

Review

The diagnosis and management of pre-invasive breast disease Promise of new technologies in understanding pre-invasive breast lesions

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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*TM, Ambion Inc., Austin, TX, USA, or RNA*later*TM 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 experi-

mental 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 cyto-keratin 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}TM (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 non-malignant 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*, angiogenesis-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.

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