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[Date: 11/02]
Identification of Estrogen Receptors and Their Role in Breast Cancer

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Breast cancer is the most common form of cancers among women. The experiments described are testing the feasibility of an invitro assay, based on dynamic alteration in gene expression of specific genes, to predict the response to drug treatment. From the previously identified set of genes, two genes PDZK1 and GREB1 were confirmed to be estrogen-responsive. We now test whether we can more accurately determine the response of tumor to the treatment with tamoxifen, an antiestrogenic drug, by analyzing the changes in expression of GREB1 and PDZK1 upon treatment with tamoxifen.
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As of August 2001, I took over Research duties from Dr. Malavika Ghosh, to whom the award number DAMD17-99-1-9834 was granted. For a brief period of time there was no research conducted due to the vacancy in the position. My current and proposed research plans are summarized below:

**Background**

The development of breast cancer has been linked to a variety of factors, including age, parity, family history and hormonal milieu. Estrogen, the female sex hormone, is a powerful mitogen and promotes neoplastic growth in mammary epithelium. *In vitro*, estrogens have been shown to modulate human breast cell via \( \alpha \) and \( \beta \) estrogen receptors (ER) [1]. While ER\( \beta \) is preferentially expressed in normal breast tissue, ER\( \alpha \) is abundantly expressed in invasive and in situ ductal carcinomas [1]. Tamoxifen, an anti-estrogenic drug is widely used for adjuvant therapy of breast cancer. ER\( \alpha \) expression is currently the best method to predict if a cancer will respond to hormonal therapy. However, 35% of the primary tumors, which are ER\( \alpha \)-positive do not respond to hormone therapy and about 10% of ER\( \alpha \)-negative tumors are hormonally responsive [2]. It would immensely beneficial to predict with a greater degree of accuracy how a breast tumor would respond to the treatment with tamoxifen, especially in the light of growing evidence of carcinogenic effects of tamoxifen. [3, 4].

Ghosh et al. identified a set of genes of estradiol-responsive genes in MCF7 cells; these genes were also repressed upon treatment with tamoxifen [5]. Two of these genes – GREB1 and PDZK1 – were confirmed to be estrogen-responsive and are expressed in association with ER\( \alpha \) in breast cancer. In order to predict a patient’s response to the drug treatment more accurately, it might be more important to examine the dynamic alteration of gene expression in response to the drug treatment as opposed to merely looking for their expression a ‘static’ time-point.

**Objectives, Method and Current Status**

1. **Optimize the conditions to examine dynamic changes in gene expression using cancer cell lines.**

   **Method:** The purpose of the method is determine the minimum number of cells required, duration of drug treatment, and best method to analyze gene expression changes in breast cancer cells. Based on an initial protocol developed by M. Ghosh, the MCF7 cells were incubated in a culture medium containing stripped fetal calf sera and lacking phenol red, so as to remove all estrogens. Cells at different concentrations (5000, 10000 and 50000
cells) are treated with β-estradiol (10 nM) or β-estradiol and tamoxifen (1 µM). The cells are treated for various time-lengths viz. 1, 2, 4, 8 and 24 hours. A control group of cells, which gets no drug treatment, is also analyzed for each cell density and time point. Total RNA is isolated at the end of the treatments and analyzed by RT-PCR for expression of GREB1, PDZK1, and pS2 genes. Expression levels of actin are also checked by RT-PCR to control for the total amount of RNA.

Current status: The assay conditions have been successfully developed using MCF7 cells, wherein 5000 cells, which is approximately the number of tumor cells expected from a small core biopsy, are sufficient to isolate reasonable amount of RNA that can be analyzed by RT-PCR. Treatment of these cells for 4 hours shows significant changes in the expression levels of target genes upon treatment with β-estradiol and tamoxifen. Thus, it will not be necessary for the tumor cells to remain viable in the cultures for long periods of time. The number of cycles, and extension and annealing temperatures for RT-PCR of each target gene has also been optimized so as to give a signal in the linear range.

2. Develop a protocol for testing breast tumor tissue for altered gene expression upon drug treatment

Method: The aim of this method is to determine conditions for culture and treatment of primary breast tumor tissue, since primary breast tumors are likely to behave differently than the cultured tumor cell lines. Fresh tumor specimens are collected from the primary breast cancers that are resected in the Stanford operating room. Tumor tissue is cut into small pieces smaller than 1mm in size. This should allow for the diffusion of oxygen into the specimen. Alternatively, the minced tissue is treated with collagenase to disaggregate the tissue and isolate tumor cells. Minced tissue or disaggregated tumor cells are distributed into culture media and treated with estradiol or estradiol and tamoxifen for 4 hours as described in method 1 above. Whenever possible, multiple replicas are performed to determine intra-sample reproducibility. After the drug treatments, total RNA is isolated and expression of GREB1, PDZK1, pS2, and ERα is analyzed by RT-PCR as above. The expression levels of target genes are compared for samples with estradiol, tamoxifen, or no treatment.

Current Status: Five primary breast tumor specimens have been analyzed so far. The first two were not treated with collagenase and showed poor intra-sample reproducibility. Treatment with 0.1% collagenase (type III) enzyme for 3-4 hours disaggregates the tissue and the tumor cells thus obtained provide a more homogenous sample and hence better intra-sample reproducibility. The sample set (five tumor specimens) has been too small to draw any significant conclusions. However, the preliminary results obtained with these
primary breast tumors indicate feasibility of the protocol to detect qualitative and semi-quantitative differences in target gene expression upon drug treatment. One of the breast tumor specimen, showed no response to tamoxifen treatment in vitro, even though it was ER-positive. This tumor likely falls into that category of ER-positive tumors that do not respond to hormonal therapy. While another tumor, which was ER-negative, did exhibit a decrease in the target gene expression in response to tamoxifen treatment. This tumor may possibly respond to tamoxifen treatment in a clinical setting.

**Future Work**

With the promising results obtained in the first few breast tumor specimens, more tumor specimens will be tested in order to draw statistically significant conclusions. In the experiments planned under this study, we expect to analyze approximately 100 tumor specimens during the time period of this grant. Since the present RT-PCR method only gives a semi-quantitative data, we will use the quantitative real time RT-PCR approach, which utilizes fluorophore-labeled oligonucleotide probes. This method is likely to be easier and more reliable in quantitating the dynamic alterations in target gene expression with drug treatment.

Clinical data will be available on the patients from whom the tumor samples were obtained. The gene expression data will be compared with the clinical parameters including age of the patient, medication use, histology of the cancer, ERα and PR expression, tumor grade and tumor stage. Patients with ERα-positive tumors are often started on tamoxifen treatment. Outcome data will be included in our analysis as and when it becomes available. Patients treated with tamoxifen will be followed for tumor recurrence by examining their medical records. There may be a trend demonstrating that tumors in which estradiol-responsive genes showed tamoxifen-induced in their expression will be more likely to respond to tamoxifen treatment in the clinical setting. Similarly, those tumors, which do not exhibit any tamoxifen-induced alterations in the expression of target genes in vitro, may be less likely to respond to tamoxifen treatment in the clinical setting. The results of this study should provide the necessary data required for the design of a clinical trial to predict the response of patients for a particular drug treatment.
References


4. Phillips DH. *Understanding the genotoxicity of tamoxifen.* 2001 (22) 839-849

5. Ghosh MG, Thompson DA, Weigel RJ. *PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer.* Cancer Res. 2000 (60) 6367-6375.
MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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Deputy Chief of Staff for Information Management