DIFFERENTIATION OF TUMOURS OF DUCTAL AND LOBULAR ORIGIN:

II. GENOMICS OF INVASIVE DUCTAL AND LOBULAR BREAST CARCINOMAS

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Breast cancer is considered to be a multifactorial disorder caused by both genetic and non-genetic factors. Different histological types of breast cancer differ in response to treatment and may have a divergent clinical course. Breast tissue is heterogeneous, with components of epithelial, mesenchymal, endothelial and lymphopoietic derivation. The genetic heterogeneity of invasive breast cancer is reflected by the wide spectrum of histological types and differentiation grades. Nevertheless, the influences of these cell types on the tumour's total pattern of gene expression can be estimated analytically. Microarrays permit total tissue analysis and provide a stable molecular portrait of tumours. Some investigations suggest differences in the gene expression profiling for ductal and lobular carcinomas. It has been reported that inactivating mutations of the E-cadherin gene are very frequent in infiltrating lobular breast carcinomas. Other than altered expression of E-cadherin, little is known about the underlying biology that distinguishes ductal and lobular tumour subtypes. However, about 8 genes have been identified differentially which are expressed in lobular and ductal cancers: E-CD, survivin, cathepsin B, TPI1, SPRY1, SCYA14, TFAP2B, and thrombospondin 4, osteopontin, HLA-G, and CHC1. Expression profiling of breast cancers can be used diagnostically to distinguish individual histologic subclassifications and may guide the selection of target therapeutics. However, future approaches will need to include methods for high throughput clinical validation and the ability to analyze microscopic samples.

BREAST CANCER AS A GENETIC DISEASE

Breast cancer is a complex genetic disease characterized by the accumulation of multiple molecular alterations. The resulting clinical heterogeneity makes current diagnostic and therapeutic strategies less than perfectly adapted to each patient. Pathological and clinical factors are insufficient to capture the complex cascade of events that drive the clinical behavior of tumours. Today, DNA arrays, by allowing simultaneous and quantitative analysis of mRNA expression levels of thousands of genes in a single assay, provide novel tools for tackling this complexity. The potential applications of microarray analysis are multiple in the cancer field and the first research results are promising. This powerful technology has been exploited to explore gene expression in breast tissue on a genome-wide scale, and it has shown that different biological subtypes of breast cancer are accompanied by differences in transcriptional programs¹.

With cDNA microarrays, the relative expression levels of tens of thousands of genes within a specific tissue sample can be measured simultaneously. Breast tissue is heterogeneous, with components of epithelial, mesenchymal,

endothelial and lymphopoietic derivation. Nevertheless, the influences of these cell types on the tumour's total pattern of gene expression can be estimated analytically. Microarrays permit total tissue analysis and provide a stable molecular portrait of tumours. Many thousands of oligonucleotides or cDNA clones can be spotted onto a single glass slide microarray and most of the genome can now be examined in a single microarray. There are two important strategies in the elaboration of slide-based microarrays. One strategy is referred to as "in situ oligo synthesis." In this approach, pioneered by the Affymetrix® Corporation, sequences of fifteen to twenty-five nucleotides can be accurately and efficiently synthesized. In an alternative approach, developed at Rosetta Inpharmatics and Agilent Technologies, an inkjet printer, rather than photolithography, is used to apply sequential rounds of synthesis, using standard phosphoramidite chemistry and this allows the construction of oligonucleotides of sixty to eighty nucleotides in length. In the other major strategy, batches of "bio-ink" are synthesized in large quantities, and then printed on a substrate, usually a treated glass microscope slide, through any of a variety of techniques, including both contact and inkjet printing¹.

Microarray technology is fundamentally changing our understanding of cancer biology at the molecular level. Use of microarrays for genome-wide expression profiling provides a more refined molecular classification of human cancers and has reinforced the notion that breast cancer is a heterogeneous disease. This knowledge has great potential for a better selection of patients in need of adjuvant therapy as well as for tailored treatment approaches². A landmark study in this area is the work of Sorlie and colleagues who proposed a new classification of breast cancer clearly separating endocrine non-responsive from endocrine-responsive disease³. Sorlie and coworkers have confirmed their results in an independent set of breast tumours, refining previously defined subtypes of breast tumours that could be distinguished by their distinct patterns of gene expression⁴. Microarray is a new technology that, although evolving rapidly, needs to be fully standardized in order to be reproducible across different laboratories. Therefore, these interesting results needed to be duplicated in an independently run study and then validated in a large, independent, prospective trial before being applicable in clinical practice⁵.

As a first step in using cDNA microarrays to identify physiologically relevant gene expression patterns in human breast tumours, *in vitro* experiments were performed using specific hormones added to breast epithelial cell cultures. By subjecting cells to different conditions, it was possible to identify "clusters" of genes. mRNA samples from grossly dissected human breast tumours were compared to the mRNA from cultured human mammary epithelial cells (HMEC). Some of the clusters of genes with distinctive expression patterns identified *in vitro* also varied substantially in their expression in the breast tumour samples. For some of the clusters of coexpressed genes, expression in the tumour samples appeared to be attributable to other, noncarcinoma cell types, including stromal cells and B lymphocytes⁶.

The study of gene expression in primary human breast tumours as in most solid tumours is complicated for two major reasons. First, breast tumours consist of many different cell types, including not just the carcinoma cells but also additional epithelial cell types, stromal cells, adipose cells, endothelial cells and infiltrating lymphocytes. Second, breast carcinoma cells themselves are morphologically and genetically diverse⁷. These features have made the study and classification of human breast tumours difficult.

Recent applications of DNA arrays in breast cancer research has been reviewed by Bertucci and co-workers⁸. These technologies were used in the analysis of breast tumour subclasses with clinical implication^{3,9}, for analysis of gene expression changes in single breast cancer cells inside the same tumour¹⁰, for analysis of expression of different gene families in breast cancer^{11,12}, and for analysis of expression in different cellular and tumour types¹³⁻¹⁶. The critical point of these studies is isolation of sufficient amount of high-quality mRNA from the fresh or frozen tumour samples. Comparison of gene expression in single tumour cells is possible using a combination of micro-

dissection and microarray. However, limited total RNA (100–200 ng) from this microdissected tissue requires the use of amplification kits to synthesize and amplify cDNA and make labelled probes^{10,15}.

Zhu et al. found that changes in gene expression associated with variation in microanatomical location of neoplastic cells can be detected within even small developing tumour masses. Comparison of gene expression changes between cancer cells at the periphery and in the centre of breast cancers was performed using a combination of microdissection and microarray analysis. Cancer cells from the two areas were pooled separately from ductal carcinoma *in situ* and invasive cancer. Triplicate analysis revealed that 22 genes had changed expression levels in the periphery relative to the central region: 15 upregulated and 7 downregulated (arbitrary threshold of 1.5-fold or greater). Differences in RNA levels were confirmed by quantitative real-time PCR for two of the genes and by changes in protein levels, detected by immunohistochemistry¹⁰.

Quantitative and qualitative analyses of mRNAs from a small number of cells are extremely important for studies on gene expression in various physiological and pathological conditions in multicellular organisms. Aoyagi et al presented an effective method for high-fidelity global mRNA amplification for *in vivo* gene expression profiling of as few as 100 cells obtained by laser-captured microdissection (LCM). This method, called TALPAT, is based on T7 RNA polymerase-mediated transcription, adaptor ligation and PCR amplification followed by T7-transcription. More than 80 % of genes were identified as more than 3-fold changed among three gastric cancer cell lines using cRNA amplified by both TALPAT and the ordinary *in vitro* T7-transcription. The reproducibility of TALPAT was validated by microarray analysis on 100 breast cancer cells obtained by LCM¹⁷.

Sotiriou et al. analyzed RNAs from breast cancer samples from patients with known clinical outcome. The results concur with those of the earlier studies, despite differences in patient populations, treatments used and technology platforms employed. This study found that the ER status of the tumour was the most important discriminator of expression subtypes, confirming that ER biology plays a central role in breast carcinogenesis. Tumour grade was found to play a secondary role. Unsupervised hierarchical clustering analysis segregated these tumours into two main clusters based on their basal (predominantly ER negative) and luminal (predominantly ER positive) characteristics; within each of these clusters smaller subgroups were identified, characterized by distinct gene expression signatures involving different oncogene-specific pathways². The molecular signature subgroups showed expected differences in survival with a better outcome in the luminal group¹⁸. The power of gene expression profiling using microarrays for distinguishing subgroups of breast cancers has been demonstrated by several research groups^{3,5,19-21}.

Expression profiling and hierarchical clustering of ductal breast cancers have identified classes of tumours

with aggressive phenotype and poor prognosis^{3,20}. Predictive models based on expression data from node-negative patients can stratify patients into groups with good prognosis versus those with poor prognosis^{5,21}. Other studies have found distinct expression patterns based on BRCA1 and BRCA2 status¹⁹. To date, however, gene expression differences based on breast cancer histology have not been reported.

E CADHERIN

There are some investigations which suggest differences in gene expression profiling in ductal and lobular carcinomas. It has been reported that inactivating mutations of E-cadherin gene are very frequent in infiltrating lobular breast carcinomas²². The human epithelial E cadherin (E-CD) gene CDH1 maps to chromosome 16q22.1. Berx and co-workers developed a comprehensive PCR/SSCP mutation screen for the human E-CD gene. These authors found harboured protein truncation mutations (three nonsense and one frameshift) in the extracellular part of the E-CD protein. Each lobular carcinoma with E-CD mutations showed tumour-specific loss of heterozygosity in the chromosomal region 16q22.1 containing the E-CD locus. In compliance with this, no E-CD expression was detectable by immunohistochemistry in these tumours²³.

Asgeirsson et al. showed that different mechanisms are involved in the altered E-CD expression seen in different subtypes of breast carcinomas. The loss of E-CD, regardless of genetic causes as an independent prognostic marker for disease recurrence, especially in node-negative breast cancer patients, irrespective of histological type. These investigators analysed DNA from the same samples for loss of heterozygosity (LOH) using three separate microsatellite markers on chromosome 16q22.1. 19 % of infiltrating ductal carcinomas showed complete loss of E-cadherin expression compared with 64 % in infiltrating lobular carcinomas. LOH was detected in 46 % of infiltrating ductal carcinomas and 89 % of infiltrating lobular carcinomas. In the infiltrating lobular carcinomas, LOH was associated with complete loss of cell membrane expression of E-CD, although a cytoplasmic expression pattern was evident. In contrast, this association was not seen in the infiltrating ductal carcinomas²⁴.

Pleomorphic lobular carcinoma of the breast is a variant of infiltrating lobular carcinoma with a poor prognosis. The pleomorphic appearance of this variant hinders its correct identification and differentiation from ductal carcinoma. Palacios et al found that in terms of E-CD inactivation, pleomorphic lobular tumours are identical to classic infiltrating lobular carcinomas and distinct from ductal tumours and therefore they should be considered a variant of lobular carcinoma of the breast, despite their aggressive behavior²⁵.

Lobular carcinoma in situ (LCIS) adjacent to invasive lobular carcinoma has previously been shown to lack E-CD expression but whether LCIS without adjacent invasive carcinoma also lacks E-CD expression and whether

the gene mutations present in invasive lobular carcinoma are already present in LCIS is not known. Vos et al report that E-CD is a very early target gene in lobular breast carcinogenesis and plays a tumour-suppressive role, additional to the previously suggested invasion-suppressive role. E-CD expression was absent in cases of LCIS and presented in ductal carcinoma in situ (DCIS) both without an adjacent invasive component. The authors demonstrated the presence of the same truncating mutations and loss of heterozygosity (LOH) of the wild-type E-CD in the LCIS component and in the adjacent invasive lobular carcinoma²⁶.

The fact that genetic heterogeneity is a hallmark of invasive breast cancer is reflected by the wide spectrum of histological types and differentiation grades in this tumour entity²⁷. DCIS and LCIS have been proposed to be direct precursors of several subgroups of invasive breast cancer. DCIS is a genetically advanced entity. There are at least two genetic pathways along which DCIS can evolve²⁸. In addition, it has been shown that most DCIS and LCIS cases share a common clonal origin with the associated invasive breast cancer^{29,30}. However, the exact genetic changes associated with invasion remained unclear. Morphologically, DCIS resembles, and often coincides with, ductal and related (tubular, cribriform, mucinous) types of invasive breast cancer, and LCIS accompanies lobular invasive carcinomas, suggesting close relations with regard to progression. Nevertheless, it is of interest that 15–25 % of cases of DCIS are associated with invasive lobular carcinoma and that an equal proportion of patients diagnosed with LCIS will develop an invasive carcinoma of the ductal subtype over the next 15–20 years³¹. The potential association between these subtypes is nevertheless a matter of debate. A major difference between ductal and lobular cancer is the expression pattern of E-CD which is almost completely absent in LCIS and lobular invasive carcinoma³².

The study of Buerger et al. suggested that invasive breast cancer is a disease with multiple cytogenetic subclones already present in preinvasive lesions. The close genetic association between well differentiated and a subgroup of intermediately differentiated DCIS and LCIS led to the hypothesis that LCIS and a subgroup of DCIS are different phenotypic forms of a common genotype. These authors investigated LCIS, some of them with associated lobular invasive carcinoma, intermediately differentiated DCIS with an associated invasive lobular carcinoma and intermediately and poorly differentiated DCIS with associated ductal invasive carcinoma by means of comparative genomic hybridisation (CGH) after microdissection and immunohistochemical staining for E-CD. LCIS was characterised by a low average rate of copy number changes, no evidence of amplifications and a high rate of gains and losses of chromosomal material at 1q and 16q, respectively. A high degree of genetic homology with well differentiated DCIS was obvious. The cases of intermediately differentiated DCIS with associated lobular invasive components and lobular differentiation revealed striking homologies and a significant difference in E-CD

expression. No specific alteration was associated with invasion³³.

The study of Shen and co-workers suggested that breast cancer progression is clonal with regard to chromosome instability but different breast cancers present distinct molecular profiles resulting from genetic heterogeneity caused by chromosome instability³⁴.

Grade I invasive ductal breast carcinomas have a specific pattern of genetic aberrations, namely gain of 1q and loss of 16q. This pattern is very similar to the changes seen in invasive lobular breast carcinomas. The gene on 16q involved in invasive lobular carcinoma is known to be E-CD (CDH1). Roylance et al investigated whether the same gene is responsible for grade I invasive ductal carcinoma, using allele imbalance analysis, mutation screening and immunohistochemistry. The results showed that despite the shared pattern of genetic aberrations seen in grade I invasive ductal carcinoma and invasive lobular carcinoma, CDH1 is not the target gene in low-grade ductal tumourigenesis³⁵.

OTHER POTENTIAL GENES

Other than altered expression of E-cadherin, little is known about the underlying biology that distinguishes the ductal and lobular tumour subtypes.

Accumulating evidences indicate that p120 catenin, a member of the E-CD/catenin adhesion complex, plays a role in tumour invasion. Sarrio and co-workers report that abnormal cytoplasmic and nuclear localization of p120 which are mediated by the absence of E-CD, characteristically occur in the early stages of lobular breast cancer and are maintained during tumour progression to metastasis. p120 may be an important mediator of the oncogenic effects derived from E-CD inactivation, including enhanced motility and invasion, in lobular breast cancer. These authors analysed breast tissue biopsies by tissue microarray. Most of the lobular tumours (88 %) showed exclusive cytoplasmic localization and 6 % also had p120 nuclear staining. Cytoplasmic p120 strongly associated with complete loss of E-CD and beta-catenin not only in lobular carcinoma and its metastases but also in atypical lobular hyperplasias. In the latter, loss of heterozygosity of E-CD gene was also observed. In ductal tumours, by contrast, reduction of p120 and E-CD in the membrane was very common (57 % and 53 %, respectively), whereas cytoplasmic p120 staining was rarely seen³⁶.

Molecular analysis demonstrated that cyclin D1 was amplified in 30 % of the comedo DCIS, 22 % of the comedo DCIS and 32 % of the adjacent invasive ductal carcinomas, 30 % of the invasive ductal carcinomas and 27 % of the invasive lobular carcinomas. Cyclin D1 was amplified in 11 % of the invasive ductal carcinomas but not in the adjacent non-comedo DCIS lesions³⁷.

Korkola et al report specific changes in gene expression which distinguish lobular from ductal breast carcinomas. These authors used cDNA microarrays to identify genes differentially expressed between lobular and ductal

tumours. Unsupervised clustering of tumours failed to distinguish between the two subtypes. Prediction analysis for microarrays (PAM) was able to predict tumour type with an accuracy of 93.7 %. Genes that were significantly differentially expressed between the two groups were identified by MaxT permutation analysis using t tests (20 cDNA clones and 10 unique genes), significance analysis for microarrays (33 cDNA clones and 15 genes, at an estimated false discovery rate of 2 %), and PAM (31 cDNAs and 15 genes). 8 genes were identified by all three related methods (E-CD, survivin, cathepsin B, TPI1, SPRY1, SCYA14, TFAP2B, and thrombospondin 4), and an additional 3 that were identified by significance analysis for microarrays and PAM (osteopontin, HLA-G, and CHC1)³⁸ (table 1).

Table 1. Genes differentially expressed between invasive ductal and lobular breast carcinomas

Genes	ILC	IDC
E cadherin	Mutation, LOH	No mutation
p120 catenin	Cytoplasmic localization	Nuclear localization
Survivin	Expression	No expression
Cathepsin B	Expression	No expression
TPI1	Expression	No expression
SPRY1	Expression	No expression
SCYA14	Expression	No expression
TFAP2B	Expression	No expression
Thrombospondin 4	Expression	No expression
Osteopontin	Expression	No expression
HLA-G	Expression	No expression
CHC1	Expression	No expression

Sorlie et al. tried to classify breast carcinomas based on variations in gene expression patterns derived from cDNA microarrays and to relate tumour characteristics to clinical outcome. cDNA microarray experiments representing cancers, fibroadenomas, and normal breast tissues were analyzed by hierarchical clustering. A novel finding was that the previously characterized luminal epithelial/estrogen receptor-positive group could be divided into at least two subgroups, each with a distinctive expression profile. These subtypes proved to be reasonably robust by clustering using two different gene sets: first, a set of 456 cDNA clones previously selected to reflect intrinsic properties of the tumours and, second, a gene set that highly correlated with patient outcome³.

Expression profiling using cDNA microarrays have redefined the molecular classification of some cancers. Comprehensive genetic analysis permits the identification of novel pathways that may determine subtle differences

in tumour phenotype. Pusztai et al. suggest that expression profiling can be used diagnostically to distinguish individual histologic subclassifications and may guide the selection of target therapeutics. These investigators analyzed bilateral cancer tissue of different histologies in each breast (pure invasive mucinous and pure invasive ductal). Mucinous phenotype was associated with expression of immunostimulatory and inhibitory genes, consistent with cellular infiltration of lymphocytes and with the expression of enzymes involved in mucin production. The panel of matrix metalloproteinases was distinctly different for mucinous and invasive tumours, suggesting that therapeutic targets to this class of compounds may need to be tailored for the varying histologies¹⁴.

For many tumours, pathological subclasses exist and these have to be further defined using genetic markers to improve therapy and follow-up strategies³⁹⁻⁴⁰. cDNA array analyses of breast cancers have been performed to classify tumours into categories based on expression patterns. Comparing purified normal ductal epithelial cells and corresponding tumour tissues, the expression of only a small fraction of genes has been found to be significantly changed. A subset of genes repeatedly found to be differentially expressed in breast cancers was subsequently employed to perform classification of normal and malignant breast specimens using cluster analysis. This identified a subgroup of transcriptionally related tumours, designated class A, which can be further subdivided into A1 and A2. Correlation with classical clinicopathological parameters revealed that subgroup A1 was characterized by a high number of node-positive tumours (14 of 16). In this subgroup there was a disproportionate number of patients who had already developed distant metastases at the time of diagnosis (25% in this subgroup, compared with 5% among the rest of the samples). Taken together, the use of these differentially expressed marker genes in conjunction with sample clustering algorithms provides a novel molecular classification of breast cancer specimens, and facilitates identification of patients with a higher risk of tumour recurrence⁴¹.

CONCLUSION

Breast cancer is a complex genetic disease characterized by the accumulation of multiple molecular alterations. Breast cancers differ in response to treatment and may have a divergent clinical course despite having a similar histopathological appearance. The genetic heterogeneity of invasive breast cancer is reflected by the wide spectrum of histological types and differentiation grades. DNA microarrays provide a systematic method for identifying key markers for prognosis and treatment response by profiling thousands of genes expressed in a single cancer. Microarray profiling of invasive breast cancers confirms striking molecular differences between ductal carcinoma specimens and suggests a new classification for oestrogen-receptor negative breast cancer. It has been reported that specific changes in gene expression distinguish lobular

from ductal breast carcinomas. Inactivating mutations of E-cadherin gene are very frequent only in infiltrating lobular breast carcinomas. Other than altered expression of E-cadherin, little is known about the underlying biology to distinguish ductal from lobular tumour subtypes. Future approaches will need to include methods for high throughput clinical validation and the ability to analyze microscopic samples.

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