Gene expression profiling in breast cancer

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introduction
With the advent of array-based technology and the sequencing of the human genome, comprehensive analysis of the transcriptional variation at the genomic level has become possible and the knowledge derived from gene expression profiling studies is already impressive in terms of improving our understanding of the basic biology of breast cancer. Thereby, these discoveries have also challenged the currently used classification of breast cancer and the existing theories around metastatic progression. The findings of some key studies and their implication for clinical practice are discussed below.

molecular classification of breast cancer
By detailing the expression levels of thousands of genes simultaneously from tumour cells and their surrounding microenvironment, gene expression profiles have provided molecular 'portraits' of breast cancer, distinguished by extensive differences of gene expression in breast cancer samples that were considered homogeneous by classical diagnostic methods. Several studies employing this technology, including one from our group, have indeed been remarkably consistent in reproducing a similar molecular classification of breast cancer [1–5]. Collectively, the conclusions were that: (i) estrogen receptor (ER) status has the strongest association with gene expression, followed by tumour grade; (ii) breast tumours can be grouped according to at least four individual subgroups: the 'basal-like' and erbB2 subgroups which are predominantly ER-negative, and two or more luminal subgroups being predominantly ER-positive; (iii) each subgroup has a distinct clinical outcome and may therefore respond differently to various therapeutics.

prognostic markers
Beside these studies aiming to provide a molecular classification of breast tumours, several independent groups have conducted comprehensive gene expression profiling studies with the aim of improving upon traditional prognostic markers used in the clinic.

In current clinical practice, the majority of patients with early breast cancer will receive some form of systemic adjuvant therapy (chemo- and/or endocrine therapy). Clinical parameters, such as lymph node status, tumour size and histological grade can provide prognostic information and are summarised in clinical guidelines, such as the National Institute of Health (NIH) [6] or the St Gallen consensus criteria [7, 8], in order to assist clinicians and patients in adjuvant therapy decision-making. However, risk stratification based on these guidelines is far from perfect and much progress is needed in identifying those patients who would really need adjuvant systemic therapy. In this context, two independent groups conducted gene expression profiling studies to identify broadly applicable prognostic markers using the 'fishing expedition' or 'top-down' approach (as illustrated by Liu [9]), which derives a prognostic model from global gene expression data from a tumour set simply by seeking gene expression profiles that are associated or correlated with clinical outcome.

The Amsterdam group first identified a 70-gene prognostic signature in a series of 78 systemically untreated node-negative breast cancer patients younger than 55 years of age, using the Agilent platform [10]. This signature included mainly genes involved in the cell cycle, invasion, metastasis, angiogenesis and signal transduction. This gene profile was then validated on a larger set of 295 young patients, including both node-negative and -positive breast tumours in treated and untreated patients from the same institution [11] and proved to be the strongest predictor for distant metastasis-free survival, independent of adjuvant treatment, tumour size, histological grade and age, both in node-negative and -positive patients.

Using a training set of 115 breast cancer patients, Wang et al. [12] identified a 76-gene signature which could be used to predict the development of distant metastases in untreated node-negative breast cancer patients of all age groups. Importantly, and in contrast to van’t Veer et al. [10], this study used the Affymetrix technology to build a classification algorithm that considered ER-positive patients separately from ER-negative patients, taking into account that the mechanisms for disease progression could differ for these two ER-based subgroups of breast cancer patients. As for the previous signature, these 76 genes were mainly associated with cell cycle and cell death, DNA replication and repair, and immune response. In the same study, they validated the prognostic ability of this signature in an additional set of 171 node-negative untreated breast cancer patients. Recently, this same group provided additional evidence for the prognostic performance of their predictor in a multi-centric cohort of 180 node-negative untreated breast cancer patients obtained from different institutions [13].
A common feature of both signatures is that when their performance in stratifying patients according to risk classification results is compared with the one of two traditional clinical risk classifications, namely the St Gallen [7, 8] and NIH criteria [9], both signatures were superior in correctly identifying the low-risk patients, suggesting a potential for reducing over-treatment in early breast cancer. However, the identification of high-risk patients could still be improved since half of these patients in fact did not recur.

A major challenge for gene expression profiling studies, especially those with clear clinical implications, is independent validation. Therefore, predictors should first be tested retrospectively in a large cohort of patients with a long follow-up period and then compared with established markers to assess their independent value.

If confirmed, the next logical step is to evaluate their prognostic power in a large prospective study. TRANSBIG (translational research network founded by the Breast International Group) decided to conduct an independent validation study of these two prognostic signatures in a series of 302 patients from five different centres, and across different statistical facilities [14, 15]. Although there was only a three-gene overlap between these signatures, both were validated in this patient cohort, even after adjustment for the clinical risk. In addition, a very interesting finding of this validation work was the heterogeneous behaviour of these gene signatures over time, which could be observed given the unusually long follow up (14 years) of the patients in this validation series. The signatures appeared to be strong predictors of the development of early distant metastases, while showing a decreased prognostic ability with increasing follow-up years. This finding, which was not observed for the clinical risk, is not entirely unexpected since the signatures were built to identify patients with distant metastases within 5 years. It suggests that different mechanisms might be associated with the development of early and late distant metastases, as already proposed by Klein and his group [16, 17].

Altogether, this 2-year intensive validation work has added to the growing evidence that gene expression signatures are of clinical relevance, especially in identifying patients at high-risk of early distant metastases, and has reinforced the belief that the time is right to proceed with the Microarray for Node Negative Disease may Avoid Chemotherapy (MINDACT) prospective trial.

The MINDACT study is a large collaborative trial conducted by the Breast International Group and the EORTC Breast Cancer Group that will randomise 6000 patients to investigate the benefit/risk ratio of chemotherapy when the risk assessment differs from that provided by the gene expression signature. This trial should then provide level 1 evidence about the clinical relevance of applying gene expression predictors to daily breast cancer patient management.

While this fishing-expedition approach may have an excellent prognostic impact, it may not carry much information about biological mechanisms, as opposed to the hypothesis-based approach. The discovery process of the latter approach starts with a specific biological hypothesis and only once a catalogue of genes has been uncovered with that particular cellular process or mechanism will the findings be linked with clinical outcome to evaluate potential clinical applicability.

Chang et al. [18] recently demonstrated the premise of this approach to gain insight into breast cancer biology. Based on the fact that wounds share many features with tumours, they identified a wound-response gene signature, whose genes appeared to be coordinately regulated in many human tumours, including breast cancer. They also found that breast cancer patients whose tumours were expressing the wound-response signature had a markedly worse clinical outcome [19]. Similar to the findings reported for the 70- and 76-gene signatures, they demonstrated that their signature improved current risk stratification based on the NIH and St Gallen guidelines and that it was able to identify a subset of low-risk patients within the clinical high-risk group. Altogether, their results pointed out a strong link between wound response and cancer behaviour on the genome scale, and also suggested that this signature would be a clinically useful tool for recognising the cancers at high risk of progression at an early stage. Therefore, these studies provided an experimental model of wound healing that could be used to study the underlying mechanisms and as a basis for developing inhibitors to the response.

Recently, our group undertook a similar hypothesis-driven approach focusing on histological grade, a well-established pathological parameter which is rooted in the cell biology of breast cancer. Our aims were to identify gene expression cassettes associated with grading and to explore whether the genetic components associated with these particular cellular states could improve breast cancer grading and its prognostic value.

Indeed, several observations and findings from recent decades provide consistent evidence that high- and low-grade tumours should be considered as distinct disease, setting the background for our study. First, pathologists have used histological grade to describe distinct breast cancer phenotypes: grade I or well-differentiated tumours are composed of polarised groups of cells that form tubular or duct-like structures, grade III or undifferentiated tumours are associated with a high mitotic activity, nuclear pleomorphism and no tubular formation, whereas grade II tumours display intermediate characteristics. In addition, high- and low-grade tumours have been correlated with the expression of different biological markers [20].

Second, there is growing evidence that tumour progression seems to occur independently of tumour grade. Roylance et al. [21] found that the long arm of chromosome 16 is lost in 65% of grade I tumours compared to only 16% in grade III tumours, implying that the majority of well-differentiated tumours do not evolve towards an undifferentiated state during tumour progression, as regain of genetic material is very unlikely. By investigating different markers in in situ and invasive breast cancer lesions, Warnberg et al. [22] suggested that the evolution from in situ to invasive cancer likely occurs independently of tumour grade.

Third, high- and low-grade tumours are also associated with a different clinical outcome profile: undifferentiated tumours being associated with the highest rate of recurrence and shorter recurrence time compared to well-differentiated tumours [23]. Additionally, clinicians are facing a real problem regarding patients carrying intermediate-grade tumours (grade II) as these tumours, which represent 30–60% of patients, are the major source of inter-observer discrepancy and display intermediate
phenotype and survival, making treatment decisions for these patients a great challenge with subsequent under or over treatment.

By examining whether histological grading was associated with distinct gene expression profiles, we were able to identify a significant cassette of 97 unique genes that were consistently differentially expressed between low- and high-grade breast carcinomas [24]. The majority of these genes were over-expressed in high-grade tumours and, as expected, they were associated with cell cycle progression and proliferation. In order to apply these findings to several external validation datasets using different microarray platforms, we developed a scoring system referred to as the gene expression grade index (GGI), which essentially quantifies the degree of similarity between the tumour expression pattern of these 97 genes and tumour grade. Intriguingly, gene expression profiles of intermediate-grade tumours look like a mixture of histological grade I and III cases rather than an intermediate between the two. Indeed, the GGI distribution of intermediate-grade tumours covered the range of the GGI values of the low and high histological grade carcinomas. Importantly, when examining the prognostic value of these molecular subtypes based on the GGI scores in the histological grade II tumour group, we found a statistically significant difference in clinical outcome, similar to the difference observed between histological grade I and III tumours.

Similar results were observed across multiple independent and heterogeneous validation series and microarray platforms, emphasising the reproducible behaviour of the genomic grade-associated genes.

Given that almost all GGI genes were associated with proliferation, we investigated how important proliferation genes are in predicting clinical outcome in previously reported prognostic gene signatures. To this end, we first applied and compared GGI with the 70-gene expression signature identified by the Amsterdam group on the same Dutch population from which the signature was derived. Despite the fact that GGI genes were selected without using clinical outcome and had to be mapped across different microarray platforms, the results were strikingly similar with respect to survival. Also, when comparing the 70- and 76-gene signatures [10, 12] with GGI in the TRANSBIG validation series reported earlier [14, 15], we observed consistent predictions of outcome, both in terms of time to distant metastasis and overall survival (data not shown).

In order to explore the implications of the joint distribution of ER status and GGI in predicting clinical outcome we examined the distribution of the GGI according to ER. We found that almost all ER-negative tumours were associated with a high GGI scores (high grade), whereas ER-positive tumours were associated with a heterogeneous mixture of gene expression grade index values. Although patients with ER-negative tumours appeared to have poorer prognosis than patients with ER-positive disease, the clinical outcome associated with ER-positive tumours was similar to that associated with all (ER-positive and ER-negative) patients considered in our study. The GGI not only separated the low- and high-risk groups better in the ER-positive population, but also the survival associated with the low GGI was substantially better than that associated with the total population. In contrast, among patients with high GGI scores, ER status was not associated with the risk of recurrence. Therefore, when GGI was known, ER status did not provide any additional information, but when ER status was known, GGI could still improve prognostic accuracy.

As described above, several microarray studies have classified breast cancer tumours based on gene expression profiles [1–5] and each of these studies has reported different subgroups within the ER-positive group. To investigate the expression of the genes in the GGI in relation to the previously reported molecular subtypes, we applied the GGI to these newly identified molecular subtypes from the data sets of Sorlie et al. [2, 3] and Sotiriou et al. [4]. Interestingly, we found that almost all ER-positive tumours previously classified as luminal-like A tumour, which had the best clinical outcome, were associated with low GGI values (low genomic grade), compared to the other luminal-like subgroups which had significantly higher GGI values and a poorer outcome [25]. These findings clearly showed that classification based on the genomic grade was comparably favourably with the molecular classification, emphasising again the role of proliferation-related genes in ER-positive breast cancer tumours.

**predictive markers**

Although most gene signatures that have been described here provide valuable information for classifying breast cancer tumours and have consistently predicted clinical outcome, the challenge is now to integrate this genomic information into prognostic models that could easily be applied in a clinical setting.

Breast cancer diagnosis and treatment decisions will continue to rely largely on classical histopathological and clinical parameters until some crucial issues have been resolved. These issues include: (i) ‘How can we compare and eventually integrate the information from these different signatures that have been identified to optimise risk stratification for breast cancer patients?’; (ii) ‘Would a combined approach of clinical and genomic data increase clinical outcome predictions?; (iii) ‘Are the technologies routinely applicable and reproducible?’.

Finally, identifying high-risk patients who would clearly need systemic adjuvant therapy is not good enough: we still do not know which therapy will be the most efficient for the individual patient. Indeed, identifying markers that could predict response to a particular drug remains a great challenge for the medical community as commonly used therapeutic agents are ineffective in many patients and side effects are common.

Several studies have already used a genome-wide approach in order to identify gene expression profiles that correlate with chemo- or hormono-sensitivity [26–28]. Although first results of the studies support the concept that predictors of anti-cancer drugs can be developed, they remain largely suboptimal. Indeed, small sample sizes have been used to build and validate these gene predictors, putting their robustness into question. Moreover, many studies suffer from methodological limitations, such as the choice of end points (clinical versus pathological response), the choice of the regimen to be studied (e.g., combination chemotherapy as opposed to single agent), and the type of population to be evaluated (e.g., the whole breast cancer...
population as opposed to a relevant molecular subgroup), as evaluating a predictor in an inappropriate cohort might lead to underestimation of its performance. Thereby, once predictors are identified, we should always investigate whether these just correlate with the natural history of the disease, predict response to cytotoxic agents in general or are really specific for a particular class of anti-cancer drug.

conclusions
Gene expression studies have great potential for improving breast cancer management and for increasing our understanding of the disease biology. There is no doubt that we are at a transition point between empirical and molecular medicine; however if we want ‘tailored’ breast cancer management to become a reality, we need adequate validation of the predictors in prospective clinical trials, such as the MINDACT trial.

references