

AD \_\_\_\_\_

GRANT NUMBER: DAMD17-94-J-4330

TITLE: Characterization of Breast Cancer Progression by Analysis  
of Genetic Markers

PRINCIPAL INVESTIGATOR: Dr. Jack Lichy

CONTRACTING ORGANIZATION: American Registry of Pathology  
Armed Forces Institute of Pathology  
Washington, DC 20306-6000

REPORT DATE: October 1995

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;  
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.

19960124 050

DTIC QUALITY INSPECTED 1

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE October 1995	3. REPORT TYPE AND DATES COVERED Annual 30 Sep 94 - 29 Sep 95	
4. TITLE AND SUBTITLE Characterization of Breast Cancer Progression by Analysis of Genetic Markers		5. FUNDING NUMBERS DAMD17-94-J-4330	
6. AUTHOR(S) Dr. Jack Lichy			
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) American Registry of Pathology Armed Forces Institute of Pathology Washington, DC 20306-6000		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited		12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) Genetic changes implicated in the etiology of breast cancer have been identified by the detection of loss of heterozygosity at specific loci. Our study utilizes a series of genetic polymorphisms detectable by the polymerase chain reaction (PCR) to look for changing patterns of LOH as breast cancer progresses from intraductal to metastatic disease. The initial phases of this work involved the identification of cases from the AFIP archives, microdissection of individual tumor components, and PCR amplification with multiple markers. To date, 248 cases have been identified for the study. Lysates have been prepared from 93 cases. Oligonucleotides were synthesized to detect the nine polymorphisms targeted for the initial screen in the grant proposal. Additional markers were identified and tested to determine ones that would be useful for the study. The 93 cases for which lysates have thus far been prepared have been analyzed for LOH at two loci on chromosome 11p15. The results demonstrate our ability to evaluate individual tumor components for LOH and validate the general approach, which will be used extensively in the next phase of the project.			
14. SUBJECT TERMS Genetics, Progression, Loss of Heterozygosity Breast cancer			15. NUMBER OF PAGES 8
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

## GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to *stay within the lines* to meet *optical scanning requirements*.

**Block 1. Agency Use Only (Leave blank).**

**Block 2. Report Date.** Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

**Block 3. Type of Report and Dates Covered.** State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

**Block 4. Title and Subtitle.** A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

**Block 5. Funding Numbers.** To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

<b>C</b> - Contract	<b>PR</b> - Project
<b>G</b> - Grant	<b>TA</b> - Task
<b>PE</b> - Program Element	<b>WU</b> - Work Unit Accession No.

**Block 6. Author(s).** Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

**Block 7. Performing Organization Name(s) and Address(es).** Self-explanatory.

**Block 8. Performing Organization Report Number.** Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

**Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es).** Self-explanatory.

**Block 10. Sponsoring/Monitoring Agency Report Number.** (If known)

**Block 11. Supplementary Notes.** Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

**Block 12a. Distribution/Availability Statement.** Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

**DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."

**DOE** - See authorities.

**NASA** - See Handbook NHB 2200.2.

**NTIS** - Leave blank.

**Block 12b. Distribution Code.**

**DOD** - Leave blank.

**DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.

**NASA** - Leave blank.

**NTIS** - Leave blank.

**Block 13. Abstract.** Include a brief (*Maximum 200 words*) factual summary of the most significant information contained in the report.

**Block 14. Subject Terms.** Keywords or phrases identifying major subjects in the report.

**Block 15. Number of Pages.** Enter the total number of pages.

**Block 16. Price Code.** Enter appropriate price code (*NTIS only*).

**Blocks 17. - 19. Security Classifications.** Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

**Block 20. Limitation of Abstract.** This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

       Where copyrighted material is quoted, permission has been obtained to use such material.

       Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

*JR* Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

       In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

*JR* For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

       In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

       In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

*JR* In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories. ....

*Jack W. Luby*      10/26/95  
\_\_\_\_\_  
PI - Signature      Date

## TABLE OF CONTENTS

	Page
Front Cover	1
SF 298 Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5
Body	6
Conclusions	7
References	8

## INTRODUCTION

The earliest events in the pathogenesis of breast cancer typically involve the loss of a normal growth regulatory mechanism by a ductal or lobular epithelial cell. Progression of the disease through the stages of intraductal proliferation to invasive carcinoma and then to metastatic disease appears to require additional alterations in growth regulatory pathways. A substantial body of evidence now supports the idea that these alterations in growth regulation result from genetic events such as point mutation, deletion, and gene amplification [1-4]. Our study aims to characterize genetic alterations in breast tumors at the various stages of tumor progression. If metastasis requires additional genetic events beyond those responsible for the intraductal and invasive components of the tumor, one should find genetic alterations in the metastasis that are not present in primary tumor. Alternatively, there may be certain genetic lesions which occur early in tumor development that can predispose a tumor to metastasize without the acquisition of additional genetic defects. The identification of such a lesion would provide an important prognostic indicator, because it would provide a means for predicting the likelihood of the development of metastatic disease in tumors identified at an early stage. The characterization of genetic changes present in individual tumor components thus offers the possibility of identifying new prognostic indicators as well as helping to elucidate the significance of genetic events to tumor progression.

The type of genetic analysis performed in our study is the amplification of polymorphic loci by the polymerase chain reaction (PCR) [5]. This technique permits the detection of loss of heterozygosity (LOH) in tumor specimens relative to normal tissue from the same patient. LOH at specific loci has been observed frequently in breast cancer. High frequency of LOH for a specific genetic marker is thought to imply the presence of a tumor suppressor gene at that locus [3, 4]. In certain cases (e.g., p53 on 17p, DCC on 18q), the loss of one copy of the tumor suppressor gene (LOH) is found in association with mutation of the remaining copy. In such cases, LOH indicates that both copies of the tumor suppressor gene have become inactivated, resulting in the loss of a normal growth regulatory pathway. The PCR methodology also permits the detection of gene amplification, assuming that amplification involves only one of the two copies of the gene present. In breast cancer, amplification of the HER2/neu oncogene is of particular interest because of potential prognostic implications [2].

The general strategy of our study involves the identification of at least 200 cases of breast cancer in the AFIP archives, the microdissection of the intraductal, infiltrating, and metastatic components present in each tumor, and analysis of each tumor component for LOH at multiple genetic loci. The results should help address questions such as when during tumor progression specific genetic lesions occur, and whether LOH at any particular locus has value in predicting the course of progression of an individual tumor.

## BODY

Progress has been made in several areas during the initial funding period of the grant. These areas include the following, each of which will be discussed separately:

1. Identification of cases from the AFIP archives
2. Evaluation of various procedures for microdissection
3. Evaluation of different labeling protocols for PCR
4. Synthesis and testing of oligonucleotides for their usefulness to this study
5. Application of two 11p15 markers to an initial group of 93 cases

1. Identification of cases: The strategy for case selection calls for selecting cases from a larger group of cases being analyzed for a variety of prognostic indicators based on certain specific criteria. The criteria include the adequate representation of individual tumor components and the presence of areas consisting predominantly of tumor cells so that the presence of LOH would not be obscured by contaminating stromal or inflammatory cells. Cases have been screened in weekly batches of 30-50 cases each. Thus far, 248 cases have been selected for use in the study.

2. Evaluation of various procedures for microdissection: Several variables were tested for their effects on the ability to recover amplifiable DNA from archival formalin fixed, paraffin embedded tissue samples. We wanted to determine the optimal approach to identifying the tumor components for microdissection. Initially, we thought that lightly staining the tissue with one of several possible stains would facilitate the identification of tumor components on the slide. However, experiments with several stains, including hematoxylin, eosin, and methylene blue, demonstrated that staining resulted in a significant degradation in the quality of DNA that could be recovered from the tissue. However, we found that the tumor components could be reliably identified on the unstained slide after deparaffinization. Other variables evaluated included the effect of several components of the buffer used for lysate preparation on the recovery of amplifiable DNA and the amount of tissue needed to generate enough lysate for the proposed analyses. The protocol which we have now adopted calls for the preparation of 5 unstained 12  $\mu$ m sections, which are deparaffinized on the slide, and one H and E stained 6  $\mu$ m section. The tumor components are first identified on the stained section and evaluated for adequacy. Regions chosen for microdissection are then identified on the unstained sections and dissected while viewing the section under the microscope. We find that tissue lysates prepared from these microdissected specimens amplify well and yield a frequency of LOH for markers at chromosome 11p15 (selected as a useful locus to test the method) as high or higher than that detected by other investigators. These preliminary results provide validation data for the microdissection method.

3. Evaluation of different labeling protocols for PCR: We compared three methods for the detection of PCR products: (1) staining with ethidium bromide, (2) internal labeling

of the PCR product by incorporation of  $\alpha$ -<sup>32</sup>P-dATP, and (3) end-labeling of the product by using a <sup>32</sup>P-labeled oligonucleotide in the reactions. Ethidium staining proved insufficiently sensitive to detect the small amount of product generated in many of our reactions. Internal labeling results in the labeling of both strands of the product. Strand separation during the running of high resolution denaturing gels therefore results in two bands for each product. We found that this would sometimes obscure the identification of heterozygotes when alleles were close together. End-labeling one of the oligonucleotides in the PCR reaction produced the cleanest results and has been taken as our standard procedure.

4. Synthesis and testing of oligonucleotides for their usefulness to this study: Small scale studies were carried out with each of the nine primer pairs proposed for use. Several were found to give clean, easily interpretable results whereas others generated high levels of background bands which made interpretation difficult. We have found that some of the markers that generate high levels of background can be used for our analysis if we detect the products with an internal probe after blotting to nitrocellulose.

5. Application of two 11p15 markers to an initial group of 93 cases: As a test of our ability to detect LOH in tumor specimens, we analyzed lysates from the 93 cases thus far dissected with two markers from chromosome 11p15, tyrosine hydroxylase (TH) and ST5. With these markers, LOH has been detected in 30-40% of informative cases, a frequency as high or higher than that observed by others at this locus. Our frequency of LOH detection may be higher than that observed previously because of the careful microdissection procedures used. We also note that, when present, LOH at 11p15 is seen in all tumor components, from the intraductal to the metastatic tumor.

## CONCLUSIONS

The initial work on this project has resulted in the establishment of an efficient system for case identification, tissue processing, and PCR amplification. The general approach proposed has been validated by an initial study of LOH at 11p15. The target of 200 microdissected cases set in the grant should be met or exceeded within the next six months. With the availability of this large bank of microdissected tumor components and the establishment of conditions for PCR at multiple markers, the characterization of LOH in these tumors should proceed rapidly in the coming year.



## REFERENCES

1. Leslie, KO and Howard, P. 1992. Oncogenes and antioncogenes in human breast carcinoma. *Pathol. Ann.* 27 (Pt. 1):321-342.
2. Yokota, J, Yamamoto, T, Toyoshima, K, Terada, M, Sugimura, T, Battifora, H, and Cline, MJ. 1986. Amplifiacion of *c-erbB-2* oncogene in human adenocarcinoma *in vivo*. *Lancet* 1:765-766.
3. Ali, IU, Lidereau, R, Theillet, C, and Callahan, R. 1987. Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. *Science.* 238:185-188.
4. Fearon, ER and Vogelstein, B. 1990. A genetic model for colorectal tumorigenesis. *Cell* 61:759-767.
5. Gruis, NA, Abel, ECA, Bardoel, AFJ, Devilee, P, Frants, RR, and Cornelisse, CJ. 1993. PCR-based microsatellite polymorphisms in the detection of loss of heterozygosity in fresh and archival tumour tissue. *Br. J. Cancer* 68:308-313.