Abstract. Completion of the human genome project has revolutionised translational medicine. High-throughput technology now permits investigators to systematically interrogate the genome, transcriptome, proteome and metabolome. It is expected that these advances will eventually be translated into new more sensitive diagnostic tests and less toxic therapeutics. A major shift is expected in clinical oncology over the next few decades as we start to move away from currently practiced, population-based approaches to personalised medicine. In this emerging approach, the molecular and pathophysiological characteristics of an individual patient and tumour will be measured and tailored therapeutic regimens will be administered based on these profiles. One of the key steps in this process will be the identification and validation of biomarkers. Whilst great advances have been made in the discovery of putative biomarkers, disappointingly few have been translated into clinically applicable assays. It is widely believed that this is due to a lack of well-designed, thorough validation studies. Here, we review the role of DNA microarrays and tissue microarrays in the validation of biomarkers in breast cancer, with emphasis on their potential application to determine mode of personalised therapy in the future.

The completion of the human genome project, allied with major technological advances in the post-genomic era, offer the opportunity to develop new and exciting approaches to the practice of clinical oncology. In particular, the potential now exists to gather increasingly complex biomedical and molecular data to develop a coherent system of personalised medicine. This could be defined as a process whereby molecular and pathophysiological characteristics of an individual patient would first be measured. Following this, a personalised therapeutic regimen would then be administered to individual patients, rather than a broader population-based approach, as is currently practiced. Technologies such as DNA microarrays, mass spectrometry-based proteomics and metabolomics have revolutionised translational research over the last decade. The fundamental premise of -omic technology is that comprehensive examination of changes in the genome (DNA), transcriptome (mRNA), proteome (proteins), or metabolome (metabolites) can provide insight into the physiology and mechanism of disease, with this having superior diagnostic and therapeutic value than that currently available. Cancer research has been to the forefront in attempts towards translation of these advanced technological approaches to clinical practice (1).

The application of DNA microarray technology, in particular, has led to an ever-growing comprehension of the complexity of the underlying pathophysiological pathways and interactions within a tumour (2, 3). Transcriptomic screens have greatly accelerated research into genotypic-phenotypic correlations, with a common aim being to elucidate the functional taxonomy of genes in both normal tissues and disease states, such as cancer. In theory, the identification of a pattern or profile of several biomarkers (representing, for example, a combination of genes, proteins, metabolites and/or a physiological response) in a given condition might bring a new dimension to disease diagnosis, classification, intervention and the assessment of therapeutic responses (4). As illustrated in Figure 1, the

Review

Contribution of DNA and Tissue Microarray Technology to the Identification and Validation of Biomarkers and Personalised Medicine in Breast Cancer

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Key Words: Breast cancer, DNA microarray, tissue microarray, personalised medicine, review.
identification and validation of new clinically applicable biomarkers will play a key role in the deliverance of personalised therapeutic regimens. Additionally, the identification of economically acceptable biomarkers that permit early diagnosis or improved risk stratification would lead to a decrease in health care costs.

It should be noted, however, that DNA microarray experiments are currently relatively expensive and produce large volumes of data that require validation. In the reduction to clinical utility, the use of tissue microarrays (TMAs) has become a common methodology to both validate and generalise the results arising from DNA microarray experiments (5).

The aim of this article is to review the role of DNA microarrays and TMAs in the identification and validation of new biomarkers in breast cancer and how this may lead to individualised treatment protocols. Particular emphasis will be placed on producing clinically applicable assays and how a number of groups have approached this extremely difficult scenario.

The Challenges of Breast Cancer

Our understanding of the underlying molecular mechanisms in breast cancer has increased exponentially over the last two decades (3, 6-10), and lies beyond the boundaries of any single review article. There are a number of useful, well-validated breast cancer biomarkers available (discussed below); however, advances in -omics approaches have started to yield an abundance of putative biomarkers, albeit with these still requiring validation.

It is now well recognised that the characteristics of an individual tumour and its life course results from multiple somatic mutations acquired over time (e.g. TP53, PTEN, RAS) and continual evolution of the responses to environmental factors (e.g., estrogen or tobacco exposure). In some situations these somatic mutations overlie inherent germline variations (e.g., BRCA1/2) (11). Conventional wisdom would now suggest that breast cancer is most probably a collection of complex inter-related diseases as represented by an immense natural heterogeneity in tumour phenotypes, disease outcomes, and response to therapies.

Conventional prognostic indicators for breast cancer include tumour size and grade, as well as lymph node and estrogen receptor (ER) status. Unfortunately, these indicators remain relatively crude and fall well short of being capable of describing the disease complexity suggested above. The challenge, as well as the opportunity, of personalised medicine lies in the capacity to develop quantitative data that can match the complexity of the disease (11). For example, due to the lack of accurate prognostic indicators, the majority of pre-menopausal

Figure 1. The Role of Biomarkers in Personalised Medicine. Biomarkers can be applied to various different areas in the clinical management of breast cancer from screening to diagnosis to detecting recurrence. Clinically applicable biomarkers that are effective at earlier points in the pathogenesis of the disease, such as risk stratification, should lead to significant decreases in health care costs.
women with node-negative disease receive adjuvant chemotherapy; however, the absolute survival benefit from treatment is only 3% at 5 years and 5% at 10 years (12). It has been estimated that 70-80% of these patients, who are inherently low-risk, would have survived without adjuvant therapy and thus avoided potentially toxic side effects.

A major challenge is to identify and thoroughly validate diagnostic and prognostic biomarkers that can accurately describe the complexity and heterogeneity ascribed to breast cancer. Additionally, accurate predictive biomarkers are required to guide current treatment protocols, as well as to guide the development and application of new targeted therapies (3).

What is a Biomarker?

The word “biomarker” is a widely used term. The Food and Drug Administration (FDA) have defined a biomarker as a factor that can be objectively and precisely measured and reflects a physiological or pathological process or a therapeutic response (13). In general, biomarkers have been classified as being either prognostic or predictive; however, they can also play a role in risk stratification, screening, aiding diagnosis and detecting recurrence (Figure 1).

A biomarker can range from being an observable clinical characteristic (e.g. patient performance status or body mass index) to quantifiable changes in the genome, transcriptome, proteome or metabolome. A clinically appropriate biomarker should act as surrogate marker/endpoint – a measurement made on a patient before, during and after therapy to determine the efficacy of the treatment. A biomarker acting as a surrogate endpoint is recognised as being able to predict clinical benefit based on epidemiologic, therapeutic, pathophysiological or other scientific evidence and is generally classified as being either prognostic or predictive (14). As previously stated, it is a widely held belief that the identification, testing and validation of clinically appropriate and economically sound biomarkers will permit the individualisation of therapy. Additionally, the use of biomarkers should enhance the way drugs are developed, as new innovative approaches will utilise the ever-accumulating knowledge of molecular mechanisms to develop better drugs for a more targeted market. At a recent EMBO Molecular Medicine Conference the importance of biomarkers in the development of personalised medicine was discussed in detail (15).

Biological therapy is the most rapidly expanding treatment modality in breast cancer and there is a concerted effort to identify agents with highly specific activities and minimal side effects (16). Biological therapeutics range from antibodies that form complexes with antigens on the surface of the cancer cell to small molecules that have been designed to inhibit critical enzymatic reactions. As the drug targets in question are usually specific to or are over-expressed in cancer cells, the new agents generally have fewer side effects than most conventional chemotherapeutic agents. Moreover, when the targeted agents are combined with single-agent chemotherapy, toxicity is only minimally increased (17).

Many of these agents have been developed following the identification of clinically appropriate biomarkers. Notably, monoclonal antibodies with neutralising activity and tyrosine kinase inhibitors have shown most promise. Herceptin (Trastuzumab), a monoclonal antibody specifically recognising the HER2 (ERBB2) receptor, was the first such agent to be approved for treatment of patients with metastatic breast cancer (18) and is currently routinely used in both the metastatic and adjuvant settings for patients with HER2-positive tumours. However, resistance to Herceptin is becoming increasingly evident and may prove to be a significant clinical dilemma in the near future. Lapatinib, an orally administered small-molecule inhibitor of the tyrosine kinase domains of HER1 and HER2, has recently been shown to have anti-tumour activity in Herceptin-refractory metastatic breast cancer (19).

It is generally agreed that the combined use of biological therapeutics with more conventional agents will lead to a decrease in drug resistance in the future. Iressa (Gefitinib), an epidermal growth factor receptor-specific tyrosine kinase inhibitor, has displayed promising data in Phase II trials, suggesting that it might act in synergy with docetaxel (20). Additionally, Iressa has also displayed promising preclinical data suggesting that it might act in synergy with tamoxifen and other hormonal agents (21, 22). Avastin (Bevacizumab), a monoclonal antibody directed against vascular endothelial growth factor, has recently shown to be active in combination with docetaxel in metastatic breast cancer (23). Furthermore, agents targeting other key cancer-relevant signalling pathways, such as the Ras pathway with farnesyl transferase inhibitors and mTOR with rapamycin analogues, are currently under investigation (24).

Identification and Validation of New Biomarkers

One of the disappointing aspects of the post-genomic era is that whilst a plethora of putative biomarkers have undergone preliminary clinical evaluations, only a small minority have received regulatory approval for clinical use from agencies such as the US FDA. Although the sequencing of the human genome is likely to have a profound influence on public health in the long-term, there have not as yet been a large number of practical advances regarding the development of new biomarkers based on this information (13). This has led to a concern that the level of investment in research is not being reflected in improved clinical outcomes, and there is particular concern that the benefits from the ‘genetic revolution’ have been slow to arrive (25).
This apparent bottleneck in transfer from putative biomarker discovery to clinical application is primarily down to a lack of rigorous validation of emerging biomarkers. In 2004, a standard template was developed to encourage the use of standards for the reporting of diagnostic accuracy for new tests, called the Standards for Reporting of Diagnostic Accuracy (STARD) initiative (26). Unfortunately, many of the studies published regarding new candidate biomarkers fail to meet these standards. In particular, the studies are often performed on small retrospective cohorts and lack power; additionally, many biomarker studies fail to include an independent validation stage, whereby the biomarker is evaluated using a second independent cohort of patients. This situation is complicated further by the fact that the validation of some of these tests is itself a new challenging aspect of the post-genomic era.

It is the authors’ opinion that the purpose of any validation study is to confirm that a previously identified biomarker has similar clinical utility in an independent cohort of patients. This is especially important regarding the validation of prognostic gene expression profiles. A key criticism regarding the validation of DNA microarray studies often cited in the medical literature is that different studies of the same disease have identified prognostic classifiers with very divergent gene sets (27). This criticism fails to recognise the fact that the purpose of a validation study is not to see whether redeveloping a gene expression profile with new data results in the selection of the same genes but is simply to identify whether a gene expression profile (or any other biomarker) provides accurate prediction for independent data (28).

The hallmark of drug development is the multi-stage clinical trials process. A similar focused, prospective, rigorously controlled multi-stage evaluation of biomarkers arising from -omic studies could facilitate and expedite the development of clinically applicable biomarkers. Using analogous terminology, Phases I-II biomarker discovery studies would be expected to show that a technology can be reliably and reproducibly applied to clinical specimens and that the estimated predictive accuracy of the proposed test falls within a range that is considered clinically useful. Phase III biomarker validation studies would then evaluate the predictor in a larger number of cases to demonstrate that clinical outcome is better when the new marker is used for decision making compared with the current standard of care (29).

**DNA Microarray Technology**

A DNA microarray is a solid support such as glass slide, silicon chip or nylon membrane on which single-stranded DNA molecules are attached at precise locations (2). Using DNA microarrays, the expression of many thousands of genes in a tumour sample can be examined within a single experiment. A number of different DNA microarray platforms have been used to generate gene expression signatures; however, the most commonly used are cDNA and oligonucleotide microarrays. In general, RNA extracted from biological samples of interest is converted into either single-stranded cDNA or cRNA, followed by hybridisation of this material to the surface of the DNA microarray and subsequent detection of probe-target interactions.

Gene expression profiling with DNA microarrays is best performed on fresh or frozen tissues, as the accuracy of the results is highly dependent on the quality of RNA used. Traditionally, investigators have used excisional biopsies; however, core needle biopsies or fine needle aspirates can also yield sufficient amounts of RNA for DNA microarray experiments (30-32). Recently, it has also been demonstrated that reverse transcription-PCR (RT-PCR) and DNA microarray profiling can be performed on RNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissues (33-35). These technological advances continue to provide investigators with an increasingly wide repertoire of choice for the tissue sampling that best suits their experimental design and may open up archival banks of FFPE tissue for gene expression profiling. The technical aspects of DNA microarray-based gene expression profiling have been extensively reviewed in the literature (2, 36-39) and lies beyond the scope of this review article. Lastly, one of the most exciting and challenging aspects of DNA microarray technology has been the need to develop new approaches for the analysis of what are extremely large datasets and complex clinical questions (40).

**DNA Microarray Studies of Human Breast Tumours**

Over 40 studies have utilised DNA microarray technology to examine gene expression profiles in human breast tumours, with more than 1,200 patients having contributed material in this way (3). Using this approach, investigators have been able to subclassify breast tumours into categories based on shared gene expression profiles and relate this to clinical parameters, including outcome. Although these studies have been informative, certain problems have also arisen. Possibly the most damning indictment of this body of work is that investigators have been slow to release data, thus limiting potential for re-analysis of data and cross-validation of gene expression signatures. Without thorough external validation, the clinical applicability of this work is debatable.

In general, there have been two main uses of DNA microarray technology in the study of breast cancer, as follows: i) Hypothesis generation to help discern molecular mechanisms of disease and improve our understanding of breast cancer biology; ii) Identification and validation of biomarkers that provide a reliable and recognisable context to classify, contrast, and improve clinical decision making. Both of these aspects will be discussed in detail below.
TMA Technology

A TMA is a collection of tissue specimens arranged on a glass slide in a grid-like fashion. TMAs were developed by Kononen et al. (41) and are a high-throughput method for the simultaneous investigation of biomarkers in multiple tissue specimens. TMAs are assembled by acquiring cylindrical cores (0.6-2.0 mm in diameter) from donor paraffin-embedded tissues and re-embedding them within a single recipient block. In this way, tissue from hundreds of specimens can be inserted into a single paraffin block. The resultant TMA block is then sectioned, with 50-75 sections being generated per block. Individual sections can then be subjected to a variety of assays. The most commonly performed assay on TMAs is immunohistochemistry (IHC) (Figure 2) however, TMA-based studies employing in situ hybridisation (ISH) for DNA and RNA are increasing in popularity. TMAs have also been used for infra-red spectroscopic (42) and protein blotting (43) studies.

TMAs and Biomarker Validation

TMAs are increasingly being used as downstream validation tools from DNA microarray-based gene expression profiling studies. The fundamental basis of all of these applications is that TMAs facilitate rapid translation of molecular discoveries to clinical applications by revealing the cellular localisation, prevalence and clinical significance of candidate biomarkers. As a TMA is only a "sampling of a sampling", the development of a putative biomarker is accomplished by repetitive validation on different patient cohorts to demonstrate its utility (44). Hewitt et al. has recommended that these cohorts should begin with the samples used for the original experiment (5). In a subsequent effort to achieve validation with utility, the sample size must expand to include other samples from the same laboratory and samples from other laboratories, followed by examination of the biomarker performance using a population-based cohort.

There are a number of different types of TMAs available to use throughout the validation process. The value of any TMA is based on the quality of the tissue embedded within the array and the extent of associated clinical and other data. Also, the clinical value of the TMA accrues over time as the final outcome of patients becomes known and multiple markers have been assessed (Figure 2a).

Although the primary objective of many groups is to determine the clinical utility of a prognostic or predictive marker, it is rarely appropriate to use valuable tissue resources at an early stage. To this end, cell pellet arrays are an ideal, renewable, cost-effective platform for optimising the antibody staining protocol, as demonstrated in Figure 2b.

Once an immunostaining procedure has been optimised and it is feasible to move onto tissue, a staged process should be employed whereby a small screening array, without requirement for extensive clinical data, is initially used, before moving on to larger cohort of consecutive tumours with long-term follow-up data. Finally, the true prognostic or predictive value of a new biomarker can be assessed on a high-quality TMA constructed from tumours removed from patients participating in a randomised control trial (discussed below).

TMA-Based Assays

As mentioned previously, the most commonly performed assay on TMAs is IHC. The complexity of immunohistochemical assays remains beyond the scope of this article. The diversity of antigen retrieval, titration of antibody, application of secondary antibody and multiple detection systems result in a multi-parameter assay that requires thorough optimisation (5). In terms of the use of TMAs for validation of DNA microarray results, the following considerations are worthy of discussion.

The most important aspect of IHC is the choice of primary antibody. The specificity of the antibody should be demonstrated by both Western blotting and IHC on cell lines (Figure 2b). It is preferable to utilise different cell lines that act as both positive and negative controls. If a negative control is not available an isotype-specific control antibody in the case of monoclonal antibodies or control serum in the case of polyclonal antibodies should be used. Cell lines can also be treated to produce controls – for example, cells can be grown in serum-free media to produce negative controls for cell cycle progression-associated proteins. It should also be noted that many antibodies, which work well for IHC, may not be suitable for Western blotting and vice versa.

A second consideration regarding IHC is the interpretation and analysis of the stained protein. The primary limitation of manual interpretation of IHC is the poor dynamic range of human scoring. Scoring models are typically qualitative in nature, e.g. positive/negative or semiquantitative i.e. using the 0, 1, 2, 3, 4 scoring systems. Additionally the manual analysis of IHC is a tedious, time consuming, subjective process to which only limited statistical confidence can be assigned due to inherent intra and inter-observer variability (42, 45).

One method of validating an antibody-based stain is to ensure that it can be assessed by a number of histopathologists simultaneously. The advent of digital slide technology has allowed this to become easily achievable (46). A digital slide is an electronic representation of a traditional glass slide, which can be laterally examined and viewed under different magnifications using a computer, in a similar fashion to viewing a glass slide using a microscope. Digital slides are accessible for review on the Internet by multiple users, who can score the TMAs simultaneously (Figure 3) (46).
Figure 2. Optimisation and Applications of Immunohistochemistry on TMAs. A) TMAs accrue value over time and allow investigators to build up IHC profiles for individual tumours, as demonstrated by the expression of six different biomarkers in a single tumour core. B) Western blotting and IHC on breast cancer cell lines to demonstrate the specificity of an antibody raised against the ER. C) Automated algorithms offer the potential to develop high-throughput quantitative scoring models for IHC, using stain-specific algorithms.
A number of sophisticated instruments provide quantitative image analysis. These include fluorescence-based approaches such as the AQUA system (HistoRx Inc, New Haven, CT, USA) (45), as well as those that allow for quantitative analysis at membranous, nuclear and cytoplasmic levels via brightfield chromogenic staining. Devices that include both image capture and analysis functions include the Aperio ScanScope (Aperio Technologies, Vista, CA, USA) and ACIS (Clarient Inc, Aliso Viejo, CA, USA) systems. Furthermore, stand-alone software can be purchased, such as Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) and TissueBase (Definiens AG, Munich). Such technologies offer the potential to devise high-throughput quantitative scoring models for IHC, which may further enhance the role of TMAs in biomarker validation (Figure 2c).

**Identification and Validation of Molecular Subtypes of Breast Cancer via the Use of DNA Microarrays and TMAs**

As outlined above, while we currently base treatment decisions in breast cancer around a number of well-established and validated biomarkers (ER, PR and HER2) and anatomical staging (tumour size and nodal status), it is becoming clear that, on a molecular level, there are distinct subsets of disease, each carrying differing survival implications for patients with breast cancer as mentioned above (28, 47-50). Identification of breast cancer subtypes by their distinct gene expression profiles initiated the first step toward formally recognising that variations in transcriptional pathways were the basis for the biological diversity often witnessed as the unpredictable survival outcomes seen in the clinical setting.

Initial work by Perou and colleagues characterised the expression of over 8,000 transcripts in 65 tumours from 42 patients using a DNA microarray (51). These investigators were able to identify four subgroups of samples with distinct gene expression patterns (i.e. ER-positive/luminal-like, basal-like, HER2-positive and normal breast) A particularly interesting finding was that ER-negative breast carcinoma contained subtypes, leading to their suggestion that basal-like and HER2-positive ER-negative phenotypes should be treated as distinct diseases. The demonstration that DNA microarrays could be used to detect new subclasses of breast cancer was very significant.

In a follow-up study of 78 human breast cancers, three fibroadenomas and four normal breast samples, the
previously defined subtypes were confirmed and related to clinical outcomes (47). This study also divided the luminal ER-positive group into two distinct subtypes, namely luminal A and B+C tumours. Luminal A tumours showed high expression of ER-associated genes, whilst luminal B+C tumours, although still ER-positive, expressed lower levels of these genes and appeared to be closely related to the HER2-positive and basal-like cluster. This was emphasised by the fact that 70-80% of the HER2-positive and basal tumours, and 67% of luminal B tumours harboured mutations of the TP53 tumour suppressor gene, whereas only 13% of luminal A tumours harboured TP53 mutations. Not surprisingly, the clinical survival of luminal A tumours was markedly better than patients with tumours of other subtypes.

Like many of the initial DNA microarray studies, these data were considerably limited in terms of sample size. In a further attempt to validate the robustness of these survival-related subclasses, Sorlie et al. (48) applied the same analysis to two independent datasets representing different patient cohorts from different laboratories, namely van’t Veer et al. (49) and West et al. (52) and the same breast cancer subtypes. In the one dataset that included survival data (49), the molecular subtypes were associated with significant differences in time-to-metastases, thus further supporting the argument that these breast tumour subtypes represent biologically distinct disease entities.

At the present time, large-scale gene expression analysis using DNA microarrays to determine the breast cancer subtype is not practical due to the need to use frozen tissue and also the cost of the experiments. TMAs are an ideal platform to develop an IHC-based surrogate of a gene expression profile and enable groups to reduce an assay to clinical utility. A number of groups have used IHC-based surrogates to validate the findings of Sorlie and Perou and colleagues outlined above.

Using TMA technology, Nielsen et al. (53) developed an immunohistochemical assay verified against gene expression profiles to identify basal-like tumours. Using breast carcinoma TMAs representing 930 patients with a 17.4 year mean follow-up, a panel of four antibodies (ER, HER1, HER2 and cytokeratin 5/6) could accurately identify basal-like tumours (53). In a follow-up study Carey et al. (54) further validated the new molecular subtypes by assigning IHC surrogates to each molecular subtype-luminal A, luminal B and basal. The study was designed to determine population-based distributions and to examine clinical associations of the molecular subtypes described above. In this case, TMAs again served as the platform for an IHC-based assay, which was revised compared to the previous study by Nielsen et al. (53). Assessment for PR expression was included and the HER2+ subtype was divided into two groups based on ER status, since HER2+/ER+ clustered separately from HER2+/ER- in hierarchical clustering analyses. The IHC profiles used in this study were basal-like (ER-, PR-, HER2-, cytokeratin 5/6+ and/or HER1+), luminal A (ER+ and/or PR+, HER2-), luminal B (ER+ and/or PR+, HER2+), or unclassified (ER-, PR-, HER2-, HER1-, cytokeratin 5/6-). Using TMAs comprised of 496 invasive breast cancer tumour samples, this group found that the basal-like subtype was more prevalent among pre-menopausal African-American women compared with post-menopausal African-American women and non-African-American women of any age. Compared with the luminal A subtype, basal-like tumours had more TP53 mutations, higher mitotic index, more marked nuclear pleomorphism and higher grade, which correlated with the initial DNA microarray study (47). Moreover, the shortest survival was seen among basal-like and HER2+/ER- subtypes, further validating the body of work (54).

This body of work provides an ideal example of how DNA microarrays and TMAs can be used together to classify tumours into distinct entities by first identifying recurrent gene expression patterns, followed by validation of these subtypes using IHC-based surrogates. More importantly, it demonstrates that, by relating gene expression patterns to clinical outcomes, one can define a clinically distinct group. It is now hoped that following the identification of specific disease-associated gene expression profiles, new therapeutic targets will be identified. For example, there is currently no specific tailored therapy for the ER-negative basal-like subgroup whom have an inherently poor prognosis. However, the Nielsen et al. study (53) identified a role for HER1 expression in the categorisation of basal-like breast cancers. There are several drugs available that act by targeting HER1, which are currently used in the treatment of lung and colon cancer predominantly. These agents, which may also provide a treatment option for the basal-like subtype, include tyrosine kinase inhibitors such as Gefitinib and Erlotinib, and the monoclonal antibody, Cetuximab, respectively. Finally, it should be again noted that it is currently now possible to identify the molecular subtype of any tumour using a relatively inexpensive well-established assay i.e., IHC.

The Use of DNA Microarrays to Predict Metastases

A seminal study that highlighted the possibilities of using DNA microarray technology for predicting breast cancer metastasis was performed by van’t Veer et al., whom used this approach to analyse the expression of approximately 25,000 genes in 78 patients (under 55 years of age) with node-negative disease (49). Using a supervised approach, these investigators were able to identify a 70-gene signature that could predict relapse-free survival over a 5 year period post-surgery more accurately than currently used clinical
and pathological prognostic factors; this signature could be used to identify those node negative patients who would benefit from adjuvant chemotherapy. If these results were confirmed and applied in the clinic, only approximately 25% of node negative breast cancer patients would be recommended to receive adjuvant chemotherapy as opposed to the 80-90% who currently receive it (55).

The 70-gene signature was validated in a follow-up study of 295 consecutive breast cancer patients (50), all of whom had stage I or II disease and were less than 53 years old. Forty-nine percent (n=144) of patients had lymph node-negative disease. Ten of the 151 patients with lymph node-negative disease and 122 of the 144 patients with lymph node-positive disease received adjuvant systemic therapy, consisting of either chemotherapy, endocrine therapy or both. Overall, 180 patients displayed a poor prognosis signature, while 115 showed a good prognosis signature. The mean 10 year survival rates for these two groupings were 54.6% and 94.5%, respectively. The estimated hazard ratio for distant metastases in the group with the poor prognosis signature, as compared with the group with the good prognosis signature, was 5.1 (95% confidence interval, 2.9 to 9.0; p<0.001). Furthermore, this ratio was similar in patients with either lymph node-negative or -positive disease. Multivariate analysis revealed that the prognosis profile was an independent factor in predicting outcome (50).

The 70-gene profile, was generated on oligonucleotide microarrays containing approximately 25,000 60-mer oligonucleotides. One could argue that, at the present time, these large-scale arrays would not be suitable for clinical practice due to their high cost for production and also their one-sample-per-chip design which would not allow for high-throughput processing of many samples on a routine basis. In an attempt to reduce to clinical utility Agendia have developed a customised DNA microarray, termed MammaPrint, which contains a limited number (1,900) of 60-mer oligonucleotides. MammaPrint is manufactured in an 8-pack format with 8 identical sub-arrays which allow for high-throughput processing. In addition, the customised array format requires less sample RNA input for labelling and hybridization, with the data processing time being also substantially reduced (56).

Initial validation studies of the MammaPrint assay have been satisfactory (56, 57). The largest of these studies has been carried out as the validation stage of the TRANSBIG/EORTC MINDACT (Microarray In Node-negative Disease may Avoid ChemoTherapy) prospective study which was designed to evaluate the role of the van’t Veer 70-gene signature in the selection of low-versus high-risk node-negative breast cancer patients. The study included 326 untreated node-negative patients from five European centres (57). Buyse et al. demonstrated that the 70 gene classifier remained a more accurate predictor of metastasis than currently used parameters, with a hazard ratio of 2.32 (95% CI=1.35-4.0) being obtained (57); this was, however, significantly lower than the original studies.

Why has this happened? Some observers have questioned the methodology of the original studies. Considering the van’t Veer group used 61 tumours in both the discovery (49) and subsequent validation (50) phases, there is a general consensus that this practice can result in "overfitting" of the data and may have inflated the discriminatory power of the signature in the validation study. Despite the fall in hazard ratio, the 70-gene classifier still emerged as an independent predictor of metastasis following multivariate analysis; and thus, the group has proceeded to a randomised prospective trial, which may well provide the first Level-I evidence for DNA microarrays in personalised medicine.

The study by Buyse et al. (57) had some limitations as a validation study. The authors used archived frozen tumour specimens, all assays were performed in a central laboratory and the study was limited to patients less than 61 years of age. Additionally, the largest limitation of the MammaPrint assay is the need to provide frozen tissue, to ensure a good yield of high-quality RNA, something which is not routinely performed in clinical practice at present.

### Predicting Response to Tamoxifen

Gene expression signatures derived from DNA microarray studies have been reported to be able to predict outcome in women with ER-positive breast cancer treated with tamoxifen better than currently used clinicopathological factors. There is an obvious clinical need as ER is not a perfect predictive marker of tamoxifen response and up to 40% of patients will fail on tamoxifen treatment, leading to incurable metastatic disease (7). aromatase inhibitors (AI) offer an alternative to tamoxifen and data from recent large prospective randomised controlled trials involving aromatase inhibitors are now emerging and herald new standards in adjuvant endocrine treatment (58, 59).

A robust gene predictor that can identify a subset of women who do poorly on tamoxifen makes them ideal candidates to study the benefits of alternative endocrine strategies, particularly upfront or early sequential aromatase inhibition and/or chemotherapy. Jansen et al. (60) used DNA microarray technology to discriminate between progressive disease (relapse within 3 to 6 months from the start of treatment) and objective response to tamoxifen in 112 patients, all of whom developed disease recurrence. This group developed a 44-gene signature using a training set of 46 tumours and validated it in an independent set of 66 tumours. This signature successfully predicted tamoxifen resistance in 27 out of 35 patients (77%) as well as time to progression of disease. The gene expression signature also performed significantly better than commonly used clinical...
parameters (menopausal status, dominant site of relapse, disease-free interval, ER and PR status) in both univariate and multivariate analyses. This study offers great potential; however, independent validation studies of the 44-gene signature are awaited.

Paik et al. reported on the Oncotype DX assay (Genomic Health), which is a quantitative RT-PCR based assay that can be applied to FFPE tissue (61). The assay is based on the expression of 21 genes that collectively can predict the risk of recurrence in lymph node negative women receiving adjuvant tamoxifen (62). This assay was derived from 250 candidate genes identified following the analysis of 447 samples from a heterogeneously-treated population (chemotherapy and tamoxifen). Twenty-one genes were chosen from this analysis, comprised of 16 cancer related genes and 5 control genes. These investigators used this multi-gene predictor to assign a "Recurrence Score" in relation to prediction of low, intermediate and high risk of distant recurrence for women receiving 5 years of adjuvant tamoxifen (± chemotherapy). The score used ranged from 0 to 100, with a score of less than 18 indicating a low risk, 18 to 31 an intermediate risk and greater than 31 a high risk of recurrence within 5 years.

The predictor was subsequently validated prospectively on an independent cohort of 675 archival patients whom had received tamoxifen only in the NSABP B-14 trial (63). Of note, none of the patients used in the initial discovery stage were used in the validation stages of the study. The validation study confirmed that the Oncotype Dx assay accurately predicted patients at high versus low risk of recurrence on tamoxifen, hence identifying those who do poorly with tamoxifen treatment (p < 0.0001).

Recently, the Oncotype Dx recurrence score (62) has been reported to be capable of predicting the magnitude of response to CMF and anthracycline/taxane chemotherapies (64, 65). Patients with a low Recurrence Score were found not to benefit from chemotherapy, whereas those with a high Recurrence Score had a larger chemotherapy benefit and a higher rate of complete pathological response. These studies suggest that the relationship between the Recurrence Score and chemotherapy benefit is not specific a certain regimen and supports anecdotal observations that there appear to be subgroups of patients whom have differing responses to chemotherapy.

The Oncotype Dx assay has a number of advantages. It has been thoroughly validated on a number of independent cohorts. The test can be easily performed on FFPE tissue, as opposed to frozen tissue which is required for the MammaPrint assay. As virtually all diagnostic tissue samples are currently processed in formalin and preserved in paraffin, this means the test is currently more likely to be used in the current clinical climate. Moreover, archival tissue can also be more easily obtained and tested. Disadvantages include the preselection of genes and a final algorithm which some have argued does not encompass more than quantitative estrogen, progesterone receptor status, proliferation and HER2/neu measurements, all currently widely available and hence provides no new biological insights into tamoxifen response (66).

Despite these criticisms, the assay is now the basis of a prospective randomised control trial, TAILORx (Trial Assigning Individualized Options for Treatment). The primary goal of the TAILORx trial will be to determine whether Oncotype DX can guide the course of treatment for women who have recurrence scores of 11 to 25 and, thus, an intermediate risk of recurrence and remain a challenging clinical dilemma. (67). If successful, the TAILORx trial should provide final validation in a prospective randomised setting and help confirm the findings outline above regarding tamoxifen response. Trial participants with a recurrence score of greater than 25 will receive chemotherapy plus hormonal therapy; women with a recurrence score of less than 11 will receive hormonal therapy alone; and women with a recurrence score of 11 to 25 will be randomly assigned to receive adjuvant hormonal therapy, with or without chemotherapy. It is expected that approximately 4,400 of the more than 10,000 expected participants will fall into the 11 to 25 range (67).

Re-Analysis of Publicly Available DNA Microarray Datasets and validation on TMAs

DNA microarray experiments are extremely expensive and as mentioned above require access to frozen tissue banks. Another method of identifying new biomarkers is to apply novel bioinformatics approaches to publicly available DNA microarray datasets. O’Brien et al. (68) applied between group analysis, a bioinformatics approach specifically tailored towards microarray data (69), to the van’t Veer dataset mentioned above (49) and identified a new cohort of putative biomarkers. They subsequently used TMAs with a view to validating their findings. One such biomarker, centromere protein-F (CENP-F), was associated with poor outcome following re-analysis of the DNA microarray data and also correlated with markers of aggressive tumour behaviour including ER negativity and high tumour grade on TMAs (68). CENP-F was also significantly associated with markers of chromosomal instability.

Another biomarker identified following the reanalysis of the van’t Veer data was carbonic anhydrase IX (CA IX), which had been previously studied in mixed cohorts of breast cancer patients all of whom had received different treatment regimens (70, 71). In an effort to fully validate the prognostic value of CA IX, Brennan et al. (72) used a TMA to analyse CA IX, protein expression in 400 stage II breast cancers from pre-menopausal women. The patients had previously
participated in a randomised control trial comparing 2 years of tamoxifen to no systemic adjuvant treatment (73). This TMA was, therefore an ideal platform to validate prognostic biomarkers and also markers associated with tamoxifen resistance or response. CA IX expression correlated positively to tumour size, grade, hypoxia-inducible factor α, Ki-67, cyclin E, and cyclin A2 expression. It was also associated with reduced relapse-free survival, overall survival and breast cancer specific survival. Moreover, multivariate analysis revealed that CA IX was an independent prognostic marker in untreated patients with one to three positive lymph nodes (72). This particular TMA has now been used to validate a number of other prognostic biomarkers, as well as a number of markers associated with tamoxifen response and resistance (Table I). This is an example of how a high-quality TMA can be used to provide important validation data for a number of markers.

Conclusion

Great advances have been made in the pursuit of personalised medicine. The post-genomic era has resulted in major changes in translational medicine, and a huge emphasis has been placed on biomarkers. However, whilst there has been great progress made in biomarker discovery in various fields, including transcriptomics, proteomics (74) and metabolomics (75), the pace of biomarker validation has not kept up with the extensive strides made in discovery. It is possible, however, to envisage a situation in the not too distant future where a tumour sample could undergo a multitude of tests using DNA microarray technology to assess prognosis, ER functional status, genomic grade and response to individual chemo- and biological therapies and thus allow for the individualisation of therapy. The incorporation and acceptance of genomic information and classifiers into clinical practice has evoked much debate. As outlined above, the key to successful translation of biomarkers into clinical settings is the establishment of robust, reproducible and informative assays that have been thoroughly validated. However, a number of important questions remain unanswered.

What extent of validation is required before clinicians believe in the reliability of the results of new assays? We have demonstrated in this article that DNA microarrays and TMAs can be used either individually, or in combination, to validate clinically applicable biomarkers and promote this process. However we also believe that prospective analysis in a randomized trials process is necessary. The results of the MINDACT and TAILORx trials will provide much needed data in this respect.

Another important question that needs to be addressed is the economic viability of gene predictors. Can DNA microarray analyses be routinely incorporated given the high cost and complex technical issues? Will the cost of these tests offset the potential savings in life lost and cost of excessive treatment, especially given the increasing price of healthcare today? How much better are gene predictors than currently widely available clinicopathological factors? These issues are complex and will not be resolved easily.

However, it should also be noted there have been major advances in our understanding of underlying mechanisms of breast cancer over the last decade. DNA microarrays and TMAs have provided valuable insights into the molecular heterogeneity of breast cancer. There is now compelling evidence that these platforms will help enable us to discover and validate biomarkers, which are necessary if we hope to provide personalised medicine in the near future.

Acknowledgements

Funding is acknowledged from Cancer Research Ireland (for part-support of D. Brennan’s post-graduate studies) and the Health Research Board of Ireland, the latter under the auspices of the "Breast Cancer Metastasis: Biomarkers and Functional Mediators” Research Programme. Support was also received from the Marie Curie Transfer of Knowledge Industry-Academia Partnership programme, Target-Breast. The UCD Conway Institute is funded by the Programme for Third Level Institutions (PRTLI), as administered by the Higher Education Authority (HEA) of Ireland.

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