Title: Proteomic Classification of Breast Cancer

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Abstract

Being a significant health problem that affects patients in various age groups, breast cancer has been extensively studied to date. Recently, molecular breast cancer classification has advanced significantly with the availability of genomic profiling technologies. Proteomic technologies have also advanced from traditional protein assays including enzyme-linked immunosorbent assay, immunoblotting and immunohistochemistry to more comprehensive approaches including mass spectrometry and reverse phase protein lysate arrays (RPPA). The purpose of this manuscript is to review the current protein markers that influence breast cancer prediction and prognosis and to focus on novel advances in proteomic classification of breast cancer.

Keywords: breast cancer, proteome, RPPA, molecular technology.

Introduction

The role of molecular markers in carcinogenesis has been extensively researched over the last few years, with the intention of facilitating early detection of different types of cancer, identification of molecular targets, improved prognostication and therapeutic response monitoring to allow early recognition of disease progression. As technologies have improved, such studies have progressed to comprehensive study of the human genome and investigation of cellular protein expression and activation. Comprehensive mapping of the functional proteome within cancer, although challenging, is closer to reality now than ever before. (1-4). With such technologic advances, the era of empiric chemotherapy as our only therapeutic option for cancer is receding. Genomic and proteomic approaches are advancing our molecular understanding of cancer and our ability to identify molecular markers for disease classification and novel molecular targets. New comprehensive genomic approaches have already facilitated genomic classification of breast and other cancers. Increasing utilization of new proteomic technologies can be expected to facilitate further advancements in research in breast and other cancers. Emerging proteomic technologies such as Reverse Phase Protein Arrays (RPPA) will allow us to increasingly elucidate functional protein networks in cancer cells, and will permit a better understanding of the defective signaling pathways in breast cancer that may be exploited for specific targeting, early diagnosis, disease classification and development of novel preventive approaches. Herein we will review the current status of breast cancer classification using conventional and novel proteomic approaches. (5-9).

Current molecular classification of breast cancer

Being a significant health problem that affects patients in various age groups, breast cancer has been extensively studied to date. Empiric management of breast cancer with chemotherapy has developed to include drugs such as anthracyclines and taxanes. The identification of oestrogen (ER) and progesterone (PgR) receptors led to the development of the first successful targeted therapy approach in breast cancer and to the recognition of two basic molecular subclasses of this disease: ER positive and ER negative. (10). Gene expression profiling has subsequently identified at least five breast cancer subtypes including HER2-positive, basal, normal-like, and at least two biologically distinct subtypes of ER positive breast cancer: luminal A and luminal B. Luminal B tumours have higher proliferation rates and poorer prognosis than luminal A tumours. (11-12).

Classic immunohistochemical protein markers and other important proteins in breast cancer

Oestrogen receptor (ER) and progesterone receptor (PgR)

Using the hormone receptors ER and PgR, breast cancers are classified as hormone receptor-positive or negative. The purpose of these tests is to determine the likelihood of benefit from endocrine therapy in patients with breast cancer. ER or PgR are defined as positive if ≥ 1% of tumour cell nuclei are immunoreactive on immunohistochemistry as per American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations. (13). For the approximately two-thirds of breast cancers that are positive for ER and/or PgR, a standard five years of adjuvant endocrine therapy reduces their risk of recurrence and death by nearly 30%. The additional role of adjuvant chemotherapy in hormone receptor-positive breast cancers is a matter of some controversy. Multiple studies have shown that anthracycline-based and other chemotherapy regimens are less effective in

patients with ER-positive as compared with ER-negative breast cancer. (14-16). Until recently, the assessment of clinical factors including histologic grade, axillary lymph node status and tumour size was the only available approach to provide a crude estimate of whether or not a patient with hormone receptor-positive breast cancer might benefit from adjuvant chemotherapy (17-18). However, this has changed with the introduction of the oncotype DX test (see later).

The lack of responsiveness of some ER-positive breast cancers to antihormonal therapy likely relates at least in part to the functionality of the ER. The classic mechanism that leads to transcriptional activation of oestrogen response genes (e.g. PgR, GATA3, EIG121, Bcl2 and insulin-like growth factor receptor 1 (IGF1R)) mediated through ER and the estrogen complex plays a pivotal role in the development of ER-positive breast cancers. It is this mechanism that is likely most effectively targeted by antihormonal therapies such as tamoxifen, and thus the presence of oestrogen response gene product proteins such as PgR in breast cancer is associated with a greater likelihood that breast cancer will be responsive to antihormonal therapy. However, in addition to this classic pathway, other mechanisms including non-genomic actions, both estrogen-dependent and estrogen-independent, have also been found to contribute to the growth of some ER-positive breast tumours. Although these mechanisms are not as well understood, some involve interactions with growth factor protein signaling pathways (e.g. Epidermal Growth Factor Receptor (EGFR) /HER2 /phosphatidylinositol-3-kinase (PI3K) signaling) and are thought to play a role in resistance to antihormonal therapy. This resistance is likely mediated not only directly through cell growth promotion by growth factor signaling, but also through enhancement of alternative ER signaling pathways. (19)

Epidermal growth factor receptor (EGFR) tyrosine kinase family

This receptor tyrosine kinase (RTK) membrane receptor family consists of EGFR, ErbB-2 (HER2), ErbB-3 and ErbB-4. HER2 overexpression has been documented to occur in as many as 20% of breast cancers. (20-21). The overexpression of HER2 protein is caused by amplification of the HER2 oncogene and is well documented as a poor prognostic factor in breast cancer. Previous studies have shown an association between the overexpression of HER2 protein and larger tumour size, high histological grade, negative ER status, distant metastases and high Nottingham Prognostic Index (NPI) score (which facilitates determination of prognosis following surgery for breast cancer; its value is calculated using three pathological criteria: the size of the lesion; the number of involved lymph nodes; and the grade of the tumour). (22-24). However, the natural history of HER2 overexpressing breast cancer has been significantly altered by the introduction of HER2 targeted therapies such as Trastuzumab and Lapatinib. (25-26). HER2 protein expression is scored from 0 to 3+ by immunohistochemistry, depending on the percentage of cells with membrane staining and the intensity of this staining. A score of 3+ represents overexpression of HER2 protein while scores of 0 and 1+ represent a lack of overexpression. With a score of 2+, fluorescent insitu hybridization (FISH) is employed to clarify whether or not the HER2 oncogene is amplified. The importance of the identification of HER2 overexpressing (amplified) breast cancers is that this is used to guide treatment with anti-HER2 targeted therapy. Central laboratory testing is highly recommended to minimize inaccurate results. For example, a 3-4% incidence of false negative results is estimated annually in the United States and this results in inadequate care of between 3000 and 5000 breast cancer patients every year. (25,27-29). However, only approximately 30% of patients with HER2 overexpressing breast cancers

actually respond to therapy with the HER2 monoclonal antibody Trastuzumab. Current determination of HER2 overexpression by immunohistochemistry or FISH assesses only HER2 overexpression and not either the phosphorylation (activation) status of HER2 or the activation status of key components of downstream HER2 signaling pathways such as the phosphatidylinositol-3-kinase (P13K) and Ras/mitogen-activated protein kinase (MAPK) pathways. The application of novel proteomic approaches to subclassify HER2 overexpressing breast cancers using this additional information will likely advance our ability to optimise classification of and targeted therapy strategies for HER2 overexpressing breast cancers. (29-31).

A positive correlation has also been found between ErbB-3 protein expression and high breast cancer grade, lymph node positivity and vascular invasion. Further, the co-existence of HER2 and c-erbB-3 overexpression is strongly associated with poor overall survival and disease free interval in breast cancer patients. This dimer forms a particularly potent signalling moiety that stimulates growth and transformation of breast cancer cells. (32-34).

Ki67 index

The nuclear non-histone protein Ki67 is a well-established cell proliferation marker in cancer, a strong predictor of tumour grade and can be used to classify breast cancers based on cancer cell proliferation rate. Ki67 plays vital roles in cell proliferation including organization of DNA in early G1 phase, binding with RNA and DNA and involvement in synthesis of ribosomes during cell division. In breast cancer, there is a correlation between the percentage of Ki67 positive cells on immunohistochemistry and nuclear grade, absence of hormone receptors, and mitotic rate. A high Ki67 index occurs in 20-50% of breast cancers. (35). Regardless of the hormone receptor status, breast cancers expressing high levels of Ki67 have

worse outcomes. (36). In addition, measurement of Ki67 index pre- and post-therapy provides an accurate surrogate for responsiveness of breast cancer to that treatment. A greater decrease in Ki67 post treatment predicts longer progression-free survival times in studies of both endocrine therapy and neoadjuvant chemotherapy. (37). A study has suggested the reliability of Ki67 in predicting early resistance to endocrine therapy following a short course of neoadjuvant endocrine treatment. (38). Increasingly, Ki67 is measured in several scenarios for clinical research, including as a primary efficacy endpoint for clinical trials, and sometimes in clinical management.

However, Ki67 index is not readily available in many cancer centres and is not routinely used in a widespread manner in clinical practice due to the enormous variation in analytical practice that markedly limits the value of Ki67 and has led many investigators to have low confidence in Ki67 determination by immunohistochemistry (IHC). As a result, an international panel of investigators has made comprehensive recommendations on preanalytical and analytical assessment, interpretation and scoring of Ki67. (39). These recommendations are geared toward achieving a harmonized methodology, create greater between-laboratory and between-study comparability, and allow earlier valid applications of this marker in clinical practice.

BRCA1 and BRCA2

BRCA1, a tumour suppressor gene and breast cancer type 1 susceptibility protein, is located in close proximity to the HER2 oncogene on the long arm of chromosome 17. Its absence in breast cancer is often caused by germline mutations and is associated with high tumour grade, hormone receptor and HER2 negativity and a high cancer cell proliferation rate. Despite its negative correlation with HER2 overexpression, breast cancers with mutated BRCA1 have a similarly poor prognosis as those

tumours that harbour HER2 overexpression. (40-41). Like BRCA1, BRCA2 mutations are associated with familial breast cancers although, in contrast to BRCA1, BRCA2 mutations are associated with less risk of ovarian cancer but confer an increased risk for male breast cancer and for several other cancers, such as prostate cancer, pancreatic cancer, non-Hodgkin's lymphoma, basal cell carcinoma, bladder carcinoma, and fallopian tube tumours. Breast cancers in carriers of BRCA1 and BRCA2 mutations often present with high nuclear grade, poorly differentiated morphology, absence of ER/PgR/HER2 expression, overexpression of cyclin E, and p53 mutations. (42-43).

p53

Located on chromosome 17p13, the first identified tumour suppressor gene and the most commonly mutated gene in human cancers, p53 plays roles in cell cycle regulation, DNA repair and apoptosis. The familial Li-Fraumeni syndrome, characterized by multiple early-onset cancers including high grade breast cancers, is associated with germline mutations in the p53 gene. The overall frequency of somatic p53 mutations in breast cancer is estimated to be approximately 20%, with higher frequencies occurring in basal, BRCA1- and BRCA2–associated and luminal B tumours. p53 mutations in breast cancer are often associated with aberrant stabilisation and overexpression of p53 protein on immunohistochemistry and are associated with high tumour grade, more advanced stage at presentation and poorer outcomes. (44-45).

PI3K, MAPK and other protein kinase signaling pathways

There is increasing clinical and preclinical evidence that intracellular membrane growth factor-regulated kinase signaling pathways may be involved in breast cancer pathogenesis and in resistance of breast tumours to antihormone and other therapies. (45-50). These pathways include the PI3K and MAPK signaling pathways

(Figure 1 (from reference 50)). These kinase signaling pathways stimulate cell growth, proliferation and contribute to oncogenesis by influencing downstream effector proteins such as cell cycle regulators (e.g. cyclins) and the cellular protein synthesis (translation) machinery (e.g. mammalian target of rapamycin (mTOR), p70S6 Kinase (p70S6K), S6 ribosomal protein and 4E Binding Protein 1 (4EBP1)). For example, it is now known that PIK3CA, the oncogene that encodes the p110 alpha subunit of the PI3K protein, is mutated in up to 40% of breast cancers, that levels of PTEN, the tumour suppressor that opposes the effects of PI3K, are decreased in a similar proportion, and that AKT2 (up to 5%) and p70S6 Kinase (10-20%) may also be overexpressed by amplification in some breast tumours. (50-53).

Src Kinase signaling activation is also a frequent occurrence in different types of cancer including breast cancer and promotes tumour invasion and progression in part through its interaction with signaling from multiple transmembrane receptor-associated tyrosine kinases, as well as other kinases, including PI3K, focal adhesion kinase (FAK) and Ras. (54-60). Src is thus being investigated as a therapy target in breast cancer. Further, Zhang et al have shown that Src is a key modulator of trastuzumab response and a common node downstream of multiple trastuzumab resistance pathways. (57)

Since protein array technologies allow comprehensive profiling of the expression and activation status of multiple protein kinase signaling pathways and their downstream effectors, these technologies are now expanding our ability to study the role and clinical significance of kinase signaling pathways in breast cancer and to subclassify breast cancers based on the activation status of these pathways (see later) (46-53).

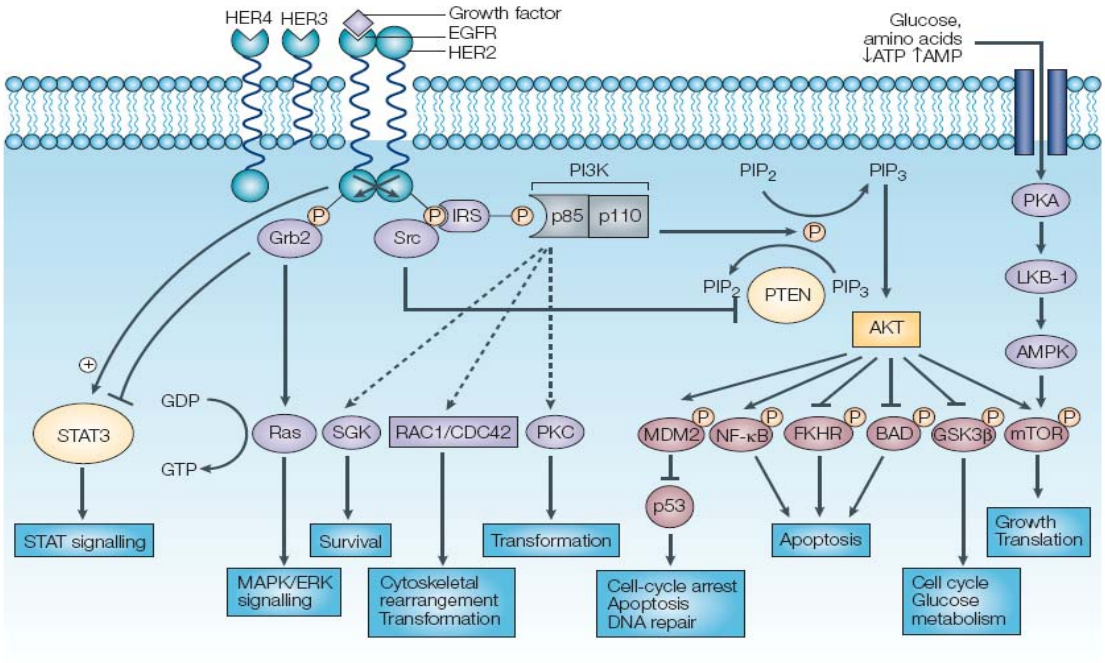

Figure 1: Schematic of signalling through the phosphatidylinositol-3-kinase (PI3K)/AKT and Ras/Mitogen-Activated Protein Kinase (MAPK) pathways. The PI3K/AKT and related pathways are important in internalizing the effects of external growth factors and of membrane tyrosine kinases. Activation of membrane kinases including epidermal growth factor receptor (EGFR) by external growth factors initiates receptor dimerization and subsequent events to activate these intracellular pathways. AKT is activated downstream of PI3K and has multiple targets. AKT and the cellular energy sensors LKB1 (STK11) and AMP-activated protein kinase (AMPK) exert opposing effects on mammalian target of rapamycin (mTOR), which is activated by AKT. ERK, extracellular signal regulated kinase; FKHR, forkhead; GDP, guanosine diphosphate; IRS, insulin receptor substrate; GSK3, glycogen synthase kinase 3; MAPK, mitogen activated protein kinase; NF-□B, nuclear factor-□B; PIP2, phosphatidylinositol-3,4-diphosphate; PIP3, phosphatidylinositol-3,4,5- triphosphate; PKC, protein kinase C; STAT, signal transducer and activator of transcription.

Cyclin D1 and other cyclins

Several cyclins play an important role in breast cancer. As part of the cyclin family, cyclin D1 protein influences the cell cycle from G1 to S phase transition and is a critical component of growth factor-induced mitogenesis in breast epithelial cells. It is overexpressed in 15% of breast cancers and in up to 50% of hormone receptor positive breast cancers where it is associated with a poor prognosis. In some cases,

cyclin D1 protein overexpression occurs as a result of cyclin D1 oncogene (CCND1) amplification and is associated with resistance to anti-hormone therapies including tamoxifen. Cyclin D1 overexpression likely occurs by different mechanisms in ER positive and negative breast cancers. High cyclin D1 expression is associated with high Ki67 expression and high tumour grade. (61-63). Other cyclins are also important in breast cancer. For example, our group has shown that breast cancer classification using protein expression levels of cyclins D1, B1 and E1 is prognostic and correlates closely with breast cancer classification by gene expression profiling. (64)

Oncotype DX Recurrence score

Oncotype DX (Genomic Health, Redwood City, CA) is a novel genomic profiling test, a 21 gene assay (table 1), which provides a novel means of predicting benefit from anti-hormonal therapy and chemotherapy in hormone receptor positive early stage node negative breast cancer. Oncotype DX essentially subclassifies these breast tumours into low, intermediate and high risk groups for distant metastases at ten years and is based on a score that is derived from the expression of 21 genes, many of which individually have been studied previously as important immunohistochemical markers in breast cancer. Using Oncotype DX, individuals with low-risk scores (<18) have excellent outcomes and can avoid chemotherapy with its attendant cost and side effects, whereas those with high-risk scores (≥30) are treated with chemotherapy and anti-hormonal therapy. The benefit of chemotherapy in patients with intermediate-risk scores is still uncertain and is being studied in the TAILOR-X clinical trial. The role of Oncotype DX in patients with lymph node-positive, hormone receptor-positive breast cancer is also being studied at present. (65-67). Oncotype DX at least theoretically demonstrates the potential power of a comprehensive

analysis of the proteome for breast cancer classification, prognostication and prediction. (48, 68).

Oncotype DX Recurrence Score Assay: 16 cancer and five references genes from three studies

Proliferation Ki67 STK15 Survivin Cyclin B1 MYBL2	**HER2** GRB7 HER2	**Estrogen** ER PgR BCL2 SCUBE2
	GSTM1	
Invasion MMP11 CTSL2	CD68	**Reference** ACTB GAPDH RPLPO GUS TFRC
	BAG1	

Table 1: The Recurrence Score result is calculated from the expression of 16 cancer-related genes and 5 reference genes that are used to normalize the expression of the former genes. (48) (Abbreviations: **STK15** serine/threonine kinase-15, **MYBL2** Myb-related protein B, **MMP11** matrix metalloproteinase-11, **CTSL2** Cathepsin L2, **GRB7** Growth factor receptor-bound protein 7, **HER2** Human Epidermal Receptor 2, **GSTM1** Glutathione S-transferase Mu 1, **CD68** Cluster of Differentiation 68, **ER** oestrogen receptor, **PgR** progesterone receptor, **ACTB** Beta-actin, **GAPDH** Glyceraldehyde 3-phosphate dehydrogenase, **RPLPO** large ribosomal protein, **TFRC** transferrin receptor)

Novel technology in molecular breast cancer classification

The limited power of single gene/protein biomarkers for prediction, classification and screening has led scientists to investigate multigene and multiprotein biomarkers. The analysis of DNA sequence and mutations and of gene expression profiles has

led to advances in the genomic classification of breast cancer. However the lack of practical proteomic technologies that can be applied to clinical specimens has meant that similar progress has not been made in the proteomic classification of breast cancer. (11,69). The following established and novel proteomic analysis technologies are currently being applied in an attempt to advance the proteomic classification of breast cancer and to identify multiprotein biomarkers with utility in classification, prognostication and prediction in breast cancer.

ELISA and Immunohistochemistry (IHC)

Enzyme-linked immunosorbent assay (ELISA) is the classical highly specific assay used for validation of specific protein biomarkers, but it is low-throughput, expensive, and requires large amounts of cellular equivalents for sensitivity. Advances in nanotechnology now allow the coupling of antibodies to fluorescent tags such as quantum dots that allow a visual resolution down to single molecules, a level of detail that is also being exploited in applications such as tumour molecular imaging (70). This has allowed, for example, the real-time in-vivo imaging of homing of HER2 specific antibodies to breast tumours in mice and represents an interesting emerging approach (71).

Immunohistochemistry (IHC) has the advantage of providing information about the spatial and cellular localization of the protein of interest in patient samples but is not truly quantitative, lacks sensitivity and its dynamic range is significantly inferior to that of the novel emerging technologies including RPPA. (72-74).

A faster alternative to traditional ELISA, Meso Scale Discovery (MSD), enables the detection of biomarkers in single and muliplex format utilizing electrochemiluminescence (ECL). However, during bridging, sensitivity can be affected by the analytes valency. Its use currently is limited to a small number of

analytes. Another flow cytometric technology, Luminex immunoassay can allow a single sample to be tested simultaneously for up to 100 different analytes within a short time frame. (92-94)

Protein mass spectrometry (MS)

Diamandis et al described the discovery of tumour biomarkers by multiple approaches including determination of secreted protein from tumour cells, testing a particular protein in sets of samples from normal and tumour specimens, applying cDNA microarrays to both normal and cancer cells to identify overexpressed genes, and the use of quantitative mass spectrometry to differentiate normal and cancerous cells. (75). Traditional mass spectrometry methods have been regarded as expensive, non-quantitative, low-throughput, and too cumbersome for large- or even medium-scale application to the classification of the proteome in human breast cancer specimens. However, since its introduction in 1950, mass spectrometry (MS) has advanced tremendously with the aid of matrix-assisted laser desorption/ionization (MALDI), electrospray ionisation (ESI), gas chromatography (GC/MS) and liquid chromatography (LC/MS). (76). More recently, biological fluids have been fractionated by chromatographic techniques and analysed by mass spectrometry. (77-78). Over the last decade, progress has been achieved in the development of MALDI-based imaging MS methods, a molecular analytical technology capable of simultaneously measuring multiple analytes directly from intact tissue sections. Several studies have demonstrated the strength of this technology for uncovering new markers that correlate with disease severity as well as prognosis and therapeutic response (79-81). Other studies have also demonstrated the utility of novel MS methods for the identification of biomarkers associated with sensitivity and resistance of breast and other cancers to various therapeutic modalities (82-85).

With the advent of triple-quadruple MS, and resultant Multiple Reaction Monitoring (MRM) assays, novel MS approaches may reach the required threshold for routine clinical analysis.

Micro-array technologies

To provide high-throughput robust assays for the analysis of clinical proteomes, protein microarray technologies were introduced (figure 2). Protein microarrays are composed of high numbers of immobilized spots in a solid matrix and are created using a robotic arrayer. They have been developed in two main formats, forward-phase protein microarrays (FPPAs) for solid phase, and reverse-phase protein microarrays (RPPAs) for liquid phase analytes including lysates from cancer cells and human tumour material. A different capture molecule such as an antibody or aptamer is immobilized in each spot in the case of FPPAs and each array is queried with one tumour sample, so multiple proteins / phosphoproteins from the same sample are measured at once. (87-89). On the other hand, with RPPA, a different individual cellular lysate (or serum or complex protein mixture) is immobilized in each array spot, and each array is queried with one antibody or affinity reagent. (74). Thus, in each RPPA array, as many as thousands of samples, (e.g. human tumour samples) can be queried in parallel for one feature detected by the probe of interest. RPPAs only require one type of antibody per analysed protein and thus do not require direct analyte tagging for detection of post-translational protein modifications (90-91). Such as phosphorylation or cleavage products once high affinity, monospecific antibodies are available. However antibody specificity must be thoroughly assessed and validated, for example by western blotting, with evidence of a single appropriately sized band in immunoblots of complex biological samples similar to those planned for array analysis. Recently, our group described a rigorous

validation process for antibodies to be used in RPPA experiments involving human breast cancer specimens. (92).

Several groups have already demonstrated the feasibility of mapping functional proteomic signaling networks using RPPA in limited fine needle aspiration (FNA) samples, in clinical human tissue material, and in laser capture microdissection primary human breast tumours and metastatic lesions. (93-96).

Several potential difficulties exist for the application of protein microarrays to analysis of functional proteomics in human breast and other tumours. For example, little is known about the stability of phosphoproteins for molecular profiling after tissue harvesting at biopsy or surgery. Espina et al explored the stability of phosphoproteins in freshly obtained human tissue specimens and found decrease over time for many phosphoproteins, to a greater degree with simultaneous inhibition of kinases and phosphatases used to stabilize phosphoproteins than with inhibition of phosphatases alone. (98-99).

Other potential limitations of the RPPA approach include detection sensitivity and the challenge of extracting reproducible data for heterogeneous tumour samples where stromal content and intra-tumour variability may impact on data reproducibility. Tyramide signal amplification (TSA) has been widely applied to increase the sensitivity of RPPAs (to nanogram levels for specific proteins). However, TSA linked with a streptavidin-biotin strategy can produce unspecific signals that may interfere with the signal of interest. Other approaches including antibody-mediated signal amplification have recently been reported to provide convenient and cost-effective approaches for the robust and specific quantification of low abundant proteins using RPPA. (7). Human tumour tissue heterogeneity is another significant challenge and one that some groups have overcome by using laser capture microdissection and/or

FNAs (see above). However, the robust application of RPPA to non-microdissected human tumour material would make the technology more widely applicable in the clinical setting. To facilitate this, we have worked with 'macrodissected' tissue sections prepared by pathologists such that tumour tissue composes at least 50% of the specimen, if this is not already the case. In ongoing work, we are also investigating the utilization of antibodies that recognize various tissue components to allow correction of RPPA data for stromal content and intra-tumour variability.

Recently, our group applied 82 validated antibodies that recognize kinase and steroid signaling proteins and their effectors to multiple sites in non-microdissected human breast cancers using RPPA after intervals of up to 24 hours on the benchtop at room temperature following surgical resection. (92). Twenty-one of 82 total phosphoproteins demonstrated time-dependent instability at room temperature, with most variability occurring at later time points between 6 and 24 h. However, the 82-protein functional proteomic "fingerprint" was robust in almost all tumours even when maintained at room temperature for 24 hours before freezing. In repeat samples taken from each tumour, intratumoral protein levels were markedly less variable than intertumoral levels. Indeed, an independent analysis of prognostic biomarkers in tissue from multiple tumour sites accurately and reproducibly predicted patient outcomes. Significant correlations were observed between RPPA and immunohistochemistry. However, RPPA demonstrated a superior dynamic range. We concluded based on this study that the robustness of RPPA and the stability of the functional proteomic "fingerprint" both facilitate the accurate and reproducible study of the functional proteome in non-microdissected human breast tumours. As stated above, studies by other groups also support the feasibility and potential utility of

comprehensive signal pathway activation profiling using RPPA for molecular analysis of microdissected human breast and other cancers. (74, 93-94, 100-103).

Multiple examples now exist of the utility of RPPAs for the study of breast cancer. Using RPPAs Dillon et al have recently demonstrated that co-activation of HER2 and AKT1 leads to breast tumour initiation and growth in mice, whereas co-activation of HER2 and AKT2 did not affect tumour growth but led to the early development of pulmonary metastases. (104). RPPA was applied by Boyd et al in the analysis of 30 breast cancer cell lines to reveal distinct pathway activation patterns between different intrinsic subtypes of breast cancer that are not obvious from gene expression studies, and that predict response to selective kinase inhibitors in vitro. (105).

Antibody name	Protein name	Company	Catalog number	Host	Dilution
14-3-3 Beta	14-3-3 Beta	Santa Cruz Biotechnology, Inc.	SC-628	Rabbit	1 in 5000
14-3-3 Zeta	14-3-3 Zeta	Santa Cruz Biotechnology, Inc.	SC-1019	Rabbit	1 in 1200
4EBP1	4E Binding Protein 1	Cell Signaling Technology, Inc.	CS 9452	Rabbit	1 in 100
4EBP1p37	4EBP1 phosphorylation at T37/T46	Cell Signaling Technology, Inc.	CS 9459	Rabbit	1 in 100
4EBP1p65	4EBP1 phosphorylation at S65	Cell Signaling Technology, Inc.	CS 9451	Rabbit	1 in 200
4EBP1pS65.mono	4EBP1 phosphorylation at S65	Cell Signaling Technology, Inc.	CS 9456	Rabbit	1 in 250
4EBP1pT70	4EBP1 phosphorylation at T70	Cell Signaling Technology, Inc.	CS 9455	Rabbit	1 in 150
AcCoA	Acetyl CoA Carboxylase	Epitomics, Inc.	1768-1	Rabbit	1 in 250
AcCoAp	AcCoA phosphorylation at S79	Cell Signaling Technology, Inc.	CS 3661	Rabbit	1 in 250
AIB1	Amplified in Breast Cancer 1	BD Biosciences	611105	Mouse	1 in 50
Akt	Protein Kinase B	Cell Signaling Technology, Inc.	CS 9272	Rabbit	1 in 250
Aktp308	Akt phosphorylation at S308	Cell Signaling Technology, Inc.	CS 9275	Rabbit	1 in 250
Aktp473	Akt phosphorylation at S473	Cell Signaling Technology, Inc.	CS 9271	Rabbit	1 in 250
alpha.actin	Alpha actin	Epitomics, Inc.	1184-1	Rabbit	1 in 200

AMPK	AMPK	Cell Signaling Technology, Inc.	CS 2532	Rabbit	1 in 250
AMPKp	AMPK phosphorylation at S172	Cell Signaling Technology, Inc.	CS 2535	Rabbit	1 in 250
AR	Androgen Receptor	Epitomics, Inc.	1852-1	Rabbit	1 in 200
β catenin	B catenin	Cell Signaling Technology, Inc.	CS 9562	Rabbit	1 in 300
BAD	BAD	Cell Signaling Technology, Inc.	CS 9292	Rabbit	1 in 200
BADpS112	BAD phosphorylation at S112	Cell Signaling Technology, Inc.	CS 9296	Mouse	1 in 200
BCL11A	BCL11A	SDI		Rabbit	1 in 1500
bcl2	bcl2	Dako	M0887	Mouse	1 in 200
BCLp70	bcl2 phosphorylation at S70	Cell Signaling Technology, Inc.	CS 2827	Rabbit	1 in 200
BIM	BIM	Epitomics, Inc.	1036	Rabbit	1 in 250
BRCA1	BRCA1	Upstate Biotechnology, Inc.	07-434	Rabbit	1 in 1000
BRCA2	BRCA2	Cell Signaling Technology, Inc.	CS 9012	Rabbit	1 in 300
caveolin 1	Caveolin 1	Cell Signaling Technology, Inc.	CS 3232	Rabbit	1 in 250
CCNB1	Cyclin B1	Epitomics, Inc.	1495-1	Rabbit	1 in 500
CCND1	Cyclin D1	Santa Cruz Biotechnology, Inc.	SC-718	Rabbit	1 in 1000
CCNE1	Cyclin E1	Santa Cruz Biotechnology, Inc.	SC-247	Mouse	1 in 500
CCNE2	Cyclin E2	Epitomics, Inc.	1142-1	Rabbit	1 in 250
CD20	CD20	Epitomics, Inc.	1632	Rabbit	1 in 125
CD31	CD31	Dako	M0823	Mouse	1 in 500
CD4	CD4	R&D Systems	MAB3791	Mouse	1 in 1500
cdk2	Cyclin dependent kinase 2	BD Biosciences	610145	Mouse	1 in 50
CDK4	Cyclin dependent kinase 4	Cell Signaling Technology, Inc.	CS 2906	Rabbit	1 in 250
cjun	Cjun	Cell Signaling Technology, Inc.	CS 9165	Rabbit	1 in 250
cJUNp73	cjun phosphorylation at S73	Cell Signaling Technology, Inc.	CS 9164	Rabbit	1 in 150
ckit	Ckit	Cell Signaling Technology, Inc.	CS 3392	Rabbit	1 in 150
cleaved caspase 7	Cleaved caspase 7 (Asp198)	Cell Signaling Technology, Inc.	CS 9491	Rabbit	1 in 150
cleaved PARP	Cleaved PARP (Asp214)	Cell Signaling Technology, Inc.	CS 9546	Mouse	1 in 250
cmyc	Cmyc	Cell Signaling Technology, Inc.	CS 9402	Rabbit	1 in 150
Collagen V	Collagen V	Santa Cruz Biotechnology, Inc.	SC-20648	Rabbit	1 in 1000
Collagen VI	Collagen VI	Santa Cruz Biotechnology, Inc.	SC-20649	Rabbit	1 in 750
COX2	COX2	Epitomics, Inc.	2169-1	Rabbit	1 in 500
COMT	Catechol-O-methyltransferase	SDI	1671	Rabbit	1 in 15000

Connexin	Connexin	SDI	1716	Rabbit	1 in 1500
CXXC6	CXXC6	SDI	2126	Rabbit	1 in 1750
E cadherin	E cadherin	Cell Signaling Technology, Inc.	CS 4065	Rabbit	1 in 200
EGFR	Epidermal growth factor receptor	Santa Cruz Biotechnology, Inc.	SC-03	Rabbit	1 in 200
EGFRp1045	EGFR phosphorylation at Y1045	Cell Signaling Technology, Inc.	CS 2237	Rabbit	1 in 100
EGFRp922	EGFR phosphorylation at Y992	Cell Signaling Technology, Inc.	CS 2235	Rabbit	1 in 100
EIG121	EIG121 / Maba1	Russell Broaddus at MDACC		Rabbit	1 in 200
EN1	Engrailed-1	SDI	2104	Rabbit	1 in 1000
ER	Estrogen receptor alpha	Lab Vision Coorporation (formerly Neomarkers)	Sp1	Rabbit	1 in 250
ERK2	Mitogen-activated protein kinase	Cell Signaling Technology, Inc.	SC-154	Rabbit	1 in 250
ERp118	ER phosphorylation at S118	Epitomics, Inc.	1091-1	Rabbit	1 in 200
ERp167	ER phosphorylation at S167	Epitomics, Inc.	2492-1	Rabbit	1 in 200
ETV6	ETV6	SDI	2101	Rabbit	1 in 400
FANCA	Fanconi anemia, complementation group A	SDI	2127	Rabbit	1 in 2000
FANCE	Fanconi anemia, complementation group E	SDI	2131	Rabbit	1 in 5000
FGFR1	Fibroblast Growth Factor Receptor 1	Santa Cruz Biotechnology, Inc.	SC-7945	Rabbit	1 in 250
FGFR2	Fibroblast Growth Factor Receptor 2	SDI	2182	Rabbit	1 in 600
Fibronectin	Fibronectin	Epitomics, Inc.	1574	Rabbit	1 in 5000
FKHRL1p318	FKHRL1 phosphorylation at S318/321	Cell Signaling Technology, Inc.	CS 9465	Rabbit	1 in 1000
Fortilin	Fortilin	Ken Fujise at UT Houston		Rabbit	1 in 3000
GABApi	Gaba aminobutyric acid pi	SDI	2103	Rabbit	1 in 200
GATA3	GATA3	BD Biosciences	558686	Mouse	1 in 200
Gelsolin	Gelsolin	SDI	1718	Rabbit	1 in 1000
GSK3	Glycogen synthase kinase 3 beta	Santa Cruz Biotechnology, Inc.	SC-7291	Mouse	1 in 1000
GSK3p21_9	GSK3 phosphorylation at S21/S9	Cell Signaling Technology, Inc.	CS 9331	Rabbit	1 in 250
HDAC7	Histone deacetylase 7	Abcam, Inc.	53101	Rabbit	1 in 400
HER2	Human epidermal receptor 2	Epitomics, Inc.	1148-1	Rabbit	1 in 250
HER2p1248	HER2 phosphorylation at Y1248	Upstate Biotechnology, Inc.	06-229	Rabbit	1 in 750
IGFBP2	IGF1 binding protein 2	Cell Signaling Technology, Inc.	CS3922	Rabbit	1 in 150
IGF1R	Insulin-like growth factor receptor 1	Cell Signaling Technology, Inc.	CS 3027	Rabbit	1 in 500
IGFRp	IGF1R phosphorylation at	Cell Signaling	CS 3024	Rabbit	1 in 200

	Y1135/Y1136	Technology, Inc.			
JAZf1	JAZf1	SDI	2138	Rabbit	1 in 1000
JNK	cjun N terminal Kinase	Santa Cruz Biotechnology, Inc.	SC-474	Rabbit	1 in 200
JNKp183-185	JNK phosphorylation at T183/Y185	Cell Signaling Technology, Inc.	CS 9251	Rabbit	1 in 150
KIT	KIT	SDI	2036	Rabbit	1 in 15000
LKB1	LKB1	Abcam, Inc.	15095	Rabbit	1 in 200
LKB1p	LKB1 phosphorylation at S428	Cell Signaling Technology, Inc.	CS 3051	Mouse	1 in 200
MALT1	MALT1	SDI	2155	Rabbit	1 in 1500
MAML2	MAML2	SDI	2136	Mouse	1 in 1500
MAPKp	MAPK1/2 phosphorylation at T202/T204	Cell Signaling Technology, Inc.	CS 4377	Rabbit	1 in 1000
MEK1	MAPK/ERK kinase 1	Epitomics, Inc.	1235-1	Rabbit	1 in 15000
MEK12p	MEK1/2 phosphorylation at T217/T221	Cell Signaling Technology, Inc.	CS 9121	Rabbit	1 in 800
MGMT	Methylguanine Methyltransferase	Chemicon	16200	Mouse	1 in 100
MLLT10	MLLT10	SDI	2116	Rabbit	1 in 400
mTOR	Mammalian target of rapamycin	Cell Signaling Technology, Inc.	CS 2983	Rabbit	1 in 400
MYH11	MYH11	SDI	2137	Rabbit	1 in 2000
Ncadherin	Ncadherin	Cell Signaling Technology, Inc.	CS 4061	Rabbit	1 in 100
NCKIPSI	NCKIPSI	SDI	2117	Rabbit	1 in 1000
NOTCH3	NOTCH3	Santa Cruz Biotechnology, Inc.	SC-5593	Rabbit	1 in 100
p110alpha	p110alpha subunit of phosphatidylinositol-3-kinase	Epitomics, Inc.	1683-1	Rabbit	1 in 500
p21	p21	Santa Cruz Biotechnology, Inc.	SC-397	Rabbit	1 in 250
p27	p27	Santa Cruz Biotechnology, Inc.	SC-527	Rabbit	1 in 500
p38	p38 MAPK	Cell Signaling Technology, Inc.	CS 9212	Rabbit	1 in 300
p38p180_2	p38 MAPK phosphorylation at T180/T182	Cell Signaling Technology, Inc.	CS 9211	Rabbit	1 in 250
p53	p53	Cell Signaling Technology, Inc.	CS 9282	Rabbit	1 in 3000
p53p	p53 phosphorylation at S15	Cell Signaling Technology, Inc.	CS 9284	Rabbit	1 in 5000
p7056 Kinase	p70S6 Kinase	Epitomics, Inc.	1494-1	Rabbit	1 in 500
p70S6Kp389	p70S6 Kinase phosphorylation at T389	Cell Signaling Technology, Inc.	CS 9205	Rabbit	1 in 200
p90RSKp	p90RSK phosphorylation at S380	Cell Signaling Technology, Inc.	CS 9341	Rabbit	1 in 400
PAI1	Plasminogen activator inhibitor-1	BD Biosciences	612024	Mouse	1 in 1000
pcmyc	cmyc phosphorylation at	Cell Signaling	CS 9401	Rabbit	1 in 150

	T58/S62	Technology, Inc.			
PCNA	Proliferating Cell Nuclear Antigen	Abcam, Inc.	29	Mouse	1 in 2000
PDK1	Phosphoinositide Dependent Kinase 1	Cell Signaling Technology, Inc.	CS 3062	Rabbit	1 in 250
PDK1p241	PDK1 phosphorylation at S241	Cell Signaling Technology, Inc.	CS 3061	Rabbit	1 in 500
PKCalpha	Protein Kinase C alpha	Upstate Biotechnology, Inc.	05-154	Mouse	1 in 2000
PKCaphap657	PKCalpha phosphorylation at S657	Upstate Biotechnology, Inc.	06-822	Rabbit	1 in 3000
PML	PML	SDI	2114	Rabbit	1 in 3000
pmTOR	mTOR phosphorylation at S2448	Cell Signaling Technology, Inc.	CS 2971	Rabbit	1 in 150
PR	Progesterone receptor	Epitomics, Inc.	1483-1	Rabbit	1 in 400
PSAT1	PSAT1	SDI	2102	Rabbit	1 in 2000
PTCH	Patche	SDI	2113	Rabbit	1 in 800
PTEN	PTEN	Cell Signaling Technology, Inc.	CS 9552	Rabbit	1 in 500
Rab25	Rab25	Courtesy Dr. Kwai Wa Cheng, MDACC	Covance	Rabbit	1 in 4000
Rb	Retinoblastoma	Cell Signaling Technology, Inc.	CS 9309	Mouse	1 in 3000
RBM15	RBM15	SDI	2139	Rabbit	1 in 2500
Rbp	Rb phosphorylation at S807/S811	Cell Signaling Technology, Inc.	CS 9308	Rabbit	1 in 250
ROPN1	Rhophilin 1	SDI	2105	Rabbit	1 in 600
S6	S6 ribosomal protein	Cell Signaling Technology, Inc.	CS 2217	Rabbit	1 in 200
S6p235-236	S6 phosphorylation at S235/S236	Cell Signaling Technology, Inc.	CS 2211	Rabbit	1 in 3000
S6p240_4	S6 phosphorylation at S240/S244	Cell Signaling Technology, Inc.	CS 2215	Rabbit	1 in 3000
SGK	Serum Glucocorticoid Kinase	Cell Signaling Technology, Inc.	CS 3272	Rabbit	1 in 250
SGKp	SGK phosphorylation at S78	Cell Signaling Technology, Inc.	CS 3271	Rabbit	1 in 250
src	Src	Upstate Biotechnology, Inc.	05-184	Mouse	1 in 200
srcp416	src phosphorylation at Y416	Cell Signaling Technology, Inc.	CS 2101	Rabbit	1 in 150
srcp527	src phosphorylation at Y527	Cell Signaling Technology, Inc.	CS 2105	Rabbit	1 in 400
stat3	Signal transducer and activator of transcription 3	Upstate Biotechnology, Inc.	06-596	Rabbit	1 in 500
stat3p705	stat3 phosphorylation at S705	Cell Signaling Technology, Inc.	CS 9131	Rabbit	1 in 500
stat3p727	stat3 phosphorylation at S727	Cell Signaling Technology, Inc.	CS 9134	Rabbit	1 in 250
stat6p641	stat6 phosphorylation at Y641	Cell Signaling Technology, Inc.	CS 9361	Rabbit	1 in 150
stathmin	Stathmin	Epitomics, Inc.	1972-1	Rabbit	1 in 500
Tau	Microtubule Associated	Santa Cruz	SC-	Mouse	1 in 150

	Protein Tau	Biotechnology, Inc.	58855		
Tau	Microtubule Associated Protein Tau	Upstate Biotechnology, Inc.	05-348	Mouse	1 in 150
TAZ	TAZ	Abcam, Inc.	3961	Rabbit	1 in 250
TAZp	TAZ phosphorylation at S89	Santa Cruz Biotechnology, Inc.	SC-17610R	Rabbit	1 in 250
Telomerase	Telomerase	SDI	1706	Rabbit	1 in 250
TopoII	Topoisomerase II	Abcam, Inc.	45175	Rabbit	1 in 100
TSC2	Tuberous Sclerosis Kinase 2	Epitomics, Inc.	1613-1	Rabbit	1 in 500
TSC2p	TSC2 phosphorylation at T1462	Cell Signaling Technology, Inc.	CS 3617	Rabbit	1 in 200
VEGFR2	KDR2 / VEGF Receptor 2	Cell Signaling Technology, Inc.	CS 2479	Rabbit	1 in 700
XIAP	X linked inhibitor of apoptosis	Cell Signaling Technology, Inc.	CS 2042	Rabbit	1 in 200
YAP	YAP	Santa Cruz Biotechnology, Inc.	15407	Rabbit	1 in 500
YB1	Y-Box Binding Protein 1	SDI	1725	Rabbit	1 in 2500
YKL40	YKL40	Ken Aldape at MDACC		Rabbit	1 in 3000

Table 2. Monospecific antibodies used to profile breast cancers with reverse phase protein arrays (RPPA). Companies: Abcam, Inc. (Cambridge, MA), BD Biosciences (San Jose, CA), Cell Signaling Technology, Inc. (Danvers, MA), Chemicon International Inc. (Billerica, MA), Dako (Carpinteria, CA), Epitomics, Inc. (Burlingame, CA), R&D Systems, (Minneapolis, MN), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), SDI (Newark, DE), Upstate Biotechnology (Millipore) Inc. (Billerica, MA).

Our group has also recently demonstrated the utility of RPPA for the proteomic classification of breast cancer. RPPA data analysis and normalization have been described in detail previously by our group. (106). By applying RPPA with 146 validated antibodies (13-55) directed against important proteins in breast cancer (table 2) to a large number of human breast tumours, and using unsupervised clustering, we identified six major subgroups of breast cancer with significantly different recurrence-free and overall survival outcomes. These six groups included a predominantly HER2 positive group and a hormone receptor (HR)-negative and HER2-negative (triple receptor-negative) group with poor outcomes, a HR positive group with a good outcome and three groups with intermediate outcomes: a HR

positive group with overexpression of proteins including cyclins B1 and E1 as well as components of the protein synthesis machinery including phosphorylated S6 ribosomal protein and 4EBP1, a group with overexpression of stromal markers including collagen VI, CD31 and caveolin1, and a group defined by up-regulation of a relatively large number of proteins and phosphoproteins that span several mechanistic pathways. Using supervised clustering, six breast cancer subgroups were similarly identified by a more compact 10-protein biomarker panel in a 712 human breast tumour training set and these subgroups were associated with markedly different recurrence-free survival (RFS) times (figure 3) (106). The structure and ability of the six subgroups to predict patient outcomes was confirmed in an independent test set of 168 human breast cancers. The 10 protein markers used were ER, PgR, bcl2, EIG121 (oestrogen induced gene 121), GATA3, cyclin B1, cyclin E1, EGFR, HER2 and HER2 phosphorylation at tyrosine 1248. A prognostic score constructed using these 10 proteins in the training set was highly associated with RFS in both training and test sets (p= 3.2E-13, for the test set). Further, there was a significant association between this prognostic score and likelihood of pathologic complete response (pCR) to neoadjuvant systemic chemotherapy (NST).

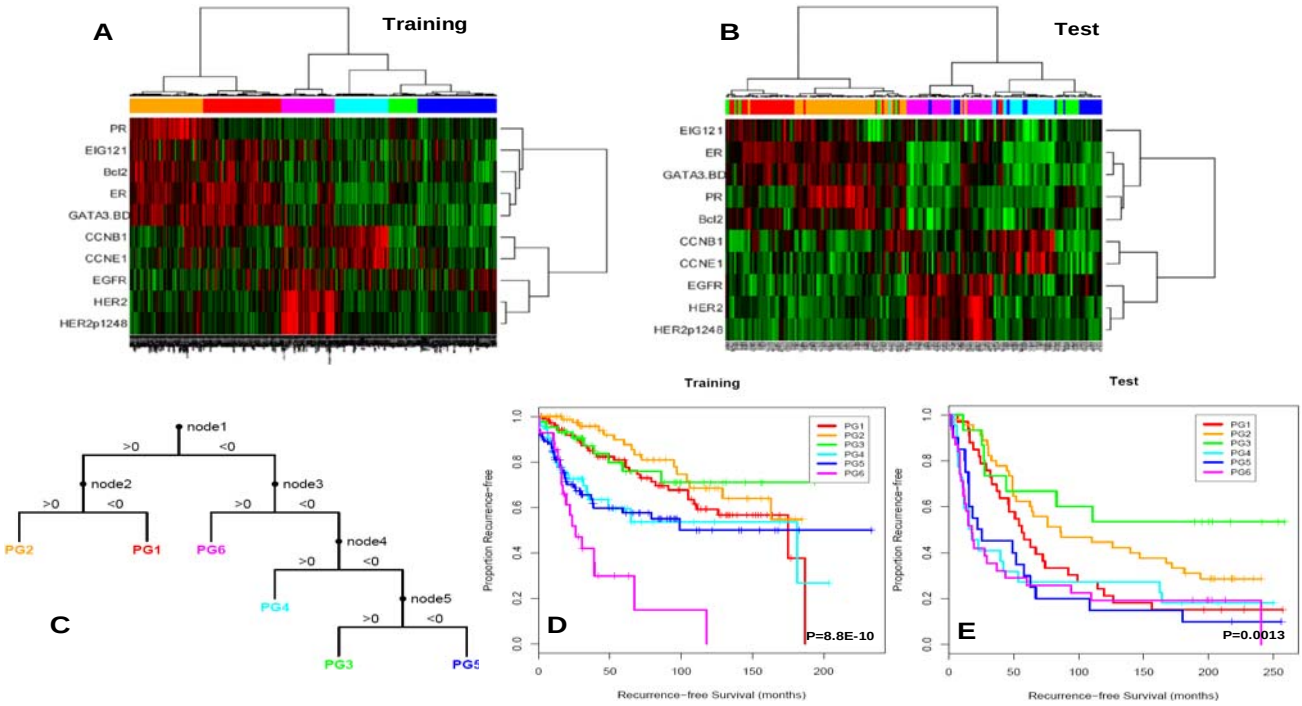

Figure 3: Supervised clustering of breast cancers with quantification data for 10 proteins derived using reverse phase protein arrays (RPPAs). RPPA data were quantified using Microvigene software (VigeneTech Inc., Carlisle, MA) and processed (including normalization) by the R package SuperCurve (version 1.01), available at "http://bioinformatics.mdanderson.org/OOMPA" (as we have detailed previously. (106-111). 712 human breast tumour samples (Training set, 1A) were clustered with the 10 markers using an “uncentered correlation” distance metric along with a linkage rule that increases the robustness of clustering. This analysis yielded six subgroups (BG1-6). 168 human breast tumor samples (Test set, 1B) were subgrouped into one of 6 groups (PG1-6) using the decision tree (1C) that was derived from the training set. Patients in the six subgroups differed significantly in their recurrence-free survival in both training (1D) and test (1E) sets. (Reference 106)

In addition, we have used RPPA to develop an accurate functional proteomic classifier of luminal A vs. luminal B breast cancers (figure 4). (92). This classifier or metric assesses ER function (ER/PgR/Bcl2 expression), HER2 levels and activity (HER2 expression and phosphorylation at Tyrosine 1248 (HERp1248)), apoptosis (cleaved caspase 7/cleaved PARP/Bcl2), protein synthesis (p70S6K expression and

S6 ribosomal protein phosphorylation), cell cycle progression (expression of cyclin B1), and stroma (collagen VI expression) using RPPA.

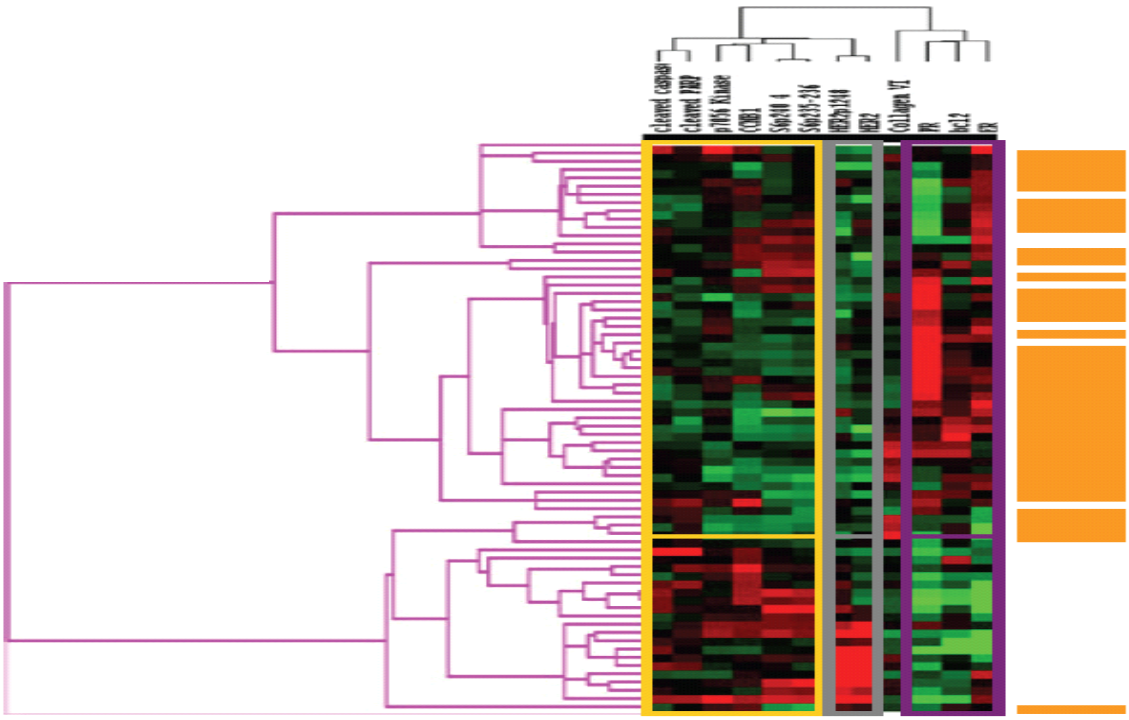

Figure 4: Hierarchical clustering analysis using 12 markers to distinguish luminal A from luminal B breast cancers. RPPA data quantification and normalization are as described in the legend of Figure 3. Luminal A tumours are designated by a brown colour to the right of the heat map. The 12 markers can be subdivided into three functional groups—a proliferation group (cleaved caspase 7, cleaved PARP, CCNB1, p70S6 Kinase, and phosphorylation of ribosomal S6 protein at serines 235–236 (S6p235–236) and 240–244 (S6p240_4)), a receptor tyrosine kinase (RTK) group (HER2/HER2p1248), and a functional ER alpha ("ERness") group (ER, PR, and bcl2). The order of these 12 protein markers from left to right at the top of panel c are: cleaved caspase 7, cleaved PARP, p70S6 Kinase, CCNBI, S6p240_4, S6p235–236, HER2p1248, HER2, Collagen VI, PR, bcl 2, ER.

Despite these recent advances, there are limitations in available quantitative proteomic technologies that need to be overcome. First, there is a limited number of affinity-capture reagents, such as high-quality antibodies, especially phospho-specific antibodies. Second, there is a necessity for development of reference standards so that proteomic profiling can be certified under Clinical Laboratory Improvement

Amendments (CLIA). Third, before the implementation of RPPA in clinical practice, standard sample collection and processing methodologies need to be established. Espina et al have described guidelines for tissue procurement for clinical research with an emphasis on quantifying phosphoproteins by RPPA. (98). For maintenance of protein stability in tissue specimens, snap freezing of tissues is ideal within seconds to minutes. Novel preservation and embedding material that preserve protein conformation and post-translational modifications need to be developed. For example, Mueller et al have originated and evaluated a novel one-step biomarker and histology preservative (BHP) chemistry that preserves the phosphorylation state of several signaling proteins at a level comparable to snap-freezing and retains formalin-like tissue histomorphology with equivalent immunohistochemistry in a single paraffin block. (97). Fourth, the cost-effectiveness of these new technologies needs to be established. (90,112). Ultimately, given that it is a moderate-to-high throughput platform suitable for screening large numbers of patient samples with multiple antibodies, the utility of the RPPA approach will likely be as a discovery tool to allow design of future diagnostic assays using platforms that can be applied to individual patient samples such as quantitative immunofluorescence or enzyme-linked immunosorbent assay (ELISA).

Conclusion

Current progress in genomic technologies has enhanced our understanding of breast cancer with the identification of specific gene expression profiles, and the discovery of assays such as Oncotype DX that assist in vital therapeutic decision making. Much progress has thus been made in genomic classification of breast cancer, with these results already impacting patient care. However, proteins are the ultimate effectors of cellular outcomes, and functional proteomic data represent an under-

evaluated information resource for the identification of useful biomarkers in and classification of solid tumours. RPPA and other emerging functional proteomic assays have the potential to provide cost- and material-effective, high-throughput, comprehensive, sensitive, and quantitative approaches to molecular classification and pathophysiology studies. RPPA, for example, allows exploration of the intricacy of cellular signaling and tumour screening, classification, prognostication and prediction based on this in a manner that cannot be accomplished by genomic studies or by older proteomic approaches such as immunoblotting or IHC. RPPA is likely to have particular significance in complementing genomic assays to classify cancers and define predictive and pharmacodynamic biomarkers for the increasing number of kinase-targeted therapies that are being investigated at present. The future application of emerging functional proteomic technologies will thus allow the identification and validation of proteomic assays that can be successfully applied to the classification of breast cancer and in screening and identification of prognostic and predictive markers.

References

1. Engwegen JY, Gast MC, Schellens JH, Beijnen JH. Clinical proteomics: searching for better tumour markers with SELDI-TOF mass spectrometry. Trends Pharmacol Sci. 2006 May; 27(5):251-9. Epub 2006 Apr 4.
2. Caetano-Anollés D, Kim KM, Mittenthal JE, Caetano-Anollés G. Proteome Evolution and the Metabolic Origins of Translation and Cellular Life. J Mol Evol. 2010 Nov 17.
3. Ming Lu, Stephen A. Whelan, Jianbo He, Romaine E. Saxton, Kym F. Faull, Julian P. Whitelegge, and Helena R. Chang. Hydrophobic Proteome Analysis of Triple Negative and Hormone-Receptor-Positive-Her2-Negative Breast Cancer by Mass Spectrometer. Clin Proteomics. 2010 September; 6(3): 93–103.
4. Solassol J, Marin P, Maudelonde T, Mangé A. Proteomic profiling: the potential of Seldi-Tof for the identification of new cancer biomarkers. Bull Cancer. 2005 Sep; 92(9):763-8.
5. Espina V, Liotta LA, Petricoin EF. Reverse-phase protein microarrays for theranostics and patient tailored therapy. . Center for Applied Proteomics and Molecular Medicine, George Mason University, Manassas, VA, USA. Methods Mol Biol. 2009; 520:89-105
6. Dupuy L, Gauthier C, Durand G, Musnier A, Heitzler D, Herledan A, Sakanyan V, Crépieux P, Reiter E. A highly sensitive near-infrared fluorescent detection method to analyze signalling pathways by reverse-phase protein array. Proteomics. 2009 Dec; 9(24):5446-54
7. Brase JC, Mannsperger H, Fröhlich H, Gade S, Schmidt C, Wiemann S, Beissbarth T, Schlomm T, Sültmann H, Korf U. Increasing the sensitivity of reverse phase protein arrays by antibody-mediated signal amplification. Proteome Sci. 2010 Jun 22; 8:36.
8. Gilabert M, Audebert S, Viens P, Borg JP, Bertucci F, Gonçalves A. Proteomics and breast cancer: a search for novel diagnostic and theragnostic biomarkers. Bull Cancer. 2010 Mar; 97(3):321-39.
9. Pietrowska M, Polanska J, Marczak L, Behrendt K, Nowicka E, Stobiecki M, Polanski A, Tarnawski R, Widlak P. Mass spectrometry-based analysis of therapy-related changes in serum proteome patterns of patients with early-stage breast cancer. J Transl Med. 2010 Jul 11; 8:66.
10. Janet L, Moyses S, Loise A. Estrogen receptors and breast cancer. epidemiologic reviews1986; vol 8: 42-59
11. Sorlie T, Perou M, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen B, van de Rijn M, Jeffrey S, Thorsen T, Quist H, Matese C, Brown O, Botstein D, Eystein Lønning P, Børresen-Dale L. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001; 98:10869–10874.
12. Cheang M, Chia S, Voduc D, Gao D, Leung S, Snider J, Watson M, Davies S, Bernard P, Parker J, Perou C, Ellis M, and Nielsen T. Ki67 Index, HER2 Status, and Prognosis of Patients with Luminal B Breast Cancer. J Natl Cancer Inst. 2009 May 20; 101(10): 736–750.
13. Elizabeth H. Hammond, Daniel F. Hayes, Antonio C. Wolff, FACP, Pamela B. Mangu, Sarah Temin. American Society of Clinical Oncology/College of American Pathologists Guideline Recommendations for Immunohistochemical Testing of Estrogen and Progesterone Receptors in Breast Cancer, J Oncol Pract. 2010 July; 6(4): 195–197.

14. Tamoxifen for early breast cancer. An overview of the randomised trials. Early Breast Cancer Trialists' Collaborative Group. Lancet. 1998; 351 (9114): 1451 – 1467.
15. Howell A, Cuzick J, Baum M, et al. Results of the ATAC (Arimidex, Tamoxifen, Alone or in Combination) trial after completion of 5 years 'adjuvant treatment for breast cancer. Lancet. 2005; 365 (9453): 60 – 62.
16. Mauri D, Pavlidis N, Polyzos NP, Ioannidis JP. Survival with aromatise inhibitors and inactivators versus standard hormonal therapy in advanced breast cancer: meta-analysis. J Natl Cancer Inst. 2006; 98 (18): 1285 – 1291.
17. Conforti R, Boulet T, Tomasic G, et al. (2007) Breast cancer molecular subclassification and estrogen receptor expression to predict efficacy of adjuvant anthracyclines-based chemotherapy: A biomarker study from two randomized trials. Ann Oncol 18:1477–1483.
18. Berry DA, Cirrincione C, Henderson IC, et al. (2006) Estrogen-receptor status and outcomes of modern chemotherapy for patients with node-positive breast cancer. JAMA 295:1658–1667.
19. Miyoshi Y, Murase K, Saito M, Oh K. Prediction of hormone sensitivity for breast cancers. Breast Cancer. 2010 Apr; 17(2):86-91.
20. Hoadley KA, Weigman VJ, Fan C, Sawyer LR, He X, Troester MA, Sartor CI, Rieger-House T, Bernard PS, Carey LA, Perou CM. EGFR associated expression profiles vary with breast tumor subtype. BMC Genomics. 2007 Jul 31; 8:258
21. Sircoulomb F, Bekhouche I, Finetti P, Adélaïde J, Ben Hamida A, Bonansea J, Raynaud S, Innocenti C, Charafe-Jauffret E, Tarpin C, Ben Ayed F, Viens P, Jacquemier J, Bertucci F, Birnbaum D, Chaffanet M. Genome profiling of ERBB2-amplified breast cancers. BMC Cancer. 2010 Oct 8; 10:539
22. Abd El-Rehim M, Pinder S, Paish C, Bell J, Rampaul R, Blamey R, Robertson J, Nicholson R, Ellis I. Expression and co-expression of the members of the epidermal growth factor receptor (EGFR) family in invasive breast carcinoma. Br J Cancer. 2004 October 18; 91(8): 1532–1542.
23. Pauletti G, Godolphin W, Press MF, Slamon DJ. Detection and quantitation of HER-2/neu gene amplification in human breast cancer archival material using fluorescence in situ hybridization. Oncogene. 1996; 13:63–72.
24. Sainsbury JR, Farndon JR, Needham GK, Malcolm AJ, Harris AL. Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. Lancet. 1987;1:1398–1402.
25. Slamon DJ, Leyland-Jones B, Shak S, et al. Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 2001; 15:783±92.
26. Paik S, Kim C, Wolmark N. HER2 status and benefit from adjuvant trastuzumab in breast cancer. 2008 N Engl J Med 358:1409–1411.
27. Moulder S. Novel cytotoxic backbones and targeted therapies: recent data and ongoing clinical trials. Journal: Clinical Breast Cancer Sept 2009 Volume: 10: S30
28. Perez EA, Reinholz MM, Hillman DW, et al: HER2 and chromosome 17 effect on patient outcome in the N9831 adjuvant trastuzumab trial. J Clin Oncol doi:10.1200/JCO.2009.26.2154
29. Jeffrey S. Ross. Human Epidermal Growth Factor Receptor 2 Testing in 2010: Does Chromosome 17 Centromere Copy Number Make Any Difference? JCO October 1, 2010 vol. 28 no. 28 4293-4295

30. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, Klos KS, Li P, Monia BP, Nguyen NT, Hortobagyi GN, Hung MC, Yu D. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. Cancer Cell. 2004 Aug;6(2):117-27
31. Crowder RJ, Lombardi DP, Ellis MJ. Successful targeting of ErbB2 receptors-is PTEN the key? Cancer Cell. 2004 Aug;6(2):103-4
32. Lemoine NR, Barnes DM, Hollywood DP, Hughes CM, Smith P, Dublin E, Prigent SA, Gullick WJ, Hurst HC. Expression of the ERBB3 gene product in breast cancer. Br J Cancer. 1992 Dec; 66(6):1116-21
33. Naidu R, Yadav M, Nair S, Kutty MK. Expression of c-erbB3 protein in primary breast carcinomas. Br J Cancer. 1998 Nov; 78(10):1385-90
34. Alimandi M, Romano A, Curia MC, Muraro R, Fedi P, Aaronson SA, Di Fiore PP, Kraus MH. Cooperative signaling of ErbB3 and ErbB2 in neoplastic transformation and human mammary carcinomas. Oncogene. 1995;10:1813–1821.
35. Gerdes J, Li L, Schlueter C, et al. Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol. 1991; 138:867–73.
36. Trihia H, Murray S, Price K, et al. Ki-67 expression in breast carcinoma: its association with grading systems, clinical parameters, and other prognostic factors — a surrogate marker? Cancer. 2003; 97 (5): 1321 – 1331.
37. Jones RL, Salter J, A'Hern R, et al. The prognostic significance of Ki67 before and after neoadjuvant chemotherapy in breast cancer Breast Cancer Res Treat 2009. 11653–68.Epub 2008 Jul 1.
38. Dowsett M, Smith IE, Ebbs SR, Dixon JM, Skene A, A'Hern R, Salter J, Detre S, Hills M, Walsh G; Prognostic value of Ki67 expression after short-term presurgical endocrine therapy for primary breast cancer. IMPACT Trialists Group. J Natl Cancer Inst. 2007 Jan 17; 99(2):167-70.
39. Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, Ellis M, Henry NL, Hugh JC, Lively T, McShane L, Paik S, Penault-Llorca F, Prudkin L, Regan M, Salter J, Sotiriou C, Smith IE, Viale G, Zujewski JA, Hayes DF; International Ki-67 in Breast Cancer Working Group. Assessment of Ki67 in breast cancer: recommendations from the International Ki67 in Breast Cancer working group. J Natl Cancer Inst. 2011 Nov 16;103(22):1656-64.
40. Olopade O. I., Weber B. I. Breast cancer genetics. Toward molecular characterization of individuals at increased risk for breast cancer: Part I. PPO Updates: Princ. Pract. Oncol., 12: 1-12, 1998.
41. Tatyana A. Grushko, M. Anne Blackwood, Phil L. Schumm, Fitsum G. Hagos, Moses O. Adeyanju, Michael D. Feldman, Melinda O. Sanders, Barbara L. Weber, and Olufunmilayo I. Olopade. Molecular-Cytogenetic Analysis of HER-2/neu Gene in BRCA1-associated Breast Cancers
42. Graeser MK, Engel C, Rhiem K, et al. Contralateral breast cancer risk in BRCA1 and BRCA2 mutation carriers. J Clin Oncol. 2009; 27: 5887–92.
43. Pankaj Taneja,1,2 Dejan Maglic,1,2,3 Fumitake Kai,1,2 Sinan Zhu,1,2,3 Robert D. Kendig,1,2 Elizabeth A. Fry,1,2 and Kazushi Inoue. Classical and Novel Prognostic Markers for Breast Cancer and their Clinical Significance. Clin Med Insights Oncol. 2010; 4: 15–34. Published online 2010 April 20.
44. Pharaoh PD, Day NE, Caldas C: Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis. Br J Cancer 1999, 80:1968-1973.

45. Smith PD, Crossland S, Parker G, Osin P, Brooks L, Waller J, Philp E, Crompton MR, Gusterson BA, Allday MJ, Crook T: Novel p53 mutants selected in BRCA-associated tumours which dissociate transformation suppression from other wild-type p53 functions. Oncogene 1999 , 18:2451-2459.
46. Shin I, Miller T, Arteaga CL. ErbB receptor signaling and therapeutic resistance to aromatase inhibitors. Clin Cancer Res 2006;12(3 Pt 2):1008s-1012s.
47. Kurokawa H, Arteaga CL. ErbB (HER) receptors can abrogate antiestrogen action in human breast cancer by multiple signaling mechanisms. Clin Cancer Res 2003;9(1 Pt 2):511S-515S.
48. Paik S, Shak S, Tang G, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med 2004;351:2817-2826.
49. Arpino G, Weiss H, Lee AV, et al: Estrogen receptor-positive, progesterone receptor-negative breast cancer: Association with growth factor receptor expression and tamoxifen resistance. J Natl Cancer Inst 2005;97:1254-1261.
50. Hennessy BT, Mills GB. Exploiting the PI3K/AKT pathway for cancer drug discovery. Nat Rev Drug Discov 2005;4:988-1004.
51. Saal LH, Holm K, Maurer M, et al. PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma. Cancer Res 2005;65:2554-2559.
52. Bellacosa A, de Feo D, Godwin AK, et al. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. Int J Cancer 1995;64:280-285.
53. Monni O, Barlund M, Mousses S, et al. Comprehensive copy number and gene expression profiling of the 17q23 amplicon in human breast cancer. Proc Natl Acad Sci U S A 2001;98:5711-5716.
54. Guarino M. Src signaling in cancer invasion. J Cell Physiol. 2010 Apr;223(1):14-26.
55. Mayer EL, Krop IE. Advances in targeting SRC in the treatment of breast cancer and other solid malignancies. Clin Cancer Res. 2010 Jul 15; 16(14):3526-32.
56. Mitra D, Brumlik MJ, Okamgba SU, Zhu Y, Duplessis TT, Parvani JG, Lesko SM, Brogi E, Jones FE. An oncogenic isoform of HER2 associated with locally disseminated breast cancer and trastuzumab resistance. Mol Cancer Ther. 2009 Aug; 8(8):2152-62.
57. Zhang S, Huang WC, Li P, Guo H, Poh SB, Brady SW, Xiong Y, Tseng LM, Li SH, Ding Z, Sahin AA, Esteva FJ, Hortobagyi GN, Yu D. Combating trastuzumab resistance by targeting SRC, a common node downstream of multiple resistance pathways. Nat Med. 2011 Apr; 17(4):461-9.
58. Campone M, Bondarenko I, Brincat S, Hotko Y, Munster PN, Chmielowska E, Fumoleau P, Ward R, Bardy-Bouxin N, Leip E, Turnbull K, Zacharchuk C, Epstein RJ. Phase II study of single-agent bosutinib, a Src/Abl tyrosine kinase inhibitor, in patients with locally advanced or metastatic breast cancer pretreated with chemotherapy. Ann Oncol. 2011 Jun 23.
59. Mayer EL, Baurain JF, Sparano J, Strauss L, Campone M, Fumoleau P, Rugo H, Awada A, Sy O, Llombart-Cussac A. A phase 2 trial of dasatinib in patients with advanced HER2-positive and/or hormone receptor-positive breast cancer. Clin Cancer Res. 2011 Nov 1;17(21):6897-904.
60. Finn RS, Bengala C, Ibrahim N, Roché H, Sparano J, Strauss LC, Fairchild J, Sy O, Goldstein LJ. Dasatinib as a single agent in triple-negative breast cancer: results of an open-label phase 2 study. Clin Cancer Res. 2011 Nov 1;17(21):6905-13.

61. Sutherland RL, Musgrove EA. Cyclins and breast cancer. J Mammary Gland Biol Neoplasia. 2004 Jan; 9(1):95-104
62. Yang C, Chen L, Li C, Lynch MC, Brisken C, Schmidt EV. Cyclin D1 enhances the response to estrogen and progesterone by regulating progesterone receptor expression. Mol Cell Biol. 2010 Jun; 30(12):3111-25.
63. Aaltonen K, Amini RM, Landberg G, Eerola H, Aittomäki K, Heikkilä P, Nevanlinna H, Blomqvist C. Cyclin D1 expression is associated with poor prognostic features in estrogen receptor positive breast cancer. Breast Cancer Res Treat. 2009 Jan;113(1):75-82.
64. Agarwal R, Gonzalez-Angulo AM, Myhre S, Carey MS, Lee JS, Overgaard J, Alsner J, Stemke-Hale K, Lluch A, Neve RM, Kuo WL, Sorlie T, Sahin A, Valero V, Keyomarsi K, Gray JW, Borresen Dale AL, Mills GB, Hennessy BT. Integrative analysis of cyclin protein levels identifies cyclin B1 as a classifier and predictor of outcomes in breast cancer. Clin Cancer Res, 2009.
65. Lyman GH, Cosler LE, Kuderer NM, Hornberger J. Impact of a 21-gene RT-PCR assay on treatment decisions in early-stage breast cancer: an economic analysis based on prognostic and predictive validation studies. Cancer. 2007 Mar 15; 109(6):1011-8.
66. Ishibe N, Schully S, Freedman A, Ramsey SD. Use of Oncotype DX in Women with Node-Positive Breast Cancer. PLoS Curr. 2011 Jul 21;3:RRN1249.
67. Lee JJ, Shen J. Is the Oncotype DX assay necessary in strongly estrogen receptor-positive breast cancers? Am Surg. 2011 Oct;77(10):1364-7.
68. Zujewski JA, Kamin LTrial assessing individualized options for treatment for breast cancer: the TAILORx trial. Future Oncol. 2008 Oct;4(5):603-10
69. Wulfkuhle JD, Edmiston KH, Liotta LA, Petricoin EF 3rd. Technology insight: pharmacoproteomics for cancer--promises of patient-tailored medicine using protein microarrays. Nat Clin Pract Oncol (2006) 3(5):256-68.
70. Medintz IL, Uyeda HT, Goldman ER, Mattoussi H. Quantum dot bioconjugates for imaging, labelling and sensing. Nat Mater (2005) 4(6):435-46.
71. Tada H, Higuchi H, Wanatabe TM, Ohuchi N. In vivo real-time tracking of single quantum dots conjugated with monoclonal anti-HER2 antibody in tumors of mice. Cancer Res (2007) 67(3):1138-44.
72. Press MF, Slamon DJ, Flom KJ, Park J, Zhou JY, Bernstein L. Evaluation of HER-2/neu gene amplification and overexpression: comparison of frequently used assay methods in a molecularly characterized cohort of breast cancer specimens. J Clin Oncol (2002) 20(14):3095-105.
73. Leader M, Patel J, Makin C, Henry K. An analysis of the sensitivity and specificity of the cytokeratin marker CAM 5.2 for epithelial tumours. Results of a study of 203 sarcomas, 50 carcinomas and 28 malignant melanomas. Histopathology (1986) 10(12):1315-24.
74. Paweletz CP, Charboneau L, Bichsel VE, et al. Reverse phase protein microarrays which capture disease progression show activation of pro-survival pathways at the cancer invasion front. Oncogene. 2001; 20:1981–9.
75. Diamandis E, Merwe D. plasma protein profiling by mass spectrometry for cancer diagnosis: opportunities and limitations. Clinical cancer research Feb 2005, Vol. 11,963-5.
76. Gross JH. Liquid injection field desorption/ionization-mass spectrometry of ionic liquids. J Am Soc Mass Spectrom. 2007 Dec; 18(12):2254-62

77. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature 2003; 422:198–207. Petricoin EF III, Ardekani AM, Hitt BA, et al. Use of proteomic patterns in serum to identify ovarian cancer. Lancet 2002; 359:572–5.
78. Stoeckli M, Chaurand P, Hallahan DE, Caprioli RM. Imaging mass spectrometry: a new technology for the analysis of protein expression in mammalian tissues. Nat Med. 2001 Apr;7(4):493-6.
79. Seeley EH, Caprioli RM. Molecular imaging of proteins in tissues by mass spectrometry. Proc Natl Acad Sci U S A. 2008 Nov 25;105(47):18126-31.
80. Aerni HR, Cornett DS, Caprioli RM. High-throughput profiling of formalin-fixed paraffin-embedded tissue using parallel electrophoresis and matrix-assisted laser desorption ionization mass spectrometry. Anal Chem. 2009 Sep 1; 81(17):7490-5.
81. Schwamborn K, Caprioli RM. MALDI imaging mass spectrometry--painting molecular pictures. Mol Oncol. 2010 Dec;4(6):529-38.
82. Bauer JA, Chakravarthy AB, Rosenbluth JM, Mi D, Seeley EH, De Matos Granja-Ingram N, Olivares MG, Kelley MC, Mayer IA, Meszoely IM, Means-Powell JA, Johnson KN, Tsai CJ, Ayers GD, Sanders ME, Schneider RJ, Formenti SC, Caprioli RM, Pietenpol JA. Identification of markers of taxane sensitivity using proteomic and genomic analyses of breast tumors from patients receiving neoadjuvant paclitaxel and radiation. Clin Cancer Res. 2010 Jan 15;16(2):681-90.
83. Girish S, Gupta M, Wang B, Lu D, Krop IE, Vogel CL, Burris Iii HA, Lorusso PM, Yi JH, Saad O, Tong B, Chu YW, Holden S, Joshi A. Clinical pharmacology of trastuzumab emtansine (T-DM1): an antibody-drug conjugate in development for the treatment of HER2-positive cancer. Cancer Chemother Pharmacol. 2012 Jan 20.
84. Zhang K, Yuan K, Wu H, Li Q, Wang Y, Chen S, Zhang L, Gu H, Fu R. Identification of Potential Markers Related to Neoadjuvant Chemotherapy Sensitivity of Breast Cancer by SELDI-TOF MS. Appl Biochem Biotechnol. 2012 Feb; 166(3):753-63.
85. Yang WS, Moon HG, Kim HS, Choi EJ, Yu MH, Noh DY, Lee C. Proteomic Approach Reveals FKBP4 and S100A9 as Potential Prediction Markers of Therapeutic Response to Neoadjuvant Chemotherapy in Patients with Breast Cancer. J Proteome Res. 2012 Feb 3; 11(2):1078-88.
86. Andersson M, Andren P, Caprioli RM. MALDI Imaging and Profiling Mass Spectrometry in Neuroproteomics. Neuroproteomics. Alzate O, editor. Boca Raton (FL): 2010: Chapter 7.
87. De Wildt RM, Mundy CR, Gorick BD, Tomlinson IM. Antibody arrays for high-throughput screening of antibody-antigen interactions. Nat Biotechnol. 2000 Sep;18(9):989-94.
88. Cahill DJ. Protein and antibody arrays and their medical applications. J Immunol Methods. 2001 Apr;250(1-2):81-91.
89. Vazquez-Martin A, Colomer R, Menendez JA. Protein array technology to detect HER2 (erbB-2)-induced 'cytokine signature' in breast cancer. Eur J Cancer. 2007 May;43(7):1117-24.
90. Sheehan KM, Calvert VS, Kay EW, Lu Y, Fishman D, Espina V, Aquino J, Speer R, Araujo R, Mills GB, Liotta LA, Petricoin EF 3rd, Wulfkuhle JD. Use of reverse phase protein microarrays and reference standard development for molecular network analysis of metastatic ovarian carcinoma. Mol Cell Proteomics. 2005 Apr; 4(4):346-55

91. Espina V, Mehta AI, Winters ME, Calvert V, Wulfkuhle J, Petricoin EF 3rd, Liotta LA. Protein microarrays: molecular profiling technologies for clinical specimens. Proteomics (2003) 3(11):2091-100.
92. Hennessy BT, Lu Y, Gonzalez-Angulo AM, Carey MS, Myhre S, Ju Z, Davies MA, Liu W, Coombes K, Meric-Bernstam F, Bedrosian I, et al: A technical assessment of the utility of reverse phase protein arrays for the study of the functional proteome in non-microdissected human breast cancers. Clin Proteomics. 2010 Dec; 6(4):129-51.
93. Wulfkuhle JD, Speer R, Pierobon M, et al. Multiplexed cell signaling analysis of human breast cancer applications for personalized therapy. J Proteome Res. 2008;7:1508–17.
94. Petricoin EF 3rd, Bichsel VE, Calvert VS, et al. Mapping molecular networks using proteomics: a vision for patienttailored combination therapy. J Clin Oncol. 2005; 23:3614 –21.
95. Ibarra-Drendall C, Troch MM, Barry WT, Broadwater G, Petricoin EF 3rd, Wulfkuhle J, Liotta LA, Lem S, Baker JC Jr, Ford AC, Wilke LG, Zalles C, Kuderer NM, Hoffman AW, Shivraj M, Mehta P, Williams J, Tolbert N, Lee LW, Pilie PG, Yu D, Seewaldt VL. Pilot and feasibility study: prospective proteomic profiling of mammary epithelial cells from high-risk women provides evidence of activation of pro-survival pathways. Breast Cancer Res Treat. 2011 Jun 7.
96. Rapkiewicz A, Espina V, Zujewski JA, Lebowitz PF, Filie A, Wulfkuhle J, Camphausen K, Petricoin EF 3rd, Liotta LA, Abati A. The needle in the haystack: application of breast fine-needle aspirate samples to quantitative protein microarray technology. Cancer. 2007 Jun 25;111(3):173-84.
97. Mueller C, Edmiston KH, Carpenter C, Gaffney E, Ryan C, Ward R, White S, Memeo L, Colarossi C, Petricoin EF 3rd, Liotta LA, Espina V. One-step preservation of phosphoproteins and tissue morphology at room temperature for diagnostic and research specimens. PLoS One. 2011;6(8):e23780.
98. Espina V, Mueller C, Liotta LA. Phosphoprotein stability in clinical tissue and its relevance for reverse phase protein microarray technology. Methods Mol Biol. 2011;785:23-43.
99. Espina V, Edmiston KH, Heiby M, Pierobon M, Sciro M, Merritt B, Banks S, Deng J, VanMeter AJ, Geho DH, Pastore L, et al: A portrait of tissue phosphoprotein stability in the clinical tissue procurement process. Mol Cell Proteomics (2008) 7(10):1998-2018.
100. Grubb RL, Deng J, Pinto PA, et al. Pathway biomarker profiling of localized and metastatic human prostate cancer reveal metastatic and prognostic signatures. J Proteome Res. 2009; 8:3044–54.
101. Sheehan KM, Gulmann C, Eichler GS, et al. Signal pathway profiling of epithelial and stromal compartments of colonic carcinoma reveal epithelial-mesenchymal transition. Oncogene. 2008; 27:323–31.
102. Ornstein DK, Gillespie JW, Paweletz CP, et al. Proteomic analysis of laser capture microdissected human prostate cancer and in vitro prostate cell lines. Electrophoresis. 2000; 21:2235–42.
103. Emmert-Buck MR, Gillespie JW, Paweletz CP, et al. An approach to proteomic analysis of human tumors. Mol Carcinog. 2000; 27:158–65.
104. Dillon RL, Marcotte R, Hennessy BT, Woodgett JR, Mills GB, Muller WJ. Akt1 and akt2 play distinct roles in the initiation and metastatic phases of mammary tumor progression. Cancer Res (2009) 69(12):5057-64.

105. Boyd ZS, Wu QJ, O'Brien C, Spoerke J, Savage H, Fielder PJ, Amler L, Yan Y, Lackner MR. Proteomic analysis of breast cancer molecular subtypes and biomarkers of response to targeted kinase inhibitors using reverse-phase protein microarrays. Mol Cancer Ther (2008) 7(12):3695-706.
106. Gonzalez-Angulo AM, Hennessy BT, Meric-Bernstam F, Sahin A, Liu W, Ju Z, Carey MS, Myhre S, Speers C, Deng L, Broaddus R, Lluch A, Aparicio S, Brown P, Pusztai L, Symmans WF, Alsner J, Overgaard J, Borresen-Dale AL, Hortobagyi GN, Coombes KR, Mills GB. Functional proteomics can define prognosis and predict pathologic complete response in patients with breast cancer. Clin Proteomics. 2011 Jul 8;8(1):11.
107. Stemke-Hale K, Gonzalez-Angulo AM, Lluch A, Neve RM, Kuo WL, Davies M, Carey M, Hu Z, Guan Y, Sahin A, Symmans WF, Pusztai L, Nolden LK, Horlings H, Berns K, Hung MC, van de Vijver MJ, Valero V, Gray JW, Bernards R, Mills GB, Hennessy BT.An integrative genomic and proteomic analysis of PIK3CA, PTEN and AKT mutations in breast cancer. Cancer Res. 2008;68:6084–91. doi: 10.1158/0008-5472.CAN-07-6854.
108. Tibes R, Qiu Y, Lu Y, Hennessy B, Mills GB, Kornblau S. Reverse phase protein array (RPPA) Validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. Mol Cancer Ther. 2006. pp. 2512–21.
109. Liang J, Shao SH, Xu ZX, Hennessy B, Ding Z, Larrea M, Kondo S, Dumont DJ, Gutterman JU, Walker CL, Slingerland JM, Mills GB.The energy sensing LKB1-AMPK pathway regulates p27kip1 phosphorylation mediating the decision to enter autophagy or apoptosis. Nat Cell Biol. 2007;9:218–24. doi: 10.1038/ncb1537.
110. Hu J, He X, Baggerly KA, Coombes KR, Hennessy BT, Mills GB. Non-parametric quantification of protein lysate arrays. Bioinformatics. 2007;23:1986–94. doi: 10.1093/bioinformatics/btm283.
111. Hennessy BT, Lu Y, Poradosu E, Liu Q, Yu S, Hall H. et al. Quantified pathway inhibition as a pharmacodynamic marker facilitating optimal targeted therapy dosing: Proof of principle with the AKT inhibitor perifosine. Clin Cancer Res. 2007;13:7421–31. doi: 10.1158/1078-0432.CCR-07-0760.
112. Liotta LA, Espina V, Mehta AI, Calvert V, Rosenblatt K, Geho D, Munson PJ, Young L, Wulfkuhle J, Petricoin EF 3rd. Protein microarrays: meeting analytical challenges for clinical applications. Cancer Cell (2003) (4):317-25.