Award Number:  DAMD17-01-1-0130

TITLE:  New Approaches for Early Detection of Breast Tumor Invasion or Progression

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REPORT DATE:  May 2002

TYPE OF REPORT:  Annual

PREPARED FOR:  U.S. Army Medical Research and Materiel Command
                Fort Detrick, Maryland  21702-5012

DISTRIBUTION STATEMENT:  Approved for Public Release;
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New Approaches for Early Detection of Breast Tumor Invasion or Progression

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Report contains appendix with two published articles and six submitted extracts.

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To assess interactions between epithelial (EP) and myoepithelial (ME) cells in association with breast tumor progression and invasion, consecutive sections were immunostained with antibodies to estrogen receptor (ER), smooth muscle actin (SMA), and other biomolecules, and then microdissected for LOH and microsatellite instability (MI) assessments. Focal losses of ER expression in EP cells and disruptions of subjacent ME layers are correlated events in ER (+) tumors, whereas focal alterations of p27 expression in EP cells and disruptions of subjacent ME layers are correlated events in ER (-) tumors, suggesting that progression or invasion of these tumors may be regulated by different mechanisms. Cells in ducts with disrupted ME cell layers showed a substantially higher proliferation rate, and a vast majority of ER (-) cells overlying disrupted ME cell layers showed a marked higher frequency and different pattern of LOH and MI, compared to adjacent ER (+) cells within the same duct. These findings are in agreement with our hypothesis that ER (-) cells overlying disrupted ME cell layer represent a more aggressive clone, and that simultaneous assessments of the immunohistochemical and genetic profiles of EP and ME cells could be a more sensitive approach for early detection of breast tumor progression or invasion.

early detection of breast tumor invasion and progression, epithelial-myoe epithelial cell interactions, breast cancer

Unclassified

Unclassified

Unclassified

Unlimited

NSN 7540-01-280-3500

17a. SECURITY CLASSIFICATION OF REPORT
17b. SECURITY CLASSIFICATION OF THIS PAGE
19. SECURITY CLASSIFICATION OF ABSTRACT
20. LIMITATION OF ABSTRACT

Unclassified
Unclassified
Unclassified

Unlimited

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102
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Introduction

To assess interactions between epithelial (EP) and myoepithelial (ME) cells in association with breast tumor progression and invasion, a double immunostaining technique with antibodies to smooth muscle actin (SMA) and estrogen receptor (ER) was used to elucidate both the ME and EP cells in mammary tissues harboring ductal carcinoma in situ. Single or clusters of EP cells with a marked diminution or a total loss of ER expression were found immediately overlying focally disrupted ME cell layers, in contrast to the dominant population of ER (+) cells within the same duct that showed no associated ME cell layer disruptions (1). This study attempted to confirm our previous findings on a larger number of cases, and to compare the immunohistochemical and molecular biological profiles of the ER (-) cells overlying disrupted ME cell layers with those of adjacent ER (+) cells and surrounding stromal (ST) cells. Since ME cell layers are physical barriers protecting the microenvironment and integrity of EP cells, and the disruption of ME cell layers is an absolute pre-requisite for breast tumor invasion, the outcomes of this project could have significant values in early detection of breast tumor progression and/or invasion.

Body

Statement of work

A total of 7 tasks were listed in the Statement of Work of the original proposal:

Task 1. To repeat our previous studies and to identify epithelial (EP) cells overlying disrupted myoepithelial (ME) cell layers (months 1-6).
   a. Select 500 female cases of ductal carcinoma in situ (DCIS) from our file with detailed information regarding age, race, and follow-up data
   b. Retrieve paraffin and frozen tissue blocks, and make 6-8 serial sections for each case
   c. Stain the first and the last sections of each case with H & E for morphological assessment
   d. Immunostain 3-4 sections from each case with antibodies to estrogen receptor (ER) and smooth muscle actin (SMA)
   e. Observe stained sections to identify cells overlying disrupted ME cell layers
   f. Select the cases with cells overlying disrupted ME cell layers

Task 2. To compare the biological behavior of cells overlying a disrupted ME cell layer with that of adjacent cells within the same duct (months 6-9)
   a. Make 40-50 serial sections for each of the selected cases
   b. Immunostain sections with different bio-markers that have been found associated with more aggressive biological behavior

Task 3. To microdissect phenotypically different EP cells and the surrounding ME and stromal (ST) cells for molecular biological analyses (months 9-12)

Task 4. To compare the frequency and pattern of loss of heterozygosity (LOH), and clonality among EP, ME, and ST cells (months 12-20)

Task 5. To assess the gene expression pattern in cells from frozen section sections with cDNA expression array technique, and to generate probes based on sequences exclusively or mainly expressed in cells overlying disrupted ME cell layers (months 20-24)
Task 6. To apply the probes to both paraffin and frozen sections, to identify the gene expressing cells and their morphologic features (months 24-32).

Task 7. To correlate the laboratory findings with that of clinical following-up data (months 32-36).

**Experimental procedures:**

Consecutive sections were made from formalin-fixed, paraffin-embedded breast tissues from patients with various grades of ductal carcinoma in situ (DCIS), and double immunostained for ER and SMA. Cross sections of all ducts lined by \( \geq 40 \) EP cells were examined for a focal ME cell layer disruption, defined as an absence of ME cells, resulting in a gap equal to or greater than the combined size of 3 EP or ME cells. A focal loss of ER expression was defined as marked diminution or a total loss of ER staining in cells immediately overlying a disrupted ME cell layer, in contrast to strong ER expression in adjacent cells within the same duct.

Consecutive sections were also immunohistochemically stained with different antibodies, as detailed in References 5-7, to assess the biologic behavior of cells associated with disrupted ME cell layers. In addition, after immunostaining for ER and SMA, cells overlying disrupted ME cell layers, adjacent ER (+) cells within the same duct, adjacent stromal (ST) cells, and other controls were microdissected for DNA extraction and assessment for loss of heterozygosity (LOH) and microsatellite instability (MI), using PCR amplification with 18 DNA markers at 6 chromosomes. The frequency and pattern of LOH and MI among samples were compared.

All above experimental procedures were carried out according to the methods described in the proposal without any major modifications. Also, all the laboratory efforts have been strictly adhered to address the issues listed in “Statement of Work”.

**Key research accomplishments**

All the laboratory procedures for Tasks 1, 2, and 3 had been completed; for Task 4 had been partially completed before April 15, 2002. Also, a majority of the completed experimental materials had been analyzed before April 15, 2002.

**Reportable outcomes**

Two abstracts that summarized the immunohistochemical findings and the principal molecular techniques for Tasks 1 to 4 were accepted for slide and poster presentations at two major international conferences, and published in Med-Line listed journals (2-3).

Six additional abstracts that addressed the issues listed in Tasks 1 to 4 of “Statement of Work” have been submitted to Era of Hope, The Department of Defense Breast Cancer Research Program Meeting, to be held in Orlando, Florida, September 25-28, 2002 (4-9).

The corresponding manuscript for each of the 8 abstracts is under preparation, and will be sent for publication before the end of the year 2002.
Conclusions

1. Of 220 ER (+) cases with a total of 5,698 duct cross sections examined so far, 94 (42.7%) contained disrupted ME cell layers with a total of 405 focal disruptions. Of these disruptions, 350 (86.4%) were subjacent to cells with focal losses of ER expression, while only 55 (13.6%) were associated with cells showing a strong ER expression (3-4). These findings are consistent with those of our previous studies (1), suggesting that focal losses of ER expression in EP cells and disruptions of subjacent ME cell layers are correlated events in ER (+) tumors.

2. Of 100 cases with various grades of ER (-) tumors evaluated so far, focal disruptions of ME cell layers were found in about a half of the cases. These focal ME cell layer disruptions, however, appeared to correlate with either a focal loss or elevation of p27 expression (5), suggesting that the progression and/or invasion of ER (-) tumors might differ from those of ER (+) tumors.

3. Several tumor suppressor gene products have been found co-expressed in ME cell layers, and a diminution or absence of these proteins correlated with an increased frequency of ME cell layer disruptions (6-7). A substantially higher cell proliferation rate was seen in ducts with disrupted ME cell layers than ducts with intact ME cell layers (6-7), suggesting that EP cells overlying disrupted ME cell layers may have a more aggressive biologic behavior.

4. As previous studies have shown that it is difficult or impossible to utilize immunostained tissues pre-treated with antigen unmasking methods for molecular analyses, an innovative antigen retrieval protocol that satisfies both immunohistochemical and subsequent molecular assessments has been developed in our laboratory (8). This protocol allows us to microdissect double immunostained cells for LOH and MI assessments, to assess the possible correlation between immunohistochemical and genetic alterations.

5. A vast majority of the ER (-) cells overlying disrupted ME cell layers showed a substantially higher frequency and different pattern of LOH and MI, compared to adjacent ER (+) counterparts within the same duct (9). In a small proportion of cases, however, ER (-) cells showed a substantially lower frequency of LOH and MI than adjacent ER (+) cells, or even displayed no distinct genetic changes (9). These findings are largely in support of our hypothesis that ER (-) cells overlying disrupted ME cell layers represent a more aggressive clone, while also suggest that a few of these cells might belong to a population involving in a normal replenishment or expansion of ducts.
References


Breast Cancer Research and Treatment

Marc E. Lippman, MD, editor-in-chief

Kluwer Academic Publishers

The Abstract is on the back
21 Persistence of occult metastatic cells in bone marrow of breast cancer patients despite systemic adjuvant treatment.

The presence of occult metastatic cells in bone marrow (BM) of breast cancer patients at the time of diagnosis indicates occult hematogenous tumor cell dissemination and increases the risk of subsequent distant disease. Currently, there are no data available on the influence of different adjuvant therapies on the survival of these cells.

We analyzed bone marrow aspirates of 161 patients without evidence of recurrence at the time of primary diagnosis and a median interval of 13 months (range: 6-74) thereafter. Carcinoma cells were detected using a standardized immunocytochemistry with monoclonal antibody A45-B/B3 directed against cytokeratin (CK).

At the time of primary diagnosis, 40 of 161 patients (25%) had a positive BM finding. Of these, 45 (28%) had a positive BM finding at the time of the second BM analysis. Among those patients with an initially negative BM finding, 21 patients (13%) had a positive BM finding at the second aspiration, while 24 patients (15%) remained BM-positive. Of the 46 patients with ITC at the time of primary diagnosis, 23 patients (50%) received adjuvant chemotherapy, 7 patients (13%) received endocrine therapy and 16 (35%) patients had no systemic treatment at all. 36% of the patients without systemic therapy (n=7) converted to a negative BM status at time of follow-up examination, while 43% of the patients with endocrine (n=4) or cytostatic (n=3) therapy became negative (P=0.70). Patients with a persistently negative BM status had a significantly better overall survival than patients with a positive BM status at the time of the second BM aspiration, both by univariate analysis (P=0.045, Log-rank) and multivariate analysis (P=0.034, Cox Regression).

In a considerable number of patients with primary breast cancer, minimal residual disease can be detected by follow-up BM analysis. Independently of systemic therapy, about half the patients remain BM-positive suggesting failure of therapy and risk of subsequent development of distant disease.

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Metastasis is the major contributor to breast cancer-related deaths. However, it is an inefficient process where only few metastatic tumors result from many cells shed by the primary tumor. Understanding the basis for this inefficiency will help in developing new approaches to prevent metastasis. Previously, we showed that murine mammary carcinoma cell lines of high (D2A1) and low (D2:OK) metastatic potential did not differ in survival in the circulation or extravasation in mouse liver. Their differences in metastatic ability thus must be due to post-extravasation events. Methods: Here we labeled the cells in vitro with fluorescent microspheres, and injected them via mesenteric vein to target mouse liver. Using a cell accounting assay and 50 micrometer thick formalin fixed tissue sections, we quantified survival of single cells and metastases immediately after injection or 3, 10, 14, 18, 21, or 77 days later. Results: We found that: 1) 85% of injected cells (both cell types) initially survived in the liver microcirculation, 2) at day 10, 70% of injected D2A1 cells remained as solitary cells, 0.06% of injected cells started to proliferate, and only 1 in 100 of those persisted in growth, 3) by day 21, although large metastases were present, <20% of D2A1 cells remained as undivided solitary cells, 4) surprisingly large numbers (80% at day 21, 50% at day 77) of undivided single cells were observed with the non-metastatic D2:OK cell line. When recovered from the tissue, at day 77, such cells were able to grow in vitro and form tumors when transplanted into the mammary fat pad. Metastasis thus resulted from growth of a subset of extravasated cells, and a surprisingly large sub-population of dormant cells may persist, even in organs where metastases are growing. If solitary cells can be activated to grow in vivo, they may represent a clinically unrecognized source of tumor dormancy. Furthermore, such cells would be especially problematic for treatment, since they would be inherently resistant to cytotoxic or anti-angiogenic therapies.

23 Genetic alterations in the progression of non-invasive to invasive breast cancer.
Li Z, Tsimelzon A, Immenni A, Mohsin SK, Hilsenbeck SG, Clark GC, Fuqua SAW, Osborne CK, O'Connell P, Allred DC. Breast Center, Baylor College of Medicine, Houston, TX.

Background: Non-invasive breast cancer is a frightening but essentially harmless disease. It is clinically important because it is very common and gives rise to most if not all invasive breast cancers (IBC's) which are potentially life-threatening. Hypothesis: Specific genetic defects occur in non-invasive breast cancer which result in the progression to invasive disease. Significance: Once identified, these genetic changes may be useful in prognostic factors to identify non-invasive lesions at high risk of progressing and, more importantly, as the therapeutic targets to prevent progression. Study Design/Methods: Frozen samples of pure ductal carcinoma in situ (DCIS; n=15), the most common (85%) type of non-invasive breast cancer, and of pure IBC's (n=15) were evaluated using cDNA expression arrays (Clontech Atlas Human Cancer 1.2 Array). To identify genes involved in tumor invasion, the samples were microdissected to retain potentially important genes from non-tumor cells (e.g. stroma) but they were screened to contain a high level of tumor cellularity (<75%). Results: Preliminary Wilcoxon analyses identified 114 genes expressed at significantly (p<0.05) different levels in DCIS compared to IBC. Many are involved in processes and pathways which make them reasonable candidates as important invasion-related genes (e.g. wnt-signaling, cell-adhesion molecules, angiogenic factors, stromal processes, protease inhibitors, etc.). Studies are ongoing to validate these data by comprehensively characterizing the expression profiles (e.g. frequency and distribution) and confirming the significance of validated genes on tissue arrays containing large numbers of clinical samples (pure DCIS, DCIS from breast with synchronous IBC, and IBC). Conclusions: A large number of genes have been identified which may be important in the progression of non-invasive to invasive breast cancer.

24 Primary bilateral breast cancers display different LOH and CGH profiles in both epithelial and stromal components.
Man YG, Moirin F, Shekita KM, Stamatakos M, Lininger RA, Kuhl's E, Braathauer GL, Tavassoli FA. Department of Gynecologic and Breast Pathology, Armed Forces Institute of Pathology and American Registry of Pathology, Washington, DC.

One of our previous studies revealed that morphologically similar cells from two sides of bilateral primary breast cancers displayed a different pattern and frequency of loss of heterozygosity (LOH) suggesting that these might be independent lesions. This study attempted to confirm previous findings. Methods: The frequency and pattern of LOH and DNA copy numbers in microdissected epithelial (EP) and stromal (ST) cells from both sides of bilateral breast cancers were compared using PCR and comparative genomic hybridization (CGH) techniques. Results: A total of 147 LOH were detected in a total of 122 paired informative loci with a combination of 9 markers. Of 147 LOH, 62 (46%) were seen in the left and 65 (44%) in the right lesions. Of 122 paired loci, 97 (80%) showed unilateral and 25 (20%) displayed bilateral LOH. Of 42 paired, microdissected samples, 32 (76%) displayed more independent, while 4 (10%) showed more concurrent LOH. In 7 selected cases, CGH changes (gains or losses) were detected in the EP component in one of three cases and in both sides of three cases. and loss of of the ST component in one side of two cases. CGH changes were distributed in the left and right lesions; in cases that showed CGH changes displayed an identical pattern or frequency of changes in either EP or ST component of both breasts. Conclusions: These results are in favor of independent lesions in most primary bilateral cancers, and further suggest that ST cells are concurrently involved or even play a role in development and progression of breast cancers.
An Official Journal of The
United States and Canadian
Academy of Pathology, Inc.

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The Abstract is on the back
monoclonal antibody for AR. Majority of the tumors, except lobular carcinoma of breast (5) and bronchoalveolar carcinoma of lung (10) were moderately to poorly differentiated. Tumors immunoreactive for >10% of nuclei were considered AR positive. AR immunoreactivity only in the cytoplasm was interpreted as negative. Review of 100 mammary carcinomas and 20% (21/105) adenocarcinomas of ovary were positive for AR. The other neoplasms did not show nuclear immunoreactivity for AR in >10% nuclei; however, several of them (72%, 50/79) did show variable cytoplasmic immunoreactivity.

Conclusions: The expression of AR in more than 10% nuclei in a metastatic tumor with unknown primary in a female favors mammary carcinoma; however, a remote possibility of ovarian primary cannot be ruled out.

161 Relative Susceptance of Androgen Receptors Compared to Estrogen and Progesterone Receptors in Mammary Carcinomas
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Background: Immunoreactivity for androgen receptors (AR) is preserved more frequently than estrone/progesterone receptors (ER/PR) in mammary carcinomas. This may be of particular value when evaluating ER/PR negative metastatic adenocarcinomas of unknown origin. The present study was undertaken to evaluate this phenomenon.

Design: We compared the prevalence of AR and ER/PR in mammary carcinomas. Formalin-fixed paraffin-embedded tissue sections from 34 cases of breast carcinoma (29 lesions were extracted from 3,233, Grade III duct infiltrating lobular carcinoma (ILC) were evaluated by immunohistochemistry for AR, ER, and PR with respective monoclonal antibodies. Immunoreactivity of >10% nuclei for AR, ER, and PR were considered positive. Immunoreactivity only in the cytoplasm was interpreted as negative.

Results: 56% of mammary carcinomas were AR positive, although 37% of these were negative for ER/PR. All ILC showed AR positivity; however, 60% of these were ER/PR negative.

AR positive (56%, 16/30) ER negative (40%, 15/30) ER positive (56%, 16/30)
88%, 14/29; ILC, 100%, 2/2 ILC, 0%, 0/5
FR- Neg FR- Neg ER/PR both Neg
12%, 3/25; ILC, 0%, 0/5

ER- Neg PR- Neg ER/PR
IHC 29% (4/14) 106% (4/14) 94% (4/14) 105% (4/15)
both Neg

Total 37% (17/46) 42% (4/9) 37% (17/46) 105% (4/15)

Conclusions: AR expression is more frequently sustained than ER/PR in mammary carcinomas, especially in lobular variant. AR is potentially useful for the evaluation of metastatic tumor of unknown origin in women.

162 Focal Loss of Estrogen Receptor (ER) Expression in ER Positive Ductal Intraepithelial Neoplasia is Associated with Disruptions of the Immediate Subjacent Myoepithelial Cell Layer
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Background: Our previous study using dual immunostaining with antibodies to ER and smooth muscle actin (SMA) revealed patchy disruptions in the myoepithelial (ME) cell layer subjacent to ER negative epithelial (EP) cells in mammary ducts with ostensibly EP proliferation.

Design: To confirm this finding on a larger scale, the same protocol was used to assess the association between ER expression and disruptions of ME cell layers on paraffin tissue sections from 125 patients with various grades of ductal intraepithelial neoplasia. The disruption of ME cell layers is defined as widening of a ME cell layer gap equal to the diameter of at least 3 EP cells in the cross section of a given duct. Focal loss of ER expression is defined as a significant reduction or complete loss of ER expression in a cluster of EP cells immediately overlying the disrupted ME cell layer, compared to strong ER expression in the remaining neoplastic cells within the same duct. The total number of the cross sections of ducts with proliferative changes was counted. All ME layers were studied. The prints were made at a magnification of 400X for immunohistochemical and morphological assessments.

Results: Of the 125 cases, 62 (49.6%) showed disrupted ME cell layers; 246 (66.6%) disruptions were detected in 223 duct cross sections. Of the 62 cases with disrupted ME cell layers, 40 (64.5%) contained less than 4 and 22 (35.5%) displayed more than 4 disruptions. Of these disruptions, 225 (91.5%) from 59 cases were associated with focal loss of ER expression and 21 (8.5%) from 9 cases were subjacent to ER positive cells. The frequency and pattern of disruptions was generally independent of the size of ducts or the degree of neoplasia. The cells overlying the ME disruptions were generally morphologically indistinguishable from adjacent neoplastic cells within the same duct on routine H&E sections.

Conclusions: These findings suggest that focal loss of ER expression might play an important role in tumor progression and that double immunostaining with SMA and ER could assist in detection of incipient cancer invasion.

Acknowledgement: This study is supported by Congressionally Directed Medical Research Programs, The Idea Award (BC001187) and Career Development Award (BC001186) to Van-guo Man, MD, Ph.D.

163 Atypical Ductal Hyperplasia in Juvenile Hyperplasty of the Breast
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Background: Atypical ductal hyperplasia (ADH) is unusual in young women and development of carcinoma has not been seen with limited follow up (Eiasson et al., Am J Surg Pathol 1992;16(3): 246-51). We sought to determine the incidence of ADH in juvenile hyperplasty encountered in a large breast pathology consultation service.

Design: The files of the Breast Consultation Service at Vanderbilt University Medical Center were searched for patients aged 16 to 25 years who were diagnosed with ADH, using strict previously published criteria, for the period 1996 to August 2001. The surgical pathology reports and corresponding hematoxylin and eosin slides were reviewed. Histologically confirmed cases of juvenile hyperplasty were selected and follow up data was reviewed.

Results: A total of 377 cases from women in this age group were received in consultation during the study period. Nineteen cases (5.0%) of ADH were diagnosed. Five of the nineteen cases were in women with juvenile hyperplasty undergoing bilateral mammoplasty, two of whom had bilateral disease and three of whom had unilateral disease. In most cases the pattern of ADH was largely lobulocentric, however in 2 cases there was limited extension of ADH into an adjacent duct. Follow-up data ranging from 2 to 6 months showed no development of carcinoma.

Conclusions: Much longer follow up is required to determine whether patients with ADH in the setting of juvenile hyperplasty are at increased risk for the development of breast carcinoma. Furthermore, caution is needed in order not to over-diagnose these cases as carcinoma in situ. A conservative approach in management, with close follow up, is indicated.

164 Pagetoid Spread in Ductal Carcinoma In Situ: Characterization and Computer Simulation
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Background: Pagetoid proliferation of neoplastic cells in ducts adjacent to foci of ductal carcinoma in situ is frequently observed in breast biopsies. However, its risk implications are unclear. If this histologic finding actually represents the spread of ductal carcinoma in situ within ducts, then pagetoid proliferation might be useful as a marker of greater extent of ductal carcinoma in situ. Pagetoid proliferation may also be useful as an indicator of the functional characteristics of tumor cells that impact clinical events.

Design: Seventy cases designated as pagetoid spread of neoplastic cells in association with ductal carcinoma in situ were obtained from the Vanderbilt Breast Consultation Service and the lesions and associated findings were characterized. A cell automata model simulating the spread of ductal carcinoma in situ was utilized to study diffusion and proliferation as independent parameters in pagetoid extension.

Results: In approximately 60% of cases, the ductal carcinoma in situ was more than 1 cm in greatest extent or was described as "extensive" and in less than 10% of cases was smaller than 0.5 cm. The vast majority of cases in which margin status was assessed showed inadequate margins of resection with 44% having transsected margins and 56% having a margin of less than 5 mm. All patterns of ductal carcinoma in situ were represented, although comedo subtype was uncommon. Computer simulations showed pagetoid spread becomes extensive when diffusion rates are multiple orders of magnitude greater than proliferation rates.

Conclusions: Ductal carcinoma in situ associated with pagetoid proliferation of neoplastic cells along the ducts was often extensive with positive or close surgical margins in excisional biopsies. The computer simulation of pagetoid spread within ducts suggests that the diffusion to proliferation ratio must be high in order for ductal carcinoma in situ to extend in this fashion.

165 Lobular Carcinoma In Situ on Core Needle Biopsy: When Should One Recommend Excision?
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Background: Core needle biopsy (CNB) is the preferred technique for evaluating breast masses and abnormal mammographic findings. The frequency of detection of noninvasive lobular lesions by CNB is increasing. Historically, the diagnosis of lobular carcinoma in situ (LCIS) has been considered a risk factor for the development of invasive carcinoma, and treatment has consisted of careful follow-up with or without tamoxifen. The purpose of this study was to review CNB material with the primary diagnosis of LCIS, atypical lobular hyperplasia (ALH), and lobular neoplasia (LN) in conjunction with clinical and radiological findings to make recommendations as to when excision is merited.

Design: The M.D. Anderson pathology database was searched from 1995 to 2001 for CNB cases with LCIS, ALH, and LN as the primary diagnosis. Microcalcifications
FOCAL LOSSES OF ER EXPRESSION IN EPITHELIAL CELLS AND DISRUPTIONS OF SUBJACENT MYOEPITHELIAL CELL LAYERS ARE CORRELATED EVENTS IN ER (+) DUCTAL INTRAEPITHELIAL NEOPLASIA

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The purpose of this study was to assess the possible correlation between focal losses of estrogen receptor (ER) expression in epithelial (EP) cells and disruptions of subjacent myoepithelial (ME) cell layers. Consecutive sections were made from formalin-fixed, paraffin-embedded breast tissues from 220 patients with various grades of ductal intraepithelial neoplasia, and double immuno-stained for ER and smooth muscle actin. Cross sections of all ducts lined by ≥ 40 EP cells were examined for a focal ME cell layer disruption, defined as an absence of ME cells, resulting in a gap equal to or greater than the combined size of 3 EP or ME cells. A focal loss of ER expression was defined as a marked diminution or a total loss of ER staining in cells immediately overlying a disrupted ME cell layer, in contrast to strong ER expression in adjacent cells within the same duct.

Of the 220 ER (+) cases with a total of 5,698 duct cross sections examined, 94 (42.7%) contained disrupted ME cell layers with a total of 405 focal disruptions (7.1%). Of these disruptions, 350 (86.4%) were associated with a focal loss of ER expression, whereas 55 (13.6%) were subjacent to cells with a strong ER expression. The frequency of ME cell layer disruptions associated with ER (-) cells was significantly higher (p < 0.01) than that associated with ER (+) cells. The frequency and pattern of ME cell layer disruptions were generally independent of the size, length, and architecture of the ducts. The cells overlying disrupted ME cell layers were often architecturally and morphologically indistinguishable from adjacent cells within the same duct on routine H & E stained sections.

This study suggests that a focal loss of ER expression among a group of ER (+) cells and disruption of the subjacent ME cell layer might be correlated events. As the disruption of ME cell layers are an absolute pre-requisite for tumor invasion, these events are possibly associated with progression and/or early invasion of the mammary tumors.

This study was supported by the US Army Medical Research and Materiel Command under DAMD 17-01-1-0129 and DAMD17-01-1-0130 to Yan-gao Man, MD., Ph.D.
FOCAL ALTERATIONS OF P27 EXPRESSION AND SUBJACENT MYOEPITHELIAL CELL LAYER DISRUPTIONS ARE CORRELATED EVENTS IN ER (-) DUCTAL INTRAEPITHELIAL NEOPLASIA

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Our previous studies, using a double immunostaining technique with antibodies to smooth muscle actin (SMA) and estrogen receptor (ER), revealed that a focal loss of ER expression and disruption of a subjacent myoepithelial (ME) cell layer were correlated events in ER (+) ductal intraepithelial neoplasia (DIN). Focal disruptions of ME cell layers were also found in various grades of ER (-) DIN. This study intended to assess whether ME cell layer disruptions in ER (-) DIN may correlate with a deregulated expression of p27, a cyclin dependent kinase inhibitor that arrests cell division.

Consecutive sections were made from formalin-fixed, paraffin-embedded breast tissues from 100 patients with ER (-) DIN. Two adjacent sections were double immunostained with [1] p27 plus SMA, and [2] SMA plus a mixture of antibodies to Ki-67, Cyclin A and D3. Cross sections of all ducts lined by ≥ 40 EP cells were examined for focal ME cell layer disruptions and focal alterations of p27 expression, defined as a marked reduction or elevation of p27 staining in cells immediately overlying disrupted ME cell layers. The cell proliferation rates in ducts with an intact and with a disrupted ME cell layer were statistically compared.

Distinct p27 immunoreactivities were seen in a vast majority of the normal ductal and lobular cells. Although the overall level of p27 expression was generally reduced with the progression of lesions and increase of tumor histological grades, a marked reduction or total loss of p27 expression was occasionally seen in normal appearing ducts, and intense p27 immunostaining was seen in some malignant tumors. In contrast, the rate of focal alterations of p27 expression seemed to be linearly correlated with the frequency of ME cell layer disruptions in both normal appearing and neoplastic ducts. Ducts with a disrupted or no distinct ME cell layer displayed a significantly higher cell proliferation rate than ducts with an intact ME cell layer.

These findings suggest that focal alterations of p27 expression and elevated rates of ME cell layer disruptions and cell proliferation might be correlated events. Since the disruption of ME cell layer is an absolute pre-requisite for tumor invasion, elucidation of the dynamic relationship of these events and the underlying mechanism may have significant diagnostic and prognostic values.

This study was supported by the US Army Medical Research and Materiel Command under DAMD 17-01-1-0129 and DAMD17-01-1-0130 to Yan-gao Man, MD., Ph.D.
CO-EXPRESSION OF MASPIN AND WILMS’ TUMOR 1 PROTEINS IN MAMMARY MYOEPITHELIAL CELLS—IMPLICATION FOR TUMOR PROGRESSION AND INVASION

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Maspin and Wilms’s tumor 1 (WT-1) proteins have been suggested as products of tumor suppressor genes, as they display inhibitory functions on tumor progression in both tissue cultures and animal models. The expression pattern and functions of these two proteins in human mammary tissues, however, have not been established. This study attempted to address these issues with an emphasis on the correlation of the proliferation rate in mammary ductal cells with the expression of these two proteins in surrounding myoepithelial (ME) cells, and with the physical integrity of ME cell layers.

Consecutive sections were made from formalin-fixed, paraffin-embedded mammary tissues from 100 patients with various grades of ductal intraepithelial neoplasia. Three adjacent sections were double immunostained with [1] smooth muscle actin plus Ki-67, [2] maspin plus Ki-67, and [3] WT-1 plus Ki-67 antibodies. The expression status of maspin and WT-1 in the same cells of each case was compared to determine the extent of co-expression of these proteins. The proliferation rates of epithelial (EP) cells in ducts with and without maspin or WT-1 expression, as well as with and without an intact ME cell layer were statistically compared.

Distinct immunostaining and the co-localization of maspin and WT-1 proteins were seen in most morphologically definable ME cells in sections from each of the 100 patients, while they were barely seen in EP or stromal cells. The expression of these proteins were closely correlated with the morphology of ME cells, but were generally independent of the size, length, or architecture of the ducts. Both morphologically normal appearing and neoplastic ducts with a reduced maspin or WT-1 expression in surrounding ME cells, or ducts with focally disrupted or no ME cell layers displayed a significantly higher cell proliferation rate than ducts with a normal maspin or WT-1 expression, and with an intact ME cell layer.

These findings suggest that maspin and WT-1 proteins may possess inhibitory functions on EP cell growth and consequently suppress progression or invasion of mammary tumors, and that maspin and WT-1 proteins might also impact the functions of ME cells. Since ME cell layers are physical barriers protecting the microenvironment and integrity of EP cells, and preventing an in situ lesion from invasion, quantitative assessments of the expression of maspin and WT-1 proteins in ME cells might have significant diagnostic and prognostic values.

This study was supported by the US Army Medical Research and Materiel Command under DAMD 17-01-1-0129 and DAMD17-01-1-0130 to Yan-gao Man, MD., Ph.D.
MORPHOLOGICALLY SIMILAR STROMAL CELLS ASSOCIATED WITH BENIGN AND MALIGNANT MAMMARY EPITHELIAL TUMORS DISPLAY DIFFERENT IMMUNOHISTOCHEMICAL AND MOLECULAR PROFILES

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Our previous studies on paraffin embedded tissues from patients with mammary and cervical carcinomas revealed high frequencies of independent and concurrent loss of heterozygosity (LOH) in microdissected epithelial (EP) tumor cells and adjacent or distant stromal (ST) cells. To confirm previous findings on a larger scale and wider spectrum, the current study attempted to compare the immunostaining pattern and the genetic profile in EP and ST cells microdissected from infiltrating syringomatous adenomas and tubular carcinomas, which are two different pathological entities, but with similar reactive background stroma.

Serial sections were made from formalin-fixed, paraffin-embedded mammary tissues from patients with above lesions, and immunostained with a panel of different antibodies. The immunostaining patterns in both the EP and ST components between two lesions were compared. Morphologically similar EP and ST cells in these lesions were microdissected for DNA extraction and assessments for LOH and microsatellite instability (MI), using PCR amplification with a panel of DNA markers at 6 different chromosomes. The frequency and pattern of LOH and MI in samples of two lesions were compared.

The cells from these two lesions displayed a substantially different immunostaining pattern to a majority of the antibodies tested, including those to tumor suppressor gene products, blood vessel components, extracellular matrix molecules, and proliferation-associated proteins. Also, both the EP and ST cells from these two lesions displayed a substantially different frequency and pattern of LOH and MI at multiple chromosomal loci, including 3p, 11p, 13p, 13q and 16q. There was no distinct LOH or MI with multiple DNA markers at chromosome 17p in the ST cells of either lesion, however.

These findings suggest that morphologically comparable ST cells associated with the benign and malignant EP lesions are bio-functionally and genetically different, but closely related with those in their EP counterparts. These findings also suggest that the functions of ST cells in both lesions are not directly subject to regulation by the p53 gene.

This study was supported by the US Army Medical Research and Materiel Command under DAMD 17-00-1-0676 to Fattaneh A. Tavessoli, MD, and DAMD 17-01-1-0129 and DAMD 17-01-1-0130 to Yan-gao Man, MD., Ph.D.
AN ANTIGEN RETRIEVAL PROTOCOL THAT SATISFIES BOTH IMMUNOHISTOCHEMICAL AND SUBSEQUENT MOLECULAR ASSESSMENTS
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Molecular analysis on DNA extracts from selected areas of immunohistochemically stained sections is a useful approach for studying the direct correlation between genetic and biochemical alterations. Immunohistochemical analyses of a variety of gene products in formalin-fixed, paraffin-embedded tissues, however, require a prior antigen unmasking treatment with enzymes or a high temperature using a microwave oven or a pressure cooker, which are found to substantially damage DNA and RNA structures, making subsequent genetic analyses difficult or impossible. This study attempted to develop a protocol that satisfies both immunohistochemical and genetic assessments.

Consecutive sections were made from formalin-fixed, paraffin-embedded breast tissues, and four adjacent sections were treated for antigen unmasking with [1] microwave irradiation; [2] pressure cooker incubation; [3] our modified protocol; [4] untreated. After immunostaining for a variety of cytoplasmic and nuclear antigens, comparable amounts of cells were microdissected from the same area in each of the four sections pretreated with the above four methods. Microdissected cells were subjected to DNA extraction and PCR amplification with a variety of DNA markers. Amplified PCR products among samples were semi-quantitatively compared.

Compared to other antigen unmasking methods, our protocol appeared to possess the following advantages: [1] better preservation of the morphological details; [2] a substantial reduction of the detachment of tissues from slides; [3] effectiveness on all antibodies tested; [4] consistently higher PCR yield; [5] ability to yield PCR products with higher molecular weights. The PCR efficiency in tissues treated with our protocol was comparable to those of both untreated and non-immunostained tissues. This protocol has been successfully used for the detection of over 30 different proteins that are known to require a prior antigen-unmasking treatment for their elucidation, the in situ detection of estrogen receptor mRNA, as well as both double immunohistochemical staining and subsequent molecular analyses.

This study was supported by the US Army Medical Research and Materiel Command under DAMD 17-01-1-0129 and DAMD17-01-1-0130 to Yan-gao Man, MD., Ph.D.
GENETIC ALTERATIONS IN ER (-) MAMMARY EPITHELIAL CELLS OVERLYING FOCALLY DISRUPTED MYOEPITHELIAL CELL LAYERS

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To observe the dynamic alterations of myoepithelial (ME) cells in association with mammary tumor progression, a double immunostaining technique with antibodies to smooth muscle actin (SMA) and estrogen receptor (ER) was used to elucidate the ME and epithelial (EP) cells in mammary biopsies harboring ductal carcinoma in situ. Single or clusters of EP cells with a marked diminution or a total loss of ER expression were found immediately overlying focally disrupted ME cell layers, in contrast to the dominant population of ER (+) cells within the same duct that had no associated ME cell layer disruptions. This study intended to test a hypothesis that these ER (-) cells may represent a more aggressive clone that genetically differs from adjacent ER (+) cells within the same duct.

Consecutive sections were made from formalin-fixed, paraffin-embedded mammary tissues from 220 patients with various grades of ductal intraepithelial neoplasia, and double immunostained for ER and SMA. The cross sections of ducts lined by ≥ 40 EP cells were examined to identify ducts with focal ME cell layer disruptions. The cells overlying disrupted ME cell layers, adjacent ER (+) cells within the same duct, adjacent stromal (ST) cells, and other controls were microdissected for DNA extraction and assessment for loss of heterozygosity (LOH) and microsatellite instability (MI), using PCR amplification with 18 DNA markers at 6 chromosomes. The frequency and pattern of LOH and MI among samples were compared.

The ER (-) cells overlying disrupted ME cell layers and the adjacent ER (+) cells displayed distinct LOH and MI in each of the 18 DNA markers, with highest frequencies at chromosomes 11p and 16q. A vast majority of the cells overlying disrupted ME cell layers showed a substantially higher frequency and different pattern of LOH and MI, compared to adjacent ER (+) counterparts within the same duct. In a small proportion of cases, however, ER (-) cells showed a substantially lower frequency of LOH and MI than adjacent ER (+) cells, or even displayed no distinct genetic changes. Overall, ER (-) cells overlying disrupted ME cell layers among different foci and cases displayed a more homogeneous genetic profile than their ER (+) counterparts within the same duct.

These findings are largely in support of our hypothesis that ER (-) cells overlying disrupted ME cell layers represent a more aggressive clone, while also suggest that a few of these cells might belong to a population involving in a normal replenishment or expansion of the duct.

This study was supported by the US Army Medical Research and Materiel Command under DAMD 17-01-1-0129 and DAMD17-01-1-0130 to Yan-gao Man, MD., Ph.D.