Endocrine resistance in breast cancer: gene expression profiling and modifications of the estrogen receptor
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Chapter 2
Estrogen receptor phenotypes defined by gene expression profiling
Estrogen receptor phenotypes defined by gene expression profiling

Marleen Kok, Sabine Linn, Marc van de Vijver


Introduction
Breast cancer is a heterogeneous disease and existing clinicopathological classifications do not fully capture the diversity in clinical disease course. Since the estrogen receptor (ER) plays a central role in the crosstalk between different signaling pathways in breast cancer, the expression of this receptor is important for the behavior of breast cancer cells and is reflected in gene expression patterns of breast tumors. High throughput analysis of gene expression of breast cancer has increased the insights in ER signaling, 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 breast cancer specimens studied to date, regardless of microarray platform or statistical approach. This chapter reviews gene expression studies either attempting to unravel the functional effect of ER or describing the gene expression profiles driven by ER in breast tumors. In addition, the development of molecular signatures predicting response to endocrine treatment will be discussed.

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 tumors to create ‘molecular signatures’ that give more insight in the biology of cancer and consequently may predict clinical outcome. Table 2.1 summarizes the current applications of gene expression profiling. There are three techniques commonly used for gene expression profiling in clinical specimens [1]. 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 300-1000 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-hybridization 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 20-80 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 hybridization kinetics. Currently, there are three approaches for the production of oligonucleotide arrays. First, the oligonucleotides can be synthesized, 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 [2] based on bead-based arrays where the oligonucleotides are attached to microbeads that are then put onto microarrays (Illumina).

Finally, the third 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 [3]. Current qRT-PCR assays can determine the expression of up to a few hundred genes simultaneously and may have an increased sensitivity compared to the array-based technology. For the analysis and interpretation of microarray data, a range of computational tools is available. The two

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<td><strong>Manufacture</strong></td>
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<td>Clontech, academic microarray facilities</td>
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basic approaches are unsupervised hierarchical clustering analysis, which orders both samples and genes on the basis of their similarity of gene expression, and supervised methods, which identify gene expression patterns that discriminate samples on the basis of predefined clinical information [4, 5]. 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 Analyses of Estrogen Receptor Function**

Estrogens are known to regulate the proliferation of breast cancer cells and to alter phenotypical properties. However, the mechanisms and pathways by which estrogens 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 estrogen-responsive target at a time.

Using gene expression profiling, Frasor and colleagues identified patterns of genes that are either stimulated or inhibited by estradiol (E2) in ER-positive MCF-7 human breast cancer cells [6]. Their findings reveal that almost 70% of the genes regulated by E2 are, in fact, downregulated. In addition they show that numerous cell cycle-associated genes as well as expression of novel transcription factors, receptors and signaling 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 estrogen receptor-targeted drugs [7, 8]. The gene expression changes induced as a response to selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene or the anti-estrogen fulvestrant indicated the agonistic and/or antagonistic actions on a large set of estrogen-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 progesterone receptor (PR)-positive breast carcinomas [9], 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 [10]. Using this approach, they identified 822 genes that were shown to be estrogen regulated. These genes were used to develop an outcome predictor, which was then validated on independent published breast cancer datasets.

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-estrogens to inactivate ER was studied [11, 12]. Phosphorylation of serine 305 in the hinge region of ER by protein kinase A (PKA) induced resistance to tamoxifen. In clinical samples, the downregulation of a negative regulator of PKA, PKA-Rlα, was associated with tamoxifen resistance. Activation of PKA by downregulation of PKA-Rlα converted tamoxifen from an ER inhibitor into a growth stimulator.

ER-mediated transcription has been intensively studied on a small number of endogenous target promoters [13, 14]. Recently, ER-binding sites were mapped in a less-biased way that did not depend on preexisting 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 [15]. Subsequently, all ER and RNA polymerase II binding sites were mapped on a genome-wide scale in breast cancer cells stimulated by E2, identifying a broad range of cis-binding sites and target genes [16]. Since the cis-regulatory elements can be located in the promoter region 50 to the gene it controls, as well as in the intron, or in the 30 region, this study found a more complete set of ER-binding sites across the genome. 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 estrogen dependence of breast cancer.

**Gene Expression Profiles Driven by Estrogen 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 2.1) [17]. Three biologically distinct subgroups of ER-negative breast tumors have been identified: the ‘basal-like’ group, which expresses cytokeratin-5 and cytokeratin-17; the ‘HER2-positive’ group, expressing several genes located in the 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 tumors 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 [18]. 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’ tumors have the best outcome. These findings have been confirmed in independent datasets [19, 20]. It has to be realized 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 [19]. Furthermore, it can be argued that these

![Figure 2.1 Molecular subtypes of breast cancer](image)

**Figure 2.1 Molecular subtypes of breast cancer**

Gene expression patterns of 85 experimental samples representing 78 carcinomas, 3 benign tumors and 4 normal tissues analyzed by hierarchical clustering of cDNA clones. 

- **A** Tumor specimens were divided into five (or six) subtypes based on differences in gene expression: dark blue: luminal A, yellow: luminal B, light blue: luminal C, green: normal breast like, red: basal like and pink: HER2+.  
- **B** Full cluster diagram scaled down, colored 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.

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analyses do not provide more information than currently given by histological grade and immunohistochemistry (IHC) for ER and HER2 of the tumor. When interpreting these observations, it is important to keep in mind that many of these correlations are expected because of the strong association between molecular class and conventional histopathological variables. The gene expression grade index (GGI), which defines histological grade based on gene expression profiles [21], could also define two ER-positive molecular subgroups (high and low genomic grade) [22]. 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 2.2 summarizes 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 tumors in more detail, Gruvberger and colleagues profiled a homogeneous group of lymph node-negative breast cancers [23]. They reported that ER-positive and ER-negative tumors 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 [24]. Out of 39 tumors 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 (see Figure 2.2). Twenty-one out of the 50 ER reporter genes as determined by Gruvberger et al. were also present in the 550 gene list [23].

Since the introduction of high throughput analysis of gene expression, several molecular signatures predicting prognosis in breast cancer patients have been developed [25-28]. 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 [20]. In addition to the degree of proliferation and histological grading, information on ER signaling is present in all prognostic signatures. Wang and colleagues included this information in the development of their prognostic test [28]. Tumors used for their discovery study were allocated to one of two subgroups stratified by ER status. Each subgroup was analyzed separately for selection of genes. Markers selected from each subgroup (60 genes for ER-positive tumors and 16 for ER-negative tumors) were combined to form a single signature to predict tumor metastasis in a subsequent independent
<table>
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<tr>
<th>Microarray type</th>
<th>Samples</th>
<th>Identified by</th>
<th>Prediction results</th>
<th>Ref</th>
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<tr>
<td>Oligonucleotide 25k, Agilent</td>
<td>98 breast tumors</td>
<td>550</td>
<td>Unsupervised clustering</td>
<td>95% of ER status (IHC) predicted correctly (training only)</td>
</tr>
<tr>
<td>cDNA array 10k ESTs</td>
<td>38 breast tumors</td>
<td>105</td>
<td>Supervised analysis</td>
<td>16/20 ER status (IHC) predicted (validation)</td>
</tr>
<tr>
<td>cDNA array 4.5k ESTs</td>
<td>38 breast tumors</td>
<td>98</td>
<td>Median difference per gene in ER+ vs ER- tumors</td>
<td>46 genes more expressed in ER+, 52 genes more expressed in ER-</td>
</tr>
<tr>
<td>cDNA array 6,728 clones</td>
<td>58 breast tumors</td>
<td>Top 100</td>
<td>Artificial neural networks models</td>
<td>100% of ER status (LBA) predicted correctly (validation)</td>
</tr>
<tr>
<td>cDNA array 8,102 clones</td>
<td>85 breast tumors and normal tissue</td>
<td>427</td>
<td>Differentially expressed between subtypes of breast tumors</td>
<td>Discrimination of ER+ (luminal) vs ER- tumors (basal, HER2, normal-like subtypes)</td>
</tr>
<tr>
<td>Oligonucleotide Hu6800 Affymetrix</td>
<td>49 breast tumors</td>
<td>Top 100</td>
<td>Correlation coefficient per gene with ER+ and ER- tumors</td>
<td>8/9 ER status (IHC) predicted correctly (validation)</td>
</tr>
<tr>
<td>Oligonucleotide 44k Agilent</td>
<td>65 breast tumors and MCF7 cell line</td>
<td>822</td>
<td>Stimulation of MCF7 cells with estradiol</td>
<td>Good discrimination of relapse-free survival</td>
</tr>
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Table 2.2 Gene expression profiling to identify genes related to ER

validation consisting of both ER-positive and ER-negative tumors. 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) characterized 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 [29]. 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 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 as depicted in Figure 2.3 (Kok
et al. unpublished data; [17, 24, 30]). 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 [27], clinical application of these tests requires further investigation. While most gene expression studies have focused on the presence or absence of ER, Creighton et al. examined RNA expression of ER+ breast cancers in relation to the presence of PR [31]. ER+/PR- breast cancer defined by gene expression profiling (i.e., tumors neither truly ER+/PR+ nor ER-/PR- but sharing expression patterns with both) tended to have 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.

**Figure. 2.2 Supervised classification by ER status** a Outline of classification system: 98 breast tumors are classified into an ER-positive and ER-negative group. b Expression data matrix across 550 optimal ER reporter genes. The contrasting patterns discriminate between tumors with an ER-negative signature (below solid line) and an ER-positive signature (above solid line). The reporter genes were ordered on the basis of their level of contribution to the classifier. Tumors are arranged according to the leave-one-out correlation coefficients to the average signatures of the classifier. The ER status, as determined by IHC and microarray, are indicated in the two right panels. Adapted by permission from Macmillan Publishers Ltd: Nature (van ’t Veer et al. copyright 2002 [24])
Figure 2.3 Relation of ER protein expression, ER mRNA levels and the corresponding expression of ER-related genes with tamoxifen response. a Scatter plot depicting ER as determined by IHC (x-axis, % of tumor cells positive) and mRNA level (y-axis, calculated using four probes for ESR1 on 44 k Agilent array) expressed in log-ratio relative to reference consisting of pool of breast tumors. Red circles indicate patients resistant to tamoxifen in metastatic disease setting (6 months benefit), green circles indicate the patients who showed a response (clinical benefit for more than 6 months). b Heatmap of 385 ER-related genes (identified by mapping the 550 ER reporter gene described by van’t Veer et al. to the 44 k Agilent array). Tumors ranked based on ER determined by IHC. Genes ranked based upon correlation coefficient with ESR1 as determined in dataset of van’t Veer et al. Yellow lines group tumors in ER negative (left), ER low (middle) and ER high (right) (Kok et al. unpublished data; [24])
Molecular Signatures Predicting Response to Endocrine Treatment

Adjuvant tamoxifen treatment reduces the breast cancer death rate with 31% in patients with ER-positive disease [32]. However, a substantial proportion of patients develop metastases despite tamoxifen treatment. Moreover, only half of the recurrences in ER-positive breast tumors respond to tamoxifen while the other half is resistant [33]. 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 focused 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 signaling can also influence sensitivity to endocrine treatment regimen. A multigene assay calculating a recurrence score (Oncotype DXTM) represents an important conceptual evolution in the diagnosis of ER-positive breast cancer [26]. 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 5 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 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 optimized for paraffin-embedded material and has been shown to accurately identify a group of patients with excellent prognosis when treated with adjuvant tamoxifen [26, 34]. Notably, the predictive power was independent of age and tumor grade or size. A disadvantage included the preselection 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 [27]. In an independent dataset of patients receiving tamoxifen, Reid et al. reported that the two-gene-index failed to detect differences in outcome [35]. Taking into account that Fan and colleagues calculated the two-gene-index using microarray data, again no association with outcome was seen [20]. However, in three other large cohorts the two-gene-index showed a relation with tumor aggressiveness and response to first-line tamoxifen monotherapy for relapse of disease [36-38]. 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 tumors HOXB13 expression was below the detection level [38]. 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 tumors [39]. The observation by Wang et al. that HOXB13 and IL17BR expression strongly correlates with the expression of ER, PgR and HER2 as determined by the routinely used immunohistochemistry supports this regulation mechanism [40]. Independent studies will reveal whether HOXB13 and IL17BR might be useful predictive markers when used instead of immunohistochemistry or add information to the standard markers. In addition, using Affymetrix Gene Chip arrays, investigators from the Jules Bordet Institute, Belgium, selected 62 genes by Cox proportional regression analysis to predict patients having an early relapse after adjuvant tamoxifen treatment [41]. In a large validation set, they were able to identify patients who will probably have more benefit from other endocrine approaches such as upfront treatment with aromatase inhibitors. 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 tumor cells to tamoxifen as well as intrinsic aggressiveness of the malignancy are measured. Furthermore, some resistant tumors 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 tumors of breast cancer patients treated with tamoxifen for their metastases [42]. In this palliative setting, tumor response can be visualized. The 81 genes were found - by supervised hierarchical clustering - to be differentially expressed between tamoxifen-sensitive and tamoxifen-resistant, ER-positive breast tumors (n=46, heatmap of genes shown in Figure 2.4). Subsequently, this response profile was tested on 66 independent cases and could select patients who had a short time to tumor progression (TTP). The genes were involved in estrogen action, apoptosis, extracellular matrix formation and immune response. Recently, these 81 genes were validated in tumor samples from another hospital using a more advanced microarray platform [43]. Combining this tamoxifen response profile with PR determined by IHC, patients with an excellent TTP could be identified. 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 aromatase inhibitor may open the door to more individualized medicine.

While adjuvant tamoxifen treatment reduces the risk of breast cancer death by 31% [32], aromatase inhibitors slightly prolong disease-free survival compared to tamoxifen [44]. In addition, a survival benefit has been shown for sequential tamoxifen and an aromatase inhibitor [45, 46]. A molecular test helping clinicians to make a choice between starting with tamoxifen, an aromatase inhibitor or rather with chemotherapy would have enormous potential for tailoring treatment. Mackay et al. conducted gene expression
Figure. 2.4 Supervised clustering of tamoxifen-resistant and -sensitive breast carcinomas (n=46) using the 81-gene signature. a Expression plot showing clusters of tumors with progressive disease and objective response. Orange bars below indicate misclassified tumors. Red indicates upregulated genes and green indicates downregulated genes. b Bars next to plot indicate genes of predictive signature (green), apoptosis (black), extracellular matrix (purple) and immune system (blue). Information includes cytoband location and references of estrogen function. Reprinted with permission from the American Society of Clinical Oncology: Jansen et al. [42]
profiling on pretreatment and posttreatment biopsies of breast cancer patients who received an aromatase inhibitor for 2 weeks before surgery [47]. Profound changes in gene expression were seen after treatment, including many classical estrogen-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 expression in genes associated with cell cycle progression, organ development, extracellular matrix regulation and inflammatory response [48]. Since most of the aromatase inhibitors are prescribed for advanced disease after adjuvant tamoxifen, Lin et al. retrospectively studied primary tumors of this group of patients and subsequently measured levels of E2-related genes using RT-PCR [49]. An algorithm combining mRNA levels of ER, PgR and BRCA1 resulted in the best predictive value. Larger datasets and samples derived from a randomized trial are necessary to enable the identification of markers or gene signatures specifically associated with aromatase inhibitor response.

Currently, the use of microarray technology in clinical practice is being tested (EORTC trial: http://www.eortc.be/services/unit/mindact/) and we speculate that a gene expression profile predicting treatment response might provide additional information on top of measurement of nuclear receptors. Thereby, endocrine treatment decisions can be tailored, e.g., starting tamoxifen, an aromatase inhibitor, or rather focus on disease control by chemotherapy. Nevertheless, most algorithms developed to predict outcome after tamoxifen are based on adjuvant treatment [26, 27, 41]. Further investigations are needed to elucidate whether these gene profiles truly predict drug response or solely prognosis. In the absence of frozen material obtained in a randomized controlled trial addressing whether a drug is effective in the adjuvant setting including a control arm with untreated patients, response to treatment can only be visualized in neoadjuvant treatment settings [50, 51] or in patients with measurable disease in metastatic disease setting [42, 43].

**Perspectives**

In a short period of time, analysis of gene expression in breast cancer has increased the understanding of ER signaling 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 tumor 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 [9, 16, 23, 24, 52, 53]. 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 transcripational co-factor or driving other co-factors, is still lacking. Furthermore, gene expression profiling is not suitable to pinpoint posttranslational 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 [17]. 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 tumors show a moderate response to chemotherapy, it will be very interesting to screen this subgroup for specific drug targets. 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 in the behavior of the disease, the relation with outcome and therapy response, accurate and robust validation of the candidate response profiles is necessary before clinical application. Standardization of technology and properly designed clinical trials performed at large scale will be essential. Moreover, the discrimination of the prognostic value of a set of genes, e.g., aggressiveness of tumor cells regardless of systemic treatment, versus the capacity to predict response to a specific drug needs more detailed investigation.

In ideal clinical practice, a single platform will be used that is able to provide prognostic (who to treat?) as well as predictive information (how to treat?). The perspective for the coming years is that the normal function of the ER and its downstream targets will continue to be unraveled and that combining this knowledge with gene expression profiling of breast cancers of patients in defined clinical settings will lead to diagnostic tests that can guide endocrine treatment, and finally to more insight in mechanisms underlying resistance to endocrine therapy that can help in developing novel treatment strategies.

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