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Global Perspectives on the Fight Against Breast Cancer

Dennis E. Chenoweth, PhD, MD Corporate Vice President, Business Development

On behalf of Dako, I would like to welcome all readers of this issue of Connection, and thank the many authors who have contributed to the success of this publication.

Dako is a global company whose mission is to lead in cell-based cancer diagnostics by providing integrated system solutions for our customers. Therefore the composition of this publication was specifically selected to reflect that global perspective, with a focus on providing information about contemporary approaches that are particularly relevant for the diagnosis and targeted therapy of breast cancer.

In our lead article, Dr. Craig Allred summarizes the literature related to measurement of both estrogen and progesterone receptors in breast cancer tissues. Drs. Bloom and Torre-Bueno provide an overview that demonstrates the power of image analysis as a tool that enables the pathologist to provide consistent scoring of semi-quantitative immunohistochemical assays such as HercepTest[®]. Dr. Philippa Saunders reviews our current understanding of the structure and function of estrogen receptor β (ER β), which is particularly important in order to assess potential responsiveness to therapy with tamoxifen and aromatase inhibitors. Yoichi Tani describes the Japanese experience with the use of pharmDxTM assays to enable targeted therapies in several different types of cancer. Dr. John Bartlett describes the basic principles, methodology and diagnostic applications employed in the emerging area of FISH diagnostics.

In addition to these articles which focus primarily on slide based assays that are predominantly utilized by anatomic pathologists, we have included an interesting review of the utility of serum based assays. In this article, Dr. Walt Carney summarizes his experience and the literature which is aimed at determining if serum HER2/*neu* levels exceeding 15 ng/mL might be useful for monitoring progression of metastatic breast cancer.

While these scientific contributions are the primary focus, I would like to point out some of the other informative articles found here. These demonstrate that Dako is uniquely capable of providing high quality diagnostic products that are employed by pathologists worldwide. This is particularly true in the area of breast cancer diagnostics, as Dako is the only global company that can deliver HercepTest[®], *HER2* FISH, serum HER2/*neu* ELISA, ER/PR pharmDx[™] and the ACIS[®]II image analysis system and HercepTest[®] image analysis algorithm.

We are committed to maintaining our leadership position in this area. In the coming year we will introduce a new series of research antibodies specifically directed toward the phosphorylated epitopes of several different signal transduction proteins, as well as an important new breast cancer antibody for detection of PTEN.

I hope you find these articles and related product information useful. In keeping with our philosophy of viewing our customers as valued partners in the fight against cancer, this publication is intended to be informative and provide important tools that can be used by pathologists around the world.

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Assessment of Prognostic and Predictive Factors in Breast Cancer by Immunohistochemistry

D. Craig Allred, MD Breast Center, Baylor College of Medicine, Houston Texas

Most decisions for treatment of breast cancer are made on the basis of prognostic factors such as tumor, nodal, and metastasis staging variables. In addition to these traditional variables, predictive factors such as status of hormone receptors are playing increasingly important roles. Pathologists today are evaluating progesterone- and estrogen- receptors primarily by immunohistochemical methods which have largely replaced the earlier biochemical ligand-binding tests.

Although many studies suggest that these immunohistochemical tests are at least as good in predicting patient outcomes as the older biochemical assays, there remain important methodological issues to resolve before immunohistochemistry achieves the clinical validation necessary to justify its routine use.

Most important among these unresolved issues are standardization of test methodology and how to interpretation of results.¹ Some laboratories have gone to considerable efforts to validate their methods of testing and evaluating results, whereas other laboratories have not adequately addressed these issues and might not even be aware of this necessity. Unless laboratories are prepared to validate their own tests or use validated procedures developed by others, they run the risk of reporting meaningless and potentially harmful results.



Estrogen receptor staining, 20x (PS4) + (IS2) = TS6

Estrogen Receptor

For the past few years our laboratory has assessed estrogen-receptor status by immunohistochemistry on formalin-fixed, paraffin-embedded tissues. The staining signal was scored by estimating the proportion of positive tumor cells and their average staining intensity. The intra- and inter-observer reproducibility of this method was more than 90 percent in our laboratory. Figure 1 depicts the method of scoring both proportion and intensity and combining these values into a comprehensive total score (TS) that weighs both factors.

Tumors with total scores (TS) of three or more were reported as "positive" on the basis of a cutpoint analysis of disease-free survival (DFS) in a study involving more than 1,900 patients.² This value separated patients into low- and high-risk subsets with approximately 15 percent difference in disease-free survival at five years. In the subset of almost 820 patients receiving no adjuvant therapy, the difference in DFS at five years was only approximately 10 percent indicating that estrogen-receptor was a weak prognostic indicator. However, as in the subset of nearly 800 patients receiving endocrine therapy (almost always tamoxifen), positive estrogen-receptor



Progesterone receptor staining, 20x (PS3) + (IS1) = TS4

status was associated with a significant improvement in DFS (about 30 percent at five years), emphasizing the strong predictive power of assessing estrogenreceptor by this method.

Progesterone Receptor

The validation of progesterone-receptor by immunohistochemistry is still evolving.3,4 In our laboratory we have performed a large clinical study involving more than 1,400 breast cancer patients. In this study immunostaining for progesterone-receptor was scored by the same method as illustrated for estrogenreceptor in Figure 1. Tumors with TS of three or more were defined as positive on the basis of a cutpoint analysis of DFS that optimally separated patients into low and high risk subsets. In the subset of 713 patients not receiving adjuvant therapy, the difference in DFS at five years was approximately five percent, suggesting a weak prognostic value. However, in the subset of 479 patients receiving adjuvant endocrine therapy (nearly always tamoxifen), positive progesteronereceptor status was associated with a relatively large improvement in DFS (about 20 percent at five years), emphasizing the strong predictive power of assessing progesterone-receptor by this method.

Allred Scoring Guideline for ER/PR pharmDx



Figure 1. Scoring Guidelines for Immunohistochemical Staining of Estrogen-receptor

Conclusions

There is still no consensus today concerning methodology for assessing progesterone- and estrogen-receptor status by immunohistochemistry. Clinical laboratories offering these tests using their own in-house methods should perform rigorous validation studies, or should follow procedures from other laboratories that have performed such studies. For laboratories where such validation studies are impractical, an alternative that is now available is to use one of the FDA-cleared immunohistochemistry tests that have been clinically validated by calibration to patient outcomes. Almost certainly a new class of immunohistochemistry tests, some of which are still in development, will be evaluated and interpreted using multivariate analysis to identify a meaningful prognostic index. That index will be more powerful than the individual factors in identifying patients at risk for disease recurrence.

Correspondence should be directed to: D. Craig Allred, MD Breast Center, Baylor College of Medicine dcallred@breastcenter.tmc.edu **Related Products**

K1903	ER/PR pharmDx™ for manual use	
K1904	ER/PR pharmDx™ for use on Dako Autostainer / Autostainer Plus	
ER/PR pharmDx [™] kits were developed and validated for use with the following companion reagents from Dako.		
Materials requ	ired, but not supplied include:	
S3006	Wash Buffer	
S2003	Dual Endogenous Enzyme Block	
S1699/S1700	Target Retrieval Solution	
	Calibrated pressure cooker with the capability of reaching and maintaining a temperature of 125° C for 5 minutes	

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So you want to go FISHing?

John Bartlett PhD Endocrine Cancer Group, Glasgow University Glasgow, Scotland

FISH, or fluorescence in situ hybridisation, is a widely-established technique which is nonetheless thought of as being challenging not only because it is a novel but also a molecular biology methodology. Yet, despite the undeniable differences between FISH and other molecular diagnostic techniques such as immunohistochemistry, there is a rapidly increasing demand for implementation and application of this technique to a growing range of clinical situations.

Perhaps the greatest challenge is that FISH is no longer in the range of methodologies used within cytogenetic and haematology laboratories, but is being applied increasingly in diagnosis in clinical pathology. In this area the most widely publicised area of FISH application has undoubtedly been in the diagnosis of HER2 status in breast cancers (Figure 1).

We would do well to remember, however, that this technique is neither limited to a single tumour type, nor confined to a single oncogene. The diagnosis of Bcr-Abl translocations in both leukemias and gastrointestinal stromal tumours (GIST) are currently also reliant on FISH diagnostics. Furthermore, there are an increasing series of FISH diagnostic panels and applications which have potential applications in bladder, prostate, lung, breast, ovarian, colorectal and gastric cancers and which could see the requirement for FISH rise exponentially over the next few years. Given that, there has been this increasing demand for information and understanding of this novel technique, I have attempted to overview the basic principles and stages of the FISH process, to provide a background to this important diagnostic technique.

In situ hybridisation was first described more than 30 years ago.¹ Subsequent progress has been dramatic, with the introduction of interphase cytogenetics in the early 1980s and non-isotopic methods in the late 1980s and early 1990s.² Fluorescence in situ hybridisation methods are relatively recent developments which have nonetheless illustrated the broad diagnostic potential of in situ techniques. The particular advantage of multicolour (2-4+) FISH, is that gene amplification (HER2 in breast cancer), gene rearrangements (BCR-Abl in leukaemias), microdeletions, chromosomal duplication and viral infections (HPV) may now be rapidly diagnosed.3-6 With the advent of multiprobe panels, exploiting 3-4 colour FISH approaches, more complex interactions between genetic alterations may be identified.

How does FISH work?

There are many conceptual, if not directly methodological, comparisons between FISH techniques and the process of immunohistochemistry, which is more widely understood. In FISH, a synthetic DNA molecule, or probe, specifically targeted against the target gene is tagged and then bound to the DNA in the tissue section and detected either directly, or following amplification of signal by secondary antibodies. The various steps in the process are readily understood by those with experience in immunohistochemistry, particularly as applied to fixed pathological specimens. Various steps are required both in the preparation of the probe and detection reagents and in the preparation of the tissue, to ensure a successful assay is performed.

Figure 1. Examples of HER2 FISH pharmDx™



The ratio of HER2 to CEN-17 is 1/1 = 1; non-amplified.



The ratio of HER2 to CEN-17 is 3/3 = 1; non-amplified.



The ratio of HER2 to CEN-17 is 3/2 = 1.5; non-amplified.



The ratio of HER2 to CEN-17 is 5/1 = 5; amplified.



The ratio of HER2 to CEN-17 is 12/3 = 4; amplified.

Methodology

Probe Selection The first requirement for FISH is a DNA probe with homology to the target gene or region of interest. Many such probes are now commercially available, greatly facilitating the application of diagnostic assays (for example, Dako *HER2* FISH pharmDxTM and *TOP2A* FISH pharmDx^{TM*} assay systems).

Probes are routinely mixed with human DNA (usually supplied with commercial probes), which suppresses cross hybridisation of probes to the large quantities of repeat DNA sequences present in normal and abnormal cells (this step can be thought of as blocking background staining).⁷

Repetitive sequence probes are of great value in FISH, as they target telomeric and centromeric repetitive DNA sequences.⁸⁻¹⁰ Sequence variations between chromosomes allow specific centromeric markers to be provided for the majority of human chromosomes.

Sample Preparation FISH is applied to isolated cells, frozen tissue sections or tissues preserved by fixation. For unfixed cells and tissues, the challenge is to sufficiently pretreat samples so that the FISH protocol, which involves high temperature and high salt washes, does not damage the sample to be evaluated. Typically this involves the use of charged slides and a brief fixation (30–60 seconds) with acid ethanol, or in exceptional circumstances, formalin buffers.

For applications involving neutral buffered, formalin fixed tissue biopsies, the technical challenge is far greater. Such samples are formalin fixed, dehydrated and saturated in paraffin wax to provide a solid support. This process causes protein denaturation due to the effects of formalin (an oxidising agent which causes protein-protein and protein-nucleic acid cross links), heat and dehydration. These steps markedly alter intracellular proteins and nucleic acid, producing a hydrophobic and crosslinked network which experience shows provides a significant barrier to the entry of macromolecules (eg, DNA probes) into the cell. The nucleic acids are masked by denatured proteins and other fixation effects. For FISH to be successful, steps must be taken to permeabilise and rehydrate tissue to allow penetration of DNA through the tissue into the cell nuclei. Such processes should be achieved with minimal tissue damage, to enable tissue morphology to be correlated with genetic aberrations.¹¹⁻¹²

Rehydration is common to most staining protocols and uses treatments to remove wax by passage through paraffin graded alcohols. Thereafter, a number of different steps may be used to increase permeabilisation of the tissue section and allow probe access. When taken to extremes. each of these will cause significant tissue damage and loss of morphology, causing the technique to fail. Conversely, undertreatment will reduce to undetectable levels the amount of probe which reaches and binds to the target nucleic acid. Each step must be critically evaluated for the benefit it provides toward reaching a successful experimental or diagnostic outcome. Steps used for tissue permeabilisation include treatment with heat, acid, detergents and reducing agents.

Acid Permeabilisation Incubation of tissue sections in acid is performed to reverse the effects of formalin fixation. Usually, incubation in 0.2N HCl is used to deproteinise tissues.^{1,13}

Reducing Agents The use of reducing agents such as sodium metabisulphite³ and sodium thiocyanate⁴ is thought to break down protein disulphide cross-links catalyzed formalin fixation,¹⁴ resulting in increased sensitivity to protease digestion and enhanced nuclear permeability. These steps, often combined with or replaced by



Hybridizer A hands-free, co-denaturation and hybridization instrument for reproducible FISH and CISH.

heat treatment (as in the Dako pharmDx[™] protocol), seek to ensure that subsequent protease digestion is optimally effective, and so reduce the exposure of tissues to proteolytic enzymes and minimize the degradation of tissue morphological structure caused by such digestion.

Each step must be critically evaluated for the benefit it provides toward reaching a successful experimental or diagnostic outcome.

Protease Digestion This step of the pretreatment process is the one which in our experience is most effective at optimizing and improving the outcome of FISH assays. As with every stage of the process, the aim is to balance tissue digestion and optimize probe access, while maintaining tissue morphology to allow correlation of results with histopathological features. The duration of protease digestion required varies according to the tissue, the batch of enzyme being used, and to a far lesser extent the degree of fixation.^{3,4,21,22}

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Breast carcinoma (FFPE) stained with HER2 FISH pharmDxTM Kit code K5331. Tumor cells show HER2 gene amplification (HER2/CEN-17 ratio \geq 2).

In all current molecular diagnostic FISH protocols, there is a tendency to underestimate the duration of protease digestion required for optimal assay performance, due to an over rigid adherence to an inadequate digestion period recommended by the probe manufacturer. This is, in our experience, the most common reason for the failure of a laboratory to establish FISH methods. We would strongly recommend digestion times be evaluated in each laboratory to optimize results.12 The importance of ensuring adequate digestion cannot be overstated, and we routinely examine sections (see 4,12,15,16), to ensure that adequate digestion has occurred prior to probe hybridization. This step is sensitive to both protease concentration and activity, and may require re-testing if protease supplies change, even from batch to batch. Although we have used both proteinase K and pepsin in the past,15,16 we currently recommend the use of pepsin for the majority of procedures.

Hybridisation/post hybridisation

Following optimisation of sample preparation for hybridisation, the components of FISH with the highest impact on specificity and quality are from highest to lowest: probe sequence and labelling, hybridisation temperature, post hybridisation washes and the buffer system used. As with antibodies in immunohistochemistry, the selection of appropriate FISH probes is the most critical component of ensuring specificity. Care must be taken to ensure that crossover with neighbouring genes is eliminated where it may compromise the assay result. Generally speaking, in diagnostic applications this quality control is performed by the kit manufacturer.

Unlike PCR, the hybridisation temperature in FISH assays is less critical, as with other in-situ hybridisation techniques. The theoretical optimal hybridisation temperature (Tm) can be calculated using a number of different approaches. However, in practice, hybridisation temperatures are designed to ensure maximum binding of the probe to DNA, and stringency is applied at the level of post hybridisation washes. Usually, tissue DNA is denatured for 5-10 minutes at 95°C, whilst blocking and probe DNA is heated for approximately 5 minutes at similar temperatures. Hybridisation between the probe DNA and the target DNA is performed, usually on a flatbed-heated stage, for anywhere between 12-72 hours.

Most problems encountered with interpretation of FISH relate to non-specific hybridisation of the probe and its subsequent detection. Unlike antibodies, the specificity of probe hybridisation to cellular DNA is governed by salt and formamide concentrations and by the temperature of post hybridisation washes.³ The objective of these washes is to remove non-specifically bound probe by destabilising DNA:DNA binding. The use of formamide enables high stringency washes to be performed at relatively low temperatures by raising the salt concentration in the aqueous phase of the buffer. Conversely systems such as the Dako pharmDx[™] kits, which avoid the use of formamide due to toxicity, use higher temperatures for these stringent washes. The optimum DNA: DNA hybridisation temperature can be defined by the following formula: T =81.5°C + 16.6 (log10[Na⁺]) + 0.41 (%G+C) - 820/I - 0.6 (%F) - 1.4 (% mismatch). In this formula [Na+] = molar salt concentration, I = probe length (bases), %F = % formamide, % mismatch = % non-complementary base pairs between hybridising strands.13-14 It is common to use a relatively low stringency during probe hybridisation and then to increase in a stepwise manner the degree of stringency in a series of post-hybridisation washes.

Interpretation of FISH results

Interpretation of FISH assays is dependent upon the critical endpoint of the assay in guestion, whether it be detection of amplification/deletion, translocations or aneusomy. In its simplest form (the detection of gene specific amplifications or deletions) the ratio of the gene to a chromosome marker can be used. In detection of translocations, the distance between two distinct signals is assessed, and interpretation is critically a matter of operator experience. It is the detection of chromosomal aneusomy, and in particular reduction to monosomy, which presents the major challenge in FISH interpretation. A simplistic approach in which the observer uses the chromosome number as a gate for abnormality is doomed to failure since there is extensive documentation of nuclear truncation in thin tissue sections. The normal chromosome copy number detected in thin sections by FISH is reduced both by this truncation artefact and by technical limitations of hybridisation methodology, which result in hybridisation efficiencies below 100%. In our extensive experience, the use of control sections of "normal tissues" is essential for the proper determination of chromosome copy numbers in tissue sections by FISH. Once the normal range is established, abnormalities may more readily be detected.4,14-17 Particular attention must be taken in situations where mixed cell populations are suspected

Accuracy, reproducibility and quality control in FISH

The fundamental question regarding the implementation of any novel technology within the clinical diagnostic portfolio must be, "does it work?" In the context of clinical medicine, this means: is it accurate, reproducible and amenable to stringent quality control procedures? There is growing evidence, at least within the area of HER2 testing, that FISH provides a viable, and in some cases superior, alternative conventional immunohistochemical to assays. Rather that revisit the entire debate surrounding HER2 testing here, we recommend the reader refer to the wide range of recent articles published on this subject (see eg, 4). However, it is

important to note that *HER2* FISH assays which do not incorporate a chromosome 17 probe, to allow assessment of the HER2/ Chromosome 17 ratio, are less accurate (see 4). This is related to the high rate of chromosome 17 aneusomy reported in sporadic breast cancers (see 4), and the current UK guidelines for HER2 testing by FISH recommend the inclusion of a chromosome 17 probe for this reason.³⁶

Accuracy With any novel diagnostic technique the priority must be its accuracy. In the area of HER2 diagnostics in breast cancer a cursory review of the literature will reveal an apparent deluge of articles illustrating different approaches to the assessment of the accuracy of HER2 FISH methodologies. However, closer scrutiny will reveal that, in fact, the vast majority of these publications compare concordance of FISH with other methods, rather than assessing accuracy.18-23 This illustrates a significant problem, since concordance between assay methods is in fact not a measure of accuracy.4 The accepted approach for evaluation of clinical diagnostic tests is to use an accepted external standard against which the performance of such tests can be evaluated. When such an approach is made, FISH is clearly shown to be a highly accurate method for determination of HER2 status.3,24

Reproducibility is also critically important, since an accurate test which cannot be reproduced in other laboratories cannot be widely applied. Whilst fixation is recognized as a major source of variability in immunohistochemistry,25 this is not in our experience a major problem for FISH. The effect of observer bias in IHC is also widely recognized²⁶⁻²⁸ and is related to the need for colour grading of signals. By contrast, even though currently all FISH-based assays are evaluated subjectively by human observers, analysis of gene and chromosome copy number by FISH requires simple counting of distinctly coloured signals, and for such analysis inter-observer errors are below 10 percent.3,29-31

Quality Control and Reference Laboratories

The data cited above suggests that there is a strong case to be made for the development of a robust external quality assurance scheme for diagnostic FISH assays. Different challenges will have to be addressed for different areas, but FISH is a technique well suited to a rigorous quality assurance approach. The inevitable expansion of FISH services in coming years further supports the need for such a system, and within the UK NEQAS (National External Quality Assurance Scheme), such a program is being established. Also within the UK, there has been to date a well organised HER2 reference laboratory scheme with the majority of FISH and IHC testing performed within three centres. An article by Dowsett et al³² illustrates the value of centralised testing in reference laboratories, pointing to the value of extensive quality assurance experience. The recommendation in Dowsett's paper that testing with such modern methodologies is likely to be most accurate when performed in laboratories with significant caseloads ("some hundreds of cases per year") is in agreement with both Paik and Roche's33-35 observations that larger laboratories tended to perform better, in their experience. Extrapolating this recommendation to routine clinical practice would result in the establishment of a network of experienced laboratories within the current clinical framework, similar to that seen in the field of clinical genetics.

Current practice and future challenges

FISH is increasingly accepted as a valuable diagnostic tool in routine diagnostic laboratories. However, its current application Different challenges will have to be addressed for different areas, but FISH is a technique well suited to a rigorous quality assurance approach.

remains limited by the small number of centres where such expertise is available. This represents a significant challenge since it is highly likely that the use of diagnostic FISH methods will expand dramatically in coming years, particularly within the field of oncology. Increased training and implementation of strict quality assurance are prerequisites if this challenge is to be met and FISH is to become more widely established as a clinical diagnostic methodology.

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K5331	HER2 FISH pharmDx™ Kit	
K5204	HercepTest® for manual use	
K5207	HercepTest® for use on Dako Autostainer / Autostainer Plus	
S2450	Hybridizer, 110V	
S2451	Hybridizer, 220V	
Recomended Counterstain		
S3301	Automation Hematoxylin for use on Dako Autostainer / Autostainer Plus	
S3302	Hematoxylin for manual use	

*Dako TOP2A FISH pharmDx[™] not available in the United States.

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Dako has introduced EnVision[™] G/2 Doublestain System, code K5361, a higher sensitivity, second generation visualization kit. The Rabbit/Mouse (DAB+/Permanent Red) system is high-sensitivity peroxidase and alkaline-phosphatase-based. It is intended for use in immunohistochemistry for the simultaneous detection of two different antigens within the same specimen in formalin-fixed, paraffin-embedded tissues or fixed cell smears.



Thyroid adenoma stained with Anti-Ki-67 Antigen, clone MIB-1, Code M7240, using HRP/DAB+ (brown color), and Anti-S100, Code Z0311, using AP/Fast Red (red color).



Thyroid adenoma stained with Anti-Ki-67 Antigen, clone MIB-1, Code M7240, using HRP/DAB+ (brown color), and Anti-S100, Code Z0311, using AP/Fast Red (red color).

PTEN

Dako has also unveiled PTEN monoclonal mouse anti-human antibody, code M3627, which identifies PTEN (phosphatase and tensin homolog deleted on chromosome ten), a tumor suppressor gene mutated in a range of cancers. It is a useful marker for clinical studies involving targeted therapies. Loss-of-function mutations in the PTEN gene lead to constitutive activation of multiple signaling pathways, including the P13K/Akt pathway, which affects cell proliferation, apoptosis and migration.



Low-PTEN-expressing breast carcinoma (FFPE) stained with Anti-PTEN, Code M3627.



High-PTEN-expressing breast carcinoma (FFPE) positively stained with Anti-PTEN, Code M3627.

Mammaglobin

New Dako antibody Monoclonal Mouse Anti-Human Mammaglobin, code M3625, for routine diagnostic use, identifies mammaglobin expression, which has been demonstrated in 80 percent of breast cancers and in normal breast epithelial cells, but its expression is limited in non-breast tissues. It is a useful tool in characterizing metastatic carcinomas of unknown primary origin.



Metastatic breast carcinoma (FFPE) positively stained with Anti-Mammaglobin, Code M3625.

Estrogen Receptor Beta: The 'New' Estrogen Receptor Implicated in Breast Cancer

Philippa Saunders, PhD MRC Human Reproductive Sciences Unit Edinburgh, Scotland

In women, estrogens have a major impact on the activity of cells within the breast, the uterus, the ovary, the brain, the bones and the immune system. In pre-menopausal women the majority of the estrogen within the body is made within the ovaries where aromatase, the enzyme required for estrogen biosynthesis, is expressed in the granulosa cells within the follicles, as well as in the corpora lutea formed after ovulation.1 After menopause, estrogen continues to be formed at locally high levels in extra gonadal sites including the breast.² Post-menopause, there is concern about the use of hormone replacement therapy (HRT), because four randomised trials that analysed the effects of HRT on the health of more than 20,000 women concluded that the excess incidence of breast cancer in 50-59 year olds using HRT was 3.3 per 1000 users.3

Estrogen receptors: structure and function

Our quest to understand how estrogens alter cell function has focused upon the expression and functional activity of the estrogen receptors (ER), which belong to a large superfamily of proteins that act as ligand-activated transcription factors.4 The first estrogen receptor was cloned from the MCF-7 breast cancer cell line in 1986⁵ its identification and the description of the different domains it contained represented a huge leap forward in our understanding. In 1996, much to many people's surprise, a second ER was cloned, initially from the rat⁶ although the cloning of the human homologue was reported in the same year.7 The first ER has now been re-named as $ER\alpha$ (ESR1) and the more recently cloned receptor is commonly known as ER β (ESR2). These proteins are the products of two genes⁸ and it is believed that they arose from a single 'ancestral' estrogen gene following a duplication event at least 450 million years ago.9

Signalling via estrogen receptors can occur in a number of different ways (reviewed in (4, 10). Classically, following binding to estrogen the ER undergoes a conformational change, dimerises with a second ER and then interacts with regulatory regions (binding sites) on DNA. Homo-dimers (ERa-ERa or $ER\beta$ - $ER\beta$), or hetero-dimers ($ER\alpha$ - $ER\beta$), can be formed depending upon which proteins are expressed in the cell.^{11,12} ER α and ER^β both bind oestradiol (E2) with high affinity. But importantly, although significant similarities do exist between the steroidbinding domains of the receptors, they exhibit different affinities for ligands such as raloxifene and tamoxifen that are used to treat breast cancer patients.^{13–15} Interestingly, studies in vitro have demonstrated that tamoxifen acts as a partial agonist on $ER\alpha$, but has a pure antagonist effect on ERβ.14

One role played by ER β may be to modulate the transcriptional activity of ER α .¹⁶ This idea has been tested using breast cancer cells, and it has been suggested that ER β may be an important regulator of proliferation and invasion.¹⁷ Direct phosphorylation of the receptors by growth factor driven pathways can also occur in breast tissue and may play an important role in steroid-ligand independent activation of tumour cells.¹⁸



Figure 1. Immunolocalization of ER β in breast cancer. Sections were stained with the monoclonal PPG5/10 antibody directed against the C-terminus of ER β . Examples of the pattern of staining in two separate cancers are shown. Note that immunopositive staining is localised in the cell nuclei and that there is variation in the intensity observed in different cells within the malignant regions.

Estrogen receptors and breast cancer

Estrogen receptor status is an important predictor of the likelihood that breast tumours will respond to hormonal therapy, such as treatment with the anti-estrogen tamoxifen.19,20 Tamoxifen is defined as a selective estrogen receptor modulator (SERM) because it acts as an estrogen antagonist in breast whereas it has been shown to have agonist activity in the uterus and in bone.^{21,22} Expression of aromatase has been detected in adipose tissue of normal breast, and reported to be elevated in breast cancer tissues.23,24 Aromatase inhibitors have been shown to reduce the amount of estrogen made by breast tissue, and have been reported to have improved efficacy compared with tamoxifen when used in patients with advanced breast cancer.25,26

By comparing the two ERs, it has been possible to identify those regions that have least sequence homology to each other. These are found in the N terminal and the hinge domains and at the extreme C-terminal end of the proteins. Antibodies have been generated to all of these regions.^{27–29}

On Western blots, monoclonal antibodies directed against the N terminus of ER β detect a number of different sizes of protein

that may correspond to both full-length and truncated isoforms.³⁰ In contrast using a monoclonal raised against a peptide at the C-terminus (clone PPG5/10) only fulllength proteins corresponding to use of different start sites were detected in tissue extracts.³¹

Immunoexpression of ER β in breast cancer tissue has been reported by a number of different groups, and the results obtained in some of these are summarised in Table 1. In most papers ERβ was detected in more than 50% of the samples tested, and the amount of ER β was not related to the amount of ERa. ERß positive/ERa negative cancers have been identified, therefore assessing tissues for ER α expression alone might miss some cases where anti-estrogen therapy could be effective.31-33 Notably an association between ERB expression and cell proliferation/expression of proliferation markers (eg, Ki-67) has been claimed.34 In a recent study an association between the degree of methylation of the ERß gene promoter and tamoxifen resistance was reported.35

Estrogen receptor beta variants

In addition to the full-length receptors a number of mRNAs encoding variant isoforms of both ER α and ER β have been identified

in breast cancers and breast cancer cell lines.^{30,36,37} Another human ER β variant, known as ER β cx (ER β 2), is identical to wild-type except at the C-terminus³⁸ and expression of this protein has also been demonstrated to occur in the breast.^{39,40} It is important to remember that the proteins encoded by these variants do not bind estrogens, and the role they might play in malignant cells is still being debated. Some authors have suggested they could act as dominant negative regulators of the full-length receptors,³⁸ and may affect disease progression and resistance to tamoxifen.⁴⁰⁻⁴²

Summary

Two ERs have been identified (ER α and ER β). Expression of ER α is an important predictor of response to therapies such as administration of tamoxifen or aromatase inhibitors. Immunoexpression of ER β has been demonstrated in more than 50% of breast tumours in a number of independent studies. ER β positive/ER α negative tumours have been identified.

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Table 1. Summary of the immunoexpression of ERß protein in breast cancers

Authors	Antibody Specificity	Result (staining of cancer samples)	Reference
Jarvinen et al 2000	C-terminal peptide, polyclonal	$ER\beta$ + 60% primary cancers (n=92) $ER\beta$ +, high background staining	43
Skilris et al 2001	N terminal domain, monoclonal	ER β + 74% various pathologies (n=65)	44
Jensen et al 2001	N terminal domain, polyclonal	$\text{ER}\beta\text{+}$ 66% (n=20) $\text{ER}\beta\text{+}$ associated with proliferation	33
Omoto et al 2001	C terminus, polyclonal	ERβ+ 59% (n=88)	45
Saunders et al 2002	C-terminal peptide, mouse monoclonal	ER β + 94% primary cancers (n=51)	31
Omoto et al 2002	3 different antibodies	Normal breast ER β +, decreased ER β + with advanced disease (n=57)	41
Murphy et al 2002	2 different antibodies	$ER\beta+$ (all forms) higher in tamoxifen sensitive tumours	46
O'Neill et al 2004	C-terminal peptide, mouse monoclonal	85%, n=118, ER β +ER α - more proliferation	34
Myers et al 2004	C-terminal peptide, mouse monoclonal	ER β + 58%, n=150, tamoxifen treated. ER β + associated with disease-free interval	47
Esslimani-Sahla et al 2004	C terminal polyclonal	N=50, tamoxifen treated, low ER β associated with tamoxifen resistance	39

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Image Analysis: A Tool to Complement Pathologists

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The clinical use of targeted pharmacologic therapies has necessitated the development of assays that aid in the selection of patients to receive therapy and to predict their responsiveness. Thus far these tests are predominantly IHC based, and their performance and interpretation present new challenges for the pathologist. Unlike qualitative IHC assays, in which the pathologist simply determines the presence or absence of a specific antigen, most of these new pharmacoDiagnosticTM assays are quantitative (or semi-quantitative) and must accurately assess the amount of antigen present. When performing a qualitative IHC test such as a cytokeratin stain, it is not unreasonable for the pathologist to optimize the stain to his or her visual preference. This is usually accomplished by altering the antigen retrieval conditions, the antibody titer or the incubation time. When performing a quantitative IHC test however, these variables cannot be altered. Typically years are spent developing quantitative assays and establishing the most robust

reagents and conditions that yield the best predictive results. In addition, they define pre-analytic variables, such as the type, length and time of fixation, as well as the scoring system to evaluate the stain. Data from clinical trials has shown that when these assays are performed in highvolume experienced clinical laboratories, in accordance with the established guidelines, excellent interlaboratory concordance can be achieved.¹⁻³

Image analysis systems are a combination of hardware and software that serves to reproducibly obtain a digital representation of a microscopic slide and allow a variety of analytic algorithms to be executed, including very accurate measurements of staining intensity. While most companies have focused their attention on creating software algorithms or sophisticated hardware, the most important component of an image analysis system is, in fact, the microscopic slide to be digitized. The principle of garbagein, garbage-out cannot be overstated. There is no point in attempting to quantify an immunostain unless one is sure that the immunohistochemical procedure has been validated and is known to be linear, or at least follow a known curve, across the range of analysis.

Much work has yet to be done in establishing the best methods for image acquisition. Whatever image capture system is used, if its purpose is to collect a digital image for subsequent analysis, procedures must be put in place to ensure the image is captured reproducibly, meaning the captured image must remain identical independent of the time it is taken. It is well known that all analog imaging systems are subject to drift over time. CCD cameras, and in fact all light gathering devices except photon counters, are inherently analog devices. A digital camera is a camera with a built-in analogto- digital converter. The problem of drift is especially acute in color systems because color is, in effect, the ratio of the different channels, and small changes in the signal in





any channel can give rise to large changes in apparent color. Sources of variation that must be considered include variation in the light source, variation in the transmission of the light, variation in the camera, and variation in the analog-to-digital conversion process. Adding further complexity, some of these factors vary at different level of magnification. Calibration must be performed to adjust these variables.

All light sources can drift, and the rate of drift can be fast enough to require daily calibration. An image collection system must either stabilize the bulb, adjust the camera gain, or collect a reference image of a knownto-be-stable calibration object to correct for this. Since the color temperature of the light source might also drift, this correction needs to be done in each channel. Care must be taken to perform this calibration at an intensity that will not saturate any channel, since saturation of the channels can give rise to a false appearance of equality.

The systems for illuminating a glass slide generally do not provide truly uniform illumination. Unless corrected, this will cause algorithms to give different results in different parts of the field of view. A system must

** The most important component of an image analysis system is the microscopic slide to be digitized.

collect calibration images of known-to-bestable, dust-free, and blank fields to have enough data to correct the captured image to that of a flat field. Since the calibration will be different at each level of magnification, this data must be collected for each objective. Since dust profoundly affects calibration, algorithms must be able to detect and correct for dust on the calibration target. Drift in the motors which control the aperture of the illuminating system can also affect calibration.

If the images are to report in absolute units of distance or area, the true magnification of the system must be calibrated. This can be accomplished by collecting images of targets with features of known dimensions. Obviously this calibration must be performed at each magnification. The algorithms using



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this calibration information should check for variations in magnification in X and Y, which can be caused by the pitch of the camera pixels not being truly equal in the two directions. Since this information is a function of the geometry of the lenses and the mechanical parts of the microscope, it will not drift as fast as other factors, but the system should be at least capable of performing this calibration at the time of manufacture and at periodic service intervals.

If the system is going to collect images that are bigger than the field of view (ie, an entire microscopic slide) and piece them together to form a larger image, the actual displacement of the stage motors must be calibrated. This can be done with absolute encoders that have traceable calibration. by imaging a target with features of known geometry as it is moved under command of a calibration algorithm, or by other methods. In principle, if overlapping images are collected, this could be done from the sample images by cross correlation of the overlapping regions. Again, this is a function of the mechanical features of the microscope and should be calibrated during periodic service.

There are several types of possible drift or unevenness in any camera. These include pixel-to-pixel variations in sensitivity, time variant changes in gain, relative drift between channels, amplifier drift, and color registration errors. All cameras must be assumed to have pixel-to-pixel variation in

sensitivity. This will include "dead" pixels as well as pixels with altered sensitivity. This will drift over time, and so should be calibrated on a periodic basis. The same algorithms that calibrate for illumination variation can be designed to perform this calibration. All cameras also perform significant analog processing of the output of the CCD. Since this can drift, it must be calibrated on a frequent, if not daily, basis. The method of calibration must take into account the fact that cameras can have intensity-dependent errors. This means that even though the output of the three channels might be matched when seeing bright white, one or more channels might have differential response to mid-range signals. This will cause a neutral gray to appear to have a tinge of some color, and can profoundly affect algorithms that use color to recognize features. This should be calibrated on a daily basis using known-to-be-stable gray or color targets.

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All color cameras create their images by separating the light into wavelength bands. This may be done by sequentially rotating different filters in front of the camera, by splitting the beam and sending it through different filters to different CCDs (a threechip camera), or by having an array of pixels with different color filters on the same CCD (a Bayer filter camera).

In each case, the alignment of the color images will not be perfect. In a rotating filter camera, tiny differences in the mounting and movement of the filters will shift the image. In a three-chip camera, the three CCDs are mounted so that the three split sections of a single beam will hit the same pixel position on all three CCDs, but this mounting is never perfect. In a Bayer camera, any given pixel only sees one color channel and the values for the other channels are interpolated from adjacent pixels. In all these situations, a sharp-edged object can develop an apparent color fringe. It may not be possible to calibrate out this error, but it will set limits on the type of analysis which can be performed at different magnifications. The designers of an image collection system and the algorithms which will be performed on the images should be aware of the magnitude of this error. At a minimum, an image collection system should provide methods to measure this factor and provide this information to developers of quantification algorithms.

It should now be obvious that acquiring images for subsequent analysis is not a trivial task, and that image acquisition is just one of the steps in an overall image analysis system. Just as it is critically important that a quantitative assay be performed in a standardized manner, and the image be acquired with a validated microscope camera system, so too the interpretation of the resulting slide must be performed in a standardized manner.

This is easier said than done since many scoring systems assess surrogate features. Even when there is a direct relationship between the intensity of an immunostain and the amount of antigen present in the tissue, like in a HER2 immunostain, manual scoring systems generally do no rely on the assessment of intensity but rather the surrogate feature of circumferential membrane staining.

The reason for this is that subtle differences in staining intensity cannot be detected by the human eye, and pathologists have differing abilities to interpret staining intensity. Instead, the manual scoring systems focuses on features that the human eye can do well, such as rough percentages and stain localization. This works in the case of HER2 because as HER2 upregulates the protein becomes circumferentially expressed on the membrane. One of the most important steps in image analysis is determining which cells to analyze. This is similar to the "gating strategy" in flow cytometry. For example, to manually assess the proliferative index of a breast carcinoma with a Ki-67 immunostain, 500 tumor nuclei would be counted and the number of positive nuclei recorded.

In selecting only the tumor nuclei and disregarding stromal cells, endothelial cells and inflammatory cells, the pathologist serves as the gating mechanism. Many image analysis systems retain the pathologist as the gating system by implementing drawing tools that pathologists can use to outline cells of interest. While it is easy to outline cells, it is a time consuming process. More sophisticated gating strategies are being developed that will include some features similar to those present in flow cytometry analysis such as gating by size, shape, antigen localization and antigen coexpression.

Can an image analysis system really make the assessment of a quantitative immunostain more accurate and consistent between pathologists? We compared the level of agreement among 10 pathologists in the assessment of the same 130 HER2 immunostained slides. Each pathologist scored the slides with a standard light microscope as 0,1+,2+ or 3+ in accordance with the HercepTest® scoring guideline, and then later with the aid of an ACIS®II image analysis system. The results were compared to the gene amplification status as assessed by fluorescent in situ hybridization. While the pathologists varied in experience, the concordance with FISH ranged from as low as 42 percent to as high as 92 percent on routine assessment. The accuracy and consistency of all ten pathologists improved significantly, ranging from 90 to 95 percent concordance with FISH, and even the most experienced pathologist showed improvement with the aid of the ACIS[®]II image analysis system. When the ROC curves were plotted for each of the 10 pathologists, if the analysis was performed with a standard light microscope, the curves had significantly different shapes, arguing that the variability was not just due to



Generates comprehensive reports for pathologists use when discussing patient test results.

differences in applying intensity thresholds but was related to something more fundamental in how each pathologist scored the cases. The ROC curves generated for each of the pathologists when using the

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ACIS®II image analysis system appeared similar, suggesting that each pathologist was now performing in an identical fashion. Other studies, using a variety of different image analysis systems, have also shown improved and more consistent HER2 scoring.⁴

Image analysis represents a significant step in standardizing the interpretation of histologic slides. The creation of an image analysis system is not an easy task. Just as it takes considerable time and effort to create and validate a pharmacoDiagnostic[™] assay, so to does it take significant effort to create and validate a clinically useful image analysis system.

These systems are meant to complement pathologists not replace them. Accordingly they should not attempt to emulate what pathologists inherently do better, but rather to aid pathologists in tasks that the human eye does not do well.

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Development of pharmacoDiagnostic[™] Tests for Molecular Targeted Therapies

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Advances in understanding the bio-molecular basis of cancers has led to the development of targeted approaches to cancer prevention and treatment. A new understanding of signal transduction pathways that regulate cell growth, differentiation and apoptosis has translated these recent discoveries in cancer biology into novel approaches for targeted cancer therapies. This yields distinctive potential therapeutic targets, and aids in identifying patients most likely to benefit from new anti-cancer agents.¹⁻⁴ Potential therapeutic targets have been found within the growth factor signaling system, and some of their receptors and tyrosine kinases are considered the most advanced in clinical development, or are already available for use.5-7 In Japan these therapies herald a promising era for oncologists and their patients, particularly in the treatment of solid-tumor cancers traditionally refractory to conventional chemotherapy and with poor prognosis. Japanese patients with breast cancer, chronic myelogenous leukemia (CML), non-small cell lung cancer (NSCLC), or gastrointestinal stromal tumor (GIST) are benefiting from these new therapies.

Along with this, there is demand for companion tests, called pharmacoDiagnostics™ (pharmDx[™]). Identification of patients bearing a target or activated target molecule is a prerequisite for determining patient eligibility, in order for these therapies to be successful.8-11 Progressive immunohistochemistry (IHC) and in situ hybridization (ISH) techniques have been providing benefits to identify tumor biology factors such as HER1 (EGFR), HER2, and KIT.^{12,13,14} The application of IHC has proven to be the most useful tool in molecular diagnosis, as well as in target identification for targeted therapies. Although these IHC assays can be easily performed on routinely formalin-fixed, paraffin-embedded (FFPE) specimens, the reproducibility of IHC staining results depends upon tissue fixation, antigenretrieval methods, and sensitivity of detection systems as well as interpretive guidelines for assessment of staining. Therefore pharmDx™ for molecular targeted therapies should provide consistent results, be practical and reproducible, and be designed as a semiquantitative assay whenever supported by the data. 9,10,15

Therapeutic strategies to target molecules and development of the targeted therapies

Signal transduction research has shown the importance of members of the human epidermal growth factor receptor (HER) family of transmembrane tyrosine kinase in a number of solid tumors.^{16,17} HER2 is an important member of the HER family, and is frequently overexpressed or amplified in some tumor types.^{8,9,10} HER2 protein overexpression or HER2 gene amplification is found in up to 30 percent of breast cancers, leading to aggressive behavior and an unfavorable prognosis. However, the overexpressed HER2 receptor protein also serves as a target for anti-HER2 humanized antibody therapy.^{18,19}



Figure 1. Overview of strategies of molecular targeted therapies for growth factor receptors While humanized or chimeric monoclonal antibodies (mABs) bind to the extracellular domain of the targets, preventing activation or inducing antibody-dependent cell toxicity (ADCC), small-molecule tyrosine kinase inhibitors (TKIs) work by inhibiting the intracellular domain of the target molecules, thus blocking downstream signaling of PI3K/Akt and Ras/Raf/MAPK.

In Japan, patients with metastatic breast cancer have benefited from the first commercially humanized monoclonal antibody, Herceptin, which was approved by the Japanese Ministry of Health, Labor, and Welfare (MHLW) in May 2001. The likelihood of tumor regression with Herceptin® therapy may be as high as 35% among patients with tumors that strongly overexpress HER2.¹⁹ The addition of chemotherapy enhances response to the humanized antibody, and treatment of appropriately selected patients with Herceptin® prolongs overall survival.^{19,20}

Gleevec/Glivec, another targeted therapy, is among the first of a new class of targeted anti-cancer agents introduced as a smallmolecule tyrosine kinase inhibitor (TKI). Blocking growth of Bcr-Abl transformed leukemic cells, it has demonstrated a long term clinical benefit for most refractory patients with Philadelphia chromosomepositive chronic myelogenous leukemia (Ph+ CML).^{21,22,23} After MHLW approval for Ph+CML in November 2001, Gleevec/ Glivec was approved for treatment of patients with unresectable and/or metastatic gastrointestinal stromal tumors (GISTs) in July 2003. Gleevec/Glivec has shrunk tumors by at least half in most patients, and stopped tumor growth entirely in other patients.14 Targeted therapeutic strategies are classified into two categories: smallmolecule tyrosine kinase inhibitors (TKIs), and humanized, or chimeric, monoclonal antibodies (mABs).1,7,24 Orally active TKIs are a promising class of targeted agents in development. These work by inhibiting the intracellular portion of the target molecules, thus blocking downstream signaling. Infusion of humanized and chimeric mABs was the earlier approach, and worked by binding to

The application of IHC has proven to be the most useful tool in molecular diagnosis, as well as in target identification for targeted therapies.

the extracellular portion of the targets, thus preventing activation or inducing antibodydependent cell toxicity (ADCC) (Figure 1).

HER1 (EGFR) and HER2 are the most widely studied HER family members. In many types of cancers, HER1 (EGFR) or HER2 expression is abnormal or upregulated, indicative of a possible role in tumorigenesis.25,26 Evidence suggests that HER1 (EGFR) and HER2 overexpression correlate with tumor progression, survival potential, and response to therapy, indicating they may be potential targets for therapeutic intervention. Although the complexities of HER-driven cell signaling are not yet fully understood, several possible points for interruption of tumorigenic cellular mechanisms have been proposed.27,28,29 HER1 (EGFR) and HER2 targeting have formed the basis of extensive and growing drug development programs (Figure 2).

 \leftarrow

2005–2007 Phase I	2004–2006 Phase II	2003–2004 Phase III	Marketed
GW-572016 Dual HER1/HER2 Reversible TKI (GSK)	PKI-166 Dual HER1/HER2 Reversible TKI (Novartis)	Erlotinib HCI (Tarceva) HER1 Reversible TK (Genentech/Roche/OSI)	Trastuzumab (Herceptin) HER2 mAB (Genentech/Roche)
2C4 HER2 mAB (Genentech)	TgDCC-EIA HER2 mAB (Targeted genetics)	MDX-210 HER2 mAB (Medarex)	Gefitinib (Iressa) Reversible TKI (AstraZeneca)
	Canertinib (CI-1033) Pan HER1-4 TKI (Pfizer)		Cetuximab (Erbitux) HER1 mAB (ImClone/BMS)
	EKB-569 HER1/HER2 irreversible TKI (Wyeth)		
	EMD-72000 HER1 mAB (Merck KGaA)		
	ABX-EGF HER1 mAB (Abgenix/Amgen)		
	h-R3 HER1 mAB (York Medical)		



Figure 3. Summary of a basic IHC assay for pharmDx[™] test

A basic IHC based on the dextran-polymeric detection is divided into four steps, including antigen retrieval, endogenous peroxidase blocking, primary antibody reaction, and visualization with dextran-polymer reagents. An enzymatic or heat-induced antigen retrieval procedure will have to be carefully selected and optimized according to the primary antibody used for the test.

pharmDx[™] assays for clinical use

Development of pharmDx[™] tests for therapeutic monoclonal antibodies (mABs)

Herceptin[®] therapy has now become a standard of care for HER2-overexpressing metastatic breast cancer, and a pharmDx[™] test for HER2 protein overexpression or HER2 gene amplification is conducted to assess Herceptin[®] eligibility. The presence or absence of overexpressed HER2 protein or HER2 gene amplification is used to differentiate patients who may have a response to the humanized antibody from patients who do not. IHC-based assays and FISH assays are available for determining eligibility for Herceptin[®] therapy, although in Japan the FISH assay is not widely used, due to practical and economic reasons.^{8.10}

HercepTest®, was approved by MHLW as an in vitro diagnostic (IVD) kit at the same time as Herceptin®. HercepTest® is an immunohistochemical staining system for semi quantitatively evaluating the expression status of HER2 protein, and includes the primary antibody and other reagents optimized for the detection of HER2 in FFPE specimens. Using the HercepTest® scoring guideline, the HER2 membrane staining is scored as 0, 1+, 2+, or 3+, according to membrane-specific intensity and pattern continuity.13,15 This methodology and scoring criteria have also been extended to the EGFR pharmDx[™] test.¹² EGFR pharmDx[™] is already used in Europe and the United States as an aid in identifying patients eligible for ERBITUX[™] therapy. This chimeric monoclonal antibody against EGFR (HER1), has been used for treating irinotecan refractory, or intolerant metastatic, EGFR expressing colon cancer.¹² HercepTest[®] and EGFR pharmDx[™] may also be utilized for other therapeutic mABs as shown in Figure 2. However scoring criteria for either test may need modification dependent on use and on data output.³⁰

In order to develop a pharmDx[™] test for new mAB therapy, it is necessary to develop a specific primary antibody against the same molecular target site as identified by the humanized, or chimeric, monoclonal antibody. Simplicity of the pharmDx[™] procedure

is critical for consistent results. Using a simple, two-step detection process,³¹ basic IHC for therapeutic mABs is designed as presented in Figure 3. The dextran-polymer detection yields an intense signal of positive staining and eliminates endogenous biotin activity that may be caused by heat-induced target retrieval.³² Target retrieval increases specificity and sensitivity of pharmDx[™] tests and provides for a more robust assay. Additionally, while developing a pharmDx[™] test for a new mAB, results are compared with clinical response data in order to validate clinical relevance.^{10,12,13}

Development of pharmDx[™] test for tyrosine kinase inhibitors (TKIs)

HER1 (EGFR) TKIs have transforming capacity in most common types of solid tumors and across tumor stages.²⁹ Up to 80 percent of non small-cell lung cancers (NSCLC) express HER1 (EGFR). The efficacy of HER1 (EGFR) TKIs in preclinical research, together with their favorable toxicity profiles, has led to their clinical development in NSCLC.33,34 Tarceva and Iressa have shown promising activity in NSCLC as single agents in phase II trials.35,36 However, randomized phase III studies of Tarceva or Iressa in combination with chemotherapy failed to demonstrate an increase in efficacy of HER1 (EGFR) TKIs over chemotherapy.^{37,38} Patients in these trials were enrolled regardless of EGFR expression levels in their tumors.

Due to the complex nature of HER1 (EGFR) TK activation and the lack of a standardized assay for measuring HER1 (EGFR) TK levels, the identification of potential responders is not as straightforward as demonstrated by the results of preclinical data. Recent studies have associated somatic point mutations in the TK domain of EGFR in a subset of NSCLC with sensitivity of the tumors to Iressa.

This suggests that EGFR mutations may define a subset of tumors that are highly dependent on activated EGFR signaling and responsive to HER1 (EGFR) TKI therapy.^{39,40} However, another study of Iressa-responsive patients lacking these mutations indicates they are not necessary in order for patients to benefit.⁴¹ Further investigation is necessary to identify useful clinical or diagnostic predictors of responsiveness to EGFR-TKIs.



Figure 4. Comparison of conventional dextran-polymer method and CSA method on immunostaining with phosphorylated HER1 (EGFR) in NSCLC

Phosphorylated HER1 (EGFR) immunostaining was performed on a Dako Autostainer, using the dextran-polymer **A** and CSA methods **B** after the heat-induced target retrieval with Tris-EDTA solution. Phosphorylated EGFR were strongly visualized by the CSA method **B**, whereas only a few tiny signals were presented by the conventional method **A**.

Potential Future Assays

An immunohistochemical assay that works on archived tumor tissues will likely be in strong demand for phosphorylated EGFR identification. Such an assay should be sensitive enough to detect phosphorylatedtyrosine residue in EGFR. In archived FFPE tissues, fixation often destroys structure or masks antibodies' binding sites, reducing the antigenicity of small peptides or epitopes.42 It may be difficult, therefore, to detect epitopes that may be sensitive to formalin fixation by conventional IHC methods. Enzymatic or heat-induced target retrieval methods may restore antigenicity and have been used successfully to detect a wide variety of antigens in FFPE tissues.43 Although the underlying mechanism of target retrieval remains unclear, it is necessary to understand the factors that influence its effectiveness. Recent studies indicate that a combination of high temperature heating and calcium-chelating are involved in antigen unmasking.44,45 It is suggested that calcium complex formation with proteins may mask epitopes. Pretreating with calcium chelating solutions such as citrate buffer (pH 6.0) or Tris-EDTA solution (pH 9.0), extracts the calcium ions from the calcium-protein complex, unmasking the epitopes. Heat-induced target retrieval is considered an easy technique, and plays an important role in enhancing the intensity

of immunohistochemical signals, especially with phosphorylated EGFR.

A sensitive detection system also optimizes phosphorylated EGFR identification. A catalyzed signal amplification (CSA) method with labeled tyramide is used for solid-phase assays, and has been adapted for IHC and ISH assays.^{31,47} This CSA technique allows a 500- to 1,000-fold increase in sensitivity of IHC signals when compared with conventional methods (Figure 4A). An optimal target retrieval method prior to signal amplification maximizes availability of phosphorylated EGFR (Figure 4B).

The CSA method can be applied to the Dako Autostainer to perform the amplification technique with minimal effort in an accurate, consistent, and reproducible manner.³² An optimal combination of heat-induced target retrieval with EDTA-based solutions, CSA, and automation approaches will be needed to develop an ideal test for the phosphorylated, activated EGFR identification.

Several clinical studies with EGFR-TKIs have been initiated to measure expression and activation of EGFR and other downstream signaling molecules such as PI3K, Akt, and MAPK and their phosphorylation in tumor tissues.^{44,45} Their objective is to establish a profile of potential responders to TKIs. pharmDx[™] tests that are able to assess expression levels, and phosphorylation/ activation of various signaling molecules will be valuable in defining subsets of patients who may potentially respond to molecular targeted therapies.

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HER2/*neu* Status is an Important Biomarker in Guiding Personalized HER2/*neu* Therapy

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An important member of the oncogene family is the growth factor receptor known as the Human Epidermal Growth Factor Receptor-2 (HER2), which is also referred to as HER2/neu or c-erbB-2. The full-length glycoprotein has a molecular weight of 185,000 daltons (p185) and is composed of the internal tyrosine kinase domain, a short transmembrane portion and an extracellular domain (ECD). The extracellular portion of the receptor protein has a molecular weight in the 97-115 kDa range and has been shown to be circulating in the serum or plasma of normal individuals and to be elevated in the circulation of patients with breast cancer.1,2,3,4

Over the last several years the HER2/neu oncoprotein has emerged as an important cellular target for the development of a variety of new cancer therapies. The method used to define the HER2/neu status is a major factor in determining who will receive these targeted therapies. The HER2/neu status can be determined by either using tissue tests or an ELISA that measures the circulating levels of serum HER2/neu. Tissue test results indicate that approximately 20-30 percent of patients with primary breast cancer have a HER2/*neu* positive tumor whereas ELISA test results indicate that approximately 45 percent (range = 23-80 percent) of metastatic breast cancer (MBC) patients have HER2/*neu* positivity, as determined by an elevated serum HER2/*neu* test.

Published studies show that the HER2/neu status of a breast cancer patient can differ based on the test methods used and the time of HER2/neu assessment. In this report, it will be shown that not all HER2/neu test results obtained from the primary breast cancer are correct and that there is a population of patients designated as HER2/neu negative by tissue tests that, in fact, have HER2/neu positive tumors by serum testing. This observation has important therapeutic implications for breast cancer patients with HER2/neu positive tumors who are not currently eligible for anti-HER2/neu therapy. In the specific case of determining HER2/neu status, it will take multiple types of HER2/neu tests applied at different times to identify patients with HER2/ neu positive breast tumors.

Methods of establishing the HER2/*neu* status of a breast carcinoma

Currently, there are two tissue tests that are FDA approved to establish the HER2/*neu* status of a patient with breast cancer and one test cleared by the FDA for monitoring changing serum levels of HER2/*neu* in patients with MBC. All three tests can identify patients with HER2/*neu* positive tumors but only the two tissue tests are approved for selecting patients for anti-HER2/*neu* therapy. The tissue tests are immunohistochemistry (IHC) and Fluorescence In Situ Hybridization (FISH).⁵ Both tests are performed on formalinfixed paraffin embedded material obtained at the time of diagnosis of primary breast cancer and require evaluation by a pathologist.

The third test, a Serum HER2/*neu* ELISA (manufactured by Oncogene Science/Bayer HealthCare and distributed by Dako) is able to reproducibly measure levels of the circulating shed extracellular domain of the HER2/*neu* oncoprotein in the serum of metastatic breast cancer patients. The test for measuring serum HER2/*neu* has been cleared by the FDA for the management and monitoring of patients with MBC who have an initial serum HER2/*neu* value above the normal cut off of 15 ng/mL.⁴

There have been numerous publications that show a strong correlation between serial changes in serum HER2/neu levels and the clinical course of patient's metastatic breast cancer, regardless of their treatment regimen. The majority of these reports have shown that serum HER2/neu levels parallel the clinical course of disease, with increasing levels being associated with progressive disease and decreasing levels being associated with response to therapy. The overall correlation between clinical course of disease and serial changes in the circulating serum HER2/neu is approximately 86 percent.⁶ Some reports⁷ have indicated that serial changes in circulating HER2/neu \leftarrow

Serum HER2/neu Levels and Clinical Status - 93 weeks



This figure illustrates the changes in serum HER2/neu levels over a 93 week period, in a metastatic breast cancer (MBC) patient receiving Herceptin-based therapy. The changes in serum HER2/neu levels parallel the clinical course of disease. Resp = Response, NED = No Evidence of disease, Prog = Progression.



Optimize patient care using HercepTest[®] and Serum HER2/neu in Breast Cancer Management.

levels may even precede the actual clinical signs of progressive disease by several months, however, this observation needs additional confirmation.

It is also interesting to note that several recent publications have suggested that the degree of decrease in serum HER2/*neu* after Herceptin-based therapy may be strongly associated with a favorable patient outcome as measured by progression-free survival.⁸⁻¹⁰

The frequency of HER2/*neu* positive breast cancers

Numerous reports measuring serum HER2/ neu have suggested that the frequency of HER2/neu positive tumors could be greater than the 20-30 percent reported with tissue tests.⁵ To better understand the frequency of HER2/neu positive tumors in breast cancer patients, we conducted a comprehensive systematic review of publications that reported elevated levels of the serum HER2/neu in breast cancer patients. We identified a total of 60 published studies, representing 7,639 breast cancer patients. Upon review of 25 studies representing 2,623 patients with primary cancer, we found that approximately 18.1 percent (range of 0-38 percent) of patients had an elevated serum HER2/neu level. We also reviewed 50 studies representing 5,044 patients with MBC; in this analysis we found that the average percent of MBC patients with an elevated serum HER2/neu level was 45.6 percent (range 23-80 percent). In 20 of the 50 publications (40 percent), it was pointed out that 50 percent or more of the MBC patients had elevated serum HER2/neu levels (reviewed in 11 & 12).

This information is in sharp contrast to the many publications since the mid-1980's that report only 20-30 percent of patients with breast cancer overexpress the HER2/neu oncoprotein in the primary tumor tissue by IHC or FISH. Collectively, the publications citing measurement of serum HER2/neu showed that the frequency of HER2/neu positive tumors in at least metastatic breast cancer patients is greater than generally known and much greater than 20-30 percent reported using tissue tests. This fact has important therapeutic implications for breast cancer patients with HER2/neu positive tumors and demonstrates the importance of clearly establishing the correct HER2/neu status of the breast tumor.

Can some breast cancer patients who are designated HER2/*neu* negative, have HER2/*neu* positive tumors?

Given that the prevalence of HER2/neu positive tumors by ELISA testing is much greater than the 20-30 percent, an obvious question to ask is whether HER2/neu negative patients are really HER2/neu negative. The answer to such a question could have serious therapeutic implications for women with MBC. To investigate this question, we searched the literature and found numerous publications (reviewed in 11) that showed the existence of a population of women where the primary breast cancer was designated HER2/neu negative by tissue testing, but who showed elevated serum HER2/neu levels in MBC. This elevation of serum HER2/neu with metastatic disease is indicative of the presence of a HER2/neu positive tumor. For example, Andersen et al., showed that 28 of the 82 (34 percent) patients who had an IHC-negative HER2/neu test on the primary breast tumor did have elevated serum HER2/neu levels at the time of MBC diagnosis.13

Currently, these patients are not considered eligible for anti-HER2/*neu* therapy. It is this denial of anti-HER2/*neu* therapy from patients who may potentially derive clinical benefit that supports the importance of establishing the correct HER2/*neu* status at the time of treatment, rather than relying upon old data or test results. If a patient is found to have an elevated serum HER2/*neu* level, then either the original tumor should be re-tested or a metastatic lesion should be tested with IHC or FISH to determine if the patient is eligible for Herceptin therapy.

Given the numbers of patients with a negative HER2/neu tissue test but an elevated serum HER2/neu level, we next asked if such an observation could also be made by comparing primary breast tumor tissue with metastatic breast tumor tissue from the same patient using IHC and/or FISH. To date, we have found four recent publications, totaling approximately 330 patients, in which the HER2/neu status was compared in the primary breast cancer tissue versus that of the metastatic breast cancer tissue of the same patient. Edgerton et al. found a 20 percent discordance between the HER2/neu status of the primary cancer tissue and the metastatic cancer tissue from the same patient. The discordance was primarily due to normal expression of HER2/neu as demonstrated by IHCnegative staining of the primary breast cancer tissue. In contrast, overexpression of the HER2/neu was observed in the paired metastatic tumor tissue.14

In a report by Gancberg et al., the HER2/neu status of primary breast tumors was compared with that of at least one distant metastatic lesion using both the IHC and FISH tests in 107 patients. Among the paired primary and metastatic tumor tissues, six percent (6 out of 100) showed discordance by IHC. In all 6 cases there was greater HER2/neu staining in the metastatic tumor tissue than in the primary tumor tissue. By FISH analysis, 7 percent (5 of 68) of the cases were discordant. Three of the five patient specimens showed amplification in the metastatic tumor tissue, but not in the primary tumor tissue. The authors concluded that the HER2/neu positive metastatic lesions with a corresponding negative primary tumor were more frequent than expected, showing that negative HER2/neu staining in primary tumors may be underestimated.15

In another study, 80 paired primary and metastatic tumor tissues were evaluated for HER2/*neu* expression. This study reported a 17 percent change from the HER2/*neu* negative status in the primary tumor tissue to a HER2/*neu* positive status in the metastatic tumor tissue.¹⁶ In a 2004 publication Regitig et al., looked at 31 paired

breast cancer samples using both IHC and FISH and found a significant increase (48 percent) in HER2/neu reactivity in the distant metastasis compared to the primary tumor.17 Zidan and colleagues reported a discordance of 14 percent between HER2 overexpression in the primary and metastatic lesion as determined by IHC. In one patient (2 percent) HER2/neu was negative in the metastatic tumor but positive in the primary tumor. In seven (12 percent) patients, HER2/neu was positive in the metastasis but negative in the primary. Three of the seven patients responded to trastuzumab. Zidan and colleagues concluded that discordance between the primary and the metastasis should be considered when making treatment decisions or in patients who have a primary breast tumor that is HER2/neu negative.¹⁸ In summary, these publications, representing more than 300 breast cancer patients, demonstrate that the HER2/neu status can be different in the primary tumor tissue when compared to the metastatic tumor tissue from the same patient.

It now seems clear that there is a population of breast cancer patients that have been categorized as HER2/neu negative by tissue testing performed on their primary tumors but who have elevated serum HER2/neu levels illustrating the presence of HER2/neu positive tumors. Although this may be a relatively small population of patients (in the 10-30 percent range), it is clearly a concern that these patients do not have access to - anti-HER2/neu therapies such as Herceptin®. In summary, our literature survey indicates that an individual patient's HER2/neu status could differ when comparing the primary tumor tissue with tissue from the metastatic sites. Discrepancies in HER2/neu status have also been reported when comparing different antibodies used in IHC (eg, HercepTest®

vs. CB11), when comparing IHC and FISH tests on the same tumor tissue or when tumor tissue is evaluated in a communitybased versus a centralized-based tissue testing environment. These examples show several possible ways in which the HER2/*neu* status may be incorrectly assessed. This may directly lead to a missed opportunity for treatment with Herceptin[®]-based therapies.

Since studies presented at the 2005 American Society of Clinical Oncology (ASCO) conference have shown promising results with Herceptin®-based therapies in the adjuvant setting, it also raises a concern about establishing the correct HER2/neu status for patients receiving Herceptin® in the adjuvant setting. To date, the emphasis has been on making sure HER2/neu negative patients are not put at risk with Herceptin®. However, the data in this report shows the importance of establishing the correct HER2/neu status so some patients do not miss the opportunity to benefit from Herceptin®. Patients who are shown to have an elevated serum HER2/neu in the MBC setting but who had a HER2/neu negative tissue test on the primary tumor, should have the original tumor tissue re-tested. In cases where the original tumor material is not available, all efforts should be made to evaluate a metastatic lesion by either IHC or FISH so as to establish the current HER2/neu status. Once a patient with MBC is shown to have a HER2/neu positive tumor, their serum HER2/neu levels can be monitored with the ELISA to manage therapy. This data also supports the use of serum HER2/neu tests in determining and monitoring HER2/neu status during the course of therapy and patient management.

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