Endocrine resistance in breast cancer: gene expression profiling and modifications of the estrogen receptor
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Chapter 11
Future perspectives
Future perspectives: lost in translation?
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-optimized cut-off value is largely unknown regarding the important clinical question: has an individual patient more benefit from tamoxifen or an aromatase inhibitor? Apparently the discovery of a biomarker related to endocrine responsiveness is relatively easy. However, translation of the findings into clinical practice seems extremely difficult.

This concluding chapter summarizes what the studies described in this thesis may have contributed to the field of predictive markers for breast cancer. Next, important challenges, limitations and impossibilities that have been experienced while working on the molecular prediction of endocrine treatment response are shared. Subsequently, we explore the fundamental and practical considerations that need to be addressed in order to identify and validate the ideal predictive marker adequately. What steps should be taken to translate the findings described in this thesis into clinical practice? Finally, an imaginary classification system is presented anticipating on the progress in the field of molecular diagnostics of breast cancer that will be made in the next decades.

Gene expression profiling

Prognosis vs. prediction
Currently, there are three commercially available genomic prognostic assays: MammaPrint®, Oncotype DX® and H/I®. In the area of predicting response to particular therapies, microarray-based studies have not yet delivered on their promise. It seems that responses to anti-cancer drugs are more difficult to predict by a molecular multi-gen expression test than prognosis is.

Randomized controlled trials vs. retrospective cohort studies
Unfortunately, frozen material for genome wide analysis is not available from patients who have been randomised between adjuvant tamoxifen and no systemic treatment. Consequently, the majority of studies attempting to find a gene expression profile that may predict tamoxifen response have been conducted using retrospective cohorts with patients who had received adjuvant tamoxifen. However, these cohort studies are biased in two different ways. First, in the adjuvant treatment setting the outcome is not only determined by drug response (prediction), but also by the intrinsic aggressiveness of the
tumor and the success of the local treatment (prognosis). As a result, in a retrospective cohort study, the true predictive value of a biomarker cannot be dissected from its prognostic value. Second, in earlier days only lymph node positive premenopausal patients received adjuvant systemic treatment and this consisted solely of chemotherapy. Adjuvant endocrine treatment was mainly reserved for postmenopausal patients with lymph node positive disease. Consequently, age and lymph node status are systematic confounders that hamper any meaningful conclusions to be drawn from such studies.

Metastatic disease vs. adjuvant treatment setting
To avoid the biases pointed out above, we have performed gene expression studies using frozen primary tumors from patients who had been treated with tamoxifen for metastatic disease. Since the metastatic lesions are in general not removed by surgery, the response to tamoxifen can be monitored in vivo. After the initial discovery of the 81-gene profile by Jansen and colleagues, we validated the 81-gene profile in the metastatic disease setting (Chapter 3). Even using a different microarray platform we were able to validate this profile in an independent dataset (Chapter 3). However, in the adjuvant treatment setting preliminary analyses did not reveal a significant association between the 81-gene profile and outcome after adjuvant tamoxifen treatment (data not shown). Obviously, this could be due to the lack of sufficiently large numbers of samples. Nevertheless, our analyses suggest that either tamoxifen response is determined by other factors in the metastatic setting versus the adjuvant treatment setting or that the differences in tumor- and patient characteristics between the advanced breast cancer patients and early breast cancer patients are responsible for the failure of our approach. In fact, the patients treated with tamoxifen for metastatic disease were younger of age and had more often LN-negative disease compared to the patients treated with tamoxifen in the adjuvant setting (illustrated in Chapter 5, table 1). This is a consequence of the treatment guidelines of those days (see above). In conclusion, the selection bias described above limits the translation of findings discovered in the metastatic disease setting to the adjuvant treatment setting. Another explanation why we may have failed in the translation of our findings to the adjuvant setting could be the subtle differences between gene expression profiles from primary tumors versus the profiles of their metastases. Of note, besides the 81-gene profile, several other gene profiles discovered in the metastatic dataset could not significantly predict outcome after tamoxifen in the adjuvant dataset (data not shown).

The future
Our experience illustrates the need for series of prospectively designed clinical studies enrolling patients whose clinical characteristics match the intended use of the test. 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
randomized between tamoxifen and an aromatase inhibitor may enable the discovery of gene profiles that predicts the response to either tamoxifen or an aromatase inhibitor.

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 technical 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 tumor 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 metabolizing enzymes.

**Modifications of ER**
True ‘from bench to bedside’ research involves the development of a biomarker that relies on a sound biological hypothesis. In chapter 6 we described our journey starting with the discovery of phosphorylation of ER at serine 305 using biophysical techniques and cell lines, via the detection of ER305p in human breast tumors, and finally presenting its value in a subgroup analysis of a randomized controlled trial. In addition, in chapter 8 the relevance of a second phosphorylation site of ER (ER118p) is shown. What steps should be taken before tests for ER305-P, ER118-P or pathways involved in the phosphorylation of ER can be used in daily clinical practice? In **figure 11.1** a simplified diagram is presented that illustrates some essential aspects that should be addressed before tests such as ER305-P, ER118-P or assays measuring activity of pathways involved in the phosphorylation of ER will reach a Level I Evidence according to the Tumor Marker Grading System (TUMGS) as presented in 1996 and updated recently by Daniel Hayes and colleagues. The biomarker ER305-P is used as an example. The TUMGS is a framework to evaluate the clinical utility of a tumor marker. Besides standard requirements that will be applicable to any tumor marker, figure 11.1 lists points of interest specific for markers making use of antibodies against phosphorylated antigens and for predictive markers that have to discriminate between tamoxifen resistance, resistance against an aromatase inhibitor, and general endocrine resistance.
Figure 11.1 How to get a test for ER305-P into the clinic?
Abbreviations: ER305-P phosphorylation of the estrogen receptor at serine 305, RCT randomized controlled trial, TAM tamoxifen, AST adjuvant systemic treatment, TMA tissue microarray, AI aromatase inhibitor

Genetic variants in drug-metabolizing enzymes
In view of the evolution of technology and the efforts to enable personalized-medicine, one can envision that in the far future every patient that walks into a hospital will get his own ‘SNP-passport’. However, a reliable and high-throughput test that can identify large-scale genomic variants is not available yet. Regarding genotype-guided endocrine therapy for breast cancers, several remaining questions need to be answered before genotypes can be used to select the most potent endocrine agent for an individual patient. First, endoxifen is the most potent metabolite from tamoxifen in vitro, however, it is largely unknown what the actual potency is of all the tamoxifen metabolites in vivo. Second, although most studies have been focusing on CYP2D6, it needs to be sorted out which alleles of which CYPs have the most influence on tamoxifen efficacy (see Chapter 9). Third, till now the relation between CYP450 genotypes and efficacy of endocrine treatment has not been explored in trials directly designed to study this important question.
Breast Cancer Stem Cells
The cancer stem cell (CSC) model proposes that the growth and progression of many cancers is driven by small populations of cancer stem cells (CSCs)(see Chapter 4). Consequently, it is very attractive to link drug resistance to characteristics of the CSC compartment in a breast tumor. While it has been stated that the CSC model rests on firm experimental foundations, some recent studies have called into question the existence of CSCs. Many fundamental questions have to be sorted out first before the CSC model may influence clinical practice. What is the proportion of CSCs in breast cancers? How to identify CSCs in human breast carcinoma? Do all subtypes of breast cancers rely on the CSCs? Or do CSCs only play a role in the undifferentiated subtypes, such a basal types, and not in luminal breast cancers, or vice versa? Although it is appealing to argue that therapeutic regimens will need to incorporate agents that target CSCs in order to achieve truly curative therapy and that drug response can be predicted on the basis of characteristics of the CSC, the bench is still too far from the bedside.

Quantification of the dominant patterns: ER and proliferation
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 tumor is ER-positive. Meta-analyses have never showed an analysis that addressed at which particular cut-off the ER was best predicting tamoxifen benefit. Already decades ago, RT-PCR based studies have shown that quantification of ER by means of measuring ESR1 mRNA levels yields additional information regarding the likelihood of response to tamoxifen in the ER-positive subgroup. However, a quantitative measurement of ER is still not used in the clinic. In chapter 5, ER-positive tumors are grouped according to the St. Gallen guidelines in ‘Incomplete Endocrine Responsive’ and ‘Highly Endocrine Responsive’ and exploratory analysis shows that those subgroups differ with regard to outcome after tamoxifen treatment. In chapter 2 (figure 2.3) the wide range of ESR1 mRNA levels even within the ER IHC 100% subgroup is illustrated. In addition, a carefully selected micro-array probe determining ESR1 mRNA levels seems to outperform the powerful 81-gene profile with respect to predicting outcome after tamoxifen treatment (data not shown). Besides ESR1 mRNA levels, tumor profiling using genes that incorporate an ERE in their promotor could be informative with regard to the assessment of endocrine sensitivity. Moreover, it has been shown that tumors with high ER levels have a poor response to chemotherapy. Future research should focus on how exactly ER activity has to be quantified.

In line with the need for quantification of ER, quantification of proliferation is an underexposed topic in translational breast cancer research. Proliferation capacity of tumor cells is determining the growth rate of tumor cells as well as the likelihood of
response to systemic treatment, in particular chemotherapy that kills rapidly dividing cells. In current clinical practice, proliferation is reflected by the tumor grading system. Besides the discrete expression of grade, the subjective scoring of mitotic rate, tubule formation and nuclear pleomorphism is a limitation. The ASCO 2007 recommendations for the use of tumor markers in breast cancer reports that the present data are insufficient to recommend the measurement of markers Ki67, cyclin D1, cyclin E, p27, p21, thymidine kinase or topoisomerase II or other proliferation markers to assign patients to prognostic subgroups. It is provocative to consider that the success of the prognostic gene expression assays is merely due to its ability to quantify proliferation capacity of the breast cancer cells. High-throughput analysis of breast tumors will help in the quantification of proliferation and this may be crucial to identify tumors that won’t benefit from chemotherapy or certain endocrine regimens. In figure 11.2 we present a futuristic molecular classification of breast tumors in which a hypothetical ‘ER Score’ and ‘Proliferation Score’ play a dominant role with regard to the treatment decisions that will be made based on a refined sub grouping of breast tumors.
Figure 11.2 Molecular classification of breast cancer - in the year 20XX. Abbreviations: AST adjuvant systemic treatment, NNT numbers needed to treat, TAM tamoxifen, AI aromatase inhibitor, SERD selective estrogen receptor downregulator, ER305-P phosphorylation of the estrogen receptor at serine 305, ER118-P phosphorylation of the estrogen receptor at serine 118, PKA protein kinase A, PAK1 p21-activated kinase, SNP single nucleotide polymorphism, EGFR epidermal growth factor receptor, HER human epidermal growth factor receptor, BRCA breast cancer, PI3KCA phosphatidylinositol 3-kinase catalytic subunit, Akt serine/threonine protein kinase Akt

Molecular classification of breast cancer in the year 20XX

Breast cancer is a heterogeneous disease. However, nowadays the WHO still classifies breast cancer as a single disease (C50). Within this C50 group a subdivision is made only based on the localization of the tumor within the breast. In figure 11.2 we present an imaginary molecular classification of breast tumors. The basis for this classification relies on molecular parameters that harbor information with regard to the likelihood of response to a certain therapy. In the year 20XX breast cancer wont be a single disease. Breast cancer will only be a general term that refers to several kinds of disease entities. And every subtype of breast cancer will be treated differently based on gene signatures, the presence of drug targets, genetic variation in drug metabolizing enzymes and the required numbers needed to treat (NNT).