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Biomarkers in Early Breast Neoplasia

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Abstract Early breast neoplasia may be defined in many ways. Any non-invasive or invasive but non-metastatic breast cancer qualifies as early neoplasia in the sense that they are non-lethal. Before we can prevent lethal breast cancer, we must gain a better understanding of the biological abnormalities underlying its development and progression. Many studies into the mechanisms of breast cancer evolution have evaluated potential precursor lesions (*e.g.*, proliferative disease without atypia [PDWA], atypical ductal hyperplasia [ADH], and ductal carcinoma *in situ* [DCIS]) for genetic alterations known to occur in fully developed invasive carcinomas. This approach has shed some light on events which may be important in early malignant transformation, including the observations that overexpression of the c-*erb*B-2 oncogene and mutations of the p53 tumor suppressor gene are present in significant subsets of DCIS, but not PDWA or ADH. This approach is limited by our incomplete knowledge of cancer genetics. However, there is more to learn by evaluating known cancer-associated genes in potential precursor lesions using established techniques such as immunohistochemistry and *in situ* hybridization. Until recently, technology could not detect unknown genetic abnormalities in microscopic lesions such as PDWA, ADH, or DCIS. Now, PCR-based techniques have the theoretical ability to detect novel tumor promoter and suppressor genes in clinical samples of these very small lesions. For example, suppressor-type genes may be detected using comprehensive mapping probes to identify loss of heterozygosity in PCR-amplified DNA extracted from a few hundred cells microdissected from either fresh or archival tissue. Differential display is another new technique with the potential to detect both tumor promoter and suppressor gene expression in very small samples. This PCR method uses short random primer pairs to amplify representative cDNA from microgram quantities of mRNA extracted from fresh tissue. Rapid progress is likely to result from applying these complementary approaches to the challenging problem of breast cancer evolution.

Key words: Breast cancer, carcinogenesis, differential display, immunohistochemistry, loss of heterozygosity, prognostic factors

Recent studies in colon cancer have demonstrated a fascinating series of genetic abnormalities closely associated with morphological tumor progression [1]. Breast cancer, where potential precursors are at least 1,000-fold smaller than comparable colonic lesions, has been unapproachable by the same experimental strategies, and this has impeded progress in understanding early breast cancer development. How-

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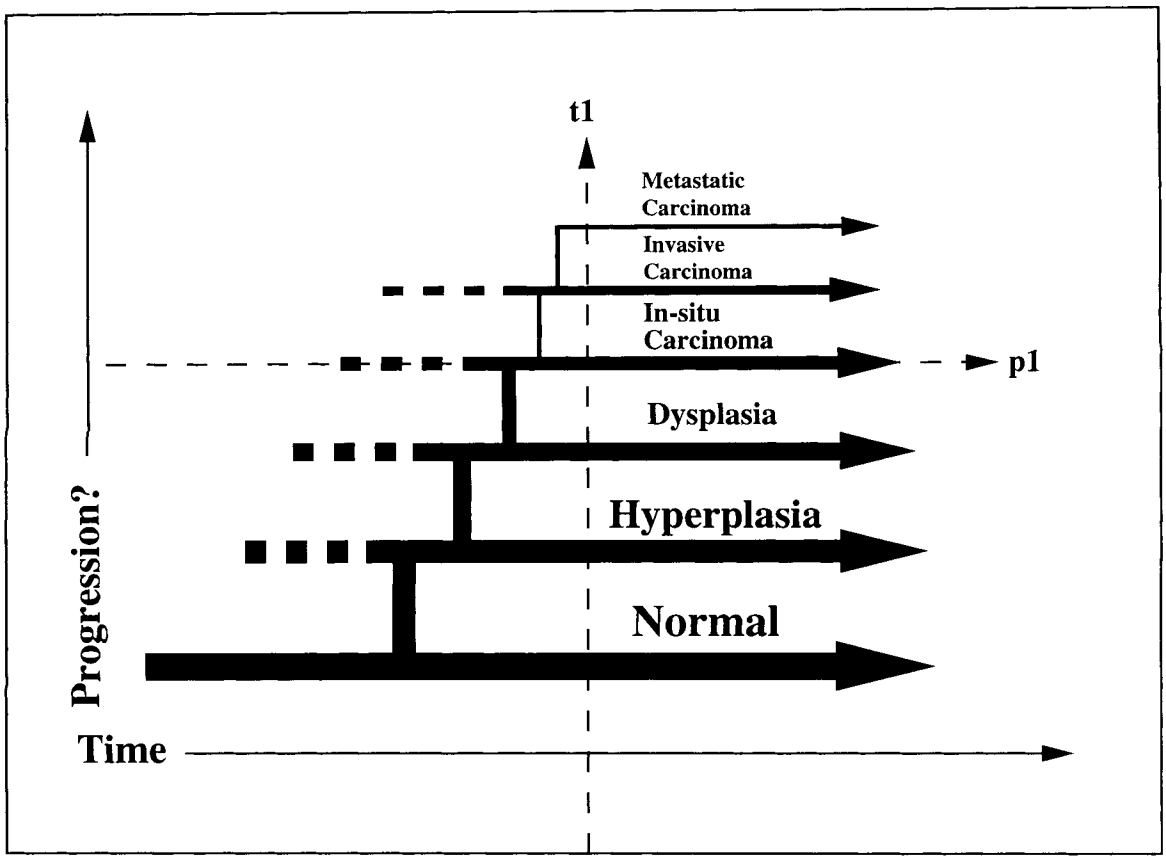

Fig. 1: Morphological model of breast cancer evolution. Ductal breast cancer is hypothesized as evolving from normal epithelium through a series of increasingly abnormal, but non-obligatory, cellular changes from hyperplasia, to dysplasia, to non-invasive carcinoma, to primary invasive carcinoma and, finally, to metastatic carcinoma.

ever, many studies are using both established and novel technology to identify the important biological events in breast cancer evolution.

MORPHOLOGICAL MODEL OF BREAST CANCER EVOLUTION

There is a reasonable morphological model of breast cancer evolution, based primarily on epidemiological evidence, which is somewhat analogous to the colon cancer paradigm. In this model, ductal breast cancer is hypothesized as evolving from normal epithelium through a series of increasingly abnormal cellular changes ranging from hyperplasia, to atypical hyperplasia or dysplasia, to non-invasive carcinoma, to primary invasive carcinoma and, finally, to metastatic carcinoma (Fig. 1). Proliferative disease without atypia (PDWA), atypical ductal hyperplasia (ADH), and ductal carcinoma *in situ* (DCIS) are proposed as examples of hyperplastic, dysplastic, and non-invasive neoplastic elements of the model, respectively (Fig. 2), although future studies may show that PDWA and ADH are really benign neoplasms rather than hyperplastic lesions. Each lesion within the model is envisioned as a non-obligatory precursor of the next in the sense that some may pursue a stable natural history, while others may progress to the next stage. The model also allows for the possibility that some lesions may arise *de novo* relative to their immediately preceding stage since many biological events in breast cancer evolution are likely to be morphologically silent.

Several epidemiological lines of evidence support elements of this model. For example, it is generally consistent with autopsy studies showing that PDWA, ADH, and DCIS are progres-

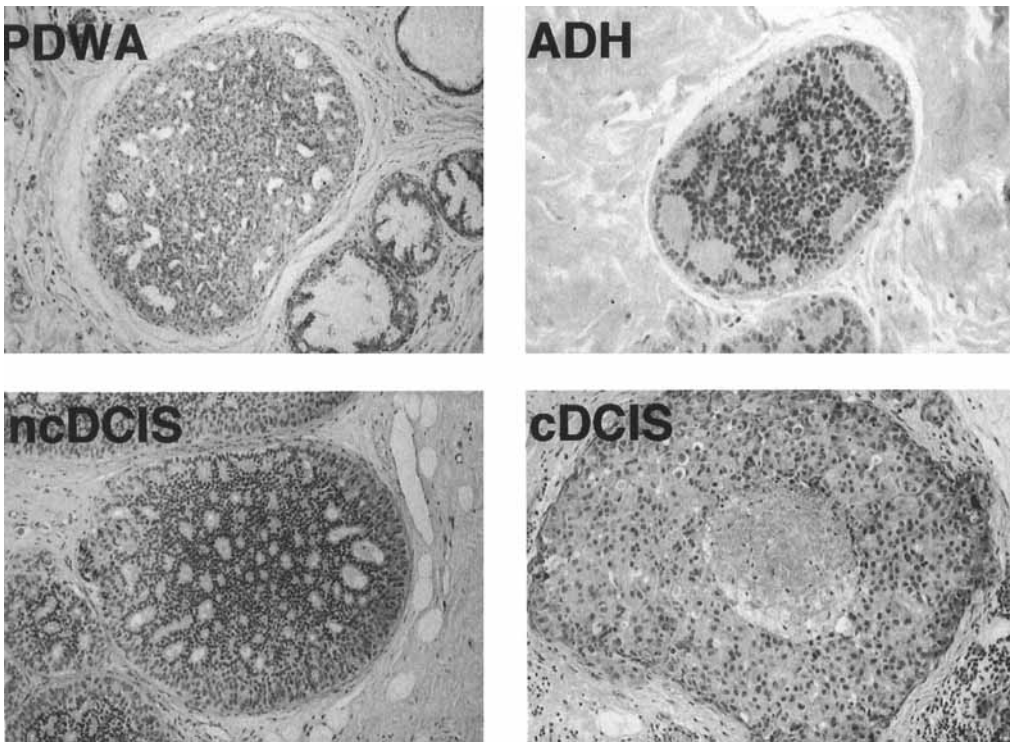

Fig. 2: Examples of proliferative disease without atypia (PDWA), atypical ductal hyperplasia (ADH), non-comedo ductal carcinoma *in situ* (ncDCIS), and comedo ductal carcinoma *in situ* (cDCIS).

sively less frequent in the breasts of women dying from causes other than breast cancer [2]. More compelling evidence is provided by studies showing progressively increasing relative risks (RR) of later developing invasive cancer in breasts with previously excised PDWA (RR = 1.2–2.0), ADH (RR = 4.0–6.0), and DCIS (RR = 10.0–12.0) [3–5]. While these studies don't demonstrate the natural history of the excised lesions, they are consistent with the concept that such lesions are markers for abnormalities left behind which have the capacity to progress to invasive cancer. In addition, lesions such as PDWA, ADH, and DCIS are concurrently observed in well over 50% of breasts containing invasive breast cancer [6,7]. While the model is undoubtedly incomplete, it represents a reasonable working hypothesis to pursue studies into the biological mechanisms of breast cancer development and progression.

KNOWN GENES IN PRECURSOR LESIONS

Several established techniques are available to study the involvement of known cancer-associated genes in potential precursors of invasive breast cancer such as PDWA, ADH, and DCIS. Immunohistochemistry (IHC) and the various modifications of *in situ* hybridization (ISH) are among the most promising because they evaluate gene expression *in situ* and *in vivo*, whether the sample under study has a mixed composition of normal and abnormal cells, or is microscopic. IHC is particularly appropriate because it is also relatively inexpensive and easy to perform.

There are at least three complementary strategies for using IHC or ISH to assess cancer-associated genes in PDWA, ADH, and DCIS. The most common evaluates gene expression in lesions unaccompanied by more advanced disease (*e.g.*, *in situ* carcinoma on pathway "p1" in Figure 1).

This approach has the theoretical ability to define the phenotypic range of a category, but is unable to distinguish specific phenotypes within the range associated with progression to the next stage.

A second strategy, which partially addresses the issue of identifying phenotypes at high risk for progression, evaluates precursor candidates excised from breasts of patients who developed invasive cancer years later. The rationale here is that the phenotype of lesions from those who eventually developed invasive disease will be different than morphologically similar lesions from those who did not. This important line of investigation has been hampered by the rarity of appropriate databases and tissue banks necessary for these studies, and the theoretical possibility that the candidate precursor lesions may have natural histories which are parallel rather than serial with invasive breast cancer.

A third closely related strategy involves assessing gene expression in potential precursors concurrent in the same breast with invasive cancer (*e.g.*, lesions on pathway "t1" in Figure 1), and comparing their phenotypes with morphologically similar lesions occurring alone (*e.g.*, *in situ* carcinoma on pathway "p1" in Figure 1). Differences in the phenotypes of these potentially overlapping pathways may identify genes important in tumor progression. Again, the databases and tissue banks necessary for these types of studies are rare, which has impeded progress.

Our current knowledge of gene expression or biomarkers associated with candidate precursors of invasive breast cancer is quite limited. The most comprehensively studied markers in this context are hormone receptors (estrogen receptors [ER] and progesterone receptors [PgR]), proliferation rate, ploidy, the c-*erb*B-2 oncogene, and the p53 tumor suppressor gene, and even this information is incomplete (Table I). For example, a few IHC studies suggest that all PDWA and ADH express high levels of ER/PgR [8,9]. In contrast, ER/PgR expression appears to be rare in comedo DCIS, but very common in non-comedo DCIS [10]. Proliferation rate, as measured by several methods, is high in comedo DCIS but low in non-comedo DCIS [11,12]. Cell cycle kinetics have been poorly studied in PDWA and ADH. Several investigations have shown that comedo DCIS is usually aneuploid while non-comedo DCIS is most often diploid [12,13]. Ploidy is not well characterized in PDWA or ADH. Several studies have also shown that amplification and/or overexpression of c-*erb*B-2 is common in comedo DCIS, rare in non-comedo DCIS, and (in a few preliminary studies) absent in PDWA and ADH [14–16]. Relatively little is know about p53 alterations in PDWA or ADH, but there are a few studies reporting up to 40% and 10% rates of mutation/overexpression in comedo and non-comedo DCIS, respectively [17,18]. Although other biomarkers have been studied in this evolutionary setting (*e.g.*, epider-

TABLE I. Biological Characteristics Associated With Potential Precursors of Invasive Breast Cancer (General Phenotype or Approximate Percent of Lesions Showing Expression/Abnormality of Marker)

			DCIS	
	PDWA	ADH	Comedo	Non-Comedo
ER	100%	100%	<50%	90%
PgR	100%	100%	<50%	90%
Proliferation rate	?	?	high	low
Ploidy	?	?	aneuploid	diploid
c-*erb*B-2	0%	0%	80%	10%
p53	?	?	40%	10%
Other	?	?	?	?

mal growth factor receptor [19], *ras* p21 [20], c-*myc* [21], integrins [22], *etc.*), the results are inconclusive. Regardless of the experimental strategy, there is a great deal more to learn about the involvement of known cancer-associated genes in potential precursor lesions.

NOVEL GENES IN PRECURSOR LESIONS

It is a near certainty that many or most of the genetic/biological events involved in breast cancer evolution are unknown. Several formidable impediments to identifying these events include uncertainty about which benign lesions represent unequivocal precursors of invasive breast cancer, the microscopic size of precursor candidates, the absence of cell lines suitable for study derived from such lesions and, until recently, the lack of technology to study gene structure and expression in clinical samples containing these microscopic lesions. It is now possible to identify novel abnormalities by assessing loss of heterozygosity (LOH) in genomic DNA and gene expression by differential display.

Recent technological modifications have made it possible to comprehensively evaluate LOH in samples as small as a few hundred cells crudely microdissected from fresh or archival tissue [23]. Thousands of mapping sites are known with sufficient allelic heterogeneity (in the context of simple sequence repeats) to demonstrate LOH over nearly the entire human genome. These sites or markers are PCR-amplified using readily available primers. The products are separated on sequencing gels to demonstrate a stoichiometric imbalance of control (normal) relative to test (PDWA, ADH, DCIS, *etc.*) DNA, thereby disclosing allelic loss and the possibility of a tumor suppressor gene at or near the site (Fig. 3).

Differential display requires microdissected fresh tissue, but has the potential to identify both induced and suppressed gene expression associated with tumor progression [24,25]. This innova-

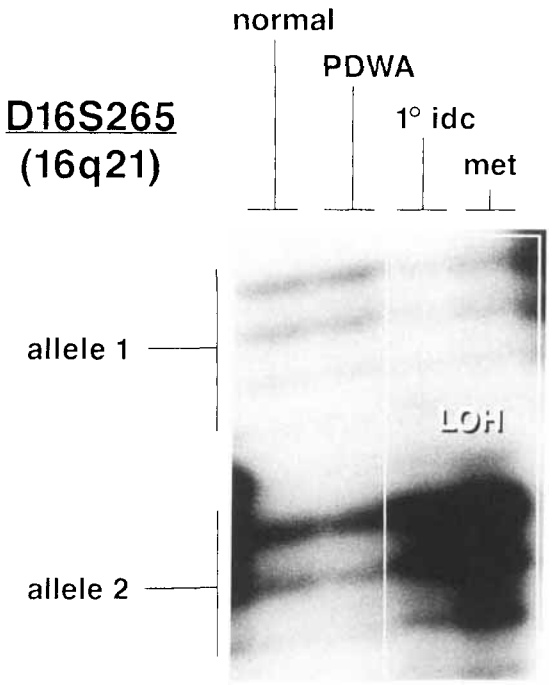

Fig. 3: Evaluation of marker D16S265 on 16q21 (27) in concurrent samples of breast tissue taken from the same patient. Loss of heterozygosity (LOH) at this site is manifested in primary infiltrating ductal carcinoma (1° idc) and metastatic carcinoma (met) by a decreased intensity of allele 1 compared to allele 2 relative to the banding pattern of normal tissue and proliferative disease without atypia (PDWA).

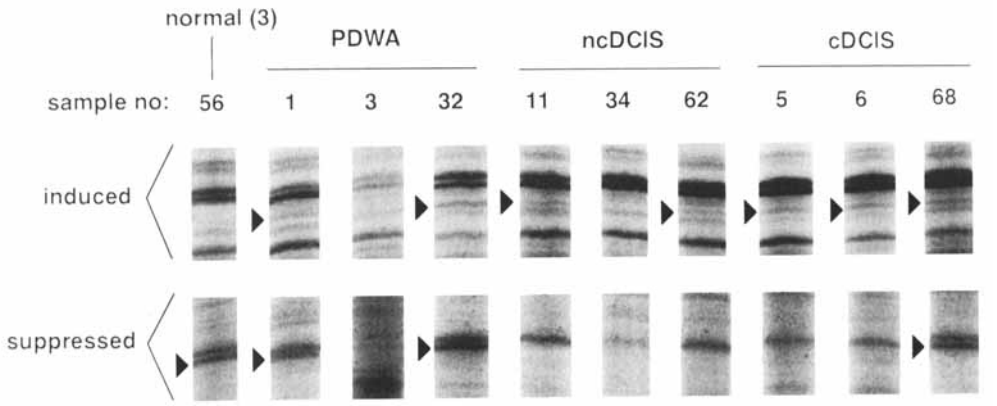

Fig. 4: Differential display of a panel of normal and abnormal breast tissues. A gene product is induced relative to normal in 2 of 3 examples of proliferative disease without atypia (PDWA), 2 of 3 non-comedo ductal carcinoma *in situs* (ncDCIS), and 3 of 3 comedo ductal carcinoma *in situs* (cDCIS). A gene product is suppressed relative to normal in 1 of 3 PDWA, 3 of 3 ncDCIS, and 2 of 3 cDCIS.

tive approach uses pairs of short, random PCR primers to amplify subsets of cDNA from microgram quantities of mRNA. Sequential amplifications with a somewhat large but manageable number of primer pairs can theoretically generate comprehensive cDNA libraries from as little as 120 μg of total RNA. Products separately amplified from individual samples using the same primers are then compared in adjacent lanes of sequencing gels to demonstrate induced or suppressed gene expression in precursor lesions relative to normal tissue (Fig. 4). Interesting bands can be directly cut from the gels, cloned, and sequenced. Novel sequences may then be used to confirm and more comprehensively characterize the involvement of these genes in tumor progression.

CONCLUSIONS

There is growing and justifiable interest in initiating breast cancer chemoprevention trials which measure surrogate endpoints. Biomarkers are likely candidates for surrogate endpoints in prevention trials, and their essential properties must include a direct link to the development of invasive cancer, a response to drug intervention which can be measured by a valid reliable assay and, most importantly, a response to therapy which predicts for decreased cancer incidence [26]. While certain biomarkers hold promise (*e.g.*, premalignant histology, high proliferation rates in lesions with premalignant histology, *etc.*), none have been demonstrated to possess all the essential properties. If prevention trials using surrogate endpoints are to be successfully implemented, our intellectual and economic resources must be focused on first identifying appropriate biomarkers.

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