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Prostate Cancer (PCa) accounts for nearly 30% of all newly diagnosed cancers among American men. Epidemiologic studies suggest that dietary factors may be important in the etiology of this disease. The objective of our research is to determine how nutritional compounds genistein, betasitosterol (SIT), and omega-6 fatty acids (FA) function as modulators of PCa. In the third and final year of this fellowship, the fellow joined the National Cancer Institute's Cancer Prevention Fellowship Program and has continued working on the DOD-PCRP Postdoctoral Fellowship project with 30% effort (with knowledge and approval from the DOD-PCRP). During this year, we finished printing our custom microarray as well as optimized the hybridization protocol. We are currently performing expression analysis using the microarrays for both the *in vitro* and *in vivo* experiments using well established cell lines. Finally, we have initiated xenograft *in vivo* experiments with a novel PCa model that was developed recently in a collaborating laboratory.

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Introduction

Prostate Cancer (PCa) accounts for nearly 30% of all newly diagnosed cancers among American men. Epidemiologic studies suggest that dietary factors may be important in the etiology of this disease. The objective of our research is to determine how nutritional compounds genistein, betasitosterol (SIT), and omega-6 fatty acids (FA) function as modulators of PCa. These three compounds belong to three distinct classes of dietary components, specifically isoflavonoid (genistein), phytosterol (SIT), and FA (omega-6 FA). Each class of compound could potentially modulate PCa in different ways and previous investigations have indicated that all three compounds may affect PCa in a dose-dependent fashion. We will determine differences in gene expression profiles at a range of doses for each compound on *in vitro* models. We will then test up to two doses on *in vivo* models to account for the complexity of tumor microenvironments.

In the first year, we began to develop the technical tools with which to investigate gene expression patterns that are modulated by the 3 dietary compounds. In the second year of study, we finished preparation of 355 cDNAs for fabricating the custom microarray for analysis of gene expression. In addition, we completed *in vitro* and *in vivo* experiments using well established PCa cell lines and the three dietary compounds in varying concentrations. In the third year, the fellow joined the National Cancer Institute's Cancer Prevention Fellowship Program and has continued working on the DOD-PCRFP Postdoctoral Fellowship project with 30% effort (with knowledge and approval from the DOD-PCRFP). During this past year, we finished printing our custom microarray as well as optimized the hybridization protocol. We are currently performing expression analysis using the microarrays for both the *in vitro* and *in vivo* experiments using well established cell lines. Finally, we have initiated xenograft *in vivo* experiments with a novel PCa model that was developed recently in a collaborating laboratory.

Here, we report our progress as it relates to the approved Statement of Work:

Task 1: To delineate the gene expression patterns of PCa *in vitro* of three nutritional compounds.

1.1 Obtain cDNAs for microarrays

From year 2, 344 cDNA clones from either in-house resources or ATCC have been prepared for our custom microarray. Genes chosen were tyrosine kinases or involved in cellular pathways with emphasis on androgen receptor (AR) and apoptosis pathways. Also included in our gene list are 3 control genes (GAPD, ACTB, and MBP) and other controls (Human Cot-1, Mouse Cot-1, Salmon Sperm DNA, and male human genomic DNA) for a total of 352 features.

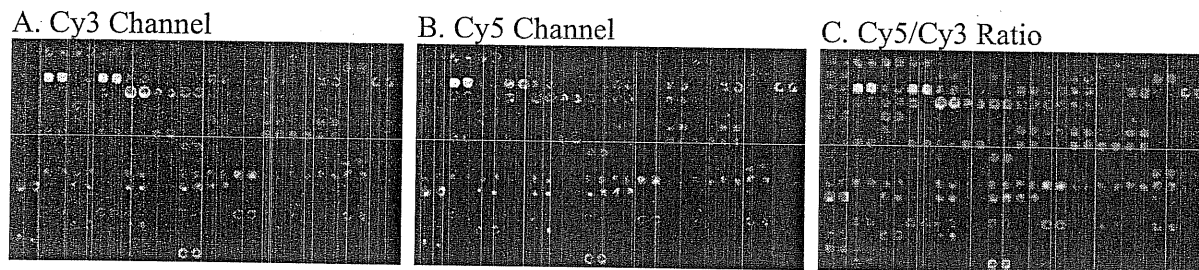
1.2 Fabricate arrays and optimize protocols

We have successfully fabricated our custom microarray with the above mentioned 352 features (in duplicate) (Table 1). For hybridization, we evaluated incorporating Cyanine 3- (Cy3) and Cyanine 5-dUTPs (Cy5) (Amersham) in the control and test samples by 2 methods: direct incorporation during reverse transcription polymerase chain reaction (RT-PCR) and during random priming (RP) after RT-PCR. Hybridization using labeled cDNA resulted in weak signals in the Cy3 probes and little or no signals in the Cy5 probes. Hybridization using RP probes resulted in much stronger signals in both Cy3 and Cy5 channels. A representative example of an array hybridization is shown in Figure 1. For subsequent array experiments, RP probe labeling will be utilized after RT-PCR of total mRNA from each sample.

Table 1: Map of the custom microarray printing

[illegible]

Figure 1: Representative hybridization patterns of RP labeled cDNAs in Cy3 channel (A), Cy5 channel (B) and Cy5/Cy3 ratio (C).



1.3 Grow cells under test conditions, isolate RNA, RT-PCR, label probes

We completed the cell culture experiments of this task in year 2. However, we were unable to replicate experiments using the test compound SIT due to difficulties in consistently dissolving SIT into solution with its appropriate vehicle (Cyclodextrin; Sigma). Because we successfully tested this compound in our *in vivo* xenograft model pilot study (Task 2), we have decided to delay the *in vitro* experiments using SIT until we are able to have appropriate resources to reliably perform the experiments.

1.4 Perform microarray experiments, analyze data with standard statistical programs and Perform multicolor spectral transcript analysis and analyze data with standard statistical programs

Due to the time needed to optimize the hybridization protocol, we are currently in the process of performing array experiments and analyzing the expression patterns from RNA isolated from the *in vitro* experiments. See Figure 2 under Task 2 for examples of array hybridizations. The multicolor spectral transcript analysis will need to be delayed until all array experiments have been completed.

Task 2: To determine if and how three select dietary components modulate the growth and gene expression of clinical PCa specimens *in vivo*.

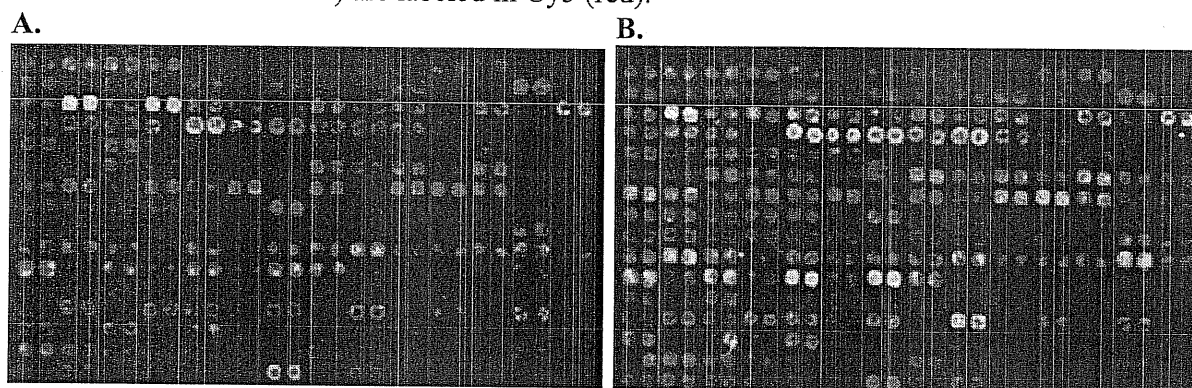
2.1 Pilot study to investigate timing of nutrient supplementation in SCID mice

In parallel with the *in vitro* experiments mentioned in Task 1, we performed *in vivo* experiments using xenograft technique on SCID mice at the co-mentor's laboratory using the same cell lines mentioned above in year 2.

2.2 Analyze tumors from pilot study

Similar to task 1.4, we are currently performing array experiments and analyzing the expression patterns from RNAs isolated from the *in vivo* experiments. Examples of hybridizations using RNAs from this pilot study are shown in Figure 2. Likewise, the multicolor spectral transcript analysis will need to be delayed until all array experiments have been completed.

Figure 2: Representative hybridization patterns of CAFTD04 derived tumors grown in SCID mice fed with control diet (A) and Genistein supplemented diet (B). All test probes are labeled in Cy3 (green) and reference probes (generated from CAFTD04 derived tumors grown in SCID mice fed with control diet) are labeled in Cy5 (red).



- 2.3 Grow 6 tumors in 21 SCID mice (2 tumors per mice) that ingest one of 7 test diets, harvest tissues, prepare tissue sections and RNAs, RT-PCR, and make probes. Test diets include control and each compound at one of 2 concentration

For this task, we found it difficult to obtain clinical samples that were large enough to be implanted into the number of mice that was initially proposed. To circumvent this problem, we decided to use a newly generated PCa model that was developed by a collaborator (Wang et al., submitted) as mentioned in the previous report. Briefly, pieces of PCa tissue from a patient were grafted into the subrenal capsule site of testosterone-supplemented male SCID mice. After 5 serial transplantations, the tissues were transferred into mouse prostates. A metastatic tumor line generated from lymph nodes, designated PCa1-met, had few chromosomal alterations, as indicated by Spectral Karyotyping. Orthotopic grafting of PCa1-met in 47 hosts led in all cases to metastases to multiple organs (lymph nodes, lung, liver, kidney, spleen and, notably, bone). Histopathological analysis showed strong similarity between orthotopic grafts and their metastases which were of human origin as indicated by immunostaining using antibodies against human mitochondria, androgen receptor, prostate-specific antigen, pan-cytokeratin, p63, cytokeratin-8 and Ki-67. This model is unique due to the fact that the xenograft implants metastasized to regions of the mouse that paralleled the locations of clinical PCa metastases (most notably lung, liver and bone) in humans. In addition, the resulting cell lines contain few cytogenetic aberrations, similar to clinical PCa and unlike most well established cell lines (i.e., PC3, LNCaP, and DU145).

When we implemented the experiments using this new model, however, the SCID mice were adversely affected in unanticipated ways. At the same time, our collaborators were having similar problems. All animal experiments were stopped so that the issue can be resolved without harm to any more animals. Recent experiments as communicated by our collaborators showed that the murine stroma, which was contained within the tumors that are regrafted, harbored a mouse virus that infected the host SCID mice. The contaminated tumor lines have since been discarded and several un-infected tumor grafts have been established. We are currently in the process of using the new and un-infected tumor lines for the purpose of this study.

2.4 *Perform microarray experiments, analyze data with standard statistical programs*

Due to the problem described above, we are currently in the process of performing the *in vivo* experiments using the newly established PCa model. Once these experiments are complete, we will analyze the expression profiles using our custom arrays.

Key Research Accomplishments for Year 2

- Fabricated a custom microarray with 352 features in duplicate.
- Optimized microarray hybridization conditions.
- Began microarray expression profiling of RNAs from *in vitro* experiments with established PCa cell lines grown in control and test media containing different concentrations of test nutritional supplements.
- Began microarray expression profiling of RNAs from *in vivo* experiments in the co-mentor's laboratory with xenografts of well established PCa cell lines in SCID mice whose diets are supplemented with various test compounds.
- Initiated *in vivo* experiments in co-mentor's laboratory with xenografts of the newly developed and un-infected PCa tumor lines in SCID mice whose diets are supplemented with various test compounds.

Reportable Outcomes

- Became a National Cancer Institute Cancer Prevention Fellow (2005-2008)
 - Beginning in July 2005, the fellow began a 1 year academic program to obtain a Masters in Public Health with an emphasis on Epidemiology at the University of California, Berkeley to supplement the fellow's training in the field of cancer prevention. Degree is expected to be conferred in May 2006.
 - Beginning in June 2006, the fellow will begin a 2 year research program at the National Cancer Institute in Bethesda, MD, which will focus on cancer prevention.

Conclusion

We have used the third year of this fellowship to fabricate our custom microarray with 352 features (in duplicate) for expression profiling of cells grown *in vitro* and *in vivo* under different dietary supplement conditions including none, genistein, SIT, and omega-6 FA. In addition, this year has been used to troubleshoot many unexpected issues such as microarray probe generation as well as viral infection of the new PCa tumor line models. Both major issues have been resolved. Microarray analysis for task 1 and the pilot study for task 2 are underway. Additionally, the *in vivo* xenograft model using the new PCa tumor lines have been initiated and expression profiling of the resulting tumors will be performed once tumors are harvested from co-mentor's laboratory.

Publications

None: pending final data analyses.