AD_____

Award Number: DAMD17-97-1-7085

TITLE: Multiplex PCR-Based Determination of Genetic Polymorphisms Involved in the Etiology of Breast Cancer

PRINCIPAL INVESTIGATOR: John R. Herron
Cosette Wheeler, Ph.D.

CONTRACTING ORGANIZATION: University of New Mexico
Albuquerque, New Mexico 87131

REPORT DATE: September 2000

TYPE OF REPORT: Final

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2000	Final (1 Aug 97 - 31 Jul 00)

4. TITLE AND SUBTITLE
Multiplex PCR-Based Determination of Genetic Polymorphisms Involved in the Etiology of Breast Cancer

5. FUNDING NUMBERS
DAMD17-97-1-7085

6. AUTHOR(S)
John R. Herron
Cosette Wheeler, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of New Mexico
Albuquerque, New Mexico 87131

E-Mail: bherron@unm.edu

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)*
Breast cancer incidence rates for New Mexico's women have risen rapidly over the last two decades, particularly in Hispanics for whom breast cancer mortality rates have doubled. Despite this fact, the causes of breast cancer in this minority population have not been adequately characterized; thus, hampering interventions to reverse these trends. We have proposed to develop novel methods to evaluate allelic polymorphisms potentially associated with breast and other forms of cancer. In the recent past, much attention has been focused on potential functional polymorphisms present in the enzymes responsible for metabolic oxidation. These enzymes play a role a role in the detoxification of xenobiotics and among others include members of the cytochrome P450 family, glutathione S-transferases class θ and μ, and N-acetyl transferases 1 and 2. The laboratory methods currently utilized to study these polymorphic enzymes are expensive, time consuming, and cumbersome when applied to large-scale epidemiological studies. We are currently developing simplified and accurate methods based on multiplex PCR and sequence-specific hybridization which will facilitate the rapid screening of each of these polymorphic loci. These methods will facilitate the large-scale epidemiological studies needed to characterize genetic variations associated with breast cancer incidence.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	14
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Genetic polymorphisms identified in phase I and phase II metabolic enzymes have been suggested to be associated with cancers of multiple anatomic sites (1-17). To date, the majority of these studies have involved epidemiological studies of polymorphisms at a single gene locus and the potential association of those polymorphisms with particular cancers(1-5,7,8,10-14,16-19); however, a few studies have examined the relationship between both phase I and phase II enzyme polymorphisms (6,9,15). Our purpose was to determine if allelic polymorphisms in CypIA1, CypIA2, CypIID6, GST θ, GST μ, NAT-1, and NAT-2 are associated with differences in risk for the development of benign breast disease (BBD) and breast cancer and do these alleles and risks differ between ethnic groups? Since each of these polymorphic metabolic enzymes have been implicated as potential risk factors in the development of various forms of human cancers including lung, bladder, colorectal, and breast cancer, it is possible that all or most of these enzymes also play an important role in the etiology of breast cancer. The intention of this study was to develop one tube multiplex PCR-based assays to amplify the relevant loci which will then be analyzed by hybridization assays as well as gel-based procedures to identify the polymorphism present in a sample set of cases verses controls.

Body

Worldwide breast cancer causes about 400,000 deaths each year (20). In the United States it is the second leading cause of cancer related mortality among women, responsible for approximately 46,000 deaths annually (21). It has been estimated that one out of every eight women in the United States who reaches the age of 85 will at some point in her life develop breast cancer (21). In 1995, this malignancy became the most common cancer diagnosed in New Mexico with 1,051 new cases diagnosed (22). In 1996, the latest year for which New Mexico data is available, that number grew slightly to 1,097 new cases remaining the most common site for malignancy in New Mexico (22). New Mexico's Hispanic (H) women have a lower breast cancer incidence rate than NHW women (73.7 per 100,000 from 1993 to 1995) representing approximately 27% of the cases diagnosed in 1996; however, incidence and mortality rates spanning the late 1950's through the late 1980's document increasing breast cancer incidence and related deaths in this population of Hispanic women. This has been postulated to result from differences in ethnicity which may be accounted for by both genetic and cultural/behavioral variations. Of the three major ethnic groups in the state, American Indians have the lowest breast cancer incidence rates (42.1 per 100,000 from 1993 to 1995) representing approximately 3% of the cases diagnosed in 1996. This study will provide an opportunity to examine and characterize genetic variations that may contribute to the observed differences in the incidence of breast cancer between these ethnic groups.

Several factors including genetic disposition, dietary habits, endocrine and chemical influences as well as many others are suspected to play a role in the development of breast cancer (23). Recently, a considerable amount of effort has been directed towards defining potential functional polymorphisms in numerous enzymes involved in the metabolism of environmental carcinogens found in the diet, tobacco

smoke and ambient air due to environmental or occupational sources(1, 23-25). Thus far, the best studied metabolic enzymes which may be associated with increased cancer risk include members of the cytochrome P450 family (CypIA1, CypIA2, CypIID6 and Cyp17), glutathione S-transferases class α,ϕ,μ and π (GSTα, GSTϕ, GSTμ, and GSTπ), and N-acetyl transferases 1 and 2 (NAT-1 and NAT-2). Functionally, the previously named enzyme families are divided into two groups known as phase I and phase II. Phase I enzymes include the cytochrome P450 family of oxidative enzymes which are generally involved in the conversion of a wide variety of compounds to reactive electrophilic metabolites. The phase II enzymes include both the glutathione S-transferases and the N-acetyltransferases which usually act as detoxifying enzymes. Genetic polymorphisms have been identified in both phase I and II enzymes and several studies have suggested that these allelic polymorphisms are associated with cancers of multiple anatomic sites(1-17). To date, the majority of these studies have involved epidemiological studies of polymorphisms at a single gene locus and the potential association of those polymorphisms with particular cancers(1-5, 7, 8, 10-14, 16-19 ,23); however, a few studies have examined the relationship between both phase I and phase II enzyme polymorphisms (6,9,15). Given that cancer susceptibility resulting from chemical exposure is likely to be determined by an individuals phenotype for multiple enzymes involved in both phase I and II functions, it is imperative that future studies examine a large spectrum of these polymorphic loci in order to adequately asses the risk of susceptibility to and potential interaction with environmentally induced cancer resulting from individual metabolic variation.

Our purpose is to determine if allelic polymorphisms in CypIA1, CypIA2, CypIID6, GST θ, GST μ, NAT-1, and NAT-2 are associated with differences in risk for the development of benign breast disease (BBD) and breast cancer and do these alleles and risks differ between ethnic groups? Since each of these polymorphic metabolic enzymes have been implicated as potential risk factors in the

development of various forms of human cancers including lung, bladder, colorectal, and breast cancer, it is possible that all or most of these enzymes also play an important role in the etiology of breast cancer. The continuation of this study will provide for a complete identification of nucleotide variations within the targeted loci. This will allow accurate risk assessment for these specific genetic polymorphisms in patients diagnosed with BBD and breast cancer. In the Western United States and specifically the Southwest, Hispanic women have lower breast cancer incidence when compared to Non-Hispanic White women.

In our original proposal, we intended to utilize samples collected in a prior case-control study. These samples were unavailable primarily as the result of a marked decrease in tumor size and thus correspondingly smaller specimens. Routine clinical testing exhausted tissue for many cancers, and pathology laboratories were increasingly reluctant to release the minute amount of tissue remaining from other cancers. This has provided us with the unique opportunity to identify and evaluate alternative sources of tissue samples. As a result, our work has experienced delays; however, we are in the process of obtaining samples through the New Mexico Women's Health Study. Thus far, a total of 497 of 1027 women ascertained with incident breast cancer have completed baseline interviews including weight history, physical activity, dietary nutrient intake, reproductive history, family history, use of oral contraceptives and postmenopausal estrogens, and personal history of cancer. Approximately 85 percent of these had blood samples, anthropometry, and body composition examinations with dual energy X-ray absorptiometry. Additionally, approximately 42 percent of the current cohort have completed food frequency questionnaires (FFQs) providing more extensive dietary information. Table 1 shows the enrollment status of this study to date.

Table 1 Patient enrollment status

	Non-Hispanic	Hispanic	Total
Total Ascertained	778	245	1027
Interview Completed	401	96	497
Blood Sample Obtainec	337	77	414
Anthropometry	350	76	426
DEXA	339	75	414
Food Frequencies	172	36	208
Refusals	106	42	148

Values listed do not total 1027 due to the following causes 1) inability to contact patient, 2) patien found ineligible for study after ascertainment, 3) physician recommended no contact, 4) patient unable to complete interview.

Experimental Methods

Samples- Samples analyzed thus far have included archival tissues from 30 cases of invasive breast carcinoma and buccal scrapes from Hispanic (n=129; 69 cases of invasive breast cancer and 60 controls) and non-Hispanic White (n=153; 73 cases of invasive breast cancer and 80 controls) women.

Extraction of DNA from paraffin-embedded tissues- DNA was extracted from paraffin-embedded tissues by standard techniques developed in our laboratory (26).

Extraction of DNA from Buffy Coats- Genomic DNA has been isolated from buffy coat specimens, for which whole blood is available, using the Puregene DNA isolation kit (Gentra Systems Inc.). Briefly, the buffy coat specimen is thawed on ice, RBC's are lysed and the corresponding white cell pellet is lysed using the kit cell lysis solution. The white cell lysis solution is then digested with

RNAse A and the proteins are precipitated using a protein precipitation solution. The sample is microcentrifuged and the supernatant containing the genomic DNA is ethanol precipitated. The DNA is washed with 70% ethanol, microcentrifuged, and the pellet is dried followed by a rehydration in Hydration Solution. Absorbances at 260 and 280 nm are taken to determine purity and concentration.

Development and optimization of multiplex PCR-based assay- We have developed procedures for amplifying all of the relevant loci including NAT-1, NAT-2, GST μ, and GST θ. We have obtained control plasmids to facilitate the optimization of the cytochrome P450 locus and we began experiments to optimize multiplex PCR-based assays.

Development and optimization of gel procedures using fluorescent primers and hybridization assays using biotinylated probes- Gel-based assays have been developed and optimized for standard primers for all of the loci previously mentioned. We have begun hybridization assays relevant to NAT-2 and we present preliminary results later in this report.

Phylogenetic computer analysis of related loci- Preliminary phylogenetic analysis has not yet provided the degree of insight we had hoped for. I have recently completed an academic course with the intent of becoming more familiar with the Genetics Computer Group (GCG) Wisconsin Package and we believe that this will facilitate our efforts in this approach.

Key Research Accomplishments

A multiplex PCR assay for NAT-1, NAT-2, GST μ and GST θ was developed. The endpoint was gel-based and could be further developed for fluorescent detection. Assays were applied to DNA obtained from archival breast cancer tissues embedded in paraffin. In addition, buccal scrapes were obtained from breast cancer cases (69 Hispanic and 73 non Hispanic Whites) as well as controls (60 Hispanics and 80 non-Hispanic Whites) and these assays were applied.

Reportable Outcomes

By definition in the guidelines regarding the format of this report, we have no significant reportable outcomes. The activity was terminated before the end of the granting period. See the body of the report and previous reports for specifics.

Conclusions

We have thus far successfully optimized the procedures for detecting polymorphisms at NAT-1, NAT-2, GST μ, and GST θ. In addition, we have analyzed a small case-control data set for NAT-2 polymorphisms in which we assigned both simple and weighted acetylation scores based on biological assignments of acetylation phenotypes. Analyses have been conducted based on individual alleles present and extended haplotypes of the targeted polymorphisms. Analyses were stratified by smoking status, race, and inferred acetylation rate. Homozygosity and heterozygosity was considered and while statistical significance was not achieved, we have identified an apparent trend which suggests that the risk of developing breast cancer increases as acetylation rates increase in nonsmokers more so than in smokers. This observation most likely indicates that the risk of developing breast cancer in smokers is defined by factors other than polymorphisms in the NAT-2 locus. It is therefore imperative that further analysis including CypIA1, CypIA2, CypIID6, GST θ, GST μ, and NAT-1 be conducted to clarify these observations regarding NAT-2 in hopes of gaining insight into the potential roles played by both phase I and phase II metabolic enzymes in the development of breast cancer.

This study will provide an opportunity to examine and characterize genetic variations that may contribute to the observed differences in the incidence of breast cancer between the two major ethnic groups present in New Mexico. Through the identification of nucleotide variations, we will have the ability to identify potential risk and protective alleles that may act as genetic predisposing factors for breast cancer. This information may also contribute to our understanding of the basic biological processes involved in breast cancer development. Our preliminary results regarding the NAT-2 locus illustrate the necessity of examining genetic polymorphisms in both phase I and phase II metabolic

enzymes as NAT-2 polymorphisms do not appear to represent a risk for the development of breast cancer in smokers.

During the course of the first twelve months of this graduate fellowship, I have completed the core course requirements for the Biomedical Sciences Graduate program as well as the Qualifying Examination and matriculated into Ph.D. candidacy. I am not permitted by my program to select a Qualifying Examination topic that is directly related to my current research interests; however, I was allowed to propose a study involving molecular epidemiology which both satisfied my examination committee and permitted me to gain a new appreciation for the power epidemiological methods provide in answering biological questions involving human disease.

References

1. Taioli, E., Trachman, J., Chen, X., Toniolo, P. and Garte, S.J. (1995) *Cancer Research* **55**, 3757-3758
2. Feigelson, H.S., Coetzeeg, A., Kolonel, L.N., Ross, R.K., Henderson, B.E. (1997) *Cancer Research* **57**, 1063-1065
3. Wrigley, E.C., McGown, A.T., Buckley, H., Hall, A. and Crowther, D. (1996) *British Journal of Cance* **73**, 763-769
4. Marie, J.P., Simonin, G., Legrand, O., Delmer, A., Faussat, A.M., Lewis, A.D., Sikic, B.I. and Zittoun, R. (1995) *Leukemia* **9**, 1742-1747
5. Katoh, T., Nagata, N., Kuroda, Y., Itoh, H., Kawahara, A., Kuroki, N., Ookuma, R. and Bell, D.A. (1996) *Carcinogenesis* **17**, 1855-1859
6. Oude Ophuis, M.B., van Lieshout, E.M.M., Roelofs, H.M.J., Peters, W.H.M. and Manni, J.J. (1998) *Cancer* **82**, 936-943
7. Lin, J.J., Han, C.Y., Bernstein, D.A., Hsiao, W., Lin, B.K. and Hardy, S. (1994) *Carcinogenesis* **15**, 1077-1081
8. Zhong, S., Wyllie, A.H., Barnes, D., Wolf, C.R. and Spurr, S.K. (1993) *Carcinogenesis* **14**, 1821-1824
9. Bailey, L.R., Roodi, N., Verrier, C.S., Yee, C.J., Dupont, W.D. and Parl, F.F. (1998) *Cancer Research* **58**, 65-70
10. Martinez, C., Agundez, J.A.G., Olivera, M., Martin, R., Ladero, J.M. and Benitez, J. (1995) *Pharmacogenetics* **5**, 207-214
11. Bell, D.A., Stephens, E.A., Castranio, T., Umbach, D.M., Watson, M., Deakin, M., Elder, J., Hendrickse, C., Duncan, H. and Strange, R.C. (1995) *Cancer Research* **55**, 3537-3542
12. Romkes, M., Chern, H., Lesnick, T.G., Becich, M.J., Persad, R., Smith, P. and Branch, R.A. (1996) *Carcinogenesis* **17**, 1057-1062
13. Kelsey, K.T., Hankinson, S.E., Colditz, G.A., Springer, K., Garcia-Closas, M., Spiegelman, D., Manson, J.E., Garland, M., Stampfer, M.J., Willet, W.C., Speizer, F.E. and Hunter, D.J. (1997) *Cancer Epidemiology, Biomarkers & Prevention* **6**, 511-515
14. Kihara, H., Kihara, M. and Noda, K. (1994) *Carcinogenesis* 15, 415-418
15. Nakachi, K., Imai, K., Hayashi, S. and Kawajiri, K. (1993) *Cancer Research* **53**, 2994-2999
16. Hamada, G.S., Sugimura, H., Suzuki, I., Naura, K., Kiyokawa, E., Iwasa, T., Tanaka, M., Takahashi, T., Watanabe, S., Kino, I. and Tsugane, S. (1995) *Cancer Epidemiology, Biomarkers & Prevention* **4**, 63-67
17. Lin, H.J., Probst-Hensch, N.M., Ingles, S.A., Han, C., Lin, B.K., Lee, D.B., Frank, H.D., Lee, E.R., Longnecker, M.P. and Haile, R.W. (1995) *Cancer Research* **55**, 1224-1226
18. Ladona, M.G., Abildua, R.E., Ladona, J.M., Roman, J.M., Plaza, M.A., Agundez, J.A.G., Munoz, J.J. and Benitez, J. (1996) *Cancer Letters* **99**, 23-28
19. Kivisto, K.T., Fritz, P., Linder, A., Friedel, G., Beaune, P. and Kroemer, H.K. (1995) *Histochemistry* **103**, 25-29
20. Jatoi, I. (1997) *Breast Cancer Screening.* Chapman & Hall
21. Herman, C.J. (1996) *Breast Cancer in New Mexico: A Handbook for Health Care Providers.* People Designs
22. Key, C.R., Davidson, A.M., Taylor, L. (1995) *New Mexico Tumor Registry: Malignancies Diagnosed (State of New Mexico).* The University of New Mexico Health Sciences Center

23. Ladona, M.G., Abildua, R.E., Ladona, J.M., Roman, J.M., Plaza, M.A., Agundez, J.A.G., Munoz, J.J. and Benitez, J. (1996) *Cancer Letters* **99**, 23-28
24. van Maanen, J.M.S., Moonen, E.J.C., Maas, L.M., Kleinjans, J.C.S. and van Schooten, F. J. (1994) *Carcinogensis* **15**, 2263-2268
25. Raunio, H., Husgafvel-Pursiainen, K., Anttila, S., Hietanen, E., Hirvonen, A. and Pelkonen, O. (1995) *Gene* **159**, 113-121
26. Greer CE, Wheeler CM, Manos MM. (1994) Sample preparation from paraffin-embedded tissues. *PCR Methods and Applications*. **33:**5113-5122.