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13. ABSTRACT *(Maximum 200 words)* The purpose of this study is to identify possible genetic risk factors that might predispose African-American women to aggressive breast cancer. Plasma samples collected from African-American women, both cases and contols would be used for genotyping, and tumor tissue samples were available from cases. The study proposed a genotypic analysis of carcinogen metabolizing genes and analysis of the p53 mutations in tumor samples to determine if associations exist between enzyme genotypes and cancer risk; and if an association exists between enzyme genotypes and p53 mutational patterns in tumors.

Unfortunately, technical problems related to sample quality and difficulties in genotyping methodology have lead the mentor for this career development grant to terminate the project. Problems with the study design under which the samples were originally collected would further complicate analysis of any data that could be obtained.

The principal invsetigator for the grant has identified a new laboratory and research project in which to continue the career development award. The plasma samples have been returned to the Howard University Cancer Center, our collaborators.

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FOREWORD

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Keith W. Crawford 5-26-98

PI - Signature Date

TABLE OF CONTENTS

Introduction Page 2

Body Page 3

Methods page 3

Results page 4

References Page 6

Appendices

INTRODUCTION

The project is entitled *Genetic Susceptibility Factors in Aggressive Breast Cancer in African-American Women and the Effects of Carcinogens and Modifiers* (DAMD17-97-1-7083). The proposed study was designed to identify possible genetic risk factors predisposing African-American women to more aggressive forms of Breast Cancer. Enzymes involved in carcinogen activation and detoxification have polymorphic variants that may display differences in functional activity. The genetic profile for these enzymes may account for differences in the manner in which carcinigens are processed. The frequency of these polymorphisms vary across racial and ethnic populations and this may contribute to differences in disease prevalence between populations, when controlling for exposures.

Caucasians have a higher prevalence of breast cancer, but African-American women present with more aggressive disease, have a worse prognosis at diagnosis, and shorter survival. When socioeconomic factors are taken into account, these survival differences persist, suggesting the involvement of biologic processes (Eley et al.,1994). Mutations in the p53 tumor suppressor gene occur in over 50% of sporadic cancers (Cole et al., 1992). P53 mutational patterns may be predictive of prognosis in breast cancer. Some studies suggest that there are differences in mutational patterns between African-Americans and Caucasians (Blasyk et al., 1994, Shiao, et al., 1995). These differences could result from the genotypic profile that determines carcinogen metabolism and DNA damage to the target genes.

This study proposed a genotypic analysis of carcinogen metabolizing genes (Cytochrome P4501A1, CYP1A2, glutathione-S-transferases, acetyltransferases) and mutational analysis of the p53 mutations in tumor samples to determine whether:

- an association exists between enzyme genotypes and cancer risk when relevant exposures are present
- an association exists between enzyme genotype and p53 mutational patterns in tumors
- a model can be constructed to mechanistically account for the above associations, if detected

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BODY

METHODS

DNA Extraction from plasma samples

Using the method of Blomeke et al. (1997), 250 ul of plasma was added to 750 ul of buffer (50 mM TRIS-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) and centrifuged at 300,00 g for 12 min (Sorvall RC M120). The pellets were resuspended in 200 ul of lysis solution (0.1 % SDS; proteinase K, 1 mg/ml; RNase 20 ug, Boerhinger-Manheim) and incubated at 65 C for 4-6 hours.. Each sample was extracted with an equal volume of buffer saturated phenol, and the resulting aeqeous phase extracted with chloroform/iso-amyl alcohol (24:1). The resulting aeqeous phase was adjusted to 3 M NH_4acetate and 10 mM MgCl. Glycogen (20 ug, Boeringer-Mannheim) and cold 2-propanol (1 ml) were added to samples, which were centrifuged for 30 min at 14,000 rpm (Eppendorf 5415) at 4 C. Pellets were rinsed with 70% ETOH, air-dried and then resuspended in 20-40 ul of distilled water.

Quantification of DNA

DNA was quantified by flourescense using a DyNA Quant 200 flourometer (Hoefer Pharmacia Biotect). Total amounts recovered from samples ranged from 200 ng to 2000 ng, with recoveries averaging 35-45% of DNA present in the sample.

Genotyping for N-Acetyl transferase 2

A Polymerase Chain Reaction (PCR)-assay was developed that amplified a 1093 bp fragment, encompassing the entire NAT2 coding sequence and part of the non-coding region (Bell et al.,1993). The assay mixture contained 10 pmoles of primers (TCTAGCATGAATCACTCTGC and GGAACAAATTGGACTTGG), 180 mM deoxynucleotide triphosphates, 10 mM Tris HCL, pH 8.3, 50 mM KCl , 30 mM $MgCl_2$, 20-100 ng of template DNA in a total volume of 100 ul. PCR cycling conditions were :
96 C (4 min), 35 cycles of 96 C (1 min), 57 C (1.5 min), 72 C(3 min); and 72 C (7 min).

NAT2 RFLP Genotyping

Four different Restriction fragment length polymorphisms (RFLP)have been reported, and restriction digests of the 1093 bp fragment produce the following patterns:
M1 *Kpn1* Presence of the mutations produces two fragments 659, 443 bp
M2 *a-Taq* presence of the mutation produces four fragments 381, 326,226,169 bp
M3 *Bam H1* presence of the mutation produces two fragments 819, 283 bp

M4 *Msp1* Presence of the mutation produce four fragments 760, 188, 94, 60 bp

For nested PCR, the reaction uses 10 ul of reaction product from the reaction described above added to 90 ul of the same reaction mix containing different primers (5'-GAACTCTAGGAACAAATTGGACTTG-3', 5'-CCATCACCAGGTTTGGGAC-3') run under the same cycling conditions. An 817 bp fragment is produced.

Results

Approximately 200 plasma samples were received from Howard University Cancer Center comprising breast cancer cases and age-matched controls. The samples were assigned LHC (Laboratory of Human Carcinogenesis) codes so the samples would remain blinded. Dr. Peter Shields, Chief of the Molecular Epidemiology section of NCI and the mentor for the training grant identified NAT2 as the first gene for us to genotype. The assay described in *Methods* was first done using our pedigree DNA sample set. This allowed experience to be acquired with the assay and a confirmation of the heritability of the polymorphisms.

When the DNA extraction procedure described above was applied to plasma samples available from the LHC lab, the recovery of DNA was comparable to that reported by Blomeke et al. based on quantification of DNA by measuring U.V. absorbance at 260 nm. However, the 260/280 ratio revealed that the seemingly high yields were due to protein contamination. The use of flourescence to quantify the DNA is a procedure specific for DNA, and not affected by protein or RNA contamination. This allowed an accurate determination of DNA concentrations. The amount of DNA recovered varied based on the quantity of cells in the plasma samples and whether they were stored properly.

PCR amplification of DNA recovered was another source of problems. In some instances, inhibitors appeared to be present in the DNA from the extraction. Reducing the amount of DNA added to the assay apparently diluted out the inhibitors and resulted in PCR product, but this was not always the case. Preheating samples at 95 C for 15 minutes prior to addition in the PCR assay was attempted to inactivate inhibitors, but this produced limited success. Nested PCR was attempted to increase PCR yield and it worked in some samples but there was a concern as to whether results could be obtained for a sufficient number Howard University samples to conduct a meaningful statistical analysis.

In discussions between Dr. Shields and Dr. Lucile Adams-Campbell, Director of Howard University Cancer Center and collaborator with the research project, concerns were raised

about the original study design under which the samples were collected (*Breast cancer and Black Women: Gene and Environmental Interactions,* NIH-RO1CA55772) and whether the questions originally posed in this grant application could be answered accurately. These issues along with the technical problems related to the genotyping assays dampened Dr. Shields's enthusiasm for the project. The data from the genotyping was absolutely pivotal to answering the questions we posed, so Dr. Shields decided to terminate his sponsorship of the project (Appendix 1). The plasma samples have been returned to Howard University.

Ms. Juanita Bourne (appendix 2) and Dr. Patricia Muldrow at Ft. Detrick have been notified of these unfortunate circumstances, and they have instructed me to submit a new Statement of Work for a new project with a different mentor to continue with the training grant. Their request is being completed.

I am currently completing a book chapter in collaboration with Dr. Shields encompassing genetic polymorphisms and cancer risk. This activity has been a valuable component of the experience in the lab and has tremendously enhanced my career development training. The manuscript will be completed prior to July 1, 1998.

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APPENDIX 1

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

National Institutes of Health
National Cancer Institute
Bethesda, Maryland 20892

MEMORANDUM

DATE: May 7, 1998

FROM: Section Chief
Molecular Epidemiology Section, LHC, DBS, NCI

SUBJECT: Status of activities and termination of breast cancer project

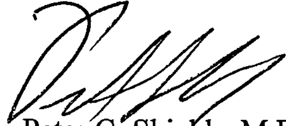
To: Kieth Crawford, Ph.D.

I would like to summarize for you our April 30, 1998 meeting. At the time I informed you of my decision to terminate our Howard University breast cancer project and collaboration as it relates to the DOD funding. This was a difficult decision for me, and I regret the inconvenience that it will cause you. The decision was based on both short-term and long-term considerations. Therefore, those activities should stop. Elise Bowman will assume the responsibility for the Howard University samples and ensure that they will be returned to Dr. Adams-Campbell or her designate.

For the interim period, we will continue your volunteer status in order to finish up the dopamine studies. You will receive appropriate credit through authorship for the genotyping of the dopamine D4 and DAT in the Mayo and UMD lung cancer studies, assuming that the analyses yield publishable data. However, the position of your authorship will be decided by me and Dr. Harris at the time that the data analysis is completed, and we can assess the relative contributions of all the authors. Your volunteer status will certainly continue until the laboratory components are completed.

Lastly, you and I are collaborating on a book chapter, and we should finish that work. You do not need volunteer status to complete this, however.

Please let me know if your new job status might impact on the completion of the above studies. And also let me know if there is anything that I can do to help you in your new job search.



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cc: Dr. Harris
Dr. Adams-Campbell
Dr. Taylor

APPENDIX 2

HOWARD UNIVERSITY

COLLEGE OF MEDICINE
DEPARTMENT OF PHARMACOLOGY

May 6, 1998

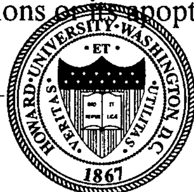
Ms. Juanita Bourne
U.S. Army Medical and Materiel Command
Breast Cancer Research Program
Fort Detrick, Maryland

Dear Ms. Bourne,

On May 1, 1997, I was formally awarded a training grant, #DAMD17-97-1-7083 for a project entitled "Genetic Susceptibility Factors in Aggressive Breast Cancer in African-American Women". The study is a collaboration between Howard University Cancer Center and the Laboratory of Molecular Epidemiology in the National Cancer Institute. The research work is conducted at NCI under the mentorship of Dr. Peter Shields. The proposed study would determine if specific polymorphisms in enzymes involved in carcinogen activation and detoxification (CYP1a1, CYP2a1, CYP2e1, NAT enzymes, GST enzymes) are associated with the development of breast cancer. The study would further investigate whether certain polymorphisms were correlated with p53 mutational patterns. One hypothesis being tested is that variability in the frequencies of certain polymorphisms between Caucasian and African-American populations may contribute to differences in the biology of tumors. p53 mutations differ in their effect on protein function and cancer prognosis, and racial differences in p53 mutation patterns have been observed. The questions posed in the study would not only identify genetic risk factors but may reveal novel pathogenic mechanisms explaining population variability in p53 mutation patterns.

Approximately 200 plasma samples from breast cancer cases and controls were received from Howard University Cancer Center. DNA was extracted from the plasma samples. Unfortunately, the quality of the DNA did not permit PCR amplification. The quality of the DNA was essential to performing genotype analysis for the enzymes of interest. Numerous strategies for improving the yield from the extractions and the PCR were tried unsuccessfully. The number of samples that produced suitable PCR product were not sufficient to allow meaningful statistical analysis after genotyping. Because our hypotheses could not be tested in the absence of the genotype data, Dr. Shields determined that the study did not merit further pursuit. The samples are being returned to Howard University.

I am writing the Agency at this time requesting permission to continue the training grant in another laboratory within the National Cancer Institute. There would be no change in the cost or the award institution. At this point, I have identified at least two laboratories within NCI that have initially expressed an interest in allowing to train with them. Dr. Alfred Johnson in the Laboratory of Molecular Biology of NCI is studying the expression of epidermal growth factor receptors on cultured breast cells during the transformation process. Dr. Kevin Gardner in the Division of Clinical Science at NCI maps the functional domains of p53 tumor suppressor protein using deletion mutations to identify regions where p53 interacts with an array of other proteins involved in its cell-cycle regulatory functions or its apoptotic functions.



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Both of these researchers are exploring possible projects that would be suitable for a training grant, and I am also formulating ideas on my own. Upon the consent of the Agency, I would like to be allowed a period of time to develop and submit a revised research plan.

In spite of the difficulties encountered, I have benefitted tremendously in the period of less than a year that I have worked under the grant. While the samples from Howard University were being collected, coded and organized in their data base, I did work on a lung cancer case-control sample set from the University of Maryland. From working with that set I successfully extracted DNA from lymphocyte samples. I performed the genotyping for two genes related to dopamine pharmacology. Both of the genes, the dopamine transporter and the D4 Dopamine receptor subtype, express Variable number tandem repeat (VNTR) polymorphisms. This work was done from the University of Maryland samples (about 250 samples) and a sample set from the Mayo Clinic (about 100 samples). We are completing analysis of the data and publications are forthcoming.

I developed PCR assays to detect restriction fragment length polymorphisms and a VNTR polymorphism in the alpha4-subunit of the neuronal nicotinic receptor. I used single strand conformation polymorphism (SSCP) analysis to genotype the nicotinic VNTR polymorphism in a pedigree set in the lab. This same method would have been used to detect mutations in p53 genes breast tumor samples from Howard University.

Other activities that have contributed to y training include:

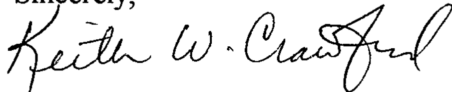
- completion of courses in Biostatistics and Epidemiology at the Johns Hopkins School of Public Health
- participation in departmental seminars of the Labarotory of Human Carcinogenesis
- participated in a symposium "Frontiers in Breast cancer" sponsored by Genequest Infosystems
- attended the American Association for Cancer Research annual meeting in New Orleans, 1998

In addition, I am co-authoring a chapter with Dr. Shields on *Cancer and Genetic Susceptibility* to be included in a book on Genetics and Disease.

Therefore, I have learned technical skills that are applicable in diverse research settings. I have also learned the most current paradigms of carcinogenesis and innovative approaches to both basic science and clinical research problems.

It is most unfortunate that the originally proposed work could not proceed as we had anticipated. I believe it would have made a contribution to our understanding of breast cancer risk factors and mechanisms of carcinogenesis. I am hopeful that the Agency will allow me to redirect my effort into a project that will be more fruitful. I can be reached at 301-402-2811. I appreciate your consideration of my request.

Sincerely,



Keith W. Crawford, Ph.D.

cc

Dr. Robert Taylor

Dept. of Pharmacology, Howard University College of Medicine