Review

The diagnosis and management of pre-invasive breast disease

Promise of new technologies in understanding pre-invasive breast lesions

Stefanie S Jeffrey and Jonathan R Pollack

Stanford University School of Medicine, Stanford, California, USA

Corresponding author: Stefanie S Jeffrey (e-mail: ssj@stanford.edu)

Published: 9 October 2003

Breast Cancer Res 2003, **5**:320-328 (DOI 10.1186/bcr655)

Abstract

Array-based comparative genomic hybridization, RNA expression profiling, and proteomic analyses are new molecular technologies used to study breast cancer. Invasive breast cancers were originally evaluated because they provided ample quantities of DNA, RNA, and protein. The application of these technologies to pre-invasive breast lesions is discussed, including methods that facilitate their implementation. Data indicate that atypical ductal hyperplasia and ductal carcinoma *in situ* are precursor lesions molecularly similar to adjacent invasive breast cancer. It is expected that molecular technologies will identify breast tissue at risk for the development of unfavorable subtypes of invasive breast cancer and reveal strategies for targeted chemoprevention or eradication.

Keywords: array comparative genomic hybridization, breast cancer, ductal carcinoma *in situ*, expression profiling, microarrays

Introduction

Over the past 5–10 years, new high-throughput technologies have been developed and applied to breast cancer research that facilitate genome-wide analyses of DNA, RNA, and proteins. Through the global analysis of normal and neoplastic breast tissue, these technologies have identified tumor-specific molecular signatures and are advancing research in breast tumor biology. They are expanding our knowledge beyond that obtained from histologic findings or studies of single genes. Consequently, these technologies are now being incorporated into clinical trials design. They offer the promise of improved diagnosis and prognostication and should assist in the identification of molecular targets for future therapeutic or preventive strategies, thereby improving our ability to care for patients with, or at risk for, breast cancer.

Although these technologies were originally used to study invasive breast cancer, they are now being extended to pre-malignant and pre-invasive disease, facilitated by other new technologies such as microdissection and nipple duct aspiration, ductoscopy, and ductal lavage. Although this field is still emerging, the results are encouraging and should impact our understanding of breast cancer development and progression.

Array-based comparative genomic hybridization – DNA analysis

The genomes of breast tumors are characterized by numerous chromosomal gains and losses (aneuploidy), as well as more localized regions of gene amplification and deletion. Such widespread DNA copy number alteration may reflect ongoing chromosomal instability [1] or a transient instability that accompanies telomere crisis [2]. Regardless, the retained, non-random genomic DNA gains and losses drive aberrant expression of oncogenes (e.g. *ERBB2*) and tumor-suppressor genes (e.g. *TP53*), contributing to the development and progression of cancer.

ADH = atypical ductal hyperplasia; cDNA = complementary DNA; CGH = comparative genomic hybridization; DCIS = ductal carcinoma *in situ*; EGFR = epidermal growth factor receptor; ER = estrogen receptor; MALDI=matrix-assisted laser desorption/ionization; MS = mass spectrometry; MS/MS = tandem mass spectrometry; PCR = polymerase chain reaction; SELDI-TOF MS = surface enhanced laser desorption/ionization time-of-flight mass spectrometry.

The specific constellation of DNA copy number alterations within a tumor or precursor lesion may provide biological insight and prognostic/predictive value. Loss of heterozygosity studies, which described allelic imbalances at specific loci on a chromosome, supported a genetic relationship between precursor lesions and invasive breast cancer [3,4].

Comparative genomic hybridization (CGH) was developed in order rapidly to map DNA copy number alteration across the genome [5]. In CGH, tumor and normal genomic DNA are labeled with two different fluorophores and co-hybridized onto normal metaphase chromosomes. The ratio of fluorescence along each chromosome provides a cytogenetic representation of DNA copy number changes in the tumor compared to normal sample. Unlike karyotyping techniques, CGH does not require tumor metaphases, and so can be readily applied to a solid tumor specimen. Furthermore, the relative stability of DNA allows for analyses of formalin-fixed, paraffin-embedded tissues. Microdissection techniques, coupled with polymerase chain reaction (PCR) amplification of genomic DNA using degenerate oligonucleotide primers, permit the analyses of small, heterogeneous pre-invasive lesions.

Comparative genomic hybridization investigations have advanced our understanding of presumptive precursor lesions including hyperplasia of usual type and atypical ductal hyperplasia (ADH) [6–9], lobular carcinoma *in situ* [10] and ductal carcinoma *in situ* (DCIS), and have, in particular, provided information about the transition from DCIS to invasive carcinoma. Comparative genomic hybridization data characterize DCIS as a genetically advanced lesion with widespread DNA copy number alteration [11,12]. Furthermore, common patterns of alteration between DCIS and adjacent invasive lesions support DCIS as a direct precursor of invasive breast carcinoma [12,13]. Analyses with CGH also suggest the existence of independent pathways of genetic evolution within DCIS [12,14,15]. Well-differentiated DCIS is characterized most frequently by loss of 16q and gain of 1q, while poorly differentiated DCIS displays localized amplifications, frequently involving 11q13 (*CCND1*) and 17q12 (*ERBB2*). Interestingly, intermediately differentiated DCIS appears to be a heterogeneous group, inclusive of both genotypes. These data suggest that poorly differentiated DCIS is not the final stage in an evolution from highly differentiated DCIS, but rather that poorly and highly differentiated DCIS are distinct genetic entities, separately evolving to invasive carcinoma. Similarities in genomic alterations suggest that highly differentiated DCIS is a precursor of more differentiated invasive carcinomas, specifically tubulo-lobular, tubular, and grade I invasive ductal subtypes, while poorly differentiated DCIS is a precursor of grade III invasive ductal carcinoma.

While CGH has provided important insight into the biology of pre-invasive lesions, its ultimate usefulness is limited by its cytogenetic mapping resolution. Array-based CGH has recently provided a high-resolution alternative to chromosome-based CGH [16–18]. In array CGH, tumor and normal genomic DNAs are differentially labeled and co-hybridized to a microarray comprising DNA elements of known chromosomal location, typically either complementary DNAs (cDNA) or large genomic DNA inserts (e.g. bacterial artificial chromosomes). The fluorescence ratio at each element on the array reflects the relative copy number for the corresponding DNA sequences in the tumor compared to a normal sample. The mapping resolution provided is at least an order of magnitude higher than chromosome-based CGH, and is limited only by the number and genomic distribution of arrayed elements. Array-based CGH also facilitates the parallel analysis of gene copy number and gene expression [18].

The high-resolution mapping afforded by array CGH has revealed in breast tumors a complexity of DNA copy number alteration across chromosomes not previously appreciated by conventional CGH [17,18]. As an example, in an array CGH study of 44 locally advanced breast tumors, several previously unrecognized regions of recurrent amplification likely to harbor important cancer genes were identified and precisely located [19].

Although no such studies have yet been reported for pre-invasive lesions, we expect the improved spatial resolution of genomic alterations afforded by array CGH to provide additional insight into the molecular pathogenesis and precursor role of these lesions, to further clarify the transition to invasive carcinoma, and perhaps to provide a basis for earlier clinical diagnosis. For the characterization of pre-invasive lesions, limited genomic DNA will be available from microdissected specimens. Although one group has reported performing array CGH using as little as 3 ng of genomic DNA [20], most array CGH protocols require 0.1–2.0 μg input DNA, and it is therefore likely that some form of whole-genome amplification will be required for the analysis of small cell numbers. It remains to be determined whether PCR with degenerate oligonucleotide primers [21] can provide sufficiently unbiased whole-genome amplification, or whether less biased methods that do not rely on exponential amplification by PCR [22] will prove more useful.

Expression profiling–RNA analysis

DNA microarrays permit the analysis of the relative expression level of thousands of genes in a single experiment. Arrays can be membrane-based or slide-based. Nylon membranes are spotted with cDNA clones and probed with radiolabeled sample. Slide-based arrays are composed of glass microscope slides specially treated with an adherent such as polylysine or aminosilane. Glass arrays

can be spotted with over 40,000 cDNA clones or presynthesized oligonucleotides using fine print tips or an ink jet printer, or prepared with oligonucleotide probes synthesized *in situ* using lithographic or ink jet technology. Slide-based arrays, which are generally probed with fluorescent dye-labeled sample, are smaller and easier to handle than membrane-based arrays for high throughput, although membrane-based arrays require less input RNA.

Tumor or breast tissue RNA is isolated from a snap-frozen specimen. In contrast to DNA, which may be extracted from tissue left at room temperature or from archival formalin-fixed tissue, RNA is less stable. Human tissue contains ribonucleases that contribute to RNA degradation, so the time between tissue devascularization and freezing at −80°C may affect both the quality of RNA and the genes that are expressed [23]. Tissue specimen of less than 0.5 cm thickness, such as core needle biopsies, may be preserved at room temperature in solutions that permeate the tissue and stabilize its RNA (e.g. RNA*later*™, Ambion Inc., Austin, TX, USA, or RNA*later*™ TissueProtect Tubes, Qiagen Inc., Ventura, CA, USA). Recently, RNA isolated from paraffin-embedded tissue has been tested and compared to fresh specimen, generally on a gene-by-gene basis using real-time quantitative PCR assays. Studies on the suitability of paraffin-embedded RNA for array-based examinations are ongoing [24]. Formalin preservation of tissue causes RNA and protein cross-linking that interfere with molecular analyses. In addition, RNA hydrolysis and fragmentation occur at the high temperatures required for paraffin embedding. Non-aldehyde-based tissue fixatives, such as ethanol and methanol, and low-melt polyester wax embedding compounds seem to hold promise, although long-term nucleic acid or protein stability are still in question and the performance of immunohistochemical staining antibodies would require reassessment. Recently developed commercial kits that facilitate the isolation of RNA from formalin-fixed paraffin-embedded tissues are undergoing testing.

For microarray experiments, either total RNA or mRNA is isolated from an experimental sample. The RNA is reverse transcribed to cDNA, directly or indirectly labeled with a fluorescent dye, and hybridized to the microarray. If RNA quantity is insufficient as a result of small tissue sample size, *in vitro* transcription-based linear amplification [25,26] may be performed. This can generate enough amplified antisense RNA, also known as complementary RNA, for array hybridization. When using cDNA microarrays, a differentially labeled reference sample is used with the experimental sample so that ratio measurements cancel out differences in hybridization kinetics and quantity of cDNA spotted on a given array. Total RNA, obtained from cell lines that reproducibly express a majority of human genes, may be used as a standard reference sample that allows comparisons among multiple experimental samples, even though they may be performed on different days and with different array print batches. By convention, the experimental (tumor) sample is labeled with a red fluorophore (Cy 5, which fluoresces at 635 nm) and the reference sample is labeled with a green fluorophore (Cy 3, which fluoresces at 532 nm). Based on the specificity and affinity of complementary base pairing, gene expression for each cDNA clone on the array is captured as signal intensities when the labeling dyes are fluoresced at the two appropriate wavelengths in an optical scanner. The measured signal intensities are normalized and a log ratio of the normalized signal intensities for the experimental sample compared to reference for each spot on the array is computed. This ratio essentially reflects the relative abundance of a particular gene in the experimental sample compared to the reference sample. The simultaneous measurement of relative gene expression of thousands of genes provides a genome-wide 'portrait' of gene expression for a tumor or other tissue. The data set is analyzed using bioinformatics tools [27,28] to identify groups of genes that may define subtypes within an experimental set according to differences in their expression profiles. Correlations of the subtypes with histologic or clinical parameters are performed with the objective of identifying groups of genes that may define characteristic features of a tumor.

Early studies of expression profiling of breast cancer were performed on cell cultures and invasive breast cancers [29,30]. Tumor specimens contained mixed cell populations: epithelial cells, stromal fibroblasts, vascular and lymphatic endothelial cells, adipocytes, and tumor-infiltrating lymphocytes and macrophages. The important signaling between epithelial and adjacent non-epithelial cells (tumor microenvironment) was captured in the molecular profile of the whole tissue, and gene expression of non-epithelial populations could be distinguished. There are now multiple studies evaluating expression profiles of invasive breast cancer using different array technologies and on different patient populations [31–34], including patients carrying *BRCA* susceptibility genes [35,36] and young breast cancer patients [37,38].

Using their transcriptional profiles, invasive breast cancers may be divided by molecular subtype into groups with different responses to systemic therapy and different survival patterns [39]. Tumor gene expression patterns from patients with locally advanced breast cancer, who were similarly treated with doxorubicin followed by tamoxifen, were distributed among five molecular subtypes. Two subtypes, denoted luminal A and B, were characterized by high relative expression of the estrogen receptor (ER) gene and other ER-associated genes, and showed cytokeratin expression patterns suggestive of luminal epithelial cell origin. The luminal subtypes comprised patients who had long-term survival, in spite of their advanced disease (luminal A), and patients with poor survival (luminal B),

reflecting either differing tumor biology or differing responses to systemic therapy, including possible tamoxifen insensitivity. The other subtypes showed relatively little expression of ER-associated genes (most were ER-negative tumors) and were divided into three subtypes: an *ERBB2* overexpressing group, a basal epithelial-like group (named for their high relative expression of basal cytokeratins), and a group that expressed normal-like genes, including genes known to be expressed in adipose and stromal tissue. The basal-like group (ER-negative and without *ERBB2* overexpression) contained high-grade tumors that were associated with high proliferation rates and 82% harbored mutations in the *TP53* gene. The expression patterns of luminal, basal, and *ERBB2*-overexpressing tumors described in this study appear to correlate with the different tumor subtypes described by others using CGH or immunohistochemistry [40,41].

Olopade and Grushko [42] suggest that tumors with *BRCA1* mutations may be consistent with a basal-like pattern of gene expression because six out of seven tumors from patients with *BRCA1* mutations stained positive for basal keratins and none showed *ERBB2* overexpression. They confirmed this in a larger study of *BRCA1*-associated tumors that showed no or low *ERBB2* amplification by fluorescence *in situ* hybridization assays [43]. This is in contrast to tumors from patients with *BRCA2* mutations that, in a limited number, appeared to have a luminal, ER-positive pattern. The findings of estrogen and progesterone receptor negativity, lack of *ERBB2* overexpression, and overall higher grade in tumors from patients with *BRCA1* mutations, compatible with a basal-like molecular phenotype, was confirmed by Lakhani and colleagues [44] in a larger series of 217 patients with *BRCA1* or *BRCA2* mutations, comparing them to 103 patients with sporadic breast cancer. They also found that breast cancers caused by *BRCA2* mutations had immunohistochemical profiles similar to sporadic breast cancers, although they were more likely to be *ERBB2* negative.

Based on the CGH work described above, it is anticipated that noninvasive precursor lesions may be characterized by similar molecular phenotypes as invasive breast cancer. Expression profiling of pre-invasive lesions, however, is technically more complex. First, it is difficult to freeze this tissue prior to diagnosis. Atypical hyperplasias or DCIS frequently present as non-palpable mammographic abnormalities (e.g. microcalcifications). Patient care necessitates that the entire surgical biopsy specimen be analyzed, without saving tissue for molecular analyses, for the following reasons: ADH and DCIS may be adjoining; DCIS requires thorough histologic examination in order not to miss areas of microinvasion; and margin status is vital for treatment decisions if DCIS or microinvasive carcinoma is identified. Therefore, the immediate freezing of surgical biopsies of mammographic abnormalities is generally not performed. However, with proper informed consent, additional core needle biopsies may be obtained at the time of mammographic stereotactic or ultrasound-directed core needle biopsy and frozen or stored in a commercial reagent that preserves both tissue architecture and RNA integrity. Using RNA*later*™ (Ambion Inc.), Ellis and colleagues [45] were able prospectively to obtain sufficient high-quality RNA for transcriptional profiling from preoperative or postoperative core needle breast biopsies.

Laser microdissection may be used to isolate pre-invasive lesions from adjacent 'normal' ductolobular tissue [46]. A purified population of epithelial or stromal cells may be obtained, and in conjunction with RNA amplification techniques [47], expression profiling of the cells can be performed. From a single modified radical mastectomy specimen, Sgroi *et al*. [48] microdissected normal epithelial cells, malignant invasive epithelial cells, and cells metastatic to an axillary lymph node and used the RNA from these specimens for studies on nylon membrane arrays containing approximately 8000 genes. Verifying gene expression with duplicate hybridizations, real-time quantitative PCR and immunohistochemistry, they confirmed the feasibility and validity of this technique. Luzzi and colleagues [49] compared the expression profiles of nonmalignant human breast epithelium and adjacent DCIS microdissected from three breast cancer patients and identified several differentially expressed genes that had been previously implicated in human breast cancer progression.

Adeyinka *et al*. [50] compared six cases of DCIS with necrosis (4 of high nuclear grade and 2 with intermediate nuclear grade) to four cases of DCIS without necrosis (all with low nuclear grade) using microdissection and 5544 spot membrane arrays. Similar to CGH studies, distinct expression changes associated with DCIS grade and morphology were found. Some of the genes that differed between the two groups included those involved in cell cycle regulation, signaling, apoptosis, and response to hypoxia. In particular, the upregulation of *AAMP*, angio-associated, migratory cell protein gene, in high grade DCIS with necrosis was demonstrated using array technology, real-time PCR, and *in situ* hybridization – a gene considered to function in migrating cells and which may be hypoxia-mediated in tumors. The four DCIS samples without necrosis demonstrated little gene expression variability, in contrast to the highly variable DCIS samples with necrosis, and consistent with the hypothesis that low-grade DCIS may represent a single molecular phenotype.

Ma *et al*. [51] compared microdissected epithelial cells captured from normal breast lobules, ADH, DCIS, and invasive ductal carcinoma. They examined 39 breast specimens, 36 containing cancer (5 of the 36 had DCIS only) and three from reduction mammoplasties. Comparing gene expression profiles of premalignant, pre-invasive, and

invasive cells to normal cells isolated from the same specimen, but distant from the tumor, or from reduction mammoplasties, they observed no consistent major transcriptional differences between ADH, DCIS and invasive ductal carcinoma from the same specimen. There were, however, distinct tumor signature differences between low-grade and high-grade tumors. Grade II tumor expression profiles were mixed, showing either low-grade or high-grade signatures. This corroborates previous limited data showing similarity between DCIS and invasive breast cancer from Porter *et al*. [52] using serial analysis of gene expression, and immunohistochemical data from Warnberg *et al*. [53] suggesting that well differentiated DCIS progresses to well differentiated invasive cancer and that poorly differentiated DCIS progresses to poorly differentiated invasive cancer. Ma *et al*. also showed that a small subset of genes whose expression increased between DCIS and invasive breast cancer, predominantly in high-grade lesions, were related to cellular proliferation/cell cycle regulation. Significantly, compared to normal epithelium, ADH appeared to be a genetically advanced lesion with an expression profile that resembled DCIS and invasive breast cancer within the same specimen. This study by Ma, Erlander, and Sgroi is the first to use transcriptional profiling to demonstrate that ADH and DCIS are direct precursors to invasive ductal carcinoma, confirming the work by Boecker [54,55] using double-immunofluorescence staining techniques, which suggested that ADH is a committed precursor lesion to different molecular phenotypes of invasive breast cancer.

Analyzing data obtained using 16,000 gene oligonucleotide arrays, Ramaswamy *et al*. [56] suggested a set of 17 genes whose common expression across multiple primary solid tumor types and their metastases identified tumors with metastatic potential. van 't Veer *et al*. [37] described a 70 gene prognosis profile in women less than 55 years of age that outperformed standard prognostic criteria in a follow-up validation study [38]. One might hypothesize that if (i) breast epithelial cells are committed to a neoplastic subtype in the ADH stage, and (ii) gene expression profiles of pre-invasive lesions presage the molecular phenotype of invasive cancers, and (iii) different molecular phenotypes of invasive breast cancer vary in their clinical outcome, then examination of pre-invasive lesions for unfavorable expression signatures may distinguish breast tissue that may ultimately evolve into metastatic breast cancer. By eradicating more aggressive subtypes of pre-invasive lesions using surgery, radiation, or targeted chemoprevention, the development and clinical outcome of invasive breast cancer might be favorably influenced.

Proteomics–protein analysis

Protein expression is the functional component that ultimately determines cellular physiology. Analysis of RNA expression alone cannot characterize all aspects of protein expression; for example, proteins may undergo post-translational modifications that can affect protein stability, activity, and subcellular localization. These differences may reveal important aspects of tumor biology.

Proteomics is the large-scale study of protein expression; its tools and techniques are still under development. The time-honored way of detecting proteins is by two-dimensional (polyacrylamide) gel electrophoresis, which sequentially separates proteins by their charge and molecular weight. Using this method, Czerwenka and colleagues [57] found multiple proteins differentially expressed in four malignant breast tissue specimens when compared to four normal tissue samples, including growth factor receptor proteins. Two-dimensional gel electrophoresis is very labor intensive and has limited resolution for the analysis of large numbers of proteins. Each protein needs to be excised from the gel, digested, and the peptide fragments further analyzed using mass spectrometry (MS) or tandem mass spectrometry (MS/MS). Two-dimensional liquid chromatography, high-performance liquid chromatography, or capillary electrophoresis use columns or multiple capillary loops containing gradients to separate proteins or protein digests on the basis of size and charge.

After protein or peptide separation, they must be ionized into a protonated gas phase prior to MS analysis. Liquids can undergo electrospray ionization and then MS or MS/MS. The advantage of MS/MS is that peptides are fragmented and then identified by specific amino acid sequences. Solids are ionized by matrix-assisted laser desorption/ionization (MALDI) or surface-enhanced laser desorption/ionization (SELDI). MALDI is a technique that mixes digested proteins with an organic acid matrix that catapults the peptides into an ionized form when irradiated by an ultraviolet laser. The peptides accelerate through an electrical field in a time of flight MS, which separates them by their mass to charge ratio (*m/z*). Reaching the detector at different times, a peptide mass profile or fingerprint is created that reflects the protein composition of the sample. Proteins are identified by comparing the peptide mass fingerprint to masses predicted by digestion of protein sequences and published in large protein databases.

SELDI is another method [58] that captures proteins from solubilized tissues or body fluids on diverse biochip surfaces using modified chromatographic techniques (affinity capture) to fractionate and isolate proteins. At the surface of the chip, retained proteins are combined with energy absorbing molecules and pulsed with a laser into a time-of-flight mass spectrometer. The use of different surfaces creates different protein binding interactions and results in different mass spectra. Since SELDI analyzes intact proteins, it is not possible to identify individual proteins from the mass spectra. Instead, each sample has a specific protein fingerprint which then is related to clinical parameters.

SELDI time of flight MS (SELDI-TOF MS) can be used to analyze proteins secreted by cells lining or extruded into the nipple ductal system. This would include secretions produced by normal, hyperplastic, and preinvasive ductal lesions. Sauter [59] found five differentially expressed proteins that were present in 75–84% of samples from women with invasive breast cancer but only 0–9% of samples from normal women. Paweletz and colleagues [60] found protein profiles that appeared to discern women with breast cancer from healthy controls. Li *et al*. [61] screened 169 serum samples from patients with cancer, benign breast disease, or healthy controls using SELDI-TOF MS. They identified a panel of three biomarkers that consistently separated stage 0–I breast cancer patients from non-cancer controls. Wulfkuhle and colleagues [62] performed the first proteomic analysis of matched normal ductal/lobular units and DCIS using laser microdissected epithelial cells from frozen tissue sections, which were separated by two-dimensional gel electrophoresis and MS. The protein profiles of microdissected epithelial cells differed from that produced from whole tissue; both strategies were used to identify 134 unique differentially expressed proteins including intracellular trafficking proteins and proteins involved in cell motility and genomic instability, suggesting that DCIS is an already advanced preinvasive lesion.

Proteins may also be analyzed using a technology that binds antibodies and/or antigens to glass microarrays. Protein binding is measured by comparative fluorescence, providing a high throughput enzyme-linked immunosorbent assay [63]. Protein array assays may be performed using biologic samples such as serum or plasma, nipple aspirate fluid, cell lysates, or, potentially, for the analysis of surface membranes of microdissected cells.

Newer activity-based protein profiling technologies (measuring enzyme activity changes not just enzyme abundance) suggest that specific enzyme activities may correlate with degree of invasiveness by matrigel assay for breast cancer and other tumor types [64]. This technique, currently used to study primary invasive breast cancers, may be applied to pre-invasive lesions if sufficient protein can be isolated from microdissected cells. The measurement of enzyme activities in precursor lesions may identify high-risk lesions and offer insight in designing prophylactic therapies that target specific molecular pathways to prevent progression to an invasive or metastatic phenotype.

Clinical impact of new technologies

The diagnosis of pre-invasive breast lesions presents a clinical dilemma for the patient and the physicians providing her care. Following a diagnosis of atypical hyperplasia or DCIS, a patient is immediately considered high risk for the future development of invasive breast cancer, although this progression will only occur in a portion of patients. Newer molecular technologies may define which patients will develop invasive breast cancer and who are at high risk for biologically aggressive disease. This could potentially alleviate anxiety and screening costs for the patient who is not at high risk. Molecular imaging technologies or breast magnetic resonance imaging may be used to identify suspicious changes (e.g. hypoxia or angiogenesis) in the breast tissue of high-risk patients [65]. Such patients may benefit from systemic [66] or surgical prophylactic therapies [67]. Targeting different molecular subtypes of breast cancer precursor lesions will probably require differing strategies; for example, some precursor lesions showing an ER-associated luminal subtype may respond to prophylactic agents that block estrogen or its production. Reduction in ER-positive invasive breast cancer by tamoxifen has already been demonstrated in breast cancer prevention trials [68]. Because basal-like invasive breast cancers overexpress epidermal growth factor receptor (EGFR) by immunostain [40] and expression profiling [Jeffrey lab, unpublished data], EGFR antagonists may prove to be a useful chemo-prophylactic therapy for precursor lesions with a basal-like molecular profile or for women with *BRCA1* mutations. A lack of response to tamoxifen chemo-prophylaxis has been demonstrated in a small number of women with *BRCA1* mutations [69], suggesting that agents useful in luminal precursor lesions do not impact basal-like lesions. The EGFR tyrosine kinase inhibitor ZD 1839 has already been shown to reduce epithelial proliferation of ER-negative/EGFR-positive DCIS implanted into immunosuppressed mice [70]. All of the data above are consistent with differentiation pathway commitment in the earliest pre-invasive stages of epithelial neoplasia. Other questions remain: whether lesions of a particular molecular subtype always herald invasive and ultimately metastatic disease; and whether developing invasive disease would be unifocal or multifocal/multicentric, thereby influencing the type of prophylactic surgery (lumpectomy versus mastectomy for excision of diseased ducts before development of invasive cancer) and decisions regarding chemo-prophylaxis. Another potential treatment approach could be introduction of pharmaceuticals via nipple duct catheterization, although intraductal dye injection demonstrates that the fluid does not always reach its intended site.

In order to link promising prognostic or predictive molecular markers of pre-invasive lesions to clinical outcome, a high throughput validation method is required. Tissue microarrays can be created from archival breast biopsies with long-term clinical follow-up. Hundreds of pre-invasive lesions can be inserted into a single paraffin block, which can be sectioned and immunostained with a variety of markers [71]. Tissue microarrays may also be assayed using RNA *in situ* hybridization techniques. Alternatively, markers may be validated by multiplexed quantitative PCR [72,73].

This article is the ninth in a review series on *The diagnosis and management of pre-invasive breast disease – current challenges, future hopes*, edited by Sunil R Lakhani.

Other articles in the series can be found at http://breast-cancer-research.com/articles/review-series.asp?series=bcr_Thediagnosis

Conclusion

The study of pre-invasive breast cancer using new technologies is still in its infancy. Array-based CGH provides higher order resolution than standard CGH and can facilitate parallel analyses between copy number changes and gene expression. This may help characterize malignant transformation and the identification of different molecular pathways that describe specific malignant phenotypes. cDNA expression profiling has already defined different molecular subtypes of invasive breast cancer that are associated with different clinical outcomes. Expression profiling of microdissected pre-invasive breast lesions shows that ADH associated with invasive breast cancer is already a genetically advanced lesion, with strong molecular similarities between ADH, DCIS, and invasive breast cancer subtypes by CGH, cDNA microarray, and immunostain analyses. Both array-based CGH and cDNA microarray analyses should identify targets for future diagnostics and novel therapies. Proteomic studies are just beginning to search for biomarkers that may form the basis of future blood, nipple aspirate fluid, or tissue diagnostic tests so that women at risk, who may benefit from close monitoring, systemic prophylaxis, or excision of diseased ducts, may be identified well before the development of any life-threatening malignant process.

Competing interests

None declared.

Acknowledgements

The authors would like to thank Vincent A Funari for his helpful comments, and Susan Overholser and Maureen H Chang for their assistance in the preparation of this manuscript.

References

1. Lengauer C, Kinzler KW, Vogelstein B: **Genetic instabilities in human cancers.** *Nature* 1998, **396:**643-649.
2. Maser RS, DePinho RA: **Connecting chromosomes, crisis, and cancer.** *Science* 2002, **297:**565-569.
3. Stratton MR, Collins N, Lakhani SR, Sloane JP: **Loss of heterozygosity in ductal carcinoma in situ of the breast.** *J Pathol* 1995, **175:**195-201.
4. O'Connell P, Pekkel V, Fuqua SA, Osborne CK, Clark GM, Allred DC: **Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci.** *J Natl Cancer Inst* 1998, **90:** 697-703.
5. Kallioniemi A, Kallioniemi OP, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D: **Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors.** *Science* 1992, **258:**818-821.
6. Werner M, Mattis A, Aubele M, Cummings M, Zitzelsberger H, Hutzler P, Hofler H: **20q13.2 amplification in intraductal hyperplasia adjacent to in situ and invasive ductal carcinoma of the breast.** *Virchows Arch* 1999, **435:**469-472.
7. Gong G, DeVries S, Chew KL, Cha I, Ljung BM, Waldman FM: **Genetic changes in paired atypical and usual ductal hyperplasia of the breast by comparative genomic hybridization.** *Clin Cancer Res* 2001, **7:**2410-2414.
8. Boecker W, Buerger H, Schmitz K, Ellis IA, van Diest PJ, Sinn HP, Geradts J, Diallo R, Poremba C, Herbst H: **Ductal epithelial proliferations of the breast: a biological continuum? Comparative genomic hybridization and high-molecular-weight cytokeratin expression patterns.** *J Pathol* 2001, **195:**415-421.
9. Jones C, Merrett S, Thomas VA, Barker TH, Lakhani SR: **Comparative genomic hybridization analysis of bilateral hyperplasia of usual type of the breast.** *J Pathol* 2003, **199:**152-156.
10. Buerger H, Simon R, Schafer KL, Diallo R, Littmann R, Poremba C, van Diest PJ, Dockhorn-Dworniczak B, Bocker W: **Genetic relation of lobular carcinoma in situ, ductal carcinoma in situ, and associated invasive carcinoma of the breast.** *Mol Pathol* 2000, **53:**118-121.
11. Moore E, Magee H, Coyne J, Gorey T, Dervan PA: **Widespread chromosomal abnormalities in high-grade ductal carcinoma in situ of the breast. Comparative genomic hybridization study of pure high-grade DCIS.** *J Pathol* 1999, **187:**403-409.
12. Buerger H, Otterbach F, Simon R, Poremba C, Diallo R, Decker T, Riethdorf L, Brinkschmidt C, Dockhorn-Dworniczak B, Boecker W: **Comparative genomic hybridization of ductal carcinoma in situ of the breast – evidence of multiple genetic pathways.** *J Pathol* 1999, **187:**396-402.
13. Aubele M, Mattis A, Zitzelsberger H, Walch A, Kremer M, Welzl G, Hofler H, Werner M: **Extensive ductal carcinoma in situ with small foci of invasive ductal carcinoma: evidence of genetic resemblance by CGH.** *Int J Cancer* 2000, **85:**82-86.
14. Buerger H, Otterbach F, Simon R, Schafer KL, Poremba C, Diallo R, Brinkschmidt C, Dockhorn-Dworniczak B, Boecker W: **Different genetic pathways in the evolution of invasive breast cancer are associated with distinct morphological subtypes.** *J Pathol* 1999, **189:**521-526.
15. Buerger H, Mommers EC, Littmann R, Simon R, Diallo R, Poremba C, Dockhorn-Dworniczak B, van Diest PJ, Boecker W: **Ductal invasive G2 and G3 carcinomas of the breast are the end stages of at least two different lines of genetic evolution.** *J Pathol* 2001, **194:**165-170.
16. Solinas-Toldo S, Lampel S, Stilgenbauer S, Nickolenko J, Benner A, Dohner H, Cremer T, Lichter P: **Matrix-based comparative genomic hybridization: biochips to screen for genomic imbalances.** *Genes Chromosomes Cancer* 1997, **20:**399-407.
17. Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y, Dairkee SH, Ljung BM, Gray JW, Albertson DG: **High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays.** *Nat Genet* 1998, **20:**207-211.
18. Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, Jeffrey SS, Botstein D, Brown PO: **Genome-wide analysis of DNA copy-number changes using cDNA microarrays.** *Nat Genet* 1999, 23:41-46.
19. Pollack JR, Sorlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE, Tibshirani R, Botstein D, Borresen-Dale AL, Brown PO: **Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors.** *Proc Natl Acad Sci U S A* 2002, **99:**12963-12968.
20. Snijders AM, Nowak N, Segraves R, Blackwood S, Brown N, Conroy J, Hamilton G, Hindle AK, Huey B, Kimura K, Law S, Myambo K, Palmer J, Ylstra B, Yue JP, Gray JW, Jain AN, Pinkel D, Albertson DG: **Assembly of microarrays for genome-wide measurement of DNA copy number.** *Nat Genet* 2001, **29:**263-264.
21. Daigo Y, Chin SF, Gorringe KL, Bobrow LG, Ponder BA, Pharoah PD, Caldas C: **Degenerate oligonucleotide primed-polymerase chain reaction-based array comparative genomic hybridization for extensive amplicon profiling of breast cancers: a new approach for the molecular analysis of paraffin-embedded cancer tissue.** *Am J Pathol* 2001, **158:**1623-1631.
22. Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J, Driscoll M, Song W, Kingsmore SF, Egholm M, Lasken RS: **Comprehensive human genome amplification using multiple displacement amplification.** *Proc Natl Acad Sci U S A* 2002, **99:**5261-5266.

23. Dash A, Maine IP, Varambally S, Shen R, Chinnaiyan AM, Rubin MA: **Changes in differential gene expression because of warm ischemia time of radical prostatectomy specimens.** *Am J Pathol* 2002, **161:**1743-1748.
24. Gillespie JW, Best CJ, Bichsel VE, Cole KA, Greenhut SF, Hewitt SM, Ahram M, Gathright YB, Merino MJ, Strausberg RL, Epstein JI, Hamilton SR, Gannot G, Baibakova GV, Calvert VS, Flaig MJ, Chuaqui RF, Herring JC, Pfeifer J, Petricoin EF, Linehan WM, Duray PH, Bova GS, Emmert-Buck MR: **Evaluation of non-formalin tissue fixation for molecular profiling studies.** *Am J Pathol* 2002, **160:**449-457.
25. Van Gelder RN, von Zastrow ME, Yool A, Dement WC, Barchas JD, Eberwine JH: **Amplified RNA synthesized from limited quantities of heterogeneous cDNA.** *Proc Natl Acad Sci U S A* 1990, **87:**1663-1667.
26. Zhao H, Hastie T, Whitfield ML, Borresen-Dale AL, Jeffrey SS: **Optimization and evaluation of T7 based RNA linear amplification protocols for cDNA microarray analysis.** *BMC Genomics* 2002, **3:**31.
27. Eisen MB, Spellman PT, Brown PO, Botstein D: **Cluster analysis and display of genome-wide expression patterns.** *Proc Natl Acad Sci U S A* 1998, **95:**14863-14868.
28. Tibshirani R, Hastie T, Narasimhan B, Chu G: **Diagnosis of multiple cancer types by shrunken centroids of gene expression.** *Proc Natl Acad Sci U S A* 2002, **99:**6567-6572.
29. Perou CM, Jeffrey SS, van de Rijn M, Rees CA, Eisen MB, Ross DT, Pergamenschikov A, Williams CF, Zhu SX, Lee JC, Lashkari D, Shalon D, Brown PO, Botstein D: **Distinctive gene expression patterns in human mammary epithelial cells and breast cancers.** *Proc Natl Acad Sci U S A* 1999, **96:**9212-9217.
30. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D: **Molecular portraits of human breast tumours.** *Nature* 2000, **406:**747-752.
31. Bertucci F, Nasser V, Granjeaud S, Eisinger F, Adelaide J, Tagett R, Loriod B, Giaconia A, Benziane A, Devilard E, Jacquemier J, Viens P, Nguyen C, Birnbaum D, Houlgatte R: **Gene expression profiles of poor-prognosis primary breast cancer correlate with survival.** *Hum Mol Genet* 2002, **11:**863-872.
32. Ahr A, Karn T, Solbach C, Seiter T, Strebhardt K, Holtrich U, Kaufmann M: **Identification of high risk breast-cancer patients by gene expression profiling.** *Lancet* 2002, **359:**131-132.
33. Gruvberger S, Ringner M, Chen Y, Panavally S, Saal LH, Borg A, Ferno M, Peterson C, Meltzer PS: **Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns.** *Cancer Res* 2001, 61:5979-5984.
34. West M, Blanchette C, Dressman H, Huang E, Ishida S, Spang R, Zuzan H, Olson JA, Jr, Marks JR, Nevins JR: **Predicting the clinical status of human breast cancer by using gene expression profiles.** *Proc Natl Acad Sci U S A* 2001, **98:**11462-11467.
35. Hedenfalk I, Duggan D, Chen Y, Radmacher M, Bittner M, Simon R, Meltzer P, Gusterson B, Esteller M, Kallioniemi OP, Wilfond B, Borg A, Trent J: **Gene-expression profiles in hereditary breast cancer.** *N Engl J Med* 2001, **344:**539-548.
36. Berns EM, van Staveren IL, Verhoog L, van de Ouweland AM, Meijer-van Gelder M, Meijers-Heijboer H, Portengen H, Foekens JA, Dorssers LC, Klijn JG: **Molecular profiles of BRCA1-mutated and matched sporadic breast tumours: relation with clinicopathological features.** *Br J Cancer* 2001, **85:**538-545.
37. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH: **Gene expression profiling predicts clinical outcome of breast cancer.** *Nature* 2002, **415:**530-536.
38. van de Vijver MJ, He YD, van 't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R: **A gene-expression signature as a predictor of survival in breast cancer.** *N Engl J Med* 2002, **347:**1999-2009.
39. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL: **Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications.** *Proc Natl Acad Sci U S A* 2001, **98:**10869-10874.
40. Korsching E, Packeisen J, Agelopoulos K, Eisenacher M, Voss R, Isola J, van Diest PJ, Brandt B, Boecker W, Buerger H: **Cytogenetic alterations and cytokeratin expression patterns in breast cancer: integrating a new model of breast differentiation into cytogenetic pathways of breast carcinogenesis.** *Lab Invest* 2002, **82:**1525-1533.
41. Jones C, Nonni AV, Fulford L, Merrett S, Chaggar R, Eusebi V, Lakhani SR: **CGH analysis of ductal carcinoma of the breast with basaloid/myoepithelial cell differentiation.** *Br J Cancer* 2001, **85:**422-427.
42. Olopade OI, Grushko T: **Gene-expression profiles in hereditary breast cancer.** *N Engl J Med* 2001, **344:**2028-2029.
43. Grushko TA, Blackwood MA, Schumm PL, Hagos FG, Adeyanju MO, Feldman MD, Sanders MO, Weber BL, Olopade OI: **Molecular-cytogenetic analysis of HER-2/neu gene in BRCA1-associated breast cancers.** *Cancer Res* 2002, **62:**1481-1488.
44. Lakhani SR, Van De Vijver MJ, Jacquemier J, Anderson TJ, Osin PP, McGuffog L, Easton DF: **The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2.** *J Clin Oncol* 2002, **20:**2310-2318.
45. Ellis M, Davis N, Coop A, Liu M, Schumaker L, Lee RY, Srikanchana R, Russell CG, Singh B, Miller WR, Stearns V, Pennanen M, Tsangaris T, Gallagher A, Liu A, Zwart A, Hayes DF, Lippman ME, Wang Y, Clarke R: **Development and validation of a method for using breast core needle biopsies for gene expression microarray analyses.** *Clin Cancer Res* 2002, **8:**1155-1166.
46. Emmert-Buck MR, Bonner RF, Smith PD, Chuaqui RF, Zhuang Z, Goldstein SR, Weiss RA, Liotta LA: **Laser capture microdissection.** *Science* 1996, **274:**998-1001.
47. Luo L, Salunga RC, Guo H, Bittner A, Joy KC, Galindo JE, Xiao H, Rogers KE, Wan JS, Jackson MR, Erlander MG: **Gene expression profiles of laser-captured adjacent neuronal subtypes.** *Nat Med* 1999, **5:**117-122.
48. Sgroi DC, Teng S, Robinson G, LeVangie R, Hudson JR, Jr, Elkahloun AG: **In vivo gene expression profile analysis of human breast cancer progression.** *Cancer Res* 1999, **59:**5656-5661.
49. Luzzi V, Holtschlag V, Watson MA: **Expression profiling of ductal carcinoma in situ by laser capture microdissection and high-density oligonucleotide arrays.** *Am J Pathol* 2001, **158:** 2005-2010.
50. Adeyinka A, Emberley E, Niu Y, Snell L, Murphy LC, Sowter H, Wykoff CC, Harris AL, Watson PH: **Analysis of gene expression in ductal carcinoma in situ of the breast.** *Clin Cancer Res* 2002, **8:**3788-3795.
51. Ma XJ, Salunga R, Tuggle JT, Gaudet J, Enright E, McQuary P, Payette T, Pistone M, Stecker K, Zhang BM, Zhou YX, Varnholt H, Smith B, Gadd M, Chatfield E, Kessler J, Baer TM, Erlander MG, Sgroi DC: **Gene expression profiles of human breast cancer progression.** *Proc Natl Acad Sci USA* 2003, **100:**5974-5979.
52. Porter DA, Krop IE, Nasser S, Sgroi D, Kaelin CM, Marks JR, Riggins G, Polyak K: **A SAGE (serial analysis of gene expression) view of breast tumor progression.** *Cancer Res* 2001, **61:** 5697-5702.
53. Warnberg F, Nordgren H, Bergkvist L, Holmberg L: **Tumour markers in breast carcinoma correlate with grade rather than with invasiveness.** *Br J Cancer* 2001, **85:**869-874.
54. Böcker W, Moll R, Poremba C, Holland R, Van Diest PJ, Dervan P, Bürger H, Wai D, Ina Diallo R, Brandt B, Herbst H, Schmidt A, Lerch MM, Buchwallow IB: **Common adult stem cells in the human breast give rise to glandular and myoepithelial cell lineages: a new cell biological concept.** *Lab Invest* 2002, **82:**737-746.
55. Boecker W, Moll R, Dervan P, Buerger H, Poremba C, Diallo RI, Herbst H, Schmidt A, Lerch MM, Buchwallow IB: **Usual ductal hyperplasia of the breast is a committed stem (progenitor) cell lesion distinct from atypical ductal hyperplasia and ductal carcinoma in situ.** *J Pathol* 2002, **198:**458-467.
56. Ramaswamy S, Ross KN, Lander ES, Golub TR: **A molecular signature of metastasis in primary solid tumors.** *Nat Genet* 2003, **33:**49-54.
57. Czerwenka KF, Manavi M, Hosmann J, Jelincic D, Pischinger KI, Battistutti WB, Behnam M, Kubista E: **Comparative analysis of two-dimensional protein patterns in malignant and normal human breast tissue.** *Cancer Detect Prev* 2001, **25:**268-279.

58. Merchant M, Weinberger SR: **Recent advancements in surface-enhanced laser desorption/ionization-time of flight-mass spectrometry.** *Electrophoresis* 2000, **21:**1164-1177.
59. Sauter ER, Zhu W, Fan XJ, Wassell RP, Chervoneva I, Du Bois GC**: Proteomic analysis of nipple aspirate fluid to detect biologic markers of breast cancer.** *Br J Cancer* 2002, **86:**1440-1443.
60. Paweletz CP, Trock B, Pennanen M, Tsangaris T, Magnant C, Liotta LA, Petricoin EF III: **Proteomic patterns of nipple aspirate fluids obtained by SELDI-TOF: potential for new biomarkers to aid in the diagnosis of breast cancer.** *Dis Markers* 2001, **17:** 301-307.
61. Li J, Zhang Z, Rosenzweig J, Wang YY, Chan DW: **Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer.** *Clin Chem* 2002, **48:**1296-1304.
62. Wulfkuhle JD, Sgroi DC, Krutzsch H, McLean K, McGarvey K, Knowlton M, Chen S, Shu H, Sahin A, Kurek R, Wallwiener D, Merino MJ, Petricoin EF, III, Zhao Y, Steeg PS: **Proteomics of human breast ductal carcinoma in situ.** *Cancer Res* 2002, **62:** 6740-6749.
63. Haab BB, Dunham MJ, Brown PO: **Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions.** *Genome Biol* 2001, **2:**research0004.1-0004.13
64. Jessani N, Liu Y, Humphrey M, Cravatt BF: **Enzyme activity profiles of the secreted and membrane proteome that depict cancer cell invasiveness.** *Proc Natl Acad Sci U S A* 2002, **99:** 10335-10340.
65. Warner E, Plewes DB, Shumak RS, Catzavelos GC, Di Prospero LS, Yaffe MJ, Goel V, Ramsay E, Chart PL, Cole DE, Taylor GA, Cutrara M, Samuels TH, Murphy JP, Murphy JM, Narod SA: **Comparison of breast magnetic resonance imaging, mammography, and ultrasound for surveillance of women at high risk for hereditary breast cancer.** *J Clin Oncol* 2001, **19:**3524-3531.
66. Powles TJ: Opinion: **Anti-oestrogenic prevention of breast cancer – the make or break point.** *Nat Rev Cancer* 2002, **2:** 787-794.
67. Hartmann LC, Schaid DJ, Woods JE, Crotty TP, Myers JL, Arnold PG, Petty PM, Sellers TA, Johnson JL, McDonnell SK, Frost MH, Jenkins RB: **Efficacy of bilateral prophylactic mastectomy in women with a family history of breast cancer.** *N Engl J Med* 1999, **340:**77-84.
68. Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N: **Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study.** *J Natl Cancer Inst* 1998, **90:**1371-1388.
69. King MC, Wieand S, Hale K, Lee M, Walsh T, Owens K, Tait J, Ford L, Dunn BK, Costantino J, Wickerham L, Wolmark N, Fisher B: **Tamoxifen and breast cancer incidence among women with inherited mutations in BRCA1 and BRCA2: National Surgical Adjuvant Breast and Bowel Project (NSABP-P1) Breast Cancer Prevention Trial.** *JAMA* 2001, **286:**2251-2256.
70. Chan KC, Knox WF, Gee JM, Morris J, Nicholson RI, Potten CS, Bundred NJ: **Effect of epidermal growth factor receptor tyrosine kinase inhibition on epithelial proliferation in normal and premalignant breast.** *Cancer Res* 2002, **62:**122-128.
71. Torhorst J, Bucher C, Kononen J, Haas P, Zuber M, Köchli OR, Mross F, Dieterich H, Moch H, Mihatsch M, Kallioniemi OP, Sauter G: **Tissue microarrays for rapid linking of molecular changes to clinical endpoints.** *Am J Pathol* 2001, **159:**2249-2256.
72. Specht K, Richter T, Muller U, Walch A, Werner M, Hofler H: **Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue.** *Am J Pathol* 2001, **158:**419-429.
73. Lehmann U, Bock O, Glockner S, Kreipe H: **Quantitative molecular analysis of laser-microdissected paraffin-embedded human tissues.** *Pathobiology* 2000, **68:**202-208.

Correspondence

Stefanie S Jeffrey, Department of Surgery, Stanford University School of Medicine, Medical School Lab-Surge Bldg, Room P214, 1201 Welch Road, Stanford, California 94305-5494, USA. Tel: (650) 723-0799; e-mail: ssj@stanford.edu