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REVIEW

Gene expression profiles of the oestrogen receptor in breast cancer

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ABSTRACT

Breast cancer is a heterogeneous disease and existing clinicopathological classifications do not fully capture the diversity in clinical disease course. Since the oestrogen receptor (ER) plays a central role in the crosstalk between different signalling pathways in breast cancer, the expression of this receptor is important for the behaviour of breast cancer cells and is reflected in gene expression patterns of breast tumours. High throughput analysis of gene expression of breast cancer has increased the insights into ER signalling, including its relation with disease outcome and therapy response. Expression of ER and its numerous downstream targets are driving patterns of gene expression and dominate unsupervised analyses in the breast cancer specimens studied to date, regardless of microarray platform or statistical approach. This paper reviews gene expression studies either attempting to unravel the functional effect of ER or describing the gene expression profiles driven by ER in breast tumours. In addition, the development of molecular signatures predicting response to endocrine treatment will be discussed.

KEYWORDS

Breast cancer, oestrogen receptor, endocrine treatment, gene expression profiling, micro-array

INTRODUCTION

Hormones have been associated with breast cancer since Beatson showed that oophorectomy resulted in tumour regression in 1896.[1] Oestrogens play a predominant role in the growth of breast cancer. The identification of the oestrogen receptor (ER) by Jensen in 1960 shifted the paradigm of steroid hormone action from an enzymatic one to a model whereby steroids interact with a receptor to elicit defined biological responses.[2,3] Oestrogens bind to the ER, leading to dimerisation, conformational change and binding to oestrogen response elements (EREs) upstream of oestrogen-responsive genes including those responsible for proliferation of the tumour cells. Approximately 75% of breast tumours express the ER.[4] Patients with an ER-positive breast tumour and who have a likelihood to develop a relapse of disease will receive adjuvant endocrine treatment. The use of endocrine manipulation covers the spectrum of metastatic disease, adjuvant and neo-adjuvant therapy. Adjuvant endocrine therapy is a major contributor to the substantial decline in breast cancer mortality.

ENDOCRINE RESISTANCE

Tamoxifen has been the mainstay of treatment for ER-positive breast cancer for more than 30 years.[5,6] Tamoxifen is a selective ER modulator (SERM) that competes with oestrogens for ER binding. An alternative strategy includes the inhibition of aromatase using aromatase inhibitors (AIs) that result in a block in the production of oestrogen.[7,8] In addition, selective ER down-regulators (SERDs), such as fulvestrant, are used in the treatment of metastatic breast cancer patients.[9]

In patients with operable ER-positive tumours, tamoxifen reduces the risk of recurrence by 41% on average.[10] With that, tamoxifen has changed the clinical management of breast cancer dramatically. However, approximately 30% of the ER-positive breast cancer patients will develop a recurrence of their disease despite five years of adjuvant tamoxifen treatment.[10] Moreover, in the metastatic disease setting, half of the ER-positive breast cancer patients will not benefit from tamoxifen.[11] Endocrine resistance

is a major problem in the clinical management of breast cancer. *Figure 1* illustrates the impact of endocrine resistance in the Netherlands.

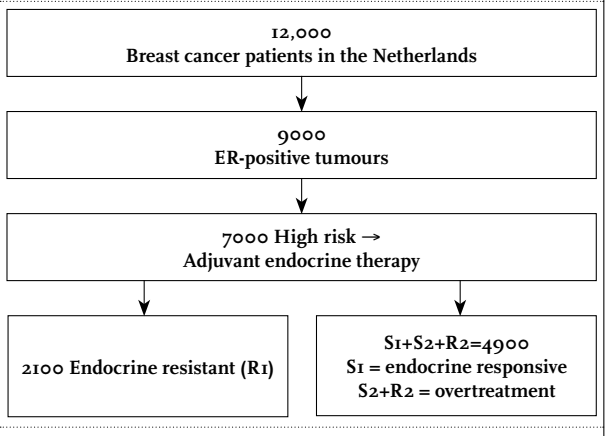
Several mechanisms may contribute to tamoxifen resistance.[8,12-15] First, genetic variations in genes coding for enzymes (cytochrome p450, CYP) that convert tamoxifen to its active metabolites can influence the effectiveness of tamoxifen. Patients with variant CYP2D6 alleles may have a higher risk of recurrence after adjuvant tamoxifen.[16,17] Secondly, a proportion of ER-positive tumours are intrinsically resistant to tamoxifen, for example due to high levels of growth factor receptors (GFRs) that may result in activation of signalling pathways in the tumour cells.[18-20] Mitogen-activated protein kinases (MAPK), protein kinase A (PKA) and p21-activated kinase 1 (PAK1) are well-characterised components of pathways that may be involved in tamoxifen resistance.[21-25] A crosstalk between the GFRs and ER has been described.[26] In addition, epigenetic and post-translational regulation of the ER may result in tamoxifen insensitivity via enhanced transcriptional activity.[27]

Thirdly, tumour growth can be stimulated by tamoxifen resulting in acquired resistance. Patients will eventually relapse despite an initial response.

Since AIs were introduced recently, it is largely unknown whether the resistance mechanisms known to be involved in tamoxifen resistance contribute to resistance to an AI as well.[8] In postmenopausal women, the only source of oestradiol (E2) is from the aromatisation of adrenal androgens. While peripheral conversion in adipose tissue contributes to detectable levels of circulating E2, local production via tumoural aromatase action results in 10- to 20-fold higher E2 concentrations in the tumour than in the serum.[28] Variations in tumour aromatase levels could therefore contribute to responsiveness to AIs. A small study suggested that the level of intratumoural aromatase activity could predict the response to the first-line AI, aminoglutethimide.[29]

Premenopausal patients who responded and relapsed after E2 withdrawal by ovarian suppression could respond to further suppression of E2 by the addition of an AI at the time of relapse.[30] This suggested that the initial resistance was due to the acquisition of an increased sensitivity to residual postmenopausal levels of E2, which could then be overcome by further reducing circulating levels of E2. Preclinical data from several laboratories support this hypersensitivity concept as a means of escape from E2 deprivation.[31,32] In part, this is caused by an adaptive increase in ER expression and function.

Figure 1. *Breast cancer patients in the Netherlands*



Proportion treated with adjuvant endocrine therapy and proportion of endocrine resistant ER-positive tumours. Numbers reflect estimates of patients per year.[4,10,117] R1 = Not cured by local treatment/Endocrine resistant (Recurrence); R2 = Cured by local treatment/Endocrine resistant (Overtreatment); S1 = Not cured by local treatment/Endocrine sensitive (Saved); S2=Cured by local treatment/Endocrine sensitive (Overtreatment).

CURRENT CLINICAL PICTURE

For premenopausal patients, tamoxifen is considered the standard adjuvant endocrine treatment. In addition, suppression of the ovarian function by means of oophorectomy or a luteinising hormone-releasing hormone (LHRH) analogue is effective, especially in women younger than 40 years.[33,34]

With regard to postmenopausal patients, recent randomised controlled trials showed that AIs are superior to tamoxifen in terms of disease-free survival (4.8% absolute difference at nine years), but failed to demonstrate a significant difference in overall survival.[35,36] Sequential tamoxifen for two or three years followed by an AI for two to three years resulted in a reduction in the risk of breast cancer recurrence and death.[37,38] The best sequence and timing for tamoxifen and AIs is still unclear.[39,40]

Almost all trials reported an increased risk of arthralgia and myalgia, as well as osteoporosis and subsequent fractures, when AIs were compared with tamoxifen.[35,36] In contrast, tamoxifen has been associated with an increased risk of endometrial cancer, especially in postmenopausal women.[37] The cardiovascular risk profile also differs between AIs and tamoxifen: thromboembolic events are more frequently seen with tamoxifen, and cardiac events are more common with AIs. Vasomotor and certain gynaecological symptoms are more frequent with tamoxifen than with AIs but quality of life, on average, appears to be similar.[35-38]

Clinicians decide whether a patient is likely to respond to endocrine treatment based on the presence of the ER and/or the progesterone receptor (PR) expression.[41] Although the predictive capacity of ER is indisputable, data on the predictive value of PR are conflicting and it could well be that PR is a prognostic as well as a predictive marker,

Box 1. *Definitions prognosis and prediction*

Prognostic marker
Any measurement available at time of diagnosis that is associated with disease-free or overall survival in the absence of adjuvant systemic therapy.

Predictive marker
Any measurement associated with response or lack of a response to a particular systemic therapy.

From: Disease of the Breast, edited by Jay Harris, © 2000.

just as the ER (definitions in *Box 1*).[10,42-44] Up till now meta-analyses have used cut-offs for ER and PR at 1% or 10% positive tumour cells. So, meta-analyses of endocrine treatment benefit have not provided the unambiguous cut-off for the percentages of ER and PR with regard to the predictive value of these markers, which is likely in a much higher range, between 50 to 100% positive tumour cells.[43,45]

Despite years of research on endocrine resistance, there are no other molecular markers, besides ER and PR, used in daily clinical practice to predict the likelihood of response to tamoxifen.[46] At present, no markers can be used to predict differential benefit from tamoxifen as opposed to AIs.

GENE EXPRESSION PROFILING TECHNOLOGY

Gene expression is a general term used to describe the transcription of information encoded within the DNA into messenger RNA (mRNA). It is assumed that for many genes there is a linear relation between the number of mRNA transcripts and functional proteins expressed in a cell. Gene expression profiling, in turn, is defined as the simultaneous measurement of the expression of a large number of genes. With gene expression profiling it has been possible to group gene transcripts of human tumours to create 'molecular signatures' that give more insight into the biology of cancer and consequently may predict clinical outcome. *Table 1* summarises the current applications of gene expression profiling. There are three techniques commonly used for gene expression profiling in clinical specimens.[47] These include gene expression profiling using two different microarray platforms (complementary DNA (cDNA) and oligonucleotide arrays) and multiplex quantitative reverse transcriptase polymerase chain reactions (qRT-PCR). On the cDNA microarray, double-stranded PCR products amplified from expressed sequences tag (EST) clones (length 300 to 1000 nucleotides) are spotted. Several ten thousands of different cDNA clones can be spotted onto the surface of a glass slide to produce a high-density cDNA array. The affixed DNA segments are known as probes. The drawback of studying gene expression using cDNA arrays is the frequent cross-hybridisation amongst homologous genes, alternative splice variants and antisense RNA. These problems have been overcome by oligonucleotide arrays, which use shorter probes of uniform length, usually 20 to 80 nucleotides. By constructing oligonucleotide arrays, complete control of the sequence is guaranteed; several different probes per gene can be spotted and many control spots provide information on contamination and hybridisation kinetics. Currently, there are four approaches for the production of oligonucleotide arrays. First, the oligonucleotides can be synthesised, purified and then printed by a robot or inkjet process onto glass slides (Agilent). Second, microarrays can be produced by *in situ* synthesis of oligonucleotides directly onto a solid surface using photolithographic technology (Affymetrix). Recently, a third technology was introduced based on bead-based arrays where the oligonucleotides are attached to microbeads that are then put onto microarrays (Illumina).[48] Finally, the fourth technique to measure gene expression in a high throughput fashion is real-time qRT-PCR, which is based on the quantification of mRNA after each round of amplification by PCR using a fluorescent reporter.[49] Current qRT-PCR assays can determine the expression of up to a few hundred genes simultaneously and may have an increased sensitivity compared with the array-based technology.

Table 1. *Gene expression profiling technologies*

	cDNA arrays	**Oligoarrays**			**Multiplex RT-PCR**
Manufacture	Clontech, academic microarray facilities	Agilent, academic microarray facilities	Affymetrix	Illumina	Taqman, Molecular Beacons, Scorpions
Probe	300-1000 nucleotide cDNA clone	60 mer oligonucleotides	20 mer oligonucleotides	50 mer oligonucleotides	~20b PCR primers
Probes per array	44,000	44,000	500,000	48,000	Up to 400
General information	Use is decreasing	Dual-channel system: expression values relative to reference	Single channel system: absolute expression values	Dual-channel system: oligos attached to beads	Most sensitive detection of mRNA levels

For the analysis and interpretation of microarray data, a range of computational tools is available. The two basic approaches are unsupervised hierarchical clustering analysis and supervised analysis.[50,51] Unsupervised hierarchical clustering analysis (or hierarchical clustering) orders both samples and genes on the basis of their similarity of gene expression. The object is to group together samples or genes that are 'close' to one another. A key component of the analysis is repeated calculation of distance measures between samples, and between clusters once samples begin to be grouped into clusters. The outcome is represented graphically as a dendogram. For example, gene expression studies using breast tumours are dominated by two main clusters: ER-negative *vs* ER-positive tumours.[52,53] In contrast, supervised analysis identifies gene expression patterns that discriminate samples on the basis of predefined clinical information such as tumour grade, disease outcome or endocrine responsiveness. Statistical analysis of expression data is complex and prone to false discoveries, e.g., identifying genes of interest just by chance. Therefore, it is crucial to validate molecular signatures in large independent series of patients before clinical application.

GENOME-WIDE ANALYSIS OF OESTROGEN RECEPTOR FUNCTION

Oestrogens are known to regulate the proliferation of breast cancer cells and to alter phenotypical properties. However, the mechanisms and pathways by which oestrogens regulate these events are only partially understood. With the sequencing of the human genome as well as the advent of microarray technology, it is now possible to investigate the complexities of ER-mediated gene transcription on a more global scale rather than studying one oestrogen-responsive target at a time. Many gene expression profiling studies have been conducted identifying E2-responsive genes, the number ranged from 100 to 1000.[54-59] The large quantitative and qualitative differences are most probably due to the use of different cell lines, treatments, microarray platforms and statistics. Collectively, expression profiles show that E2 influences a large variety of targets including genes involved in cell cycle and proliferation, apoptosis and transcriptional regulation.

Using gene expression profiling, researchers identified patterns of genes that are either stimulated or inhibited by E2 in ER-positive MCF-7 human breast cancer cells.[56,60,61] In addition they show that numerous cell cycle-associated genes as well as expression of novel transcription factors, receptors and signalling pathways are modulated by E2, many of which could play roles in mediating the effects of E2 on breast cancer proliferation. Subsequently, to better understand the actions of endocrine treatment, microarray analysis was performed after exposure of breast cancer cells to different ER-targeted drugs.[62,63] The gene expression changes induced as a response to SERMs such as tamoxifen and raloxifene or the anti-oestrogen fulvestrant indicated the agonistic and/or antagonistic actions on a large set of E2-regulated genes. Although the regulation of the majority of E2-regulated genes is either partially or fully reversed by SERMs and fulvestrant, differences can be observed among these ligands in their balance of agonistic, partial antagonistic or fully antagonistic activities on E2-regulated genes. In addition, in 2006 Oh and colleagues used this strategy to classify ER or PR-positive breast carcinomas, applying supervised analysis (significant analysis of microarray data 'SAM', software for expression data mining) on gene expression data of ER-positive MCF-7 cells treated with E2.[64,65] Using this approach, they identified 822 genes that were shown to be E2 regulated. These genes were used to develop an outcome predictor, which was then validated on independently published breast cancer datasets. Also, Musgrove *et al*. used their E2-induced gene signatures to predict survival in tamoxifen-treated patients.[60]

Translational research performed at the Netherlands Cancer Institute, the Netherlands, showed that combining *in vitro* experiments with gene expression analyses of clinical breast cancer samples can improve the understanding of ER function in cancer patients. Using fluorescence resonance energy transfer (FRET) that detects changes in the conformation of ER, the efficacy of anti-oestrogens to inactivate ER was studied.[24] Phosphorylation of serine 305 in the hinge region of ER by PKA induced resistance to tamoxifen. In clinical samples, the downregulation of a negative regulator of PKA, PKA-RIα, was associated with tamoxifen resistance. Activation of PKA by downregulation of PKA-RIα converted tamoxifen from an ER inhibitor into a growth stimulator. To further test whether ERαS305-P is indeed associated with PKA in human breast tumours, Michalides and colleagues evaluated gene expression of tumours known to have a phosphorylated ERα at serine 305. Nineteen pathways were differentially expressed in ERαS305-P-positive tumours and these pathways were enriched for pathways that include one or more PKA subunits.[25]

Whereas oestrogens exert their effects by binding to nuclear ER and directly altering transcription, they can also initiate extranuclear signalling through activation of kinase cascades. Madak *et al*. investigated the impact of E2-mediated extranuclear-initiated pathways on global gene expression.[66] Their findings document that E2 action initiated outside the nucleus stimulates the transcription and expression of a significant (~25%) portion of the total number of E2-regulated genes.

ER-mediated transcription has been intensively studied on a small number of endogenous target promoters.[67,68] Recently, ER-binding sites were mapped in a less-biased way that did not depend on pre-existing concepts of classic promoter domains and subsequently several new features of ER-mediated transcription were identified, such as the facilitation of ER binding to chromatin leading to gene transcription.[69] A number of proteins have been identified as ER co-factors using chromatin immunoprecipitation (ChIP), which has revolutionised our understanding of ER action.[70] Unbiased ChIP-microarray (ChIP-chip) work identified a total of 3665 ER binding sites throughout the entire genome.[69,71] A similar genome-wide approach mapped 1234 ER binding sites across the genome.[72] Combining this unique resource with gene expression data from breast cancer patients, it correctly predicted that the genes co-expressed with the ER and thereby identified important and previously unexplored regions of the genome that could be the critical regulators of the oestrogen dependence of breast cancer.

GENE EXPRESSION PROFILES DRIVEN BY OESTROGEN RECEPTOR

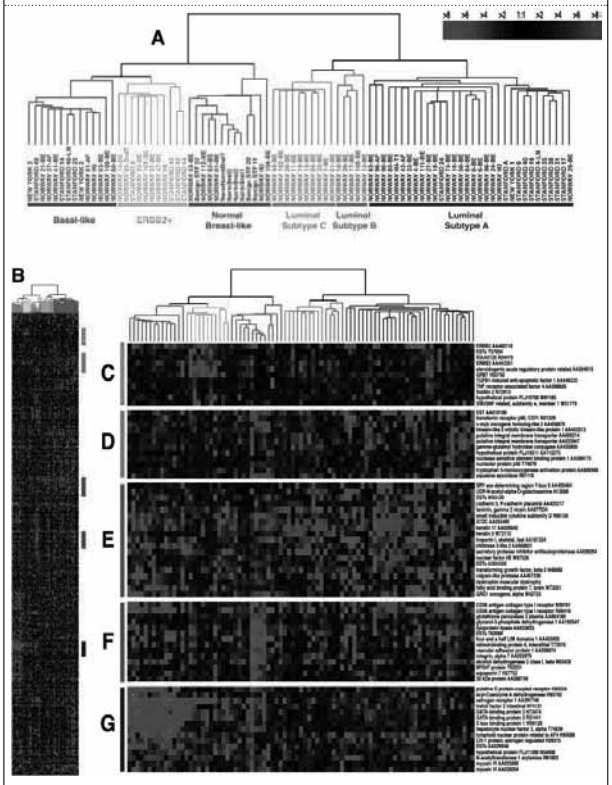
The first large-scale study of gene expression profiling in breast cancer was performed by Perou and colleagues who showed that based on overall gene expression profiles, breast carcinomas can be subdivided into five molecular subtypes (*figure 2*).[52] Three biologically distinct subgroups of ER-negative breast tumours have been identified: the 'basal-like' group, which expresses cytokeratin-5 and cytokeratin-17; the 'HER2-positive' group, expressing several genes located in the human epidermal growth factor receptor 2 (HER2) amplicon including HER2 and the gene encoding for growth factor receptor-bound protein 7 (GRB7); and the 'normal-breast-like' group, which expresses genes usually expressed in normal breast. The ER-positive tumours that were originally found to be a single group have in subsequent studies been separated into at least two distinct groups: the 'luminal A' subtype, which expresses high levels of cytokeratin-8 and cytokeratin-18 and other breast luminal genes, and the 'luminal B' subtype, expressing low levels of these genes.[73] Importantly, these five subtypes also represent clinically distinct subgroups of patients. For example, the ER-negative 'basal-like' and 'HER2-positive' subtypes are associated with a shorter overall and disease-free survival, whereas the ER-positive 'luminal A' tumours have the best outcome. These findings have been confirmed in independent datasets.[74,75] It has to be realised that classifications generated by hierarchical clustering may be unstable. For example, adding more breast cancer samples resulted in a changed dendrogram, as demonstrated by the disappearance of the luminal C subtype.[74] Furthermore, it can be argued that these analyses do not provide more information than currently given by histological grade and immunohistochemistry (IHC) for ER and HER2 of the tumour. For example, recently Cheang *et al*. showed that expression of ER, PR, HER2 and Ki67 determined by IHC appear to distinguish luminal A from luminal B breast cancer subtypes.[76]

The gene expression grade index (GGI), which defines histological grade based on gene expression profiles, could also define two ER-positive molecular subgroups (high and low genomic grade).[77,78] Despite tracking a single biological pathway, these subgroups were highly concordant with the previously described luminal A and B classifications.

Subsequent studies confirmed that there are large-scale gene expression differences between ER-positive (most 'luminal-like') and ER-negative (most 'basal-like') cancers. *Table 2* summarises different studies describing the dominant gene expression pattern in breast carcinomas driven by ER. To study the characteristics of ER-positive and ER-negative breast tumours in more detail, Gruvberger and colleagues profiled a homogeneous group of lymph node-negative breast cancers.[79] They reported that ER-positive and ER-negative tumours display remarkably different molecular phenotypes. To gain insight into the genes of this dominant expression signature, Van 't Veer *et al*. associated gene expression data with ER expression as determined by IHC.[53] Out of 39 tumours stained negative for ER by IHC, 34 clustered together. By this unsupervised approach, known ER target genes formed a cluster with the ER gene (ESR1). Supervised classification showed that 550 genes optimally reported the dominant pattern associated with ER status; reporter genes included cytokeratin-18, bcl-2, HER3 and HER4. Twenty-one out of the 50 ER reporter genes as determined by Gruvberger *et al*. were also present in the 550 gene list.[79]

Since the introduction of high throughput analysis of gene expression, several molecular signatures predicting prognosis in breast cancer patients have been developed.[80-83] All classifiers have been developed using different microarray platforms and approaches to select genes. Consequently, a direct comparison between the various gene lists generated is difficult. However, these different gene sets show significant agreement in the outcome predictions for individual patients and are probably tracking a common set of biological phenotypes.[75] In addition to the degree of proliferation and histological grading, information on ER signalling is present in all prognostic signatures. Wang and colleagues included this information in the development of their prognostic test.[83] Tumours used for their discovery study were allocated to one of two subgroups stratified by ER status. Markers selected from each subgroup (60 genes for ER-positive tumours and 16 for ER-negative tumours) were combined to form a single signature to predict

Figure 2. *Molecular subtypes of breast cancer*



Gene expression patterns of 85 experimental samples representing 78 carcinomas, three benign tumours and four normal tissues analysed by hierarchical clustering of cDNA clones. a Tumour specimens were divided into five (or six) subtypes based on differences in gene expression: luminal A, luminal B, luminal C, normal breast-like, basal-like and HER2+. b Full cluster diagram scaled down, bars on the right represent the inserts present in c–g. c HER2 amplicon. d Unknown cluster. e Basal epithelial cell-enriched cluster. f Normal breast-like cluster. g Luminal epithelial gene cluster containing ER.

tumour metastasis in a subsequent independent validation consisting of both ER-positive and ER-negative tumours. This result supports the idea that the extent of heterogeneity and the underlying mechanisms for disease progression could differ for the two ER-based subgroups of breast cancer patients. In addition, Dai *et al*. showed within a subset of young patients (<55 years) characterised by relatively high ER expression for their age (i.e., the ER/age high group) that the occurrence of metastases is strongly predicted by a homogeneous gene expression pattern almost entirely consisting of cell cycle genes.[84] By combining information on expression of ER with clinical variables such as age at diagnosis, a subgroup of patients was identified in which expression of proliferation-associated genes is a very strong predictor of outcome. All the above findings describe the marked influence of ER and its numerous targets on gene expression in breast cancer. Expression of ER drives patterns of gene expression and dominates unsupervised analyses in the breast cancer specimens studied to date, regardless of microarray platform or statistical approach. mRNA levels of ER (gene name ESR1) show strong correlation with protein expression.[52,53,85] Although there is preliminary evidence that quantitative mRNA levels of ESR1 and gene lists containing ER target genes could be predictive for outcome after endocrine treatment, clinical application of these tests requires further investigation.

In 2010, Dunbier and colleagues were the first to show a relationship between the expression of E2-dependent genes in ER-positive breast carcinoma and basal levels of E2 in plasma.[86] Their data challenge the view and strongly suggest that differences in plasma E2 levels between patients have a significant influence on the behaviour of breast tumours.

Table 2. *Gene expression profiling using human breast tumours to identify genes related to oestrogen receptor (ER)*

Microarray type	Samples	ER-related genes	Identified by	Prediction results	Reference
Oligonucleotide 25k, Agilent	98 Breast tumours	550	Unsupervised clustering	95% of ER status (IHC) predicted correctly (training only)	53
cDNA array 10k ESTs	38 Breast tumours	105	Supervised analysis	16/20 ER status (IHC) predicted (validation)	85
cDNA array 4.5k ESTs	38 Breast tumours	98	Median difference per gene in ER+ vs ER- tumours	46 genes more expressed in ER+, 52 genes more expressed in ER-	109
cDNA array 6,728 clones	58 Breast tumours	Top 100	Artificial neural networks models	100% of ER status (LBA) predicted correctly (validation)	79
cDNA array 8,102 clones	85 Breast tumours and normal tissue	427	Differentially expressed between subtypes of breast tumours	Discrimination of ER+ (luminal) vs ER- tumours (basal, HER2, normal-like subtypes)	52,73
Oligonucleotide Hu6800 Affymetrix	49 Breast tumours	Top 100	Correlation coefficient per gene with ER+ and ER- tumours	8/9 ER status (IHC) predicted correctly (validation)	110
Oligonucleotide 44k Agilent	65 Breast tumours and MCF7 cell line	822	Stimulation of MCF7 cells with oestradiol	Good discrimination of relapse-free survival	64

IHC = immunohistochemistry.

While most gene expression studies have focused on the presence or absence of ER, Creighton *et al*. examined RNA expression of ER-positive breast cancers in relation to the presence of PR.[87] ER+/PR- breast cancer defined by gene expression profiling (i.e., tumours neither truly ER+/PR+ nor ER-/PR- but sharing expression patterns with both) tended to have a poor outcome and this was not observed when using the IHC assays to determine ER and PR status. This shows that gene expression profiles may provide a clinically relevant tool to assess PR levels for diagnostic or therapeutic purposes.

MOLECULAR SIGNATURES PREDICTING RESPONSE TO ENDOCRINE TREATMENT

Adjuvant tamoxifen treatment reduces the breast cancer death rate by 31% in patients with ER-positive disease.[10] Gene expression studies have consistently confirmed the heterogeneity of ER-positive breast cancer and may provide insights into the mechanisms of response to endocrine treatment.

Current research efforts are focusing on the discovery of molecular signatures that might identify those patients most responsive to tamoxifen. The expression of ER does not guarantee functional activity and other molecular events unrelated to ER signalling can also influence sensitivity to endocrine treatment regimens. A multigene assay calculating a recurrence score (Oncotype DXTM) represents an important conceptual evolvement in the diagnosis of ER-positive breast cancer.[81] This RT-PCR-based assay was derived from 250 candidate genes selected by a literature search of the most important microarray studies in breast cancer. For the recurrence score, out of these 250, 16 genes were selected as well as five control genes. This assay measures ER mRNA levels in a quantitative and reproducible manner and also measures expression of several downstream ER-regulated genes (PR, bcl2 and SCUBE2) that probably contain information on the functionality of ER. The same assay also quantifies HER2 expression and proliferation-associated genes (Ki67, cyclin B1 and survivin). This RT-PCR-based test has been optimised for paraffin-embedded material and has been shown to accurately identify a group of patients with excellent prognosis when treated with adjuvant tamoxifen.[81,88] A disadvantage included the pre-selection of genes and a subsequent algorithm that may not encompass more than quantitative ER and PR levels, proliferation and HER2 expression, all currently easy to test and hence may provide no new biological insights into tamoxifen response. Another study, conducted in 60 ER-positive breast carcinomas treated with adjuvant tamoxifen, suggested the utility of a two-gene index of HOXB13 and IL17BR in identifying a subset of patients who are at risk for relapse of disease.[80] In an independent dataset of patients receiving tamoxifen, Reid *et al*. reported that the two-gene index failed to detect differences in outcome.[89] Taking into account that Fan and colleagues calculated the two-gene index using microarray data, again no association with outcome was seen.[75] However, in three other large cohorts the two-gene index showed a relation with tumour aggressiveness and response to first-line tamoxifen monotherapy for relapse of disease.[90-92] In studies of relatively small sample size, a model based on analysis of only two genes is much more likely to be sensitive to technical differences or patient selection. Further, in a substantial proportion of ER-positive tumours HOXB13 expression was below the detection level.[92] Rodriguez *et al*. showed by functional experiments that HOXB13 is an ER target gene and that its repression is mediated by DNA methylation in ER-positive tumours.[93] The observation by Wang *et al*. that HOXB13 and IL17BR expression strongly correlates with the expression of ER, PR and HER2 as determined by the routinely used IHC supports this regulation mechanism.[94] Independent studies will reveal whether HOXB13 and IL17BR might be useful predictive markers when used instead of IHC or add information to the standard markers.

In addition, using Affymetrix Gene Chip arrays, investigators from the Jules Bordet Institute, Belgium, selected 181 genes by Cox proportional regression analysis to predict patients having an early relapse after adjuvant tamoxifen treatment.[95]

While the recurrence score and two-gene index might be very helpful in predicting the likelihood of relapse of disease, a major limitation of these tests is that tamoxifen is prescribed as adjuvant treatment. A disadvantage of assessing response in the adjuvant setting is that both the response of tumour cells to tamoxifen and intrinsic aggressiveness of the malignancy are measured. Furthermore, some resistant tumours will not recur because they were already cured by surgery and radiation. The proportion of this group of patients is unknown.

In contrast, Jansen and colleagues discovered, using cDNA microarrays, an 81-gene signature in tumours of breast cancer patients treated with tamoxifen for their metastases.[96] In this palliative setting, tumour response can be visualised. Subsequently, this response profile was tested on 66 independent cases and could select patients who had a short time to tumour progression (TTP). The genes were involved in oestrogen action, apoptosis, extracellular matrix formation and immune response. Recently, these 81 genes were validated in tumour samples from another hospital using a more advanced microarray platform.[97] It is provocative to speculate on the predictive value of this tool if used for adjuvant treatment decisions. Identification of a subset of patients who might have more

chance to be cured by tamoxifen instead of an AI may open the door to more individualised medicine.

While adjuvant tamoxifen treatment reduces the risk of breast cancer death by 31%, AIs slightly improve disease-free survival compared with tamoxifen.[98] In addition, a survival benefit has been shown for sequential tamoxifen and an AI.[37,99] A molecular test helping clinicians to make a choice between starting with tamoxifen, an AI or rather with chemotherapy would have enormous potential for tailoring treatment. Mackay *et al.* conducted gene expression profiling on pre-treatment and post-treatment biopsies of breast cancer patients who received an AI for two weeks before surgery.[100] Profound changes in gene expression were seen after treatment, including many classical E2-dependent genes (TFF1, CCND1, PDZK1 and AGR2) as well as a prominent decrease in the expression of proliferation-related genes. Using a similar approach, Miller and colleagues identified letrozole-induced changes in gene expression associated with cell cycle progression, organ development, extracellular matrix regulation and inflammatory response.[101-103] With regard to the steroidal anti-oestrogen AI exemestane, Harvell and colleagues identified 50 genes that can predict response or intrinsic resistance to neoadjuvant exemestane treatment.[104] This study showed upregulation of a lipogenic pathway in non-responsive tumours that may serve as a marker for intrinsic resistance. Subsequently Harvell *et al.* demonstrated that an AI alone alters gene expression five times more than an AI in combination with tamoxifen, and is 11 times more effective in modifying expression of E2-regulated genes.[105] Moreover, *in vitro* studies suggest that gene profiles unique to AI resistance are inherently different from tamoxifen resistance profiles.[106] Larger datasets and samples derived from a randomised trial are necessary to enable the identification of markers or gene signatures specifically associated with AI response.

FUTURE PERSPECTIVE

The published literature is awash with examples of biomarkers promising to predict responses to endocrine therapy in breast cancer. However, only two molecular markers, ER and PR, have become standard measurements in the management of breast cancer patients with regard to assessment of endocrine sensitivity. Moreover, even their exact predictive value, e.g. sensitivity and specificity at a well-optimised cut-off value, is largely unknown regarding the important clinical question: has an individual patient more benefit from tamoxifen or an AI? Apparently the discovery of a biomarker related to endocrine responsiveness is relatively easy. However, translation of the findings into clinical practice seems extremely difficult. In the majority of clinics the endocrine dependence of a breast carcinoma is simply rated as ER-positive or ER-negative. Around the world several cut-offs are used to determine whether a tumour is ER-positive. Meta-analyses have never showed an analysis that addressed at which particular cut-off the ER was best at predicting tamoxifen benefit. Although the presence or absence of ER is widely used to guide therapy, less attention has been paid to the quantitative aspects of ER. Thirty years ago, McGuire and colleagues observed that the response of metastatic disease to endocrine treatment was directly related to the level of ER expression.[107] The Oxford overview analysis has extended this to primary disease showing a greater proportional reduction in recurrence rate with tamoxifen treatment in high *vs* low ER-positive tumours.[108] However, a quantitative measurement of ER is still not used in the clinic. Besides ESR1 mRNA levels, tumour profiling using genes that incorporate an ERE in their promoter could be informative with regard to the assessment of endocrine sensitivity. Future research should focus on how exactly ER activity has to be quantified.

In a short period of time, analysis of gene expression in breast cancer has increased the understanding of ER signalling and the diversity of ER-positive and -negative breast cancer subtypes. However, there are still many questions remaining that could be answered by continuing research using gene expression profiling of human tumour samples. The advantage of microarray technology is that thousands of genes can be studied at the same time instead of focusing on a single gene of interest. Regarding the genes responding to activation of ER, several lists of either putative ER targets or genes correlating with ER expression have been published.[53,64,71,80,109,110] However, currently there is no consensus on the comprehensiveness of these gene sets. A complete overview of genes also including processes in which ER is influencing gene expressing by functioning as a transcriptional co-factor or driving other co-factors, is still lacking. Furthermore, gene expression profiling is not suitable to pinpoint post-translational modifications of ER or epigenetic regulation by ER by binding to chromatin.

While the description of breast cancer phenotypes in distinct molecular subtypes, as first portrayed by Perou and colleagues, has been exciting, further refinement of subdivision of ER-positive breast cancer is needed.[52,111] How to define the group of patients with a very good outcome for which systemic treatment can be safely omitted? And since some ER-positive tumours show a moderate response to chemotherapy, it will be very interesting to screen this subgroup for specific drug targets.[108,112-114] If these can be identified, clinicians can offer endocrine treatment combined with targeted therapy.

Although the high throughput analysis of gene expression of breast cancer cells has increased the insights into the behaviour of the disease, the relation with outcome and

therapy response, accurate and robust validation of the candidate response profiles is necessary before clinical application. Standardisation of technology and properly designed clinical trials performed on a large scale will be essential. Moreover, the discrimination of the prognostic value of a set of genes, e.g., aggressiveness of tumour cells regardless of systemic treatment versus the capacity to predict response to a specific drug needs more detailed investigation.

Currently, whole genome analyses require frozen material. The isolation of sufficient and high-quality mRNA from formalin-fixed paraffin-embedded (FFPE) material will allow the analysis of the complete genome from archived material. Besides, it saves the complex logistics of the storage of frozen material. Important challenges for the future include the implementation of a technically robust gene expression technology in daily clinical practice, and to combine multiple separate predictive tests into a single assay to improve cost-effectiveness. In an ideal world, a breast tumour will be profiled using a single microarray resulting in information on prognosis, endocrine resistance, chemo-sensitivity, expression of drug targets and genetic variation in drug metabolising enzymes.

Series of prospectively designed clinical studies enrolling patients whose clinical characteristics match the intended use of the test are needed. Since endocrine treatment has an undisputed efficacy, a trial incorporating a study arm that withholds adjuvant endocrine treatment for intermediate-high risk ER-positive breast cancer patients is impossible to conduct. However, collecting material from patients randomised between tamoxifen and an AI may enable the discovery of gene profiles that predict the response to either tamoxifen or an AI. In the MINDACT trial novel gene expression signatures predicting clinical response in patients treated with sequential tamoxifen-letrozole *vs* letrozole alone will be compared.[115] In addition, the trans-ATAC has been set up as a follow-up of the ATAC trial to try to identify the molecular characteristics of tumours of patients that benefit more from anastrozole than tamoxifen and pinpoint the resistance mechanisms that still allow many patients to relapse.[116]

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