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GRANT NUMBER DAMD17-94-J-4446

TITLE: Breast Cancer Training Program

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CONTRACTING ORGANIZATION: University of Illinois
Chicago, Illinois 60612-7205

REPORT DATE: October 1996

TYPE OF REPORT: Annual

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1996	Annual (30 Sep 95 - 29 Sep 96)

4. TITLE AND SUBTITLE
Breast Cancer Training Program

5. FUNDING NUMBERS
DAMD17-94-J-4446

6. AUTHOR(S)
Tapas K. Das Gupta, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Illinois
Chicago, Illinois 60612-7205

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, 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*

Objective: To draw together in a common environment doctoral students from different basic science disciplines and provide them with a strong multidisciplinary background in breast cancer research.

Approach: 1) Provide future basic scientists (predoctoral students) in a given discipline (e.g., physiology, biochemistry, etc.) a stable multidisciplinary foundation from which to develop cutting-edge research in breast diseases, resulting in a dissertation dealing with a specific breast cancer research topic. 2) From these doctoral (Ph.D.) students, develop a cadre of postdoctoral fellows who will devote their entire professional careers to the field of breast cancer research and will be able to generate their own funding through competitive grants and contracts. It is expected that this group of researchers will swiftly and effectively provide translational (i.e., clinically relevant) bench research results into the clinical arena.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
39

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

FOREWORD

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

Tapas K. Das Gupta

PI - Signature Date

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Reprint:

Waltz MR, Pandelidis SM, Pratt W, Barnes D, Das Gupta TK, Gendler SJ, Cohen EP. Alteration of Microsatellite DNAs in the Neoplastic Cells of Breast Cancer Patients. Submitted.

(Abstract) Shamon LA, Mehta RR, Das Gupta TK, Pezzuto JM. Evaluation of Natural Products as Inhibitors of HER-2/neu. 87th Annual Meeting of AACR, Washington, DC, April 20-24, 1996

(Abstract) Shamon LA, Mehta RR, Das Gupta TK, Pezzuto JM. Natural Product Inhibitors of Her-2/neu tyrosine kinase. 37th Annual Meeting of the American Society of Pharmacognosy, Santa Cruz, CA, July 27-31, 1996

5. Introduction

The purpose of this grant is to draw together in a common environment doctoral students from different basic science disciplines and provide them with a strong multidisciplinary background in breast cancer research.

We have tried to provide the milieu of a multidisciplinary basic research forum for training the future generation of basic scientists so that they can develop an appropriate basis from which to pursue clinically relevant (i.e., translational) research in breast cancer. We have also tried to provide a stable multidisciplinary foundation from which to develop cutting-edge research in breast diseases, resulting in a dissertation dealing with a specific breast cancer research topic.

6. Body

The tasks listed in the grant proposal are:

Task 1: Provide to predoctoral fellows in various disciplines (e.g., Physiology, Biochemistry) a multidisciplinary basic foundation and emerging knowledge in breast cancer biology.

Task 2: Develop a well-focused dissertation theme related specifically to breast cancer.

Task 3: Generate a cadre of well-trained doctoral students (Ph.D.s) who will devote their professional careers to the field of breast cancer research and will be able to generate their own funding through competitive grants and contracts.

Task 4: Provide the milieu of a multidisciplinary basic research forum for training and educating clinical scientists, so that they can develop an appropriate basis from which to pursue clinical research.

To date (i.e., during the first two years of this proposal), we have established a participating faculty committee for selection of the three graduate students funded by the grant. Although, in the original application, we proposed to select nine predoctoral students for the fellowship, the requested funding was reduced to support only three students. Therefore, we currently are supporting only three students with the fellowships.

The selection committee consists of Dr. Das Gupta (chairman) and Drs. E.P. Cohen (Department of Microbiology and Immunology), R.L. Davidson (Department of Genetics), R.R. Mehta (Department of Surgical Oncology), M.B. Mokyr (Department of Biochemistry), P. Raychaudhuri (Department of Biochemistry), and I.G. Roninson (Department of Genetics). This committee has selected the first three graduate students from the candidates nominated by each basic science department to be supported by this training grant. The candidates selected are Lisa Shamon, Margaret R. Waltz, and Lavanya Lall. These three were chosen from a pool of 22 candidates from all the basic science departments. The paramount reasons for selecting these three graduate students were their commitment to breast cancer research, their grades, and, finally, the disciplines in which they were pursuing their graduate studies. Ms. Shamon is a student in the Department of Medicinal Chemistry and Pharmacognosy in the College of Pharmacology; Ms. Waltz is a student in the Department of Microbiology and Immunology, College of Medicine; and Ms. Lall is in the Department of Genetics.

These graduate students are not only taking all the courses necessary to fulfill the requirements of the parent department, but also completing a

course offered by the program faculty as a prerequisite for the Breast Cancer Research Fellowship. This is a specially designed course, titled "Basic Concepts in Cancer Biology." The objective of this course is to provide the predoctoral students with a larger vista in cancer biology, so that these students evolve into mature cancer biologists. Currently, this course is being offered in the form of a seminar once a month (2 hours). Developing this program, we planned a 3- to 4-credit hour course. However, with three students in the program, it is very difficult to execute it as a University graduate-level course. We requested to initiate a course with the Graduate College; however, our request was turned down due to the small number of students interested at that time. Thus, currently, the course is organized as a seminar. These seminars are mandatory for the fellows, and all faculty members (basic scientists and clinical faculty members) are expected to attend. Also, the fellows have to present their research work as a formal seminar. Margaret Waltz and Lisa Shamon already presented their work in this series. The workshops are on informal basis. For example, Lisa Shamon spent time in Dr. Constantinou's laboratory learning topoisomerase assays and in Dr. R.R. Mehta's laboratory learning immunocytochemistry. Miss Lall spent time in Dr. R.G. Mehta's laboratory learning carcinogen transformation of human breast epithelial HBL 1000 cells. This association not only teaches the fellows other techniques, it also allows them to be part of a publication if one is generated from this effort. We plan to increase the seminar series to twice a month by January, 1997.

Each of these trainees has also developed a research program dealing with some aspect of breast cancer, under the respective preceptorship of Drs. John M. Pezzuto (Department of Medicinal Chemistry & Pharmacognosy), Edward A. Cohen (Department of Microbiology and Immunology), and Richard L. Davidson (Department of Genetics). The trainees' dissertations will be written about the research performed in breast cancer. Dr. Das Gupta is a member of the respective thesis committees of these three graduate students. Descriptions and progress reports on their research activities are provided below.

Research Projects of Lisa Shamon (Preceptor: Dr. J.M. Pezzuto)

Title: Natural Products in the Chemoprevention and Chemotherapy of Breast Cancer: Antimutagens and Inhibitors of HER-2/neu Proto-oncogene

Description

The HER-2/neu proto-oncogene encodes for a 185-kD protein with intrinsic tyrosine kinase activity. Oncogenic effects are manifested when the protein is overexpressed, occurring in approximately 25%-30% of invasive breast carcinomas and in >80% of comedo ductal carcinoma *in situ* (comedo-DCIS) (1). Although the mechanism is not fully understood, overexpression of HER-2/neu has been associated with aggressive disease, increased probability of tumor recurrence, and poor patient survival rates (2,3). Thus, the presence of HER-2/neu in both preinvasive and invasive disease makes this protein a rational target for antitumor agents. In order to screen for potential inhibitors of HER-2/neu, 300 compounds were selected from an inventory of natural product isolates. In this project, several *in vitro* and *in vivo* methods were used to screen for inhibitors of HER-2/neu and to follow-up specific leads with more in-depth mechanistic studies.

To summarize, three human breast carcinoma cell lines developed in our departmental laboratory (UISO-BCA-1, UISO-BCA-2, and MAXF-401) were used to evaluate the effectiveness of these agents in an *in vitro* cytotoxicity assay. UISO-BCA-1 and UISO-BCA-2 were established from pleural effusions of post-menopausal breast cancer patients (4). MAXF-401 was established from a xenograft originating from a lung metastasis (5). UISO-BCA-1 and MAXF-401

overexpress HER-2/neu, whereas UISO-BCA-2 expresses only basal levels. Test agents which preferentially inhibited the growth of UISO-BCA-1 and MAXF-401, as compared to UISO-BCA-2, were subsequently evaluated for their ability to inhibit the tyrosine kinase activity of HER-2/neu. This strategy led to the identification of dehydrocostus lactone and episteganangin, among others, as potential antineoplastic agents which may act via inhibition of HER-2/neu. Experiments are currently underway to more fully elucidate the mechanism of action of these compounds in relation to HER-2/neu.

Both compounds inhibited autophosphorylation of HER-2/neu in a dose-dependent manner. To determine the specificity of these agents toward other kinases, both were evaluated for effects on protein kinase C activity. Neither compound had any effect on the basal enzymatic activity of protein kinase C. However, dehydrocostus lactone did increase the enzymatic activity of phorbol ester-induced PKC. In addition, neither compound demonstrated an inhibitory effect against the tyrosine kinase activity of a crude membrane fraction from HL-60 human leukemia cells, suggesting a specificity for at least the epidermal growth factor receptor subfamily of tyrosine kinases. A dose-dependent decrease in the tyrosine phosphorylation of HER-2/neu in UISO-BCA-1 cells was observed after exposure to either dehydrocostus lactone or episteganangin. Both compounds are currently under evaluation for activity against solid tumors (UISO-BCA-1 cells) implanted into nude mice. Immunohistochemical studies are planned in order to determine any *in vivo* effect of the test agents on HER-2/neu.

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Baek, N.-I., Chung, M.-S., Shamon, L., Kardono, L.B. S., Tsauri, S., Padmawinata, K., Pezzuto, J.M., Soejarto, D.D., and Kinghorn, A.D. Selligueain A, a Novel Highly Sweet Proanthocyanidin from the Rhizomes of Selliguea feii. 34th Annual Meeting of the American Society of Pharmacognosy, San Diego, CA, July 18-22, 1993.

Shamon, L.A., Mehta, R.G., Moon, R.C., and Pezzuto, J.M. A Correlative Approach to the Discovery of Antimutagens that Demonstrate Cancer Chemopreventive Activity. 34th Annual Meeting of the American Society of Pharmacognosy, San Diego, CA, July 18-22, 1993.

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Shamon, L., Lee, I.-S., Kinghorn, A.D., and Pezzuto, J.M. Evaluation of the Cytotoxic Mechanism Mediated by the Diterpene Lactone 13-Methoxy-15-oxozopatlin. International Research Congress on Natural Products and 35th Annual Meeting of the American Society of Pharmacognosy, Halifax, Nova Scotia, July 31-August 4, 1994.

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Shamon, L.A., Mehta, R.R., Das Gupta, T.K., and Pezzuto, J.M. Natural Product Inhibitors of HER-2/neu Tyrosine Kinase. 37th Annual Meeting of the American Society of Pharmacognosy, Santa Cruz, CA, July 27-31, 1996.

Jang, M.S., Cai, L., Lee, S.K., Suh, N., Gerhauser, C., Shamon, L., Kinghorn, A.D., Mehta, R.G., Moon, R.C., and Pezzuto, J.M. Discovery of a Novel Dietary Cancer Chemopreventive Agent that Inhibits Cyclooxygenase Activity. 37th Annual Meeting of the American Society of Pharmacognosy, Santa Cruz, CA, July 27-31, 1996.

MEETINGS ATTENDED:

Thirtieth Annual Medicinal Chemistry Meeting-in-Miniature, Iowa City, IA, May 17-19, 1992.

Tenth Marquette Life Sciences Symposium, Milwaukee, WI, March 12-13, 1993.

Thirty-fourth Annual Medicinal Chemistry Meeting-in-Miniature, Minneapolis, MN, April 23-25, 1993.

Functional Foods for Health (UIC/UIUC Joint Program), Second Annual Retreat, Monticello, IL, May 10-12, 1993. Abstract presented.

Sixth Annual Chicago Signal Transduction Symposium, Chicago, IL, May 18, 1993.

34th Annual Meeting of the American Society of Pharmacognosy, San Diego, CA, July 18-22, 1993. Abstracts presented (2)

Third Drug Discovery and Development Symposium, San Diego, CA, July 22-24, 1993.

206th Annual Meeting of the American Chemical Society, Chicago, IL, Aug. 22-28, 1993. Abstract presented.

Thirty-second Annual Medicinal Chemistry Meeting-in-Miniature, Chicago, IL, April 20-22, 1994. Meeting co-chair.

Functional Foods for Health (UIC/UIUC Joint Program), Third Annual Retreat, Monticello, IL, May 8-10, 1994.

International Research Congress on Natural Products and 35th Annual Meeting of the American Society of Pharmacognosy, Halifax, Nova Scotia, July 31 -August 4, 1994. Abstracts presented (2).

Functional Foods for Health (UIC/UIUC Joint Program), Fourth Annual Retreat, Itasca, IL, May 22-24, 1995.

36th Annual Meeting of the American Society of Pharmacognosy, Oxford, MS, July 23-27, 1995.

87th Annual Meeting of the American Association for Cancer Research, Washington, D.C., April 20-24, 1996. Abstracts presented (2).

Functional Foods for Health (UIC/UIUC Joint Program), Fifth Annual Retreat, Monticello, IL, May 20-22, 1996.

Histopathobiology of Neoplasia, The Edward A. Smuckler Memorial Workshop, Keystone, CO, July 21-28, 1996.

37th Annual Meeting of the American Society of Pharmacognosy, Santa Cruz, CA, July 27-31, 1996. Abstracts presented (2).

Research Projects of Margaret R. Waltz (Preceptor: Dr. E.P. Cohen)

Title: The Role of the Polymorphic Epithelial Mucin (PEM) in the Progression of Mammary Carcinomas.

Description

The polymorphic epithelial mucin (PEM) is a high molecular weight glycoprotein which is aberrantly expressed by breast carcinoma cells. The function of PEM in tumor formation and progression has not been elucidated, although a number of studies indicate that expressed PEM may serve as a target

for immunotherapy. The basis for this hypothesis is that tumor-expressed PEM is under-glycosylated relative to the normal glycoprotein, which results in the exposure of new peptide sequences that may then be presented to the immune system. PEM expression is also highly tissue-specific, allowing for direction of the immune system to those tissues expressing aberrant PEM. In the current description of research, we aim 1) to outline experiments designed to investigate the potential of PEM as a target for immunotherapy of breast tumors and 2) to address the question of autoimmunity which may result from sensitization of the immune system to a self-antigen.

PEM-expressing mouse tumors cells

To study the role of PEM in tumor formation, we are using E3 cells, a derivative of the 410.4 cell line which was established from a spontaneously arising mammary tumor in Balb/c mice. E3 cells have been modified to express human PEM by cotransfection of 410.4 cells with a plasmid encoding PEM and a plasmid carrying the resistance gene for hygromycin. When maintained in hygromycin, E3 cells stably express high levels PEM, as verified by fluorescence activated cell sorting (FACS) analysis.

The innoculum of E3 cells required to cause a tumor in 100% of Balb/c mice is larger than that of 410.4 cells (10^5 as compared with 10^3). And, though their in-vitro growth rates are the same, E3 tumors grow more slowly than 410.4 tumors in vivo. These findings suggest that expression of PEM by E3 cells results in partial rejection of the cancer cells, and supports a role for PEM as a target for immunotherapy. However, the role of PEM as a tumor antigen in E3 cells is complicated by the fact that it is a xenogeneic antigen, a human protein expressed in mouse cells. As such, PEM should elicit an immune response and cause rejection of the cells expressing it. To study PEM as a self antigen in mouse cells, use of transgenic mice, which express PEM endogenously, is required.

Transgenic mouse model

We have obtained *MUC1*-transgenic mice (*MUC1* is the gene encoding PEM) from the lab of Dr. Joyce Taylor-Papadimitriou at the Imperial Cancer Research Fund in London, England. In these mice, human PEM is expressed as a self antigen, with specific expression in breast and other tissues.

Prior evidence indicates that mouse tumors modified to secrete cytokines, such as interleukin-2 and interferon-gamma, become immunogenic and stimulate specific antitumor immunity. In analogy to this work, we plan to investigate the effect of cytokine-secretion by E3 cells on their immunogenicity in transgenic mice. To this end, E3 cells have been transfected with plasmids encoding IL-2, IL-4, IFN-gamma, and TNF-alpha. The immunogenic properties of these constructs will be studied to determine the cytokine or combination of cytokines which best enhances the anti-E3 response. This will be determined by 51-Chromium release assays using 51-Chromium-labeled E3 as the target cell. This information will point to the cytokine with the greatest therapeutic benefit for patients with breast cancer.

Knockout mouse model

Muc-1 (Muc-1 is the mouse homologue of human *MUC-1*) knockout mice have been created by Sandra Gendler at the Mayo Clinic, Scottsdale, AZ. These mice have been found to develop normally and are healthy and fertile. This is thought to be due to the expression of compensatory mucins which are expressed in the absence of Muc-1. The nature of these compensatory mucins and their ability to serve as tumor antigens has not been investigated. We are currently in the process of acquiring these animals from Dr. Gendler and plan

to characterize the Muc-1 compensatory epitopes antigens expressed by their tumors.

Genetic alteration of the *MUC-1* locus is a prognostic indicator in human breast cancer

A final aspect of our work toward understanding the role of PEM in the pathogenesis of breast cancer uses paired (tumor and blood) human DNA samples to study genetic alterations of the *MUC-1* locus by PCR and Southern blotting. We found that 63% of breast cancer patients have an amplification of the *VNTR* region of one of their *MUC-1* alleles, and that 24% of patients have instability of the microsatellite (CA repeat) located within intron 6 of the *MUC-1* gene. We also found no association between the patients who have an alteration at the *VNTR* region and those having an alteration in the microsatellite within the locus. From this, we concluded that these alterations represent different mechanisms of genetic change. While the amplification of the *VNTR* region may be mediated by homologous recombination, instability of the microsatellite may be due to a failure of the repair machinery to recognize and correct errors or replication.

Of special interest is the finding that 14% (2/14) of breast cancer patients without an alteration of the *VNTR* unit within the *MUC-1* gene have died. In contrast, 43% (10/23) of patients with an alteration of the *VNTR* have died. This difference is statistically significant ($p<.05$). I am currently analyzing additional specimens to increase the number of cases analyzed and the statistical significance of this finding. If the preliminary finding is borne out in the expanded study, alteration of the *VNTR* and PEM will prove a valuable prognostic indicator for patients with breast cancer.

Publications during the period of this fellowship

Waltz, M.R., Pandelidis, S.M., Pratt, W., Barnes, D., Swallow, D.M., Gendler, S.J., Cohen, E.P. Alteration of Microsatellite DNAs in the Neoplastic Cells of Breast Cancer Patients. (Submitted, manuscript enclosed).

Sun, T., Kim, T.S., Waltz, M.R., Cohen, E.P. (1995) Interleukin-2 secreting mouse fibroblasts transfected with genomic DNA from murine neoplasms induce tumor-specific immune responses that prolong the lives of tumor-bearing mice. Cancer Gene Therapy 2(3):183-190.

Prior publication

Waltz, M.R., Pullman, T.N., Takeda, K., Sobieszczyk, P., Refetoff, S. (1990) Molecular basis for the properties of the thyroxine-binding-globulin-slow variant in American Blacks. J. Endocrinological Investigation 13:343-349.

Meetings

1996 Annual Meeting of the American Association for Cancer Research April 20-24, 1996. Washington, D.C. Poster: "Non-random alterations of microsatellite DNAs in the neoplastic cells of breast cancer patients"

Histopathobiology of Neoplasia July 9-16, 1995. Keystone, CO Poster: "Non-random alterations of microsatellites in breast cancer"

Nature Genetics DNA Integrity and Instability April 6-7, 1995. Chicago, IL

3rd International Workshop on the Carcinoma-Associated Mucins August 7-11, 1994. Cambridge, England Poster: "Alterations in mucin by PCR and Southern blotting"

Research Projects of Lavanya Lall (Preceptor: Dr. R.L. Davidson)

Title: Sequence-specific mutagenesis in human oncogenes

Description

Thymidine (dT), the naturally occurring nucleotide involved in DNA synthesis, has been shown to be mutagenic because it can inhibit the ribonucleotide reductase catalyzed reduction of CDP to dCDP, eventually leading to a decrease in the intracellular dCTP/dTTP ratio. Subsequently, thymine, the nucleotide in excess, is able to mispair with template guanine, leading to a GC->AT transition at the next round of replication. Mutations induced by dT are highly sequence-specific: they occur preferentially in sequences containing runs of two or more adjacent guanine residues, and specifically at the 3'G of such runs of adjacent guanines. Such sequence-specific mutagenesis seems to be relevant to mutations in certain human oncogenes.

Examples of mutationally activated human oncogenes include the ras genes and the p53 tumor suppressor gene. In the case of the ras oncogenes, studies on mutagenesis by chemical carcinogens in rodents have frequently found these genes to possess activating point mutations in codons 12, 13, and 61--the first two of which contain GG doublets. A significant number of these mutations arose by GC->AT transitions at the 3'G of the GG doublet. This same sequence specificity has been observed in certain human tumors. In human pancreatic and colorectal cancers, a significant number of all activating ras mutations occurred at the 3'G of the GG doublet in codon 12.

Analyses of spontaneously occurring and carcinogen-induced rodent mammary tumors showed that the primary mutations were single GC->AT transitions at the 3'G of the GG doublet in codon 12 of their ras oncogenes. In our laboratory, it had been previously shown that, when cultured mouse cells were subjected to high concentrations of thymidine (dT), GC->AT transitions occurred preferentially at the 3'G of GG doublets in the bacterial *gpt* gene that had been stably integrated in the mouse genome. Our *in vitro* experiments, described in this report, showed that such sequence specificity of dT mutagenesis can be accounted for by the greater preference for dT misincorporation opposite the 3'G of the GG doublet in codon 12 of mammalian ras genes. Since this is the exact same mechanism of activation of mammalian ras genes, this is directly relevant to mutagenesis by high dT concentration in breast cancer pathogenesis. Using the same dT misincorporation experiments, we also showed that the 3'G of the GG doublets in codons 187 and 278 of the human p53 gene, which are frequent sites of mutation in human breast cancers, show preferential mispairing with dT. Based on our ras results, this suggests a similar mechanism of activation of the human p53 gene leading to, and thus directly relevant to, human breast cancer.

In human breast cancers, ras mutations are not frequently encountered. Instead, the most commonly occurring genetic aberrations involve the p53 tumor suppressor gene. Data were compiled from 10 studies of human breast tumors, and 91 tumors with base substitution mutations leading to amino acid changes were identified. An analysis of these 91 tumors revealed 11 unequivocal cases involving base substitution mutations occurring at GG doublets where mutations at either the 3'G or the 5'G led to an amino acid substitution. Of these 11 cases, 10 represented tumors that arose due to mutational activation of the p53 gene by a GC->AT transition at the 3'G of the GG doublets. This seems to suggest a similarity among the mutational activation of ras genes at the GG doublet in codon 12 leading to tumor formation, the mutational activation of the human p53 tumor suppressor gene in some cases of breast cancer, and the sequence specificity that we have observed for mutagenesis by thymidine.

In addition, we conducted a new literature survey of p53 mutations in human breast tumors. Almost 23% of the known p53 mutations implicated in human breast cancer occur at GG doublets of codons 196, 245, 248, 249, 278, and 282 of the gene. More specifically, 46% of these mutations occurred at the 3'G of these GG doublets. Of interest is the fact that these six codons have been identified, along with codons 175, 213, and 273, as the predominant "hot spots" of all known p53 mutations. Thus, 66% of the "hot spots" of all p53 mutations comprise GG doublets, and 84% of the p53 mutations implicated in human breast cancers occurred at "hot spot" codons, with at least 50% of the mutations involving GC->AT transitions. These data suggest that the preferential mispairing of dT at the 3'G of GG doublets in the human p53 gene, leading to GC->AT transition mutations, may play a larger role in human breast cancer than previously thought. Conceivably, the sequence-specific-directed mispairing of dT and GG doublets may represent a general mechanism of sequence-directed base mispairing, and this could account for other types of mutations leading to breast cancer.

Experiments were set up to test whether sequence-specific base mispairing can account for the preferential occurrence of mutations at the 3'G residue of the GG doublets in codon 12 of the human Ha-, Ki-, and N-ras genes, and those in certain mutation "hotspots" in the human p53 gene. For this, an IN VITRO MISINCORPORATION ASSAY was used. Briefly, single stranded DNA templates containing the GG doublets of interest were separately annealed to end-labeled primers designed such that the next base extension off one primer was opposite the 3'G of the template GG doublet and the next base extension off the other primer was opposite the 5'G. Single-base extension reactions were performed in the presence of dTTP (as the only nucleotide) and an exonuclease-free Klenow polymerase that lacks both the 3'->5' and 5'->3' exonuclease activities. For any given dTTP concentration, dT was found to misincorporate with greater efficiency opposite the 3'G than the 5'G of the ras and p53 GG doublets of interest. To generate a similar extent of dT misincorporation opposite the 5'G versus the 3'G of the N-ras GG doublet in codon 12, a 25-fold higher dTTP concentration was required. In the case of the GG doublets in codon 12 of Ki- and Ha-ras and codons 187 and 278 of the p53 gene, 20-fold, 3-fold, 6-fold, and 5-fold higher dTTP concentrations, respectively, were required to generate a similar extent of dT misincorporation opposite the 5'G versus the 3'G. Control experiments in which dCTP, the correct nucleotide, was provided as the only nucleotide showed no significant difference between incorporation of dC opposite the 3'G versus the 5'G of the ras and the p53 GG doublets.

COMPETITION EXPERIMENTS were set up in order to determine if the 3'G/5'G differential observed in the above experiments remained if the correct nucleotide, dCTP, was also provided along with the incorrect nucleotide, dTTP. With increasing concentrations of cold dTTP in the presence of a fixed concentration of labeled dCTP, both Ki-and N-ras showed progressive decreases in the incorporation of the labeled dC opposite both 3'G and 5'G. This indicated progressive increase in misincorporation of dT opposite these guanine residues even in the presence of the correct base. For any given concentration of dTTP, in the presence of labeled dCTP, misincorporation was consistently higher opposite 3'G versus 5'G. Overall, the dTTP concentration necessary to attain a given level of competition in the presence of labeled dCTP differed at least 30-fold opposite the 3'G versus the 5'G of the N- and Ki-ras genes. Thus, the sequence specificity of misincorporation remained even in the presence of the correct base.

In the earlier experiments, dT misincorporates with greater efficiency opposite the 3'G rather than the 5'G of the GG doublets in codon 12 of the human Ha-, Ki-, and N-ras and codons 187 and 278 of the human p53 genes. This could result either from stimulation of dT misincorporation opposite the 3'G

by the 5'G and/or from suppression of dT misincorporation opposite the 5'G by the 3'G. To test these possibilities, two template pairs were used in the previously described misincorporation assays, providing dTTP as the only nucleotide. In one pair, one template was identical to wild type N-ras, and the other differed from it by changing the 5'G to an A. The other pair had C as the upstream base flanking the 5'G, but in one of them, the 3'G was replaced by a T. When the 5'G of the doublet was replaced by an A, dT misincorporation opposite the adjacent guanine significantly decreased compared to that opposite the same guanine residue flanked by an upstream G (i.e., in a GG doublet). In the second template pair, dT misincorporation opposite the 5'G of the GG doublet was consistently lower than when this same G was flanked by a downstream T instead of a G. Thus, each 3'G and 5'G of the GG doublet seem to have a role (either stimulatory or suppressive) in influencing the extent of dT misincorporation opposite the other.

In the misincorporation experiments, the extent of the 3'G/5'G differential varied among the three ras genes, being lowest in Ha-ras (3-fold) and highest in N-ras (25-fold). This difference seemed primarily due to higher dT misincorporation opposite the 5'G of the Ha-ras GG doublet as compared to that of N-ras. Since the base immediately downstream of the 5'G in both genes is 3'G while the base immediately upstream differs, these different upstream bases might possibly have a role in affecting the extent of dT misincorporation opposite the 5'G of the codon 12 GG doublets.

To test this, four synthetic templates were made based on sequences around codon 12 of Ki-ras. One was the wild type Ki-ras sequence which has T upstream of the 5'G. In the other three, this was changed to A, G, or C. These were used in a misincorporation assay, providing dTTP as the only nucleotide. With increasing concentrations of dTTP, misincorporation of dT opposite the 5'G was consistently highest when there was an upstream C, followed by T and G, and it was least when A was the upstream base. These results exactly correspond to those obtained in the initial misincorporation assays with Ha-, Ki- and N-ras. Thus, these results, along with the previous experimental observations, may further define a role of the immediately upstream base in governing the extent of dT misincorporation opposite the 5'G of the ras codon 12 GG doublets.

The above results could indicate the effect of the upstream flanking base in determining the efficiency of dT mispairing with the 5'G of the GG doublet. On the other hand, they could also indicate the effect of the upstream base on the stability of the G:T mispair once it had formed. To test the latter possibility, a primer was synthesized that, when annealed in separate reactions to the same four templates used in the earlier experiment, led to the formation of a 5'G:T mismatch. These templates were then used in separate single-base extension reactions providing the correct nucleotides (dATP, dTTP, dCTP, and dGTP respectively). Correct extension beyond the preformed 5'G:T mismatch was influenced by the upstream flanking base. With increasing dNTP concentrations, correct extension was highest when C was the base upstream of the 5'G, followed by T and G, and it was least when A was the upstream base. Since the pattern of these results was identical to that of the previous experiment, they both might reflect the same effect, namely the effect of the upstream flanking base on the stability of the 5'G:T mismatch.

CONCLUSIONS

Our experiments have shown a preference for misincorporation of dT opposite the 3'G of the GG doublet in codon 12 of the ras and in codons 187 and 278 of the human p53 genes. This preference remained even in the presence of the correct nucleotide (dC). This pattern corresponds with the most frequent mode of activation of the human ras genes, through GC->AT transitions

at the 3'G of the codon 12 GG doublet. Thus, our data suggest that dT-induced mutations may be a source of mutations in the ras and p53 genes leading to various human cancers. We have also shown that the bases immediately upstream and downstream of guanine residues determine the efficiency with which they mispair with dT. This in turn may be used to predict the susceptibility of various guanine residues to be mutated in different sequence contexts within the DNA molecule and in different oncogenes.

PUBLICATIONS

Sequence specific mutagenesis in human oncogenes. Lall, L. and Davidson, R.L. Manuscript in preparation.

MEETINGS ATTENDED

Presented a poster at the Annual U.I.C. Molecular Biology Retreat, Lake Geneva, Wisconsin, 1995.

7. Conclusions

We are pleased with the projects developed by the trainees and the rewarding results and promising future of each project. The results of this research have been presented at national scientific meetings and submitted to peer-reviewed journals for publication.

We have chosen trainees from widely varied fields (Medicinal Chemistry & Pharmacognosy, Microbiology and Immunology, and Genetics), to attack the problem of breast cancer from many directions. All three trainees are women, affirming our commitment to recruiting more minorities and women into the translational research arena.

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9. Appendix

Waltz MR, Pandelidis SM, Pratt W, Barnes D, Das Gupta TK, Gendler SJ, Cohen EP. Alteration of Microsatellite DNAs in the Neoplastic Cells of Breast Cancer Patients. Submitted.

(Abstract) Shamon LA, Mehta RR, Das Gupta TK, Pezzuto JM. Evaluation of Natural Products as Inhibitors of HER-2/neu. 87th Annual Meeting of AACR, Washington, DC, April 20-24, 1996

(Abstract) Shamon LA, Mehta RR, Das Gupta TK, Pezzuto JM. Natural Product Inhibitors of Her-2/neu Tyrosine Kinase. 37th Annual Meeting of the American Society of Pharmacognosy, Santa Cruz, CA, July 27-31, 1996

Alteration of Microsatellite DNAs in the Neoplastic Cells of Breast Cancer Patients.

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Running head: Alterations of microsatellite DNA in breast cancer

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Supported by grant number PDT441 awarded by the American Cancer Society to Dr. Cohen.

We thank Larisa Stross for expert technical assistance.

Abstract

Paired DNA samples from the neoplastic and non neoplastic cells of 118 patients with the sporadic, non familial form of breast cancer were analyzed for evidence of genetic alteration at each of five microsatellite loci, and at the polymorphic epithelial mucin locus (MUC1) gene. The analysis of the microsatellite loci was carried out by the polymerase chain reaction (PCR), using oligonucleotide primers that flanked the regions of interest. The variable number tandem repeat (VNTR) region of the MUC1 gene was analyzed by Southern blotting. The microsatellite loci investigated were D17s579, at 17q21, D18s34 at 18q21, D1S104 at 1q21, APO-A2 at 1q21 and a microsatellite within the MUC1 gene at 1q21. At least 87% of patients were heterozygotic at one or more locus. The results revealed an alteration of at least one locus in the neoplastic, but not the non neoplastic cells of 30.0% of the patients. Instability of microsatellite DNA was present in at least one locus in 17.5% of patients. The VNTR region within the MUC1 gene was altered in 61.4% of informative cases. There was no significant association, however, between changes at the VNTR region and an alteration of microsatellite DNA at any of the loci investigated. In 16.5 % of cases, hMSH2, a replication error repair (RER) gene mapped to 2p16, was altered. A C to T transition at the -6 position of the splice acceptor site, a conserved region, was detected in each of the altered cases. The hMSH2 locus was altered in both the neoplastic and non neoplastic cells of the same patients. There was no association between an alteration of the hMSH2 locus and the presence of microsatellite instability in this group of patients.

Introduction

Recent evidence indicates that an accumulation of genetic changes at specific loci within an individual somatic cell are responsible for conversion to the malignant phenotype (Fearon and Vogelstein, 1990). Subsequent genetic changes in the malignant cells as the tumor progresses may lead to the appearance of cell-derivatives with metastatic capability and resistance to drugs commonly used for chemotherapy.

The discovery of mismatch repair genes in mammalian cells (Fishel *et al.*, 1993; Leach *et al.*, 1993; Papadopoulos *et al.*, 1994; Bronner *et al.*, 1994), homologous to mismatch repair genes described previously in E. coli (Levinson *et al.*, 1987) and in yeast (Strand *et al.*, 1993) provided insight into a mechanism that might be responsible for the genetic changes. The data indicate that mutations in genes responsible for error repair during DNA replication lead to the accumulation of numerous genetic alterations. The hMSH2 gene, a replication repair gene mapped to chromosome 2p16 (Fishel *et al.*, 1993; Leach *et al.*, 1993) is one of several recently described mismatch repair genes.

The first indication of the replication error repair (RER^+) phenotype in cancer patients was the discovery of widespread alterations in microsatellite DNAs in malignant cells (Aaltonen *et al.*, 1993). Microsatellites are di-, tri, or tetranucleotide repeats present thoughout the genome (Weber and May, 1989). Their highly polymorphic nature, precise chromosomal localization, and relative ease of detection by the PCR render them ideal for the detection of genetic instabilities in neoplastic cells. Alterations in microsatellite DNAs have been found in colorectal carcinoma cells of patients with hereditary non-polyposis colon cancer (HNPCC) (Thibodeau *et al.*, 1993; Aaltonen *et al.*, 1993) and in the neoplastic cells of a small proportion of patients with bladder (Gonzalez-Zuluetta *et al.*,1993), endometrial (Risinger *et al.*, 1993) or gastric cancer (Peltomaki *et al..*, 1993). Whether or not alterations in microsatellite DNAs are present in the malignant cells of breast cancer

patients, and their possible relationship to mutations in the hMSH2 gene, is uncertain.

Here, we compared each of five polymorphic microsatellite loci in the neoplastic cells of breast cancer patients with the analogous loci in the non neoplastic cells of the same individuals. In total, 118 patients with the sporadic form of the disease were investigated. The analysis was performed by the PCR, using oligonucleotide primer DNAs that flanked the regions of interest. Three of the five microsatellite loci investigated were chosen because of their known association with disease. The locus at D17S579 is closely linked to the BRCA1 locus, involved in hereditary early-onset breast cancer (Hall *et al.*, 1992). Also, LOH at D17S579 has been demonstrated in sporadic breast cancers (Futreal *et al.*, 1992). D18s34 is linked to the DCC gene (Weber and May, 1990), a suspected tumor suppressor gene. Previously, Thompson *et al.*, (1993) detected LOH at the DCC locus in 31% of patients with non familial breast cancer. The third microsatellite investigated lies within intron 6 of the MUC1 gene, a site of frequent alteration in sporadic breast cancer. (Gendler et al., 1990). Two other loci at 1q21, APO A2 and D1s104, are not known to be primarily involved in breast cancer. locus (Pratt *et al*, unpublished) at 1q21. The results indicated that one or more of the microsatellite loci was altered in the neoplastic, but not the non neoplastic cells of 30.0% of the patients. The variable number tandem repeat region (VNTR) within exon 2 of the MUC1 gene was also investigated. Finally, the hMSH2 gene, a mismatch repair gene, was analyzed for alteration in the paired DNA specimens.

Materials and Methods.

Human tissue samples.

DNA was extracted from the neoplastic and non neoplastic cells of 118 patients with the sporadic, non familial form of breast cancer. Fresh solid tumor tissues, taken during the usual course of the patient's treatment, were dissected free of fat and stored in liquid nitrogen. DNA from the tumor was prepared from the tissue pellets used for estrogen and progesterone receptor analysis. This method conserved a portion of the neoplasm for subsequent use and ensured that the DNA was isolated from non-necrotic portions of the specimen. Peripheral blood buffy coat cells obtained at the time of surgery were used as the source of DNA from non neoplastic cells from the same individuals. The histological type and grade of the tumor were determined in stained paraffin-embedded sections, prepared according to conventional techniques.

Preparation of high molecular weight DNAs from paired neoplastic and non neoplastic cells of the same individuals with breast cancer.

High molecular weight DNAs were obtained from the neoplastic and non neoplastic cells of the same breast cancer patients. Frozen tumor tissues (approximately 0.3 gms) were disaggregated in a Braun (Melsuugen, Germany) Mikro-disembrator II, and the DNA was isolated from the cells in an Applied Biosystems (Foster City, CA) 340A DNA extractor. Non neoplastic cells from the patient's peripheral blood were lysed with water before addition to the extractor. After dialysis, the DNA concentrations were measured spectrophotometrically. Before the various analyses were performed, aliquots of undigested DNA from the paired samples were subjected to electrophoresis through 0.7% agarose gels, to ensure that they were undegraded.

Analysis of microsatellite DNAs from paired neoplastic and non neoplastic specimens of the same patients.

Analyses of each of five microsatellite loci were performed by the PCR, using oligonucleotide primers that flanked the region of interest. The loci investigated and the primer sequences are presented in Table 1. A 50 ul reaction mixture contained approximately 300 ng of genomic DNA from neoplastic or non neoplastic cells, 10X reaction buffer (consisted of 500 mM KCl, 100 mM Tris-HCl, pH 9.0 and 1% Triton X-100), 2mM $MgCl_2$, 1.25 mM of each dNTP, 50 pMol of each primer, 3 uCi α-^{32}P-dCTP and 0.5 units Taq polymerase (Promega, Madison,WI). The samples were overlaid with oil, placed in a thermocycler (Perkin-Elmer, Norwalk, CT) and subjected to 27 cycles of 94^0, 45 sec; 55^0, 45 sec; 72^0, 45 sec. The PCR products were separated in 8% polyacrylamide denaturing gels and then exposed to XAR-5 film (Kodak, Rochester, NY) at -70^0 for 1 to 3 days before the films were developed and analyzed.

In some instances, the studies were performed by the "Booster" PCR method. In the initial reaction, a 50 µl reaction mixture consisting of 300 ng genomic DNA in reaction buffer (0.55 mM $MgCl_2$, 50 mM each dNTP, 2.5% formamide, 50 pMol each primer and 0.5 units Taq polymerase) were overlaid with oil and cycled 14 times at: 94^0, 1 min.; 45^0, 1 min.; 72^0, 1 min. For the second phase, 50 ul of a mixture containing the reaction buffer, 2.35 mM $MgCl_2$, 150 uM each dNTP, 2.5% formamide, 50 pMol each primer,10 µCi α-^{32}P dCTP and 0.5 units Taq polymerase were added and the reaction was continued for another 20 cycles.

The autoradiographs were examined to determine if deletions, partial deletions, or instability was evident at each microsatellite locus in tumor DNA. .Instability of microsatellite DNA was indicated by a gain or loss of base pairs in one or both alleles, or the presence of additional bands in DNA from the neoplastic but not non neoplastic cells of the same individual.

Analysis of the VNTR region of the MUC1 gene at 1q21 in the neoplastic and non neoplastic cells of breast cancer patients.

Southern blotting was used to detect possible alterations in the VNTR region of the MUC1 gene in the neoplastic cells of the breast cancer patients. Approximately 10 μg of paired tumor and normal DNAs were digested to completion with Hinf1 (Gibco BRL, Gathersburg, MD), according to the directions of the supplier. Afterward, the digested DNAs were fractionated in 0.7% agarose gels. Hind III digested lambda DNA (Gibco BRL, Gathersburg, MD) was used as a fragment size reference. After fractionation, the DNAs were denatured in alkali and transferred to nylon membranes (Biodyne, Pall, Glen Cove, NY), according to methods described previously (Gendler*et al.*, 1990). After transfer, the membranes were baked for 1 hr at 80^{0} under vacuum, and then hybridized with a probe homologous to the VNTR region at exon 2 of the MUC1 gene (Gendler *et al.*, 1987). The probe was labeled to high specific activity with [α-^{32}P]-dCTP by the random priming method (Feinberg and Vogelstein, 1984). To reduce non specific binding, the hybridization reactions were performed at 42^{0} in the presence of non specific DNA (herring testis, Promega, Madison, WI) in a buffer containing 5X SSC (1X SSC is 150 mM NaCl, 15 mM trisodium citrate, (pH 7.0) and 50% formamide. After incubation, the filters were washed extensively, first at 55^{0} with 2 X SSC/0.1% NaDoSO4, and then at 55^{0} with 0.1 X SSC/0.1% NaDoSo4. The washed filters were exposed to XAR-5 film (Kodak, Rochester, NY) at -70^{0} for 1 to 3 days before the films were developed and analyzed.

Analysis by SSCP of the hMSH2 locus in the paired neoplastic and non neoplastic specimens of the breast cancer patients.

The analysis of the hMSH2 locus in the paired specimens was performed according to the method described by Orita *et al* (1989), using oliogonucleotide primers specific for codons 668-736 of the hMSH2 locus (Leachet *al.*, 1993). The primers were:

PF CGC GAT TAA TCA TCA GTG,
PR GGA CAG AGA CAT ACA TTT CTA T (Fishel*et al.*, 1993).

The reaction mixture consisted of 200 ng genomic DNA, 5 µl of 10X reaction buffer, 1.5 mM $MgCl_2$, 50 pMol of each primer, 25 nMol of each nucleotide, 10 µCi [α-^{32}P]-dCTP and 2.5 Units Taq polymerase in a total volume of 50 ul. The reaction mixtures were cycled 27 times, at 94^0, 45 sec; 57^0, 45 sec; and 72^0, 45 sec for each cycle. Afterward, 6 ul of the mixture was heated to 95^0 for 3 minutes before it was placed in a neutral 7% polyacryamide gel containing 10% glycerol. The gels were subjected to electrophoresis at room temperature for 6 hrs at 50 watts. Afterward, they were dried and then exposed to XAR-5 film (Kodak, Rochester, NY) at -70^0 for 1 to 3 days. Sequencing of the PCR products was performed using a double strand DNA Cycle Sequencing System (Gibco/BRL, Gaithersburg, MD), according to the directions of the supplier.

Results

Alteration of microsatellite DNAs in breast cancer cells.

Paired DNA specimens from the neoplastic and non neoplastic cells of 118 patients with breast cancer were analyzed for alterations at each of five microsatellite loci. The loci and their chromosomal localizations were D17s579, at 17q21, D18s34 at 18q21, D1s104 at 1q21, APO-A2 at 1q21 and a microsatellite within the MUC1 gene at 1q21. Primer sequences are given in Table 1. Each of the patients was heterozygotic at one or more of the loci, and was therefore suitable for analysis.

A deletion, partial deletion or a change in size of one allele in the neoplastic, but not non neoplastic cells of the same patient, or the presence of additional allelic bands in the neoplastic cells was taken as an indication of an alteration. Representative examples are shown in Figure 1. At least one of the five loci examined was altered in 30.0% of informative cases. Six percent of the patients revealed an alteration at more than one locus. The incidence of an alteration at D17s579 and the microsatellite within the MUC1 gene was significantly ($p < .001$) higher than that of the other loci (Table 2).

Microsatellite instability was found in at least one microsatellite locus in 17.5% of patients. Instability at more than one locus was present in the tumor DNA of one patient. Complete or partial deletions of one allele was found in the neoplastic cells of 14.3% of cases at D17S579 and 12.1% of cases at the microsatellite within the MUC1 locus.

The use of the primers outside the microsatellite within the MUC1 locus resulted in the presence of secondary bands. Alteration of the secondary bands was found in the neoplastic but not non neoplastic cells of seven patients.

Alterations of the VNTR region of the MUC1 locus in the neoplastic but not non neoplastic cells of breast cancer patients.

The VNTR region within the coding region of the MUC1 gene (Figure 2), was analyzed by Southern blotting to determine if an alteration in this region was accompanied by changes at the microsatellite within the locus. Conceivably, the same molecular defect responsible for alterations in the microsatellites could affect the coding region of the MUC1 gene as well.

Paired DNA samples from the neoplastic and non neoplastic cells of 70 breast cancer patients were investigated. Fifty seven patients (81.4%) were heterozygous and were therefore informative. There was LOH in three cases (5.1%). In 32 patients, (56.1%) one of the two alleles was amplified in the neoplastic, but not the non neoplastic cells, as indicated by a hybridization signal of relatively greater intensity (Figure 1). The larger of the two alleles was amplified in 23 (40.3%) of the informative cases. In one specimen, a recombination event that included the MUC1 gene had taken place in the malignant, but not in the non malignant cells of the same individual (Figure 1).

DNAs from the neoplastic cells of patients with an alteration at the VNTR region were investigated for alterations at each of the five microsatellite loci, including the microsatellite within intron 6 of the MUC1 gene. The results failed to indicate a significant association between changes at the VNTR region within the coding region and alteration in microsatellites either within or without the gene ($p \geq .067$).

Alterations of the hMSH2 locus in the neoplastic and non neoplastic cells of breast cancer patients.

The hMSH2 locus is a site of alteration in patients with HNPCC (Fishel *et al.*,1993). To determine if an analogous alteration was present in the hMSH2 locus in the neoplastic or non neoplastic cells of breast cancer

patients, the paired DNA-extracts were investigated by SSCP, using primers for codons 668-736 of the hMSH2 locus (Leach *et al.*, 1993). This portion of the gene is conserved across species and is believed to code for the DNA binding region of a mismatch binding protein. The results indicated that an alteration of the hMSH2 locus was present in 16 of 97 cases examined (16.4 %). In each instance, however, an alteration in the neoplastic cells was accompanied by the same alteration in the non neoplastic cells of the same individual. It is likely that the alterations represented a polymorphism or a germ line mutation. A typical result is indicated in Figure 3.

The region (codon numbers 668 to 736 and flanking sequences; corresponding to bps 2072 to 2208) was sequenced in patients revealing an alteration by SSCP. As indicated (Figure 4), a T to C substitution at the -6 position of the splice acceptor site was found. It was present in both the neoplastic and non neoplastic cells of the same individuals. Eight paired specimens which failed to indicate an alteration at the hMSH2 locus by SSCP were sequenced as well. No alterations were detected in these cases.

We made an attempt to associate changes at the hMSH2 locus with alterations in the microsatellite DNAs in neoplastic cells from the same individuals. There was no significant association between a change in the hMSH2 gene and a change in any of the microsatellite loci investigated. The one patient whose tumor demonstrated instability of microsatellite DNA at two of the five loci investigated did not have an alteration at the hMSH2 locus.

Discussion.

Microsatellite DNA was first described in humans and has been found in almost all other eukaryotes investigated. In humans, 76 percent of microsatellites are repeats of A, CA, AAAN, AAN or AG, in decreasing order of frequency. The repeated bps are found in 5' and 3' untranslated regions, and introns (Tautz and Renz, 1984). The repetitive regions are

normally stable and the number of base pairs in the repeat is highly polymorphic. The segregation of alleles of heterozygous parents is considered to be unambiguous. These highly polymorphic microsatellite loci have proved useful in the development of genetic maps and in the detection of chromosomal aberrations in disease.

Here, we described the results of a comparative analysis of five microsatellite loci along with the VNTR region of the MUC1 gene in malignant and non malignant cells of 118 breast cancer patients. The investigation was an extension of prior studies in which we found that the MUC1 gene was frequently altered in patients with primary breast carcinomas (Gendler *et al.*, 1990).

As reported by others, we too found LOH in the region of the BRCA1 locus. In this instance, the neoplastic cells of 14.3% of patients with sporadic breast cancer demonstrated LOH. Thus, an alteration at this locus is not limited to patients with familial neoplasia.

Analysis of the microsatellite within the MUC1 gene revealed alterations in the secondary bands in 7 of 99 informative cases. Weber and May (1990) stated that the formation of secondary bands is a consequence of PCR, and not secondary to genetic mosaicism. We included changes in secondary bands in our overall analysis of alterations at the CA repeat within the MUC1 gene.

We were unable to detect an association between alterations in the VNTR portion of the MUC1 gene with mutations in the microsatellite within the locus. Sixty one percent of patients revealed a deletion or amplification of the MUC1 gene, and 23.3% of patients had an alteration of the microsatellite within the gene. However, in those instances in which one portion of the gene was altered, the other portion had a no greater than random likelihood of exhibiting a change. Most changes within the VNTR region of the MUC1 gene were amplifications. Given the proximity of the CA repeat at intron 6 of the gene, one would also predict a similar change there. Amplification of a microsatellite by the PCR would be difficult to detect given

that the PCR is not generally considered to be a quantitative technique. Also, amplifications of the MUC1 gene may be limited to the VNTR region. Deletions of the CA repeat at intron 6 of MUC1 in a tumor were not accompanied by deletions at the VNTR region. Perhaps what appeared to be amplifications of the VNTR region in one allele of a tumor, could have actually represented a deletion of the opposite allele with background contamination by non neoplastic DNA.

Investigators have described specific genetic changes in HNPCC and other cancers in the HNPCC syndrome.(Leach et al., 1993; Fishel et al., 1993; Peltomaki et al.,1993; Papadopoulos et al.1994; Bronner et al. 1994) The RER+ phenotype has not only been found in HNPCC colon cancers, but also in sporadic tumors which are part of the HNPCC syndrome. (Peltomaki et al, 1993).

Whether or not breast cancer is part of the HNPCC syndrome is a uncertain. Lynch and others (Lynch et al 1991) report no excess of breast cancer cases in HNPCC kindreds as compared to the normal population, while Itoh and others (Itoh et al.,1990) report a five-fold incidence of breast cancer in HNPCC families.

Peltomaki and others (Peltomaki et al., 1993) found no sporadic breast cancers which demonstrated the RER+ phenotype. They did not find a single incident of microsatellite instability among 7 microsatellite loci examined in 84 breast cancers.

In our investigation of sporadic breast cancer, microsatellite instability was occasionally detected; however most of the alterations detected were losses of heterozygocity Only one of our tumors would fit the RER+ phenotype as defined by Aaltonen and others. (Aaltonen et al., 1993) These investigators defined the RER+ phenotype as microsatellite instability in at least 2 of 7 microsatellites.

The microsatellite instability we detected appears different than that demonstrated in tumors of the HNPCC syndrome.(Aaltonen et al., 1993; Gonzalez-Zuluetta et al., 1993; Petolmaki et al., 1993; Risinger et al., 1993) In those tumors, one sees expansions from the two original alleles of a microsatellite to multiple alleles which represent inadequately repaired DNA replication errors resulting from multiplications of the original error-prone clone. The microsatellite instability we detected only on rare occasions showed expansions. Usually what we saw were changes in size of one of the alleles, or just on or two additional bands.(Figure 1) Wooster and others (Wooster et al., 1994) also found similar sorts of changes when they examined microsatellites in breast cancer.

Fishel *et al.* (1993) and Leach *et al.* (1993) found that abpproximately 60% of patients with colon cancer with the RER+ phenotype had mutations of the hMSH2 gene. In an analogous manner, we attempted to detect changes at the hMSH2 locus in breast cancer patients. No significant association between an alteration at the hMSH2 locus and alterations of the microsatellite DNAs were detected. The mutation we detected, a T to C substitution at the -6 position of the splice acceptor site, was present in 16 of 97 cases. Since it was present in both the neoplastic as well as non neoplastic cells of the same individual, it was likely a polymorphism unrelated to the disease. Leach *et al.* (1993) found the same mutation in 2 of 20 tumor-free individuals. The one patient in our series whose tumor fit Aaltonen's definition of the RER+ phenotype did not have an alteration of the portion of the hMSH2 gene we studied.

It is likely that the rare microsatellite instability we detected results from a different mechanism than seems to be responsible for the widespread instability seen in cancers of HNPCC and the HNPCC syndrome. Our results confirm the high frequency of alteration in the region of the BRCA1 locus and in the region of the MUC1 gene in sporadic breast cancer.

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TABLE 1

SEQUENCE OF PRIMERS USED FOR ANALYSIS OF MICROSATELLITES

MICROSATELLITE	PRIMER SEQUENCE	REFERENCE
D17S579	5'-AGT CCT GTA GAC AAA ACC TG 5'-CAG TTT CAT ACC AAG TTC CTA	Hall et al.,1992
D18S34	5'-CAG AAA ATT CTC TCT GGC TA 5'-CTC ATG TTC CTG GCA AGA AT	Weber and May,1990
APO-A2	5'-GGT CTG GAA GTA CTG AGA AA 5'-GAT TCA CTG CYG TGG ACC CA	Weber and May,1989
D1S104	5'-ATC CTG CCC TTA TGG AGT GC 5'-CCC ACT CCT CTG TCA TTG TA	Weber et al.,1990
MS within MUC1	5'-AGG AGA GAG TTT AGT TTT CTT GCT CC 5'-TTC TTG GCT CTA ATC AGC CC	Pratt, unpublished

TABLE 2

FREQUENCY OF ALTERATION IN BREAST CANCER CELLS OF MICROSATELLITE DNAs AT MUC1 AND FOUR OTHER LOCI

	MUC1	D17S579	D18S34	D1S104	APOA2
# of cases	104	101	94	74	70
# of informative cases	100 (96.1%)	90 (89.1%)	81 (87.2%)	65 (87.8%)	70 (100%)
Total # of changes	24 (23.3%)	17 (18.7%)	5 (6.2%)	3 (4.7%)	2 (2.8%)
Deletion	5	9	3	0	0
Partial Deletion	7	4	1	0	0
Change in size	4	4	1	2	1
2o band alteration	7	0	0	0	0
Additional bands	1	0	0	1	1

LEGENDS

Figure 1: Representative examples of alterations of microsatellite DNAs and the MUC1 locus
B represents blood (non neoplastic) DNA, and T represents tumor DNA.

Figure 2: MUC1 gene
The variable number tandem repeat region (VNTR) is located within exon 2 of the gene. The CA repeat is located within intron 6 of the gene.

Figure 3: Detection of hMSH2 alteration by SSCP
Lanes 1 and 2 are the blood and tumor DNA (respectively) of a patient with unmodified hMSH2, as indicated by analysis by SSCP. Lanes 3 and 4 represent blood and tumor DNA (respectively) of a patient with an alteration of hHMSH2.

Figure 4: Sequencing of a portion of the hMSH2 gene
Arrow denotes change of T (left gel) to C (right gel) at the -6 position of splice acceptor site in hMSH2.