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Award Number: W81XWH-11-1-0086

TITLE: Epigenetic Control of Tamoxifen-Resistant Breast Cancer

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REPORT DATE: January 2013

TYPE OF REPORT: Revised Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE January 2013		2. REPORT TYPE Revised Annual Summary		3. DATES COVERED 15 December 2011 – 14 December 2012	
4. TITLE AND SUBTITLE Epigenetic Control of Tamoxifen-Resistant Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-11-1-0086	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Kristin Paczkowski Williams E-Mail: kep@chem.umass.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Massachusetts Amherst, MA 01003-9333				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT 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.					
15. SUBJECT TERMS Methylation, Breast Cancer, tamoxifen resistance					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER (include area code)

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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).

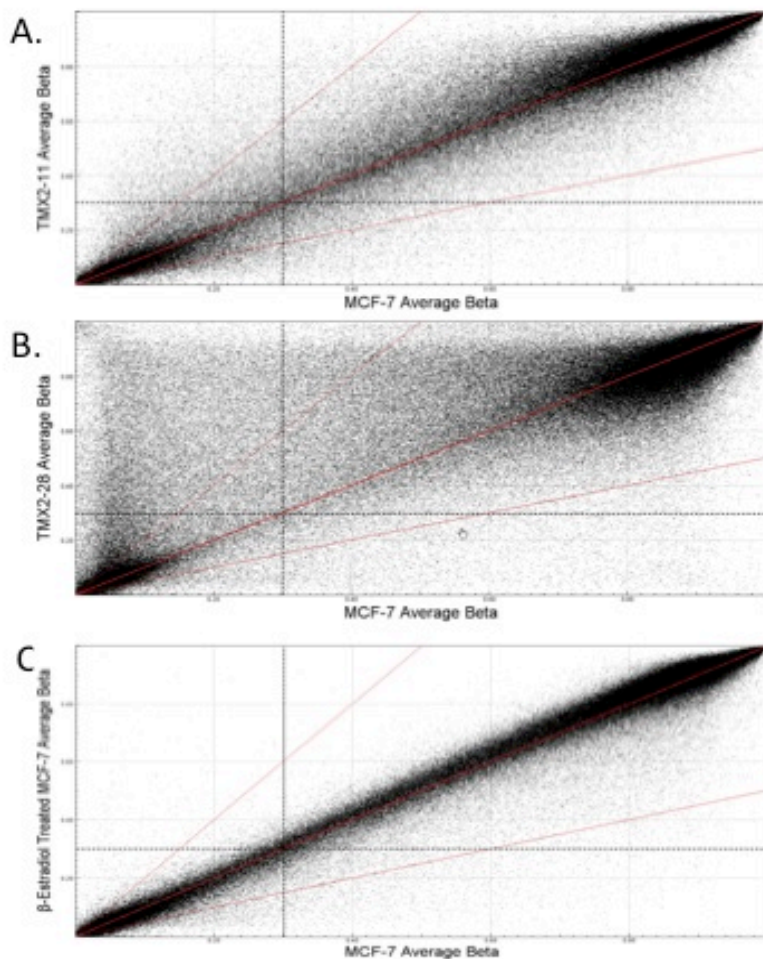


Figure 1. Scatter plots indicate genome-wide methylation changes in Tamoxifen-resistant lines compared with the parental. A) TMX2-11 and B) TMX2-28 C) 10^{-10} M $17\text{-}\beta\text{-E}_2$ treated MCF-7 were compared with the parental line, MCF-7 in Genome Studio to determine the overall changes in methylation. Dashed lines: Average beta value of 0.3; Red lines: 2-fold change in average beta values.

Table 1. CpG site methylation changes in tamoxifen-resistant cell lines as compared to the parental line.

	<i>TMX2-11/MCF-7</i>	<i>TMX2-28/MCF-7</i>	<i>MCF-7 E₂/MCF-7</i>	<i>TMX2-11 and TMX2-28/MCF-7</i>
Increased Methylation*	4,039	33,752	128	3,130
Decreased Methylation**	2,593	5,252	1,698	203
No Change in Methylation	472,153	436,113	479,003	431,909

*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.

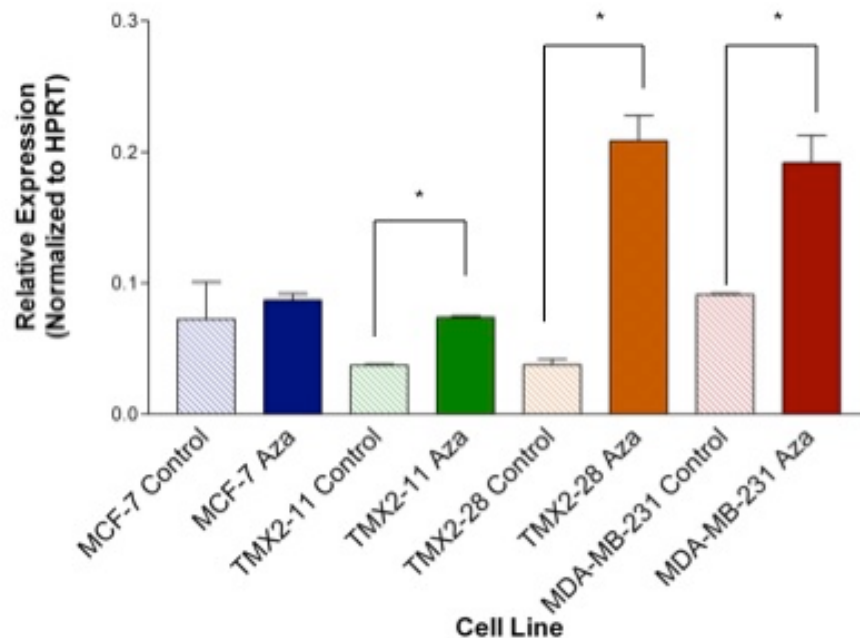


Figure 2. 5-Aza-2'deoxyctidine restores TMX2-11 and TMX2-28 ZNF350 expression levels. Relative mRNA expression levels of ZNF350 as measured by qRT-PCR. Significance of control to 5-Aza treated samples was determined by student's paired t-test. ZNF350 expression: TMX2-11, p-value 0.0013; TMX2-28 p-value 0.0087; MDA-MB-231 p-value 0.0422. No significant decrease in expression was found between MCF-7, TMX2-11 and TMX2-28 (p-value 0.2918).

Task 3: I have successfully treated MCF-7, TMX2-11, and TMX2-28 cells with the demethylase, 5-azadeoxycitidine and have purified RNA, DNA, and protein. I analyzed the expression of one gene, ZNF350 and found that after treatment with 5-azadeoxycitidine 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-azadeoxycitidine. No change growth rates were seen in TMX2-11 or MCF-7 (Figure 3).

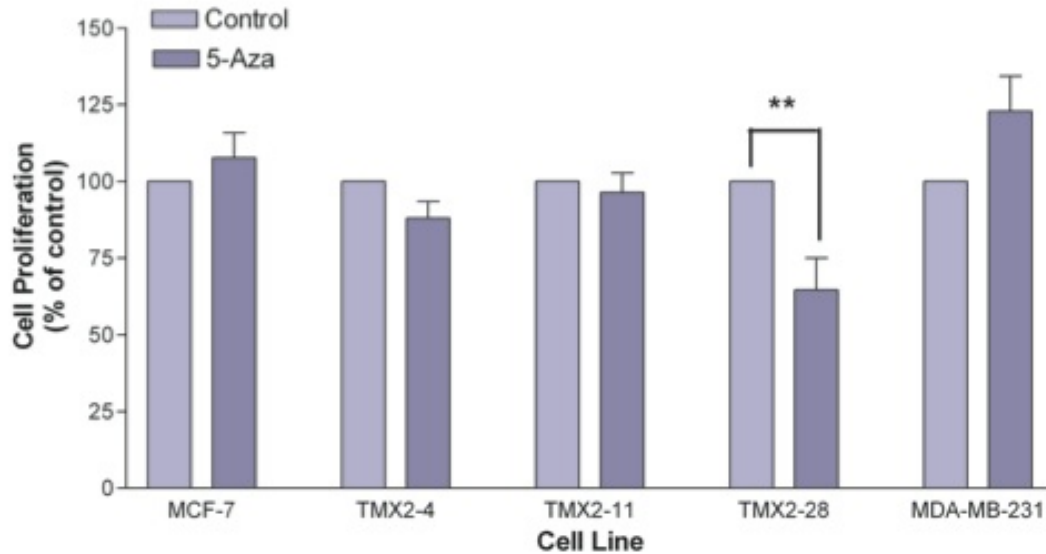


Figure 3. 5-Aza-2'deoxyctidine treatment decreased cell proliferation in TMX2-28. Cells were treated with 2.5 μ M 5-Aza for 4 days and cell proliferation was assessed by MTS assay and read at 490 nm. 5-Aza results are represented as percent of control. TMX2-28 cell proliferation was decreased by 36% ($\pm$ 10%) in the 5-Aza treated samples.

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



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Epigenetic Changes in Breast Cancer Cells Associated With Acquired Tamoxifen-Resistance

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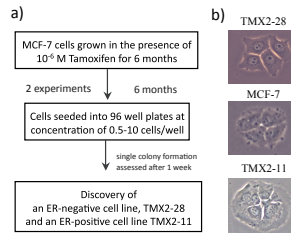
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New York State Department of Health

INTRODUCTION

- Roughly 75% of all breast cancers express estrogen receptor alpha (ERα) and most are sensitive to the anti-estrogen, Tamoxifen.
- Approximately 1/3 of all women treated with Tamoxifen develop recurrence within five years¹; a greater understanding of Tamoxifen-resistance is needed.
- TMX2-11 and TMX2-28, Tamoxifen-resistant clones of the ERα-positive breast cancer cell line, MCF-7, have acquired resistance through prolonged exposure to the drug².
- Phenotypes of the Tamoxifen-resistant cell lines vary.
 - TMX2-28 are ERα-negative, invasive and express basal-like cytokeratins³.
 - TMX2-11 are ERα-positive, non-invasive and non-migratory.

Figure 1. a) To identify the molecular mechanisms of Tamoxifen-resistance in breast cancer, we are using Tamoxifen-selected derivatives of the MCF-7 breast cancer cell line: TMX2-11 and TMX2-28. **b)** Despite their invasive behavior, TMX2-28 retains a morphology similar to the non-invasive MCF-7 cells.



- Aberrant DNA promoter methylation, a major epigenetic mechanism by which gene expression is altered in cancer, is thought to play a role in this resistance.
- DNA from MCF-7, TMX2-11, and TMX2-28 cells was analyzed on the Illumina Human Methylation 450 BeadChip platform to probe mechanisms of Tamoxifen resistance.
- The objective of this research is to elucidate the role of DNA promoter methylation in Tamoxifen-resistance.

HYPOTHESIS

We hypothesized that promoter methylation plays a role in both ER-positive and ER-negative acquired Tamoxifen-resistance.

RESULTS

Tamoxifen-selection results in altered DNA methylation patterns

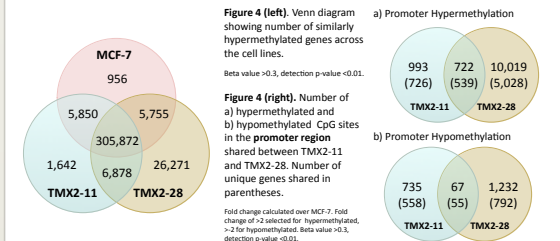
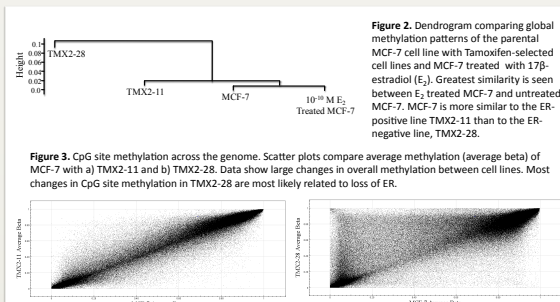
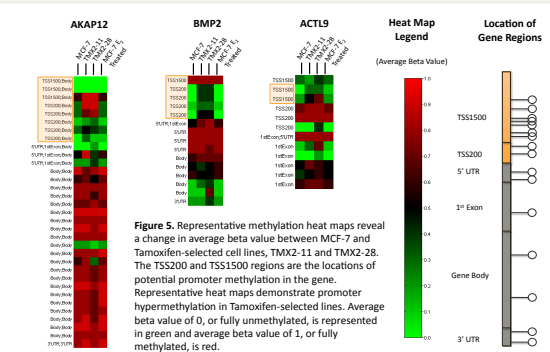


Table 1. Biological pathways and the genes affected by Tamoxifen selection. DAVID Bioinformatics Database (NIH) was used to determine the biological pathways affected in the 539 hypermethylated and the 55 hypomethylated genes in the promoter region shared by TMX2-11 and TMX2-28. Ten of the 30 pathways with hypermethylated genes (top) and the single pathway with hypomethylated genes (bottom) are presented.

Biological Pathway	Hypermethylated Genes
G-protein coupled receptor protein signaling pathway	GABRE, ORSP3, ORSH3, CCK2, AKAP2, DGK, ORJ2, TAC1, FSHR, OR4D6, OR153, RGS13, OR2M5, EDNRB, GRM3, PRPF, GRM8, OR5V1, OR515, OR5B2, OR5A3, GAP43
cell surface receptor linked signal transduction	ORH4, CCK2, AKAP2, ORJ2, TAC1, EDNRB, OR5A3, OR5B2, GABRE, BMP2, ORSP3, DGK, FSHR, OR4D6, OR2M5, OR153, RGS13, GRB10, GRM3, OR5L1, PRPF, GRM8, OR5V1, OR5H15, GAP43
sensory perception of chemical stimulus	ORSP3, GRM8, OR5V1, ORH3, OR5H15, OR5A3, OR5B2, ORJ2, OR4D6, OR153, OR2M5
neurological system process	ORSP3, ORH3, SIK3, AS21, TAC1, ORJ2, OR4D6, OR153, OR2M5, GRM3, GRM8, OR5V1, OR5H15, OR5B2, OR5A3
regulation of neurological system process	EDNRB, GRM3, LZTSL1, GRM8, TAC1
regulation of transcription from RNA polymerase II promoter	MAGED1, BMP2, HELL, IL17, SIK3, HXB89, GF11, RORA, HDAC9, CTF2
regulation of adenylate cyclase activity	EDNRB, GRM3, GRM8, FSHR
defense response	BMP2, PRPF, CCK2, IL17, DEFB118, TAC1, DEFB116, DEFB115, HDAC9
regulation of cyclase activity	EDNRB, GRM3, GRM8, FSHR
regulation of lyase activity	EDNRB, GRM3, GRM8, FSHR
Biological Pathway	Hypomethylated Genes
oxidation reduction	XDH, CYB, HMOX2, VAT1L, PYROD1, NIMRA1, LDH

RESULTS

Changes in promoter methylation between Tamoxifen-selected cell lines and MCF-7



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 it will lead to discovery of new therapeutic targets and improved treatment.

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