Award Number: DAMD17-01-1-0129

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

PRINCIPAL INVESTIGATOR: Yan-Gao Man, M.D., Ph.D.

CONTRACTING ORGANIZATION: American Registry of Pathology
Washington, DC 20306

REPORT DATE: August 2005

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
**REPORT DOCUMENTATION PAGE**

<table>
<thead>
<tr>
<th>1. REPORT DATE</th>
<th>2. REPORT TYPE</th>
<th>3. DATES COVERED</th>
</tr>
</thead>
<tbody>
<tr>
<td>01-08-2005</td>
<td>Annual Summary</td>
<td>23 Jul 2001 – 22 Jul 2005</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4. TITLE AND SUBTITLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Approaches for Early Detection of Breast Tumor Invasion or Progression</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5a. CONTRACT NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>5b. GRANT NUMBER</td>
</tr>
<tr>
<td>DAMD17-01-1-0129</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>6. AUTHOR(S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yan-Gao Man, M.D., Ph.D.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>American Registry of Pathology</td>
</tr>
<tr>
<td>Washington, DC 20306</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.S. Army Medical Research and Materiel Command</td>
</tr>
<tr>
<td>Fort Detrick, Maryland 21702-5012</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>12. DISTRIBUTION / AVAILABILITY STATEMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approved for Public Release; Distribution Unlimited</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>14. ABSTRACT</th>
</tr>
</thead>
<tbody>
<tr>
<td>This project assessed potential roles of myoepithelial (ME) cells in breast tumor invasion, and found that a subset of pre-invasive breast tumors contained focally disrupted ME cell layers. Compared to adjacent cells within the same duct, cell clusters overlying these focal ME layer disruptions showed several unique features, including a loss of estrogen receptor expression, a significantly higher rate of proliferation, genetic instability, expression of tumor invasion and metastasis related genes, and infiltration of leukocytes. Together, our findings suggest that [1] focal ME layer disruptions might represent an early sign of tumor invasion, [2] cell clusters overlying focal ME layer disruptions might represent precursor of invasive lesions, and [3] focal ME layer disruption may result from a localized degeneration of aged or injured ME cells and resultant immune reactions. This project is completed ahead the schedule. The outcomes of this project are expected to generate a total of 98 scientific publications (74 have been published or accepted for publication).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>15. SUBJECT TERMS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breast tumor invasion; Breast tumor progression; Myoepithelial cells; Early detection; Precursor of invasive breast cancer; Estrogen receptor expression; inflammatory cell infiltration</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>16. SECURITY CLASSIFICATION OF:</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. REPORT U</td>
</tr>
<tr>
<td>b. ABSTRACT U</td>
</tr>
<tr>
<td>c. THIS PAGE U</td>
</tr>
<tr>
<td>17. LIMITATION OF ABSTRACT</td>
</tr>
<tr>
<td>18. NUMBER OF PAGES</td>
</tr>
<tr>
<td>19a. NAME OF RESPONSIBLE PERSON</td>
</tr>
<tr>
<td>19b. TELEPHONE NUMBER (include area code)</td>
</tr>
</tbody>
</table>

<p>| |</p>
<table>
<thead>
<tr>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Form 298 (Rev. 8-98)</td>
</tr>
<tr>
<td>Prescribed by ANSI Std. Z39.18</td>
</tr>
</tbody>
</table>
Table of Contents

Cover............................................................................................................1
SF 298........................................................................................................2
Introduction................................................................................................4
Body..............................................................................................................4-5
Key Research Accomplishments............................................................ 6
Reportable Outcomes...............................................................................6
Conclusions..............................................................................................6
References..............................................................................................6-13

Appendices.........................................................................................6-13 A total of 74 published or accepted papers and abstracts and 5 submitted manuscripts (total about 400 gages) are available upon request.
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. 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

a: 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)

Completed: The outcomes had been published (please see attached “References-publications: # 4,5 (papers), and #1-8 (abstracts).)

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)

Completed: The outcomes had been published (please see attached “References-publications: # 9,10, 12 (papers), and #1-8 (abstracts).

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

Completed: The outcomes had been published (please see attached “References-publications: # 4,5 (papers), and #9-16 (abstracts).

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

Completed: The outcomes had been published (please see attached “References-publications: # 6-10 (papers), and #16-27 (abstracts).

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)

Completed: The outcomes had been published (please see attached “References-publications: # 14-19 (papers), and #28-50 (abstracts).
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)

**Completed:** The laboratory procedures have been completed and the outcomes are in the process of summarization for publication (please see attached “References-Scientific papers near completion or in preparation, #1-24).

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

**Completed:** The laboratory procedures have been completed and the outcomes are in the process of summarization for publication (please see attached “References-Scientific papers near completion or in preparation, #1-24).

**b: Experimental procedures:**

Consecutive sections were made from formalin-fixed, paraffin-embedded breast tissues from over 400 patients with various grades of ductal carcinoma in situ (DCIS), and double immunostained for ER and SMA. 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 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.

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 a panel of DNA markers at 6 chromosomes. The frequency and pattern of LOH and MI among samples were compared.

Consecutive sections were also prepared from frozen breast tissues of patients with DCIS and invasive ductal carcinomas (IDC), and were double immunostained for ER and SMA. Immunostained sections were examined for ER expression and focal ME cell layer disruptions. ER (-) cells overlying disrupted ME layers and adjacent (+) cells within the same duct in DCIS, along with morphologically and immunohistochemically similar cells in IDC, were microdissected for RNA extraction, using the RNA extraction kits from Arcturus Engineering, Inc (Mountain View, CA). The RNA extracts were subjected to RT PCR amplification. The gene expression profiles among samples were compared, using the software and reagents from Affymetrix, Inc (Santa Clara, CA) and SuperArray Bioscience Corporation (Frederick, MD).

A total of 7 biotin-labeled probes and detection kits from our collaborators, DAKO Corporation (Carpinteria, CA), and Sigma (St. Louis, MO) had been used in both paraffin-embedded and frozen sections from selected cases. The experimental procedures had been completed and several manuscripts are in preparation to report the results (see “References”-Scientific papers near completion of in preparation).

The clinical follow-up data from 50 cases with focally disrupted ME cell layers had been compared to those from 50 cases without ME cell layer disruptions, and several manuscripts are in preparation to report the results (see “References”-Scientific papers near completion of in preparation).
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 to 7 had been completed, and the outcomes have been either published or in the process of preparation for publication (see below).

The outcomes of this project have generated 74 published or accepted research papers (n=21), abstracts (n=50), and figures (n=3), as well as 24 submitted (n=5), to be submitted within a month (n=2), and partially completed (n=17) research papers.

Based on his own and other findings, this PI has proposed a new hypothesis for breast and prostate tumor invasion. The hypothesis and supportive data have been recently published in several peer-reviewed journals, including Breast Cancer Research, Breast Cancer Research and Treatment, Experimental Cell Research, Cancer Detection and Prevention, and Applied Molecular Morphology & Immunohistochemistry (see attached “References”: Scientific papers published, accepted, and submitted #5, 10, 12-14).

Several molecules exclusively or mainly expressed in ER negative cell clusters overlying focally disrupted ME cell layers have been identified and characterized, and is in the process for potential development of early detection or therapeutic agents.

**Reportable outcomes**

A total of 98 research papers (n=45), abstracts (n=50), and figures (n=3) are expected to be generated by this projects (see the “References” below).

**Conclusions**

1. Tasks 1-7 listed in the proposal have been completed.
2. A total of 98 publications are expected to be generated by this project.
3. The outcomes are in a total agreement with the original hypotheses in the proposal.
4. Several new molecules associated with tumor progression or invasion have been identified.

**References**

**A. Honors and Awards:**

1. A invited speaker at the Department of Chemistry and Biochemistry at Florida State University in June, 2003
4. A distinguished lecturer at Department of Defense, Center for Prostate Disease Research, October 6, 2004.
6. Invited reviewer for Cancer Therapy in 2004 (one manuscript).

B. Research grants:
1. Author and recipient of AFIP/ARP joint research initiative grant (05AA) in 2005
2. Author and recipient of “Hypothesis Development Award” (PC051308) from Congressionally Directed Medical Research Program in 2005
3. Co-investigator and co-recipient of a grant from Susan Komen Breast Cancer Foundation in 2005

C. Patent:
Co-invent of a filed patent (with Dr. Patricia E. Berg of the George Washington University)

D. Publications:
a. Scientific papers published, accepted, and submitted:
12. Yousefi M, Mattu R, Gao C, Man YG. Mammary ducts with and without focal myoepithelial cell layer disruptions show a different frequency of white blood cell infiltration and growth pattern:
Implications for tumor progression and invasion. A1MM 13:30-37, 2005


22. Man YG, Zhao CQ. Cell clusters overlying focal myoepithelial layer disruptions and budded derivatives have different estrogen receptor expression profiles: implications for treatment and prevention. Submitted


25. Weisz J, Shearer DA, Frauenhofer E, Man YG, McCaffery. Divergent effect of progression of breast cancer from the in situ to the invasive stage on the expression of the retinoic acid biosynthetic enzyme retinaldehyde dehydrogenase 2 (RALDH2): Implications for chemoprevention and treatment of breast cancer. Submitted (under revision)


b. Scientific papers near completion or in preparation

1. Man YG, Schwartz AM. Berg PE. Expression of BP1, a homeobox gene, correlates with prostate
tumor progression and invasion
2. Man YG, Simmens SJ. Focal myoepithelial cell layer disruptions induced tumor and stromal cell alterations correlate with an elevated frequency of invasion.
5. Man YG. Impacts of mast cell infiltration into breast epithelia on cell proliferation and gene expression
6. Man YG, Gao CL, Gardner WA. Impacts of mast cell infiltration into prostate epithelia on cell proliferation and gene expression
7. Man YG. Solid cell clusters with unusual morphologic and immunohistochemical features in pre-invasive breast tissues: Seeds for invasive and recurrent tumors?
8. Man YG. Focal degeneration of aged or injured myoepithelial cells and resultant immunoreactions trigger onset of breast tumor invasion
9. Man YG, Zeng X. Elevated protein expression in stromal cells near focally disrupted myoepithelial cell layers
10. Man YG, Zeng X. Elevated protein expression in stromal cells near focally disrupted prostate basal cell layers
11. Man YG. Co-current and independent protein profiles in cells overlying focally disrupted myoepithelial cell layers and adjacent stromal cells
12. Man YG. Co-current and independent protein profiles in cell clusters overlying focally disrupted basal cell layers and adjacent stromal cells
13. Man YG. Differential frequency and pattern of T-lymphocyte and mast cell infiltration in benign and malignant breast tumors with and without myoepithelial cell layers
14. Man YG. Unique profile of loss of heterozygosity in prostate tumors with an aberrant basal cell layer
15. Man YG. Genetically different primary bilateral breast tumors show similar signs of progression and invasion
16. Man YG, Chen XL, Gardner WA. Prostate ducts and acini with and without focal basal cell layer disruptions have a different profile of androgen receptor expression.

c. Scientific abstracts published and accepted
1. Man YG, Shekitka KM, Saenger JS, Tai L, Bratthauer GL, Chen PY, Tavassoli FA. Focal loss of estrogen receptor (ER) expression in ER positive ductal intraepithelial neoplasia is associated with disruption of the immediate subjacent myoepithelial cell layer. Mod Pathol 15(1):42A, 162, 2002
3. Man YG, Strauss B, Saenger JS, Tai L, Bratthauer GL, Chen PY, Tavassoli FA. Genetic alterations in ER (-) mammary epithelial cells overlying focally disrupted myoepithelial cell layers.


24. Man YG, Shen T, Zhao YG, Sang QX. Morphologically comparable prostate acini and ducts with and without focal basal cell layer disruption have a different cell proliferation rate: Implications for tumor invasion. FASEB 18(5): A1193, 2005
35. Sang QXA, Zhao YG, Man YG. Mechanism of human prostate cancer invasion: Basement


43. Man YG. CD8 and mast cell tryptase positive cells are preferentially associated with focal myoepithelial cell layer disruptions: implications for breast tumor invasion. Proceedings of Department of Defense Breast Cancer Research Program Meeting P38-13,263, 2005

44. Man YG. Focal degenerations in surrounding structures and infiltration of immunoreactive cells are a potential trigger for invasion of breast and other epithelium-derived tumors. Proceedings of Department of Defense Breast Cancer Research Program Meeting P10-7: 75-76, 2005

45. Man YG. Genetically different primary bilateral breast tumors show similar signs of potential progression and invasion. Proceedings of Department of Defense Breast Cancer Research Program Meeting P10-8: 76, 2005

46. Man YG. CD8 and mast cell tryptase positive cells are differentially distributed in benign and malignant breast tissues with and without myoepithelial cell layers. Proceedings of Department of Defense Breast Cancer Research Program Meeting P10-9: 76, 2005


d. Other publications:

2. Man YG, Burgar A. Triple immunohistochemical detection of Ki-67, ESA, and SMA in breast carcinoma. The cover-page for the entire 2004 issues of Pathology- Research & Practice