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TITLE: Epigenetic Control of Tamoxifen-Resistant Breast Cancer

PRINCIPAL INVESTIGATOR: Kristin Paczkowski Williams

CONTRACTING ORGANIZATION: University of Massachusetts
Amherst, MA 01003-9333

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THE OBJECTIVE IS TO UNDERSTAND THE ROLE THAT EPIGENETICS, SPECIFICALLY METHYLATION, PLAYS IN ANTIESTROGEN RESISTANT BREAST CANCER. THE GOAL OF THIS STUDY IS TO IDENTIFY GENES DIFFERENTIALLY METHYLATED BETWEEN ACQUIRED TAMOXIFEN RESISTANT CELLS (TMX2-28 AND TMX2-11) AND THEIR PARENT STRAIN (MCF-7) THROUGH THE USE OF THE ILLUMINA HUMANMETHYLATION450 BEADCHIP. A PANEL OF GENES SHOWING CHANGES IN METHYLATION BETWEEN THE TAMOXIFEN RESISTANT CELL LINES, TMX2-11 AND TMX2-28 AND THE PARENTAL LINE, MCF-7 WERE SELECTED FOR FURTHER ANALYSIS. ONE GENE, ZNF350, WHICH WAS HYPERMETHYLATED IN BOTH TMX2-11 AND TMX2-28 BUT NOT IN MCF-7, SHOWED SIGNIFICANT INCREASE IN EXPRESSION WHEN TREATED WITH A DEMETHYLASE. ADDITIONALLY, PRELIMINARY TREATMENT ASSAYS SHOW A DECREASE IN THE PROLIFERATION RATE OF TMX2-28 WHEN TREATED WITH A DEMETHYLASE.
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**Introduction:**

The objective is to understand the role that epigenetics, specifically methylation, plays in antiestrogen resistant breast cancer. I hypothesize that both hyper- and hypomethylation of DNA plays a major role in the etiology of antihormone resistant breast cancer. The goal of this study is to identify genes differentially methylated between acquired tamoxifen resistant cells (ER-negative TMX2-28, ER-positive TMX2-11, and TMX2-4) and their parent strain (MCF-7) through the use of the Illumina HumanMethylation450 BeadChip. I also aim to determine whether treatment using methylases or demethylases reverses the methylation profiles in cells, potentially indicating its therapeutic value in tamoxifen resistant breast cancers. Furthermore, I will use breast cancer tissue specimens to determine whether genes found differentially methylated in breast cancer cell lines and believed to be involved in antiestrogen resistance occur *in vivo*. Results from this study are expected to show that the epigenetic profiles of tamoxifen resistant and sensitive cells differ and that this molecular mechanism will make a good therapeutic target for women with tamoxifen resistant breast cancer.

**Body:**

Task 2: I have analyzed the results from the HumanMethylation 450K BeadChip and found that a considerable number of CpG sites are differentially methylated between the tamoxifen resistant clones, TMX2-11 and TMX2-28 and the parent MCF-7 cell lines (Figure 1 and Table 1). I am currently designing real-time RT-PCR primers to determine whether expression levels are changed in genes of interest and pyrosequencing assays to confirm methylation results. One gene, ZNF350 is trending towards a decrease in expression between MCF-7 and the two tamoxifen resistant cell lines (Figure 2).
Table 1. CpG site methylation changes in tamoxifen-resistant cell lines as compared to the parental line.

<table>
<thead>
<tr>
<th></th>
<th>TMX2-11/MCF-7</th>
<th>TMX2-28/MCF-7</th>
<th>MCF-7</th>
<th>TMX2-11 and TMX2-28/MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased Methylation*</td>
<td>4,039</td>
<td>33,752</td>
<td>128</td>
<td>3,130</td>
</tr>
<tr>
<td>Decreased Methylation**</td>
<td>2,593</td>
<td>5,252</td>
<td>1,698</td>
<td>203</td>
</tr>
<tr>
<td>No Change in Methylation</td>
<td>472,153</td>
<td>436,113</td>
<td>479,003</td>
<td>431,909</td>
</tr>
</tbody>
</table>

*Increased methylation: >2-fold change, >0.3 beta-value of TMX2-11, TMX2-28, or E2 treated MCF-7; **Decreased methylation: <-2-fold change, >0.3 beta-value in MCF-7; No change in methylation: <2-fold change in all lines. Detection p-value of ≤ 0.01 was used to distinguish statistically significant methylation changes.
Task 3: I have successfully treated MCF-7, TMX2-11, and TMX2-28 cells with the demethylase, 5-aza-deoxycytidine and have purified RNA, DNA, and protein. I analyzed the expression of one gene, ZNF350 and found that after treatment with 5-aza-deoxycytidine there is a significant increase in the expression in both TMX2-11 and TMX2-28, but not in MCF-7 (Figure 2). Preliminary treatment assays show that TMX2-28 has a significant decrease in cell growth when treated with 5-aza-deoxycytidine. No change growth rates were seen in TMX2-11 or MCF-7 (Figure 3).
Task 4: IRB approval was submitted to Baystate Medical Center for the human breast carcinoma tissue work and I have begun communications with Dr. Otis and a pathology resident, Rahul Jawale, at Baystate Medical Center to obtain the human breast tissue samples needed for the study.

Task 6: Preparing first manuscript based on the HumanMethylation 450K data. It is to be submitted to BMC Cancer.

**Key Research Accomplishments:**

**Training Accomplishments:**

- Continued collaboration with Dr. Sallie Smith-Schneider, Pioneer Valley Life Sciences Institute; Dr. Douglas Anderton, University of South Carolina; Dr. Brian Pentecost, New York State Department of Health; and Dr. Christopher Otis, director of Surgical Pathology Baystate Medical Center. I also began collaborating with Dr. Maxwell Lee, National Institutes of Health on the HumanMethylation 450 BeadChip data
- Current and active member of AACR
- Weekly meetings with my mentor Dr. Kathleen Arcaro about my research and progress
- Weekly participation in Cancer & Chemoprevention journal club, Molecular and Cellular Biology Colloquium and seminar, and Vet and Animal Science seminar

Research Accomplishments:

- Analyzed methylation data from HumanMethylation 450K BeadChip and determined which genes were differentially methylated between tamoxifen resistant and tamoxifen sensitive cell lines
- Treated cell lines with demethylase, purified RNA, DNA and protein for future analyses
- Started designing real-time RT-PCR and pyrosequencing assays for genes of interest
- Began growth assay treatment studies on cell lines using demethylase and tamoxifen alone and in combination
- Submitted IRB approval from Baystate Medical Center to collect formalin-fixed paraffin embedded human breast cancer tissue samples
- Discussed which tissue samples are needed with Dr. Otis and the pathology resident who will be assisting me with the scoring of slides
- First manuscript based on the HumanMethylation 450K BeadChip work is in preparation

Reportable Outcomes:

I have analyzed the methylation data from the HumanMethylation 450 BeadChip and obtained a list of genes that are differentially methylated between the tamoxifen resistant and sensitive cell lines and serve as potential genes of interest. The gene ZNF350 was found to have a significant increase in expression in TMX2-11 and TMX2-28, but not in MCF-7 when treated with the demethylating agent, 5-azacytidine. Preliminary growth assays using a demethylase and tamoxifen indicate that TMX2-28 has a significant decrease in growth rate when treated with these compounds. A poster based on the HumanMethylation 450 BeadChip data was presented at two poster sessions: Capital Region Cancer Research Group New Frontiers Symposium 2012 and the Wadsworth Center New York State Department of Health Poster Day.

Conclusion:

In the second year of this study I analyzed the methylation data from the HumanMethylation 450K BeadChip and found genes of interest that will be analyzed for expression. I treated cells with a demethylase and purified RNA, DNA, and protein for future analysis. Preliminary growth assay treatment studies were completed using a demethylase and tamoxifen alone and in combination. Additionally, I submitted IRB approval for the human breast cancer tissue sample work that will be completed. I have also continued my training by interacting with
my mentor on a weekly basis, collaborating with both scientists and physicians, and attending weekly journal clubs and seminars. In my final year, I expect to complete the remaining tasks, pyrosequencing, real-time RT-PCR analysis, immunohistochemistry, and cellular growth treatment assays.

References: none

Appendices: Capital Region Cancer Research Group New Frontiers Symposium 2012 poster
Epigenetic Changes in Breast Cancer Cells Associated With Acquired Tamoxifen-Resistance

Kristin E. Williams¹, Douglas L. Anderton², Maxwell Lee³, Brian T. Pentecost⁴, Kathleen F. Arcaro⁵

¹Molecular and Cellular Biology Program, University of Massachusetts, Amherst, MA; ²SADIR, University of Massachusetts, Amherst, MA; ³Laboratory of Population Genetics, CCR/NCI/NIH, Bethesda, MD; ⁴Wadsworth Center, New York State Department of Health, Albany, NY; ⁵Department of Veterinary and Animal Science, University of Massachusetts, Amherst, MA

INTRODUCTION

- Roughly 75% of all breast cancers express estrogen receptor alpha (ERs) and most are sensitive to the anti-estrogen, Tamoxifen.
- Approximately 1/3 of all women treated with Tamoxifen develop recurrance within five years; a greater understanding of Tamoxifen-resistance is needed.
- TM2X-11 and TM2X-28, Tamoxifen-resistant clones of the ERα-positive breast cancer cell line, MCF-7, have acquired resistance through prolonged exposure to the drug.
- Phenotypes of the Tamoxifen-resistant cell lines vary.
  - o TM2X-28 are ERα-negative, invasive and express basal-like cytokeratins.
  - o TM2X-11 are ERα-positive, non-invasive and non-migratory.

RESULTS

Tamoxifen-selection results in altered DNA methylation patterns

Figure 9. (A) DNA methylation patterns of the promoter MCF-7 with TM2X-11 and TM2X-28. Data show large changes in overall methylation between cell lines. Most changes in CpG site methylation in TM2X-28 are most likely related to loss of ER.

Figure 8. DNA methylation patterns of the promoter MCF-7 with Tamoxifen-selected cell lines and MCF-7 treated with 100 ng/mL of IGF-1. Greater and diversity is seen between the untreated MCF-7 and untreated MCF-7: MCF-7 treatment shows partial to similar to the ER-negative line, TM2X-28.

Table 1. Biological pathways and the genes affected by Tamoxifen selection. Tightly co-localizes with the TSG101 and TSLX highly methylated genes in the promoter region shared by TM2X-11 and TM2X-28. Ten of the 20 pathways with hypermethylated genes (top) and the single pathway with hypomethylated genes (bottom) are presented.

<table>
<thead>
<tr>
<th>Pathway Description</th>
<th>Genes Involved</th>
</tr>
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<tbody>
<tr>
<td>Estrogen signaling</td>
<td>ERα, ERβ, ESR1, ESR2</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>BAD, BAX, BCL2</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>CDK4, CDK6, CYP1A1</td>
</tr>
<tr>
<td>DNA damage reparation</td>
<td>ATM, TP53</td>
</tr>
<tr>
<td>DNA methylation</td>
<td>SAMHD1, DMBT1</td>
</tr>
</tbody>
</table>

CONCLUSIONS & FUTURE DIRECTIONS

- The three cell lines share similar hypermethylation patterns in 87% of assayed CpG sites across the genome.
- A subset of CpG sites (1.2%) was identified in which the two Tamoxifen-selected lines share a promoter methylation pattern distinct from the MCF-7 parent line.
- Further pathway-analysis reveals the hypermethylated genes are involved in processes relevant to acquired Tamoxifen-resistance including cell signaling, adhesion, transcriptional activation and repression, differentiation, proliferation, and apoptosis.
- Pyrosequencing and real-time RT-PCR will be used in future studies to probe the role of the identified gene sets in both Tamoxifen-resistant tumors and selected lines.
- Clearly, greater knowledge of the molecular modifications accompanying Tamoxifen-resistance is needed, as this will lead to discovery of new therapeutics targets and improved treatment.

REFERENCES & FUNDING SOURCES


FIGURE CAPTIONS

Figure 2. Gene expression of Tamoxifen-selected breast cancer cell lines. (A) TIGA7, VEGFA, and TFGH are highly expressed in both Tamoxifen-resistant lines. (B) Up-regulation of TGFα and TGFβ in Tamoxifen-resistant lines compared to untreated MCF-7 is shown. (C) Down-regulation of proliferation and cell cycle genes in Tamoxifen-resistant lines is shown.

Figure 3. Heat map showing the percentage of methylation in each CpG site in the promoter region of each gene.

Figure 4. Heat map showing the percentage of methylation in each CpG site in the promoter region of each gene.