diverse types from a single specimen, have the potential to streamline testing algorithms that might otherwise become intractably complicated. To accommodate future needs of oncology patients, the IVD companion diagnostic model will need to adapt and evolve.

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