Microarrays in primary breast cancer – lessons from chemotherapy studies

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Abstract

Current development in molecular techniques has extended the opportunities to explore genetic alterations in malignant tissue. There is a need to improve prognostication and, in particular, to understand the mechanisms of treatment resistance in different tumours. Gene analyses by microarrays allow concomitant analyses of several genes in concert, providing new opportunities for tumour classification and understanding of key biological disturbances. This paper outlines our continuing studies exploring prognostic and, we hope, predictive factors in breast cancer therapy.

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Introduction

Numerous prognostic factors have been studied in relation to breast cancer, but the biological knowledge that may be extracted from individual parameters is limited. First, many of these factors act in concert. Thus, multivariate analyses incorporating several factors generally reveal the prognostic impact of only two or three parameters, because of co-expression of individual parameters (Battaglia *et al.* 1988, Berger *et al.* 1988, Fisher *et al.* 1988, Fisher *et al.* 1993, Howat *et al.* 1983, Søreide *et al.* 1992). Secondly, because of this co-variance, prognostic factors do not necessarily reflect biological function. Thus, expressions of the oestrogen and the progesterone receptors are associated with a good prognosis in breast cancer, whether or not the patient receives adjuvant endocrine therapy (Harvey *et al.* 1999, Vollenweider-Zerargui *et al.* 1986). From a theoretical point of view this is unexpected, taking into account that ligand stimulation of the oestrogen receptor by oestradiol creates a potent mitogenic signal in cancer cells. Accordingly, the prognostic impact of oestrogen receptor expression is probably the result of its correlation to other parameters, such as low histological grade, slow growth rate (Singh *et al.* 1988), or other parameters.

There are several ways in which we might improve the way we are studying the biology of cancers *in vivo*.

Prognostic factors are limited by the fact that they often contain a 'predictive element'. The most important prognostic factor in breast cancer – expression of lymph node metastases – does not seem to be predictive of resistance or sensitivity to treatment (the reduction in the hazard ratio obtained with adjuvant chemotherapy or endocrine therapy seems to be approximately the same in lymph-node-positive and lymph-node-negative patients (Abe *et al.* 1998, Clarke *et al.* 1998). However, this is not the case for other parameters such as expression of the oestrogen receptor (Clarke *et al.* 1998) or mutations in the *TP53* gene (Geisler *et al.* 2001). From a therapeutic prospective, the major goal is to identify those patients whose tumours are sensitive and thus may benefit from treatment. An alternative to studying individual parameters is to look for several genetic alterations in concert, to better understand the functional network between individual parameters. This paper briefly views our current experience in applying such techniques to primary locally advanced breast cancer treated with neoadjuvant chemotherapy.

Clinical procedure

In 1991, we implemented our first procedure in which patients with locally advanced breast cancer (T_3/T_4, or N_2

**mRNA from Sample 1
(Reference Sample)**

**mRNA from Sample 2
(Experimental Sample)**

**Reverse transcribe each sample
using a different fluorescent
nucleotide (Cy3 or Cy5)**

**Mix the complex probes together and
hybridize overnight**

**Scan and
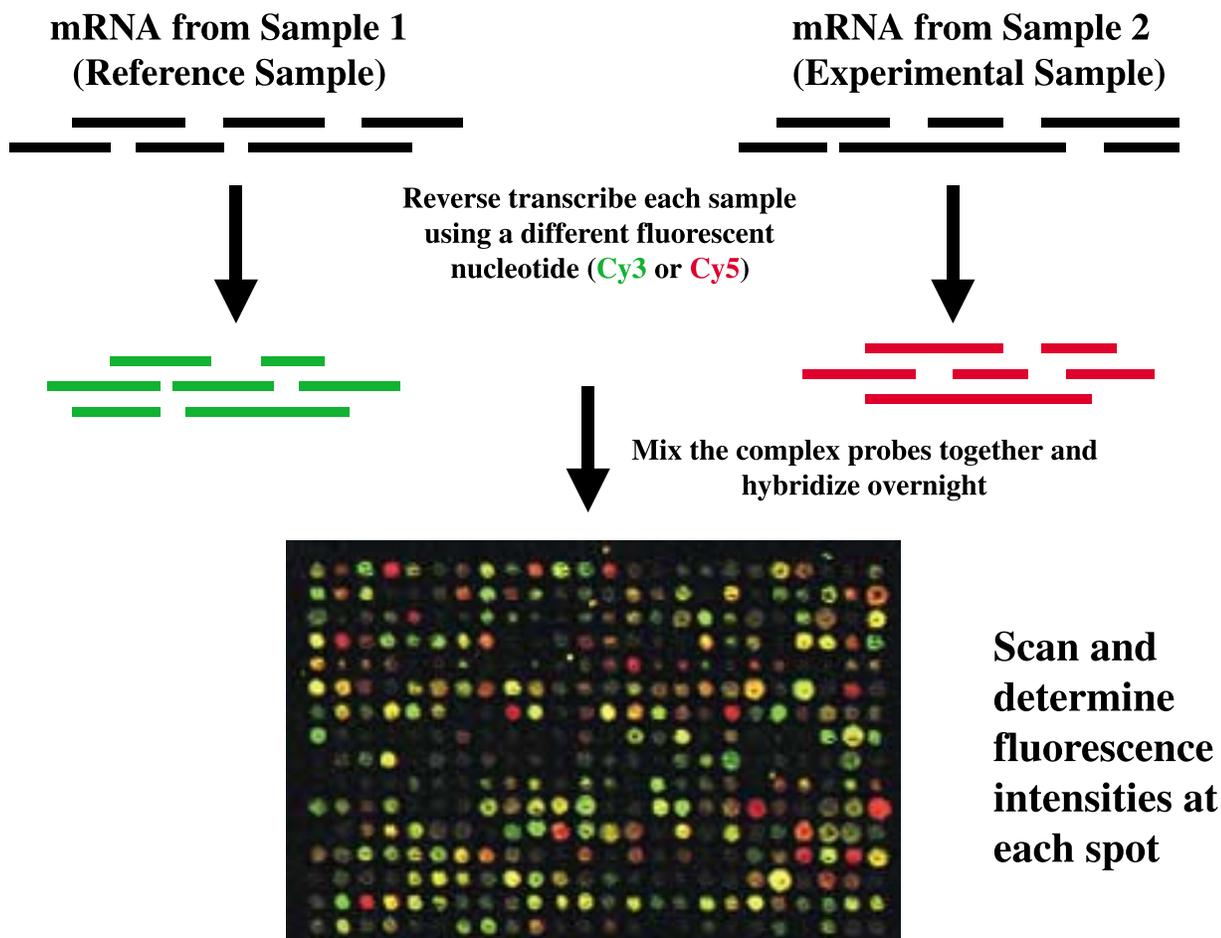determine
fluorescence
intensities at
each spot**

Figure 1 Two-colour fluorescent hybridization for the analysis of gene expression.

tumours, or both) were treated with primary chemotherapy followed by local treatment (Aas *et al.* 1996). The bulk of these patients were senior patients with a median age of 64 years. Accordingly, we applied the drug regimen commonly used for metastatic breast cancer in our department at that time – namely, weekly doxorubicin monotherapy (dose 14 mg/m^2). The scientific aim of this study was to explore biological markers in relation to chemoresistance. Accordingly, we preferred using a monotherapy regimen, in order to correlate these parameters to the effects of a particular drug.

Importantly, in this study we collected tumour samples before therapy by incisional biopsies and after chemotherapy (aimed at 16 weeks in accordance with the treatment procedure) when the patient underwent final surgery. In each case, tumours were collected and immediately snap-frozen in the operating theatre. Samples were stored in liquid nitrogen until required for processing.

Microarray techniques and analysis

The methods used have been outlined elsewhere (Alizadeh *et al.* 2000, DeRisi *et al.* 1997, Eisen & Brown 1999, Ross *et al.* 2000). cDNA prepared from mRNA in the experimental samples were labelled with Cy5, while the reference standard was made from a pool of mRNA isolated from 11 different cultured cell lines and labelled with Cy3 and the mixture was hybridised to microarrays with 8102 genes (Fig. 1). The microarray analysis was performed at the Stanford University Department of Genetics (P O Brown and D Botstein).

Results and discussion

In a previous study using this material (Aas *et al.* 1996), we showed that particular mutations in the *TP53* gene affecting the DNA-binding domains L2 and L3 predicted resistance to doxorubicin monotherapy. In a later extension of the study
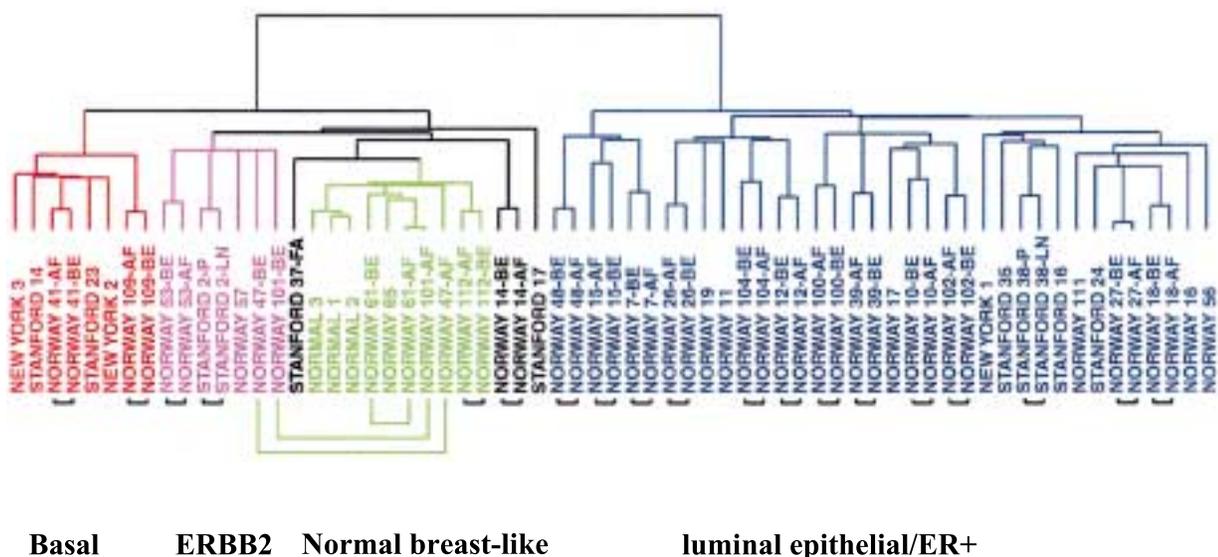
Basal   **ERBB2**   **Normal breast-like**   **luminal epithelial/ER+**

Figure 2 Square brackets indicate pairs. ER + , oestrogen receptor positive.

(Geisler *et al.* 2001), we confirmed the correlation between *TP53* mutations and resistance to therapy. In addition, we found that overexpression of the c-erbB-2 protein correlated with doxorubicin resistance. Notably, we observed a strong correlation between these two parameters, questioning the role of c-erbB-2 as an independent predictive factor for chemoresistance to doxorubicin given as a weekly 'low-dose' regimen.

To study the gene expression profile of primary breast cancers, we analysed 40 samples from this material (20 pairs, each consisting of a before- and an after-treatment sample) together with 44 other samples (Perou *et al.* 2000). Different sets of genes were selected from the total set of expression data. The first set consisted of 1753 genes (about 22% of the 8102 genes analysed), the transcripts of which varied in abundance by at least fourfold from their median abundance in the sample set, in at least three of the samples. Another set of genes called the 'intrinsic gene list' consisted of 496 cDNA clones. These genes were selected to show significantly greater variation among tumours from different patients than between paired samples from the same tumour. On the basis of the pattern of gene expression in these 84 samples using the different gene lists, the tumours could be classified into distinct groups by hierarchical cluster analysis. The patterns of expression observed provided a remarkably distinctive molecular portrait of each tumour. The analysis of the tumours that had been sampled twice (before and after a 16-week course of doxorubicin chemotherapy) showed a remarkable similarity. Thus the pattern of gene expression observed in the two tumour samples from the same individual were almost always more similar to each other than either was to any other sample (see below). Sets of

co-expressed genes were identified for which variation in mRNA levels could be related to specific features of physiological variation, or to variation in the cellular constituents of the tumours, limiting the need for microdissection.

The tumours could be classified into four distinctive groups that were distinguished by pervasive differences in their patterns of gene expression. These four groups were:

(1) An *ERBB2* cluster group expressing high levels of ERBB2 as well as several other genes, many of them located in the ERBB2 amplicon.
(2) 'Normal-like' breast cluster, expressing many genes characteristic of the normal breast epithelium.
(3) 'Basal-cell-like' cluster. This cluster expresses the genes characteristic of the basal breast cells, in particular with respect to certain keratins (keratin 5 and 17).
(4) 'Luminal-cell-cluster'. This cluster was characterised by a relatively high expression of many genes known to be expressed by breast luminal epithelial cells, including the oestrogen receptor.

The tumours in groups 1–3 where characterised by low or absent expression of the oestrogen receptor. Tumours in the basal-cell-like cluster (group 3) had high expression of gene characteristics for basal cells, including keratin 5/6, keratin 17, B4 and laminin. The fourth cluster, the so-called luminal cell cluster was characterised by expression of the oestrogen receptor in addition to several other genes, including breast cancer oestrogen regulated proteins such as LIV-1 protein, GATA-binding protein 3, prolactin receptor and carnitine palmitoyl-transferase II. For tumours belonging to either the basal-cell-like cluster or the luminal cell cluster,

A significant observation pertaining to the similarity in gene expression was that the 'paired samples' collected from the same tumour revealed a remarkable reproducibility with respect to gene profiling. Despite the fact that these samples were collected randomly from different parts of the tumour, that sample collection was separated by a time interval of 16 weeks, and that the patient had received chemotherapy in between, there was a remarkable consistency in gene expression between the two samples. Thus, when the tumours where classified by hierarchical clustering using the 1753 gene list, in 15 of the 20 pairs the two samples from the same tumour clustered next to each other, revealing a greater degree of similarity of gene expression (Fig. 2). Regarding the five pairs of samples for which such a similarity was not observed, in three cases this was characterised by a more 'normal-like' gene expression in the second sample. Notably, these three tumours were all collected from responders, meaning that the amount of tumour tissue compared with normal tissue could be significantly reduced in the second sample, which may explain this observation.

Continuing work and aims for future studies

Currently, we have extended the number of samples and are in the process of correlating profiles of gene expression to clinical outcome. A current observation is that the luminal group of tumours may be sub-divided into at least two sub-categories (luminal type A and B) with different gene expression profiles. By selecting tumours from our series of locally advanced cancers receiving uniform therapy for survival analysis, we were able to correlate tumour classification to clinical outcome (relapse-free and overall survival). As expected, the three oestrogen receptor-negative classes (basal-like, erbB-2-like and normal-cell-like tumours) were all associated with a poor outcome. Most interestingly, the two luminal sub-classes exhibited a significant difference in outcome with respect to relapse-free and overall survival, suggesting that this sub-classification may have novel clinical implications (Sørlie *et al.*).

In addition to the materials mentioned above, we have further analysed samples from another chemotherapy study of primary breast cancers, using a combined regimen of 5-fluorouracil and mitomycin, mainly in senior patients. Currently, we are correlating gene expression profiles in both series to clinical outcome, in particular with respect to drug responsiveness. We (Geisler *et al.* 2001) and others (Paik *et al.* 1998, Kandioler-Eckersberger *et al.* 2000) have shown that mutations in the *TP53* gene, and c-erbB-2 expression, correlate to resistance to chemotherapy in patients with breast cancer, but, importantly, we also observed tumours that expressed primary resistance to doxorubicin therapy despite harbouring wild type *TP53*. Most important, we also observed patients harbouring *TP53* mutations affecting the

DNA-binding domain who nevertheless responded to therapy (Geisler *et al.* 2001). Our current hypothesis is that, among patients expressing primary chemoresistance despite wild-type *TP53*, other disturbances in the p53 pathway may account for this phenotype. In addition, the finding that *TP53* mutations may be compensated for suggests that redundant mechanisms may be involved. Thus, a major aim of our current study programme is to evaluate other alterations in chemoresistance, in addition to *TP53* mutations. Clearly, drug resistance *in vivo* is a complex process likely to involve several genetic alterations and perhaps this also involves the patient's own genetic makeup. Accordingly, we hope that microarray studies of expression of several genes, together with complementary gene sequencing, could be a valuable tool for exploring this complex diversity.

Acknowledgements

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