# Cell Survival miRNAs (29a, 29c, and 221) and Pre-metastatic Prostate Cancer

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## INTRODUCTION

*Prostate Cancer (PCa)*

- The leading cancer diagnosis American men.
- Indolent PCa is effectively treated with radiation and surgery
- Metastatic disease ,however, has poor survival due to limited treatment options and lack of prognostic tools that predict metastasis.
- Thus, the need to find miRNA that track metastatic disease is imperative.

*MiRNAs and their Role in Apoptosis and Cancer*

- MiRNAs are responsible for altering the expression of mRNAs that regulate the biological pathways essential to the 6 hallmarks of cancer, including cell survival.
- In particular, overexpression of miR that recognize and inhibit pro-apoptotic mRNA may lead to tumor escape of apoptosis, accumulation of cellular damage, increased proliferation, increased tumor growth and metastasis.
- A few miRNA such as miR34-a, miR125-b, and 488* have been shown as important regulators in cell survival.
  - MiR34-a promotes apoptosis by inhibiting *BCL-2* protein translation preventing bcl-2 mediated degradation of p53.
  - *In vitro and in* vivo studies show that miR125-b works against apoptosis by binding 3 pro-apoptotic genes:*P53, PUMA,* and *Baki*. MiR125 also decreases mitochondrial release of cytochrome C and caspase 3 activity.
  - *In vitro,* MiR488* overexpression decreases tumor growth by decreasing the number of cells that undergo apoptosis.

## CLINICAL RELEVANCE

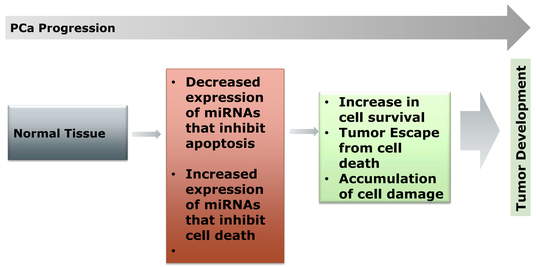
- Detection of serum based miRNAs associated with apoptosis may help to:
  - improve the early prediction of pre-metastatic or metastatic PCa
  - Identify new therapeutic targets for the treatment of metastatic PCa
  - Help clinicians predict patients who may benefit from aggressive PCa treatments.

## OBJECTIVE

- To determine whether apoptosis-associated miRNAs are differentially expressed in serum collected from age and race-matched PCa patients with bone-specific metastatic (stage IV), pre-metastatic PCa (stage III), or non-advance disease when compared to disease-free participants (n =5).

## HYPOTHESIS

- In comparison to disease-free men, we propose men diagnosed with prostate cancer will have:
  - lower serum levels of miRNA that suppress apoptosis and/or
  - higher levels of miRs that suppress cell death
- We further postulate a stage-dependent expression pattern exists.
  - Specifically, men with more advanced disease will have greater dysregulation as compared to their indolent diseased counterparts.




## METHODS

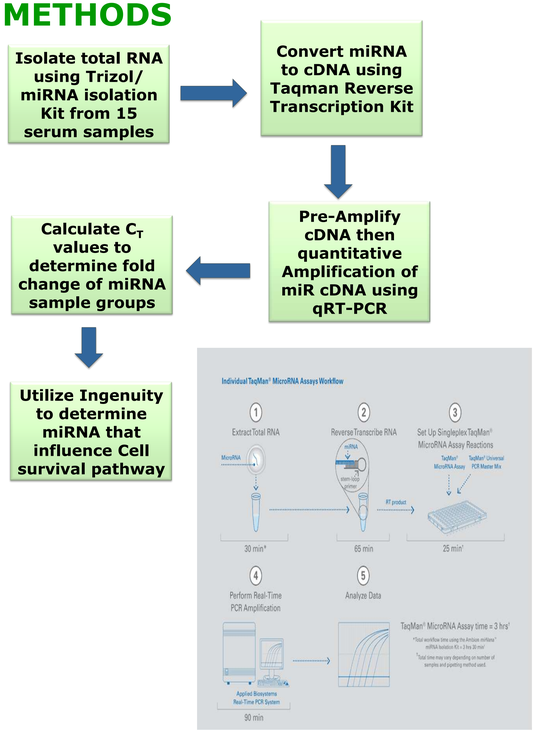



**Table 1- Population Characteristics**

| | Patients | Controls |
|---|---|---|
| # Serum samples | 15 | 5 |
| Mean Age (SD), yrs | 66.2 (6.46) | 66.2 (5.89) |
| Age Range, yrs | 47-72 | 56-71 |
| Sex, n(%) | | |
| Men | 15 (100%) | 5 (100%) |
| Race, n (%) | | |
| Caucasian | 15 (100%) | 5 (100%) |
| Smoking, n (%) | | |
| Non-smoker | 5 (33.3) | --- |
| Smoker | 10 (66.7) | --- |
| PSA | | |
| <4 ng/ml | | |
| ≥4 ng/ml | 11 | 5 |
| Missing values | 4 | |
| Tumor classification, n(%) | | |
| Adenocarcinoma | 9 (60) | |
| Unknown | 6 (40) | |

**Table 2. Differentially Apoptosis-associated miRNAs in cases and controls**

| Serum miRNA | P-value | Fold-change (case vs ctrl) | Increased or Decrease in expression | PCa Stage |
|---|---|---|---|---|
| 29c | 0.038 | 2.3 | Increase | Stage III |
| 221 | 0.042 | 8.1 | Increase | Stage III |
| 29a | 0.064 | 0.187 | Decrease | Stage IV |

**Table 3. miRNAs involved in Apoptosis**

| miRNA | mRNA Target | Source |
|---|---|---|
| Let-7c | CASP3, HRAS, TGFBR1, KRAS | Tsang et al., 2008, Oh et al., 2010, Ingenuity |
| miR-141 | TGFB2,TCF4, SOX5 | Burk et al., 2008 |
| miR-29c | TNFAIP1,CDC 42 | Wang et al. 2011,Park et al. 2009, Ingenuity |
| miR-221 | CASP10, FOS, TBK1 | Ichimura et al., 2009, Pineau et al., 2010 Ingenuity |
| miR-29a | MCL1, CDC42 | Lima et al., 2011,Park et al., 2009, Ingenuity |

## CONCLUSIONS

- Relative to disease-free men
  - miRNAs 221 and 29c were over-expressed by 2.3-8.1 fold in the serum collected from men with Stage III PCA (P = 0.038-0.142).
  - miRNAs 29a had a marginal 81.3% reduction in serum from men with Stage IV disease (P = 0.064)

## FUTURE DIRECTIONS

- Future experiments will identify and validate other miRNAs that influence apoptosis using a larger population set.
- Use miRNA mimic or inhibitors *in vitro and in vivo* to elucidate the impact of miRNA- 29a, -29c, and -221 on mRNA targets and PCa bone-specific metastasis.

## ACKNOWLEDGEMENTS

NCI R25 Cancer Education Grant to D.W. Hein (CA134283); Our Highest Potential" in Cancer Research Endowment to LRK.

# Are Cell Adhesion Associated Micro-RNAs Linked With Metastatic Prostate Cancer?

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## INTRODUCTION

*Prostate Cancer as a Public Health Problem*

- Prostate cancer (PCa) remains one of the leading causes of cancer-related deaths among American men.
- In adavnced stages of the disease, metastasis of tumor cells occurs through the process of angiogenesis to the bone.
- Mortality rises markedly as bone metastatic cancers are typically aggressive and often unresponsive to conventional therapy.
- Studies over the last few years have shown the inadequacies associated with the Prostate Specific Antigen (PSA) test, the current gold standard for Pca detection which often fails to adequately detect and stage the disease.
- Solving this problem requires the development of biomarkers that would aid the accurate detection, staging of the disease, and ultimately clinical management of the disease

*Micro-RNAs as Effective Cancer Biomarkers*

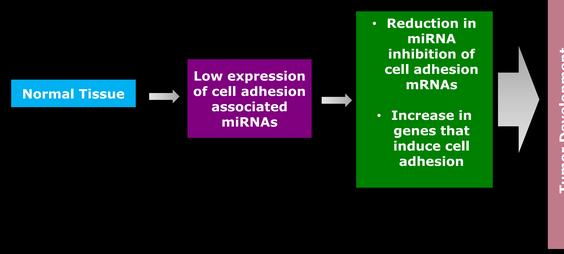
- Limited studies suggest microRNAs (miRNAs) show great promise as PCa biomarkers.
- MiRNAs are small RNA molecules that exert a host of post-transcriptional effects by binding to complementary sequences on messenger RNA (mRNA) transcripts resulting in translational repression, target degradation, and gene silencing.
- Their relative stability and resistance to RNase degradation in addition to the ease with which they are detected in biological samples make them great prognostic tools for Pca metastasis.
- While several studies have been done with tissues and other cell lines, very little work has been done with serum which could provide a less invasive means of detecting disease.

## OBJECTIVES

- To determine whether cell adhesion associated miRNAs are differentially expressed in serum collected from age and race-matched PCa patients with bone-specific metastatic (stage IV), pre-metastatic PCa (stage III), or non-advance disease when compared to disease-free participants (n =5).

## Hypothesis

- We hypothesized that individuals with advanced stage PCa would have lower levels of miRNAs that influence cell adhesion.




## CLINICAL RELEVANCE

- The findings of our study may serve as a foundation to for future studies to identify and validate miRNA as non-invasive biomarkers for bone specific PCa metastasis.
- The establishment of biomarkers that track with pre-metastatic and metastatic PCa will help guide new aggressive treatment strategies toward individuals susceptible to metastatic disease.
- Ultimately, investigation of these biomarkers will improve early detection of pre-metastatic and metastatic PCa in patients.

## METHODS

**Step 1**
- Separation of serum into 250 µl aliquots
- Denaturation of serum using TriZol Reagent LS
- Isolation of total RNA using miRNA miRVana Isolation Kit

**Step 2**
- Quantitation of Total RNA using a Nanodropper
- miRNA selection using In Silico tools(Ingenuity, KEGG, BioCarta)

**Step 3**
- Serum miRNA profile expression analysis (Taqman Array Human miRNA cards)
- Data analysis

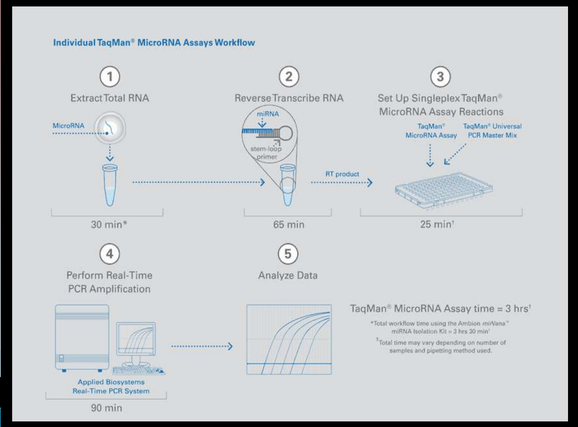



**Table 2. Differentially expressed serum miRNAs in cases and controls**

| Serum miRNA | P-value | Fold-change (case vs ctrl) | Increase/ Decrease in expression | Disease Stage Comparisons |
|---|---|---|---|---|
| 185 | 0.204 | 7.82 | Increase | Stage I vs. Controls |
| 29a | 0.064 | 0.187 | Decrease | Stage IV vs. Controls |
| 133a | 0.198 | 0.026 | Decrease | Stage IV vs. Controls |
| 145 | 0.074 | 0.094 | Decrease | Stage IV vs. Controls |
| 197 | 0.064 | 0.136 | Decrease | Stage IV vs. Controls |

**Table 3. miRNAs involved in Cell Adhesion using Ingenuity**

| Pathway | miRNA | mRNA Targets |
|---|---|---|
| Cell Adhesion | | |
| | let-7a | CCND1 |
| | 128 | TGFBR1 |
| | 129-5p | SOX4 |
| | 132 | RB1 |
| | 133a | CASP9 |
| | 143 | BCL2, KRAS |
| | 145 | MYC |
| | 146a | CHUK |
| | 149 | E2F1 |
| | 155 | TCF7L2 |
| | 195 | BCL2, CCND1, WNT3A, ZYX |
| | 185 | CDC42, RHOA |
| | 18a | CDKN1A, E2F1 |
| | 193a-3p | CCND1 |
| | 197 | ACVR1, TSPAN3 |
| | 219-5p | PLCG2 |
| | 222 | CDKN1B, DIRAS3, PIK3R1, PTEN |
| | 26a | PTEN |
| | 29a | ARPC3, CDC42, PIK3R1 |
| | 34a | BCL2, CCND1, CREB1, HDAC1 |
| | 375 | PDPK1 |
| | 200b | PLCG1 |

**Table 1. Characteristics of study population**

| | Patients | Controls |
|---|---|---|
| # Serum samples | 15 | 5 |
| Age | | |
| Range | 47-72 | 56-71 |
| Mean (SD) | 66.2 (6.46) | 66.2 (5.89) |
| Sex, n(%) | | |
| Men | 15 (100%) | 5 (100%) |
| Race, n (%) | | |
| Caucasian | 15 (100%) | 5 (100%) |
| Smoking, n (%) | | |
| Non-smoker | 5 (33.3) | NA |
| Smoker | 10 (66.7) | NA |
| PSA | | |
| <4 ng/ml | | |
| ≥4 ng/ml | 11 | 5 |
| Missing values | 4 | |
| Tumor classification, n(%) | | |
| Adenocarcinoma | 9 (60) | |
| Unknown | 6 (40) | |

## CONCLUSIONS

Relative to disease-free individuals:

- Several cell adhesion-associated miRNAs detected in the serum of patients with stage IV disease were down-regulated
  - miRNAs-133a, 197 as well as miRNAs-145 and 29a, which were marginally significant (P-value = 0.064-0.074).
- However, one cell adhesion-associated miRNA detected in the serum of patients with stage I disease was up-regulated
  - miRNA-185, but it was not significant.
- Overall the cell adhesion-associated miRNAs found in the study were down-regulated among men w/ bone-specific metastasis.

## FUTURE DIRECTIONS

- We will validate differentially expressed serum miR targets individually using Taqman Singleplex miRNA assays.
- Determine miR and mRNA signatures using archival tissue.
- Use miR mimics and inhibitors to determine whether miRNAs regulate the expression of mRNAs that regulate cell migration, motility or metastasis.
- Establish a co-culture bone metastasis model to determine whether miRNA inhibitors/mimics will modify: adhesion of tumor cells to bone; bone cell's susceptibility to tumor invasiveness, or release of chemokines, growth hormones, or osteoporin.

## ACKNOWLEDGEMENTS

Grant/Research Support: NCI R25 Cancer Education Grant to D.W. Hein (CA134283); Our Highest Potential" in Cancer Research Endowment to LRK.

# Utilizing the Humoral Response to Early-stage Lung Cancer as a Potential Biomarker for Early Diagnosis

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## Introduction

Lung cancer is the major cause of cancer-related deaths worldwide, accounting for about 1.3 million deaths annually. Approximately $10.3 billion is spent on lung cancer treatment in the United States each year. Often, clinical symptoms only appear during later stages of cancer, or a diagnosis is made after an x-ray is done for other medical reasons. Various screening strategies have been tested for detection of early stage lung cancer but only one cumbersome technique (low-dose computed tomography) has shown any significant reduction in lung cancer mortality. Though this method can detect smaller nodules than conventional CT scans, there is also an associated high number of false-positives, necessitating additional tests.[1] Autoantibodies have been found in patient serum up to five years before spiral CT scans were able to detect non-small cell lung cancer nodules, suggesting the possibility of earlier detection via autoantibodies.[2]

We have developed an inexpensive and fast alternative blood test that we believe will detect antibodies that appear early in the development of lung cancer. The test involves flow cytometric analyses of A549 (human lung adenocarcinoma) cells incubated with dilute patient serum and a secondary anti-human IgG antibody (tagged with the fluorescent tag, R-Phycoerythrin).

In very early tests of this system, we found that two of four serum samples from stage I non-small cell lung cancer patients were positive for antibodies against A549 cells. Additional tests with samples from later stages of lung cancer development have confirmed that antibodies can be detected throughout lung cancer stages 1-3.
A potential strength of this approach is that it is a broad-spectrum screen that will identify antibodies against a variety of cell surface antigens. In addition to detecting lung cancer in its earliest stages, our technique also offers the exciting possibility of identifying novel human lung cancer antigens, extracellular as well as intracellular, that may serve as targets for future therapy.

## Materials & Methods

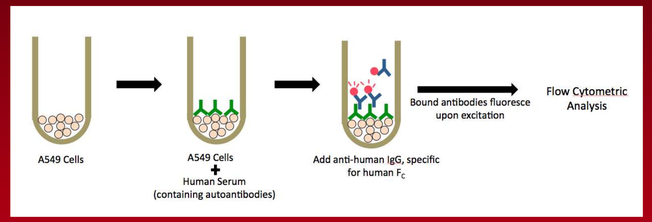
**Extracellular Antigen Detection**

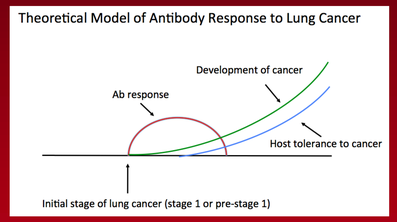
1) Grow 1x10$^5$ A549 human adenocarcinoma cells in 100 ul staining buffer (PBS + 1% Fetal Bovine Serum). Add human serum to a final dilution of 1:50 and incubate on ice for 30 minutes.
2) Wash cells twice at 1500 RPM for 5 minutes with 2 ml of staining buffer.
3) Add anti-human IgG, $F_C$ fragment specific (conjugated with PE) to a final dilution of 1:100 and incubate on ice for 30 minutes in the dark.
4) Wash cells twice with 2 ml of staining buffer.
5) Resuspend cells in 500 ul of staining buffer and test for antibody binding via flow cytometry.

**Intracellular Antigen Detection**

1) Grow up 1x10$^5$ A549 human adenocarcinoma cells and incubate in 100 ul fixing solution for 30 minutes at room temperature.
2) Wash with 2 ml of permeabilization buffer (diluted 1:10 in $dH_2O$).
3) After washing, add human serum to a final dilution of 1:50 and incubate for 30 minutes at room temperature.
4) Follow steps 2-5 of the extracellular protocol described below except use permeabilizationbuffer instead of the staining buffer and all steps should be conducted at room temperature.

## Theoretical Framework behind Autoantibody Detection




## Results

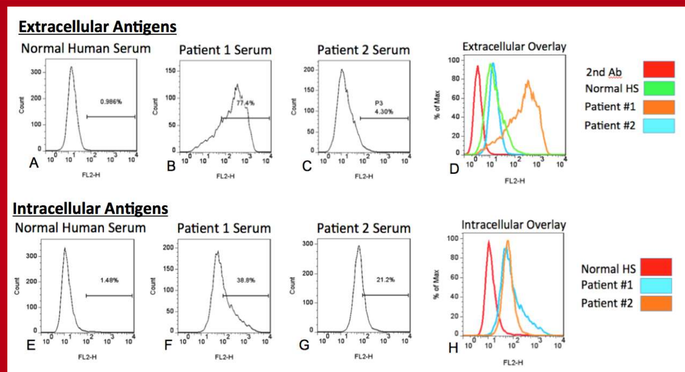



**Figure 1. Detection of autoantibodies via flow cytometry using an anti-human IgG antibody, specific for $F_C$, indicates the production of IgG antibodies in early stage lung cancer.** Results from the initial experiment (n=4) indicate that it is possible to detect autoantibodies against non-small cell lung cancer in 50% of the serum samples using our novel method of detection. The results also show that extracellular antigens (A-D) as well as intracellular antigens (E-H) can be detected.

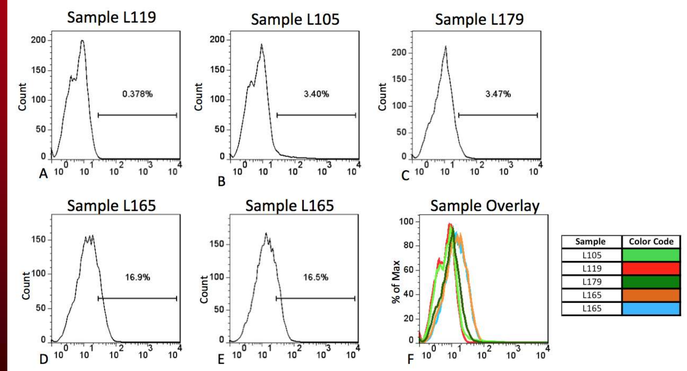



**Figure 2. Detection of autoantibodies in later stages of non-small cell lung cancer reveals a lower titer of antibodies present in human serum during later stages of cancer development.** Results from additional testing (n=15) indicate that antibodies are detectable in some (A-D), but not all serum samples. There is also evidence of reproducibility within samples (D-E).

**Table 1. Percent Positive Cells for autoantibody binding from early stage lung cancer serum to extracellular/intracellular antigens on A549 cells.**

| | | Extracellular | Intracellular |
|---|---|---|---|
| Serum Sample | Stage of Cancer | % Cells Positive | % Cells Positive |
| Normal | N/A | 0.986 | 1.480 |
| Patient 1 | 1 | 77.400 | 38.800 |
| Patient 2 | 1 | 4.300 | 21.200 |

**Table 2. Percent Positive Cells for autoantibody binding from late-stage lung cancer serum to extracellular antigens on A549 cells.**

| | | Extracellular |
|---|---|---|
| Serum Sample | Stage of Cancer | % Cells Positive |
| L105 | 2 | 3.400 |
| L119 | 3 | 0.378 |
| L179 | 3 | 3.470 |
| L165 | 3 | 16.900 |
| L165 | 3 | 16.500 |

## Conclusions

The results indicate that it is possible to detect autoantibodies in early, as well as late, stages of non-small lung cancer development that react to antigens found on the surface of A549 (human lung adenocarcinoma) cells using an anti-human IgG antibody. As seen in Fig. 1A, background from normal human serum is low compared to autoantibodies that can be detected in the serum from a patient with stage 1 lung cancer (Fig. 1B). Although it is evident that there is variability in antibody response as detected by our system (50% of serum samples from stage 1 did not register a response), we believe this phenomenon could be explained by the repression of MHC class I molecules on tumor cell surfaces. This currently remains to be validated due to the difficulty of acquiring paired serum/tissue samples from patients diagnosed with non-small cell lung cancer.

Repeated tests on several samples (Figs. 2D-2E and other samples not shown) have confirmed that the results are reproducible within the samples. This suggests that the reason for the variability between samples from the same stages (Figs. 1B-1C [stage 1], and Figs. 2A, 2C, 2D [stage 2]) remains to be elucidated. Tests on additional serum samples will provide a more comprehensive overview of the IgG response that can be detected with our method. Higher dilutions of serum have been tested (data not shown) which resulted in a reduced response from the cancer patient serum, but not in the normal serum. This suggests that specific, rather than non-specific, binding is occurring between the autoantibodies and the extracellular antigens on A549 cells.

Our results indicate that our technique allows the detection of intracellular antigens (Figs. 1E-1H) as well as extracellular antigens (Figs. 1A-1D). This method presents the exciting possibility of being able to identify novel cancer antigens that may serve as targets for future drug development.

## Future Work and Limitations

The limitations of this method include the inability to detect autoantibodies from non-small cell lung cancer patients in which the cancerous cells have lost their MHC class I molecules. It has been shown that about 38% of non-small cell lung cancer tissues will lose their MHC class I molecules.[3] Further complicating this technique, it is difficult to properly analyze the decrease in autoantibody response throughout the four stages of lung cancer development without previous knowledge of the degree to which the cancer cells have begun to repress MHC class I molecule expression.

Future work will include testing additional serum samples that we currently have access to as well as testing all samples against an IgM secondary antibody to determine if class switching may be occurring during the early stages of cancer. We intend on further searching for serum samples from sources that include information on MHC class 1 molecule status (present or absent) or for paired serum/tissue samples. In the future, we also intend on identifying and isolating novel cancer antigens using this technology via proteomics approaches.

## References

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2. Zhong, Li, PhD, *et al*. “Profiling Tumor-Associated Antibodies for Early Detection of Non-small Cell Lung Cancer.” *Journal of Thoracic Oncology* 1.6(2006): 513-519.
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## Acknowledgements

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# Gene Expression in Breast Carcinomas from Patients with Ethnical Differences

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**Abstract:**

**Background:** African American women often exhibit more aggressive breast cancer and have a higher mortality rate than Caucasian women. Differences in cultural and socioeconomic status are possible explanations for the higher mortality rate and more advanced stage of breast cancer. However, numerous studies suggest that differences in insurance coverage and socioeconomic status do not explain the observed differences observed in clinical behavior of breast carcinomas of black and white patients, suggesting a biological basis. The goal was to ascertain if dissimilarities occur in gene expression of breast carcinoma biopsies of white and black patients, and further to evaluate if these gene expression differences were related to cancer behavior.

**Materials and Methods:** Using an IRB-approved biorepository and database, gene expression levels were compared in tissue biopsies from white and black patients utilizing microarray analyses of LCM-procured breast carcinoma cells and assessed with clinical information. These studies generated a candidate gene list, which included TRAPPC2L, CRYBB2P1 and PDHA1 to be validated by qPCR. Then frozen tissue sections from de-identified patients diagnosed with primary breast carcinoma and metastasis were utilized. Total RNA was extracted from frozen intact tissue sections with the RNeasy® Mini Kit (Qiagen Inc.). Integrity of RNA was analyzed using the Bioanalyzer 2100 (Agilent Technologies). Total RNA was reverse transcribed using iScript (Biorad). Primers were designed using Primer Express (Applied Biosystems) and Primer Blast (NCBI). RNA quantification and analyses were performed in triplicate by qPCR in duplicate wells using the ABI Prism® 7900HT (Applied Biosystems) with Power Sybr® Green (Applied Biosystems) for detection. Relative gene expression was calculated using the ΔΔCt method with cDNA prepared from Universal Human Reference RNA (Stratagene) as both a calibrator and a standard for quantification of RNA using β-actin (ACTB) as a reference gene. T-tests, box and whisker and Kaplan-Meier plots were performed in Graph Pad Prism. Pearson correlations and Cox regressions were performed in SPSS Statistics 20. The gene interactions were evaluated with Ingenuity IPA.

**Results:** Examination of candidate gene expression levels from microarray analyses revealed that CARD11 (P=0.001), TRAPPC2L (P<0.001), CRYBB2P1 (P<0.0001) and PDHA1 (P<0.0001) exhibited significant differences in breast carcinomas of African American patients compared to those of Caucasian patients. Of these genes, only PDHA1 expression was correlated with overall survival (P=0.05) when the entire population of 245 breast carcinoma patients was stratified by median gene expression level without regard to race. Only PDHA1 expression assessed by microarray was correlated with overall survival (P=0.04) of white patients when stratified by race and gene expression level. From the expression levels of 20 genes found most significant by T-test of the microarray data, PDHA1, CRYBB2 and TRAPPC2L were investigated further by qPCR. Using a platform comparison of gene expression levels of candidate genes from qPCR and microarray, CRYBB2 was significantly correlated (P=0.01), while PDHA1 was insignificantly correlated (P=0.22). From analyses of tumor marker protein results, significant differences were detected in estrogen and progestin receptor gene expression levels in tissue biopsies when comparing white and black patients and white and hispanic breast carcinoma patients.

**Conclusions**: Expression levels of CARD11, PDHA1, TRAPPC2L and CRYBB2P1 were significantly different in breast tissue biopsies of African American patients when compared to those of Caucasian patients. Of the four genes, only PDHA1 gene expression levels of LCM-procured carcinoma cells were significant when correlated with overall survival of the entire population (n=245) regardless of race. Furthermore, PDHA1 expression was correlated with overall survival when only white patients were considered. Although dissimilarities in gene expression levels were observed in black and white patients, preliminary evaluation of a limited gene subset to personalize prognosis assessment requires additional studies related to a patient's ethnical background.

## Introduction:

Numerous studies have shown that African American women often have a more aggressive breast cancer and have a higher mortality rate than Caucasian women. A difference in cultural and socioeconomic status is one explanation for African Americans presenting a higher mortality rate and a more advanced stage of breast cancer than Caucasian patients. However, some studies have shown that differences in insurance coverage and socioeconomic status do not explain the observed differences seen between blacks and whites (1). Through this information, it is valid to suspect biological differences affecting breast cancer in black and white patients. Breast tumors may be classified using five immunohistochemical (IHC) tumor markers: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) (2). Estrogen and progesterone receptors play an important role in predicting prognosis and response to endocrine therapy in breast cancer. Hormone receptor-negative breast tumors are associated with poorer survival, whereas tumors that have a lobular history are associated with better survival (1). Almost two thirds of ER- positive patients respond favorable to endocrine therapy; less than ten percent of ER-negative patients exhibit a favorable response to endocrine therapy (1). Previous studies have shown that African Americans are more likely to have ER-negative, PR-negative breast tumors (2). This study primarily focused on the discrepancies in gene expression involved in white and black breast cancer patients.

## Materials and Methods

### Tissue Preparation & RNA Extraction

Using an IRB-approved study, frozen tissue sections from de-identified patients diagnosed with primary breast carcinoma and metastasis were utilized. Total RNA was extracted from frozen intact tissue sections with the RNeasy® Mini Kit (Qiagen Inc., Valencia, CA). Integrity of RNA was analyzed using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Total RNA was reverse transcribed using iScript (Biorad, Hercules, CA).

### Gene Expression Analyses

Primers were designed using Primer Express (Applied Biosystems) and Primer Blast (NCBI). RNA quantification and analyses were performed in triplicate by qPCR in duplicate wells using the ABI Prism® 7900HT (Applied Biosystems, Foster City, CA) with Power Sybr® Green (Applied Biosystems) for detection. Relative gene expression was calculated using the ΔΔCt method with cDNA prepared from Universal Human Reference RNA (Stratagene, La Jolla, CA) as both a calibrator and a standard for quantification of RNA using β-actin (ACTB) as a reference gene.

### Statistical Analysis

T-test, Kaplan Meier Plots, and Tumor Marker Analyses were performed in Graph Pad Prism. Pearson Correlations were performed in SPSS Statistics 20. The Gene Interactions were performed through Ingenuity IPA.

Figure 1: Kaplan-Meier Plots of Black vs White Patients without Regard to Gene Expression Levels

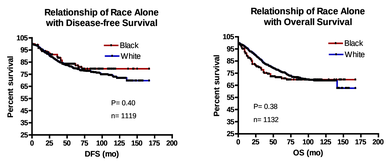
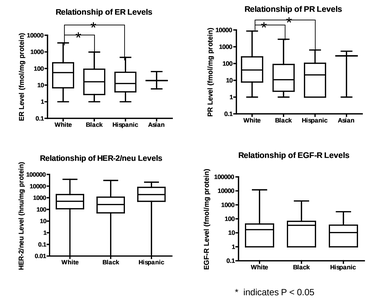



Figure 2: Box and Whisker Plots of Tumor Marker Levels of Patients with Differing Ethnical Heritage



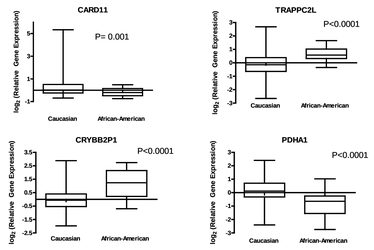

* indicates P < 0.05

Table 1: Results of T-Test of Microarray Data Comparing Gene Expression Levels in Patients with Differing Racial Backgrounds

(Most Significant Genes are Shown)

| Gene ID | T-test (P-value) |
|---|---|
| PSPH | 7.07 E-11 |
| TRAPPC2L | 5.36 E-10 |
| PCSK4 | 4.61 E-08 |
| DPH2 | 4.18 E-07 |
| CARD11 | 4.87 E-07 |
| SLIT3 | 5.90 E-07 |
| PLEKHA8 | 9.12 E-07 |
| PDHA1 | 9.37 E-07 |
| CRYBB2P1 | 9.37 E-07 |
| HAMP | 9.78 E-07 |
| SLC22A1 | 1.20 E-06 |
| CST5 | 1.39 E-06 |
| DCSTAMP | 1.53 E-06 |
| GLS2 | 2.67 E-06 |
| WDR48 | 2.87 E-06 |
| A4GNT | 4.68 E-06 |
| CGB1 | 4.88 E-06 |
| KRTAP3-3 | 5.60 E-06 |
| POLRIA | 5.66 E-06 |
| PNO1 | 8.02 E-06 |

Figure 3: Box and Whisker Plots of Expression Levels of Candidate Genes from Microarray




The box represents gene expression levels within the second and third quartiles of values observed. The horizontal line within the box represents the median expression level, while the whiskers extend to the lowest and highest expression level for each gene.

Figure 4: Kaplan-Meier Plots of Patients Stratified by Levels of Expression of the Candidate Genes without Regard to Race (n = 245)

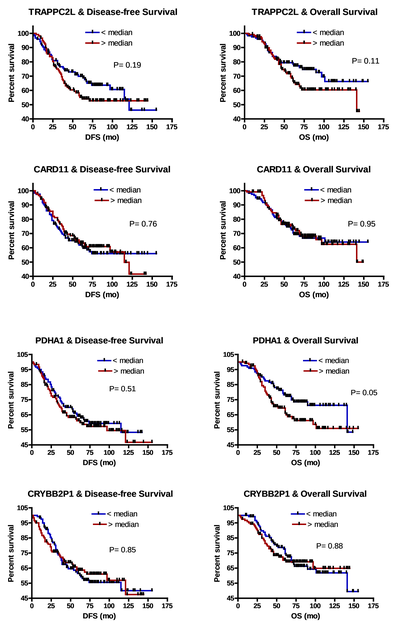



Figure 5: Kaplan-Meier Plots of Patients Stratified by Levels of Expression of the Candidate Genes & Patient Race (n = 245)

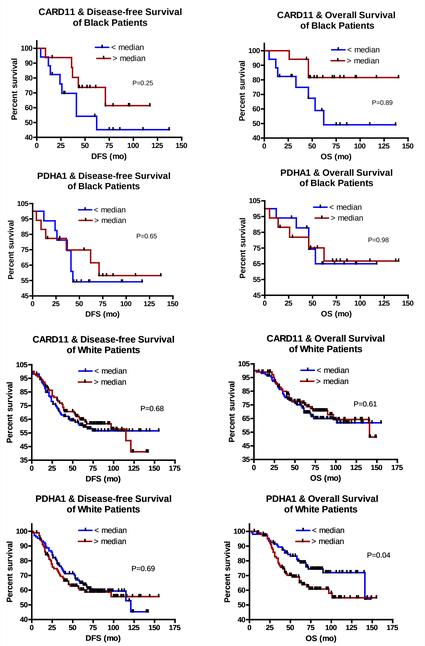



Figure 6: Comparison of Expression Levels of Candidate Genes Measured by Microarray and qPCR (n = 46)

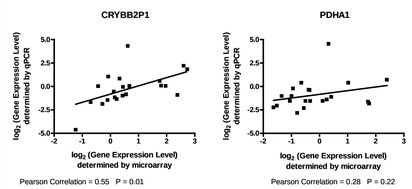



Figure 7: Kaplan-Meier Plots of Patient Survival in Relation to Gene Expression Levels Measured by qPCR (n = 46)

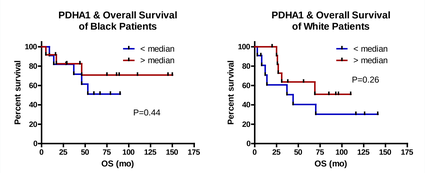
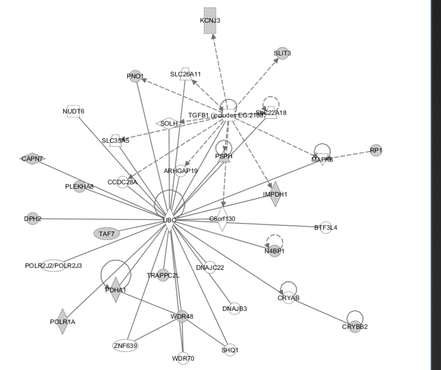



Figure 8: Gene Interactions Identified using Ingenuity Pathway Analysis (Genes of Interest indicated by Shaded Areas)




## Conclusions:

- Patient survival did not appear to be correlated with race when the overall population was considered (n = 1132).
- ER and PR protein levels were significantly different in carcinomas of white, black and hispanic patients, but no differences were observed in HER-2/neu or EGF-Receptor protein levels.
- Comparisons of gene expression levels in breast carcinomas from patients with different ethnical backgrounds identified 475 genes with P < 0.01 and 116 genes with P < 0.001 using microarray data.
- Candidate genes from microarray data were analyzed further: CARD11, PDHA1, TRAPPC2L and CRYBB2P1 expression levels were significantly different in tissue biopsies when compared to patient ethnical heritage.
- PDHA1 gene expression in LCM-procured carcinoma cells was significantly correlated with overall survival of the study population regardless of race.
- PDHA1 expression in LCM-procured carcinoma cells was correlated with overall survival only in white patients.
- CARD11, TRAPPC2L and CRYBB2P1 expression did not appear to be correlated with patient survival in patients of different ethnical heritage.
- Gene expression was also evaluated by qPCR in a subset of patients for further analysis.
- Evaluation of a limited gene subset suggests additional studies are warranted to determine the relationship of a breast cancer patient's ethnical background to personalize prognosis assessment.

## References:

1. Li CI et al. Differences in Breast Cancer Hormone Receptor Status and Histology by Race and Ethnicity among Women 50 Years of Age and Older. Cancer Epidemiol Biomarkers Prev 11:601-607, 2002.
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6. Wittliff JL et al. Expression of estrogen receptor-associated genes in breast cancer cells procured by laser capture microdissection. Jensen Symp Abs 81, 2003.

## Acknowledgements:

AMB was supported by a fellowship from the NCI R25 grant support University of Louisville Cancer Education Program NIH/NCI (R25-CA134283).

# *In Silico* Screening for Novel Human Arylamine *N*-Acetyltransferase 1 Inhibitors

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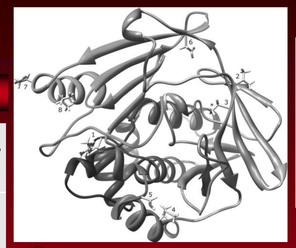


Ribbon diagram of human NAT1 enzyme

## Abstract

Human arylamine *N*-acetyltransferase 1 (NAT1) is a phase II xenobiotic-metabolizing enzyme that plays an important role in the deactivation and bioactivation of many environmental carcinogens such as, 4-aminobiphenyl (ABP) which is found in cigarette smoke. Arylamines, such as ABP can either be *N*-acetylated (deactivation) by NAT1, or if first acted upon by a cytochrome P450 enzyme, they can be *O*-acetylated (bioactivated) by NAT1. Once bioactivated, these compounds form arylnitrenium ions leading to DNA adducts. If not repaired by nucleotide excision (NER), these DNA adducts can lead to mutagenesis and cancer initiation. Human NAT1 is therefore an ideal molecular target for cancer therapy. Compound 10, an effective inhibitor of human NAT1 was previously discovered using *in silico* screening. This compound has been shown to decrease human NAT1 activity, ABP-induced DNA adducts, cell proliferation, and cell invasion in human breast adenocarcinoma cells. The objective of this project was to identify novel small molecule inhibitors of human NAT1 that were even more potent than compound 10 by building upon the previous *in silico* studies through the utilization of an updated ZINC library and also by performing a similarity search to compound 10. The two new *in silico* searches performed identified 161 potential NAT1 inhibitors, 35 of which were tested for their ability to inhibit human NAT1 *in vitro*. From this initial screening, compounds 63, 66, 72, 86, and 95 were chosen as lead compounds because they experimentally inhibited human NAT1 greater than 75% *in vitro*. We determined the $IC_{50}$ values of these lead compounds to be 66.1, 5.74, 161, 100, and 102 μM, respectively. The most potent inhibitor, compound 66, was chosen for further experiments. Compound 66, inhibited human NAT1 recombinantly expressed in yeast (*in vitro* $IC_{50}$ = 5.57 μM) and endogenously expressed in human breast cancer cells (*in situ* $IC_{50}$ = 2.44 μM). Compound 66 was tested against human NAT2 (*in vitro* $IC_{50}$ = 12.8 μM) to determine which isozyme of NAT it preferentially binds to, with the results showing a preference for human NAT1, our molecular target. [NCI R25-CA134283]

## Hypothesis

Inhibition of human NAT1 activity will decrease carcinogen metabolism and cancer progression.

## Objective

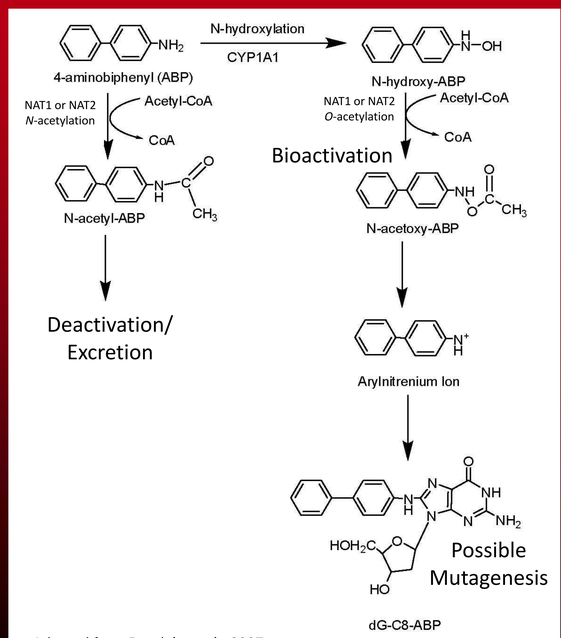
- To identify potent novel small molecule inhibitors of the molecular target human NAT1 that will suppress carcinogen activation and cancer progression.

## Introduction

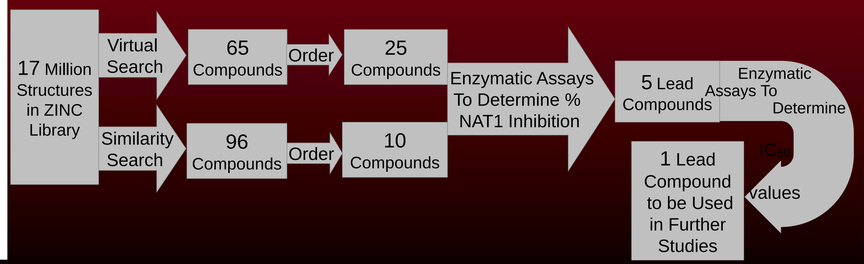
- Human arylamine *N*-acetyltransferase occurs in two isozymes, NAT1 and NAT2. These isozymes bioactivate environmental carcinogens such as 4-aminobiphenyl (ABP).
- Human NAT1 is a proposed molecular target for novel small molecule inhibitors because it has been found to be overexpressed in urinary bladder, breast, colorectal, and lung cancers. This overexpression has been linked to enhanced growth properties and etoposide (cancer medication) resistance in human breast cancer cells. Here, we describe the identification of novel and effective NAT1 inhibitors.

## Methods

- Two *in silico* searches for small molecule inhibitors that bind to the active site of human NAT1 were performed.
- The *in silico* screening program Surflex-Dock 2.3 was used to conduct a virtual search that screened small molecule inhibitors that had been added to the ZINC library since the previous search had been conducted. Sixty-five compounds were identified in the virtual search as potential inhibitors, scored, and ranked based on their active site association.
- The *in silico* screening program Surflex-Sim was used to conduct a similarity search that screened compounds that were structurally similar to compound 10, a small molecule inhibitor that was discovered in a previous screening and was found to be an effective inhibitor of human NAT1. Ninety-seven compounds were identified in the similarity search as potential inhibitors, scored, and ranked based on their active site association.
- Based on their commercial availabilities, 35 compounds were tested for their ability to inhibit NAT1 activity. The $IC_{50}$ values of the most efficacious inhibitors (compounds 63, 66, 72, 86, and 95) were determined *in vitro* against human NAT1.
- Further studies were completed using the most potent inhibitor of the lead compounds, compound 66.




Adapted from Bendaly et al., 2007.



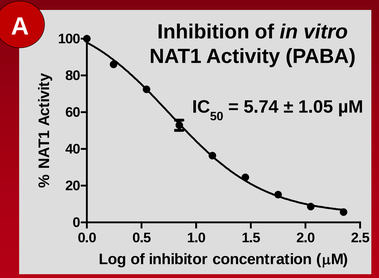

## Results

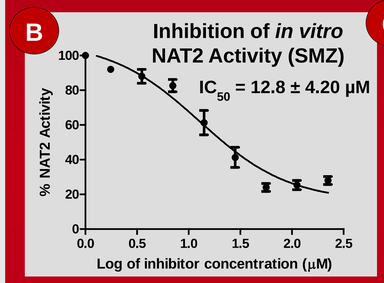
| Compound # | Screening Source | % NAT 1 Inhibition |
|---|---|---|
| 61 | Virtual | 0.00% |
| 62 | Virtual | 40.4% |
| **63** | **Similarity** | **89.2%** |
| 64 | Similarity | 32.6% |
| 65 | Similarity | 46.1% |
| **66** | **Similarity** | **93.1%** |
| 67 | Virtual | 14.3% |
| 68 | Virtual | 0.00% |
| 69 | Virtual | 33.3% |
| 70 | Virtual | 23.8% |
| 71 | Virtual | 38.1% |
| **72** | **Similarity** | **78.1%** |
| 73 | Virtual | 0.00% |
| 74 | Similarity | 13.9% |
| 75 | Similarity | 35.3% |
| 76 | Virtual | 12.7% |
| 77 | Virtual | 0.00% |
| 78 | Virtual | 0.00% |
| 79 | Virtual | 0.00% |
| 80 | Virtual | 0.00% |
| 81 | Virtual | 0.00% |
| 82 | Virtual | 21.5% |
| 83 | Virtual | 0.00% |
| 84 | Similarity | 0.00% |
| 85 | Virtual | 70.1% |
| **86** | **Virtual** | **76.8%** |
| 87 | Virtual | 0.00% |
| 88 | Virtual | 27.6% |
| 89 | Virtual | 0.00% |
| 90 | Virtual | 4.7% |
| 91 | Similarity | 70.9% |
| 92 | Virtual | 32.2% |
| 93 | Virtual | 0.00% |
| 94 | Similarity | 55.4% |
| **95** | **Virtual** | **79.4%** |

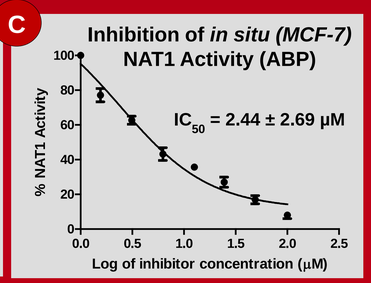
***TABLE 1*: Percent inhibition of human NAT1 activity *in vitro*. The compounds in red inhibited human NAT1 activity greater than 75%.**

| Compound # | *In vitro* NAT1 $IC_{50}$ (μM) |
|---|---|
| 63 | 66.1 ± 7.56 |
| **66** | **5.74 ± 1.05** |
| 72 | 161 ± 43.6 |
| 86 | 100 ± 9.56 |
| 95 | 102 ± 17.0 |

***TABLE 2*: Lead compounds $IC_{50}$ *in vitro* against human NAT1 (PABA). The compound in red is the most potent, and therefore used for further experiments.**




**FIGURES A-C: *A*. *In vitro* NAT1 activity was determined using 100 μM acetyl coenzyme A and 300 μM p-aminobenzoic acid (PABA) with varying concentrations of compound 66 (0 – 100 μM) in yeast lysates that express recombinant human NAT1. *B*. *In vitro* NAT2 activity was determined using 100 μM acetyl coenzyme A and 300 μM sulfamethazine (SMZ) with varying concentrations of compound 66 (0 – 100 μM) in yeast lysates that express recombinant human NAT2. C. In situ NAT1 capacity was determined in human breast cancer cells (MCF-7) that express endogenous NAT1. These cells were treated with media supplemented with 6.67 μM ABP or PABA with varying concentrations of compound 66 (0 – 100 μM). Reaction products were collected and analyzed using high pressure liquid chromatography (HPLC) to determine the amount of acetylated product.**

## Conclusions

- We have identified 5 novel inhibitors of human NAT1 and have experimentally determined their IC50 values *in vitro*.
- Based on these data, compound 66, is the most effective and potent NAT1 inhibitor. Compound 66 was also an effective inhibitor of endogenous NAT1 activity in MCF-7 (human breast cancer cells) using in situ assays. These data show promising results for further studies.
- Further *in situ* studies are needed using compound 66, to evaluate the compounds ability to inhibit cell invasion, proliferation, and also metastasis which are all hallmarks of cancer.

## Acknowledgements

Partially supported by the University of Louisville Cancer Education Program (NCI R25-CA134283).

# Echinomycin Decreases Cell Viability of Pancreatic Adenocarcinoma Cells through Inhibition of Autophagy

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[1] Middle Tennessee State University, [2] Department of Medicine, University of Louisville

## ABSTRACT

Objective: Pancreatic carcinomas are the fourth leading cause of cancer death in the United States. Because pancreatic cancers respond poorly to conventional chemotherapy, investigation of new drug treatment options is needed. We hypothesized that echinomycin, an inhibitor of hypoxia-inducible factor-1 (HIF-1), would significantly decrease cell viability of pancreatic cancer cell lines in a hypoxic environment.

Methods: Highly metastatic pancreatic adenocarcinoma cell lines S2VP10 and S2CP9 and the non-metastatic pancreatic adenocarcinoma cell line MiaPaCa2 were treated with echinomycin in normoxic (20% $O_2$) and hypoxic (5% $O_2$) conditions. Following treatment with 1 nM, 5 nM, 10 nM, 20 nM, and 40 nM echinomycin, cell viability after 24 hours, as a measure of ATP, was compared to an untreated normoxic control in all cell lines. Mechanism of induced cell death by echinomycin was evaluated using cell stress and apoptosis profiling. Proteins from treated cell lines and untreated control cell lines were harvested and analyzed by western blotting to evaluate changes in the autophagy pathway.

Results: In all three cell lines treated with 10 nM of echinomycin, cell viability at twenty-four hours was less than 50% in comparison to the normoxic control. Accumulation of prominent autophagosomes was evident in treated cell lines twelve hours after treatment. Cell stress array revealed a prominent increase in heat shock protein 60 (HSP60) in treated S2VP10 cells in comparison to untreated S2VP10 cells. Although apoptosis array revealed extremely high levels of pro-caspace-3, cleaved caspace-3 did not increase in treated S2VP10 cells. In all treated cell lines, levels of the autophagy marker protein light chain 3-II (LC3-II) were greatly increased in comparison to untreated cell lines.

Conclusion: Our results indicate that echinomycin inhibits cell viability of metastatic pancreatic cancer cells through inhibition of autophagy rather than through apoptosis. Treatment with echinomycin in a hypoxic environment resulted in higher levels of cellular death in comparison to normoxic conditions. Echinomycin has potential to be used in combination drug therapy as a treatment for pancreatic adenocarcinoma.

## MATERIALS AND METHODS

### Cell Lines

Highly metastatic pancreatic adenocarcinoma cell lines S2VP10 and S2CP9 and the non-metastatic cell line MiaPaCa2 were maintained in RPMI or DMEM cell culture medium supplemented with 1% L-Glutamine and 10% FBS.

### Treatment and Cell Viability

Cells were treated with 1 nM, 5 nM, 10 nM, 20 nM, and 40 nM of echinomycin and incubated in normoxic (20% oxygen) and hypoxic (5% oxygen) conditions. Following treatment for 24 hours, cell viability, as a measure of ATP, was compared to an untreated normoxic control in all cell lines with an ATPLite™ Luminescence Assay System.

### Protein analysis

The mechanism of induced cell death under hypoxic conditions for untreated S2VP10 control cells and S2VP10 cells treated with echinomycin for 24 hours was then evaluated using R&D Systems human cell stress array and apoptosis arrays in untreated S2VP10 control cells and S2VP10 cells treated with 5 nM (cell stress array) or 10 nM (apoptosis array) echinomycin for twenty-four hours under hypoxic and normoxic conditions. Proteins from untreated control cell lines and cell lines treated with 10 nM echinomycin for twelve and twenty-four hours were harvested. Proteins harvested after twenty-four hours of echinomycin treatment were analyzed for the autophagy marker protein LC3-II by Western blotting. Proteins harvested after 12 hours of echinomycin treatment were analyzed by Western blotting for both the LC3-II protein and the autophagy marker protein autophagy protein five (Atg-5).

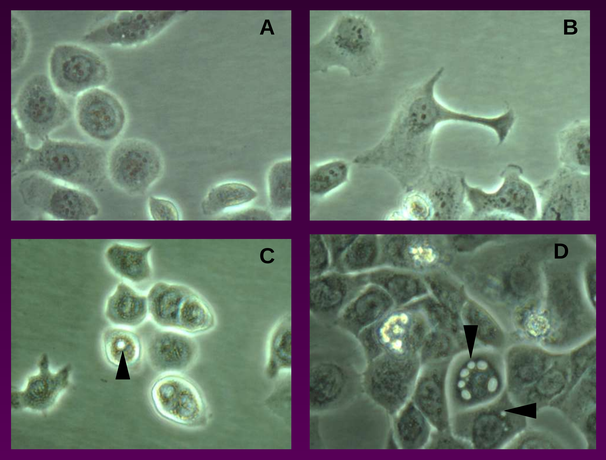


**Figure 1. Cellular morphology 12 hours after echinomycin treatment.**
Untreated S2VP10 cells in normoxic conditions, 20% oxygen, (A) or hypoxic condtions, 5% oxygen, (B) after twelve hours. S2VP10 cells treated with 10 nM echinomycin in normoxic conditions (C) or hypoxic conditions (D) after twelve hours. Black arrows indicate the presence of prominent autophagosomes.

## Results

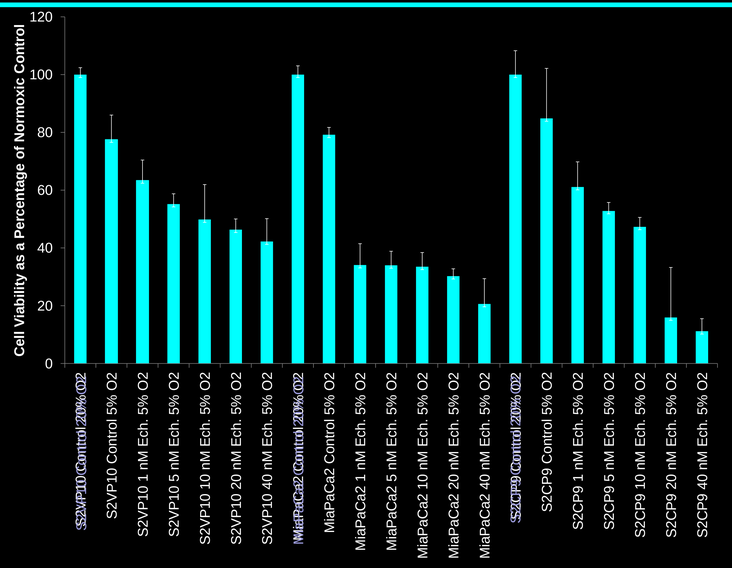


**Figure 2. Cell viability after 24 hours of echinomycin treatment.**
S2VP10, S2CP9, and MiaPaCa2 cells treated with echinomycin or untreated control cells were placed in both normoxic (20% oxygen) and hypoxic (5% oxygen) conditions for twenty-four hours. Viability of cells placed in hypoxic conditions, as a measure of ATP, was compared to an untreated normoxic control in all cell lines.

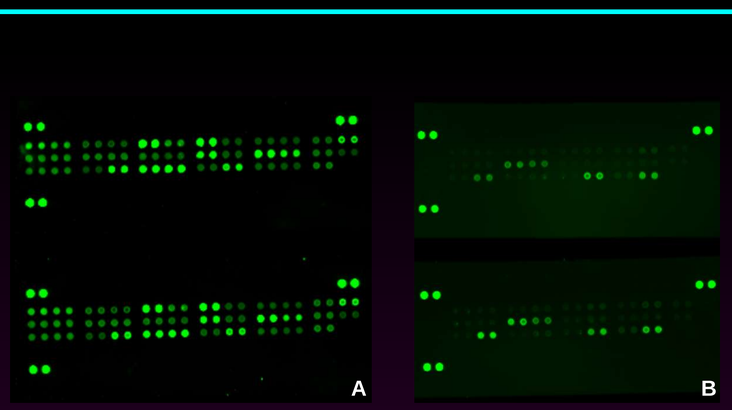


**Figure 3. Human apoptosis array (A) and cell stress array (B) membranes.**
Proteins harvested from untreated control S2VP10 cells in hypoxic conditions and S2VP10 cells treated with 10 nM echinomycin for twenty-four hours in hypoxic conditions were incubated on nitrocellulose membranes containing binding sites for thirty-five different apoptosis-related proteins. (B) Proteins harvested from untreated control S2VP10 cells in hypoxic conditions and S2VP10 cells treated with 5 nM echinomycin for twenty-four hours in hypoxic conditions were incubated on nitrocellulose membranes containing binding sites for twenty-six cell stress related proteins.

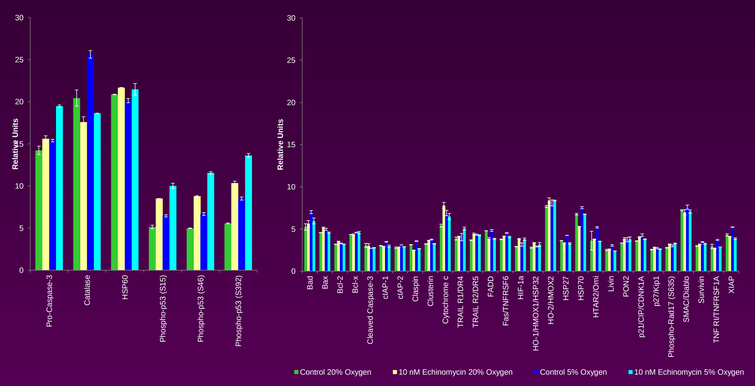


**Figure 4. Human apoptosis array.**
Proteins harvested from untreated S2VP10 control cells placed in both normoxic and hypoxic conditions for 24 hours and S2VP10 cells treated with 10 nM echinomycin for 24 hours in both normoxic and hypoxic conditions were incubated on nitrocellulose membranes containing binding sites for thirty-five different apoptosis related proteins. Relative levels of proteins were compared to standard reference spots on the membranes.

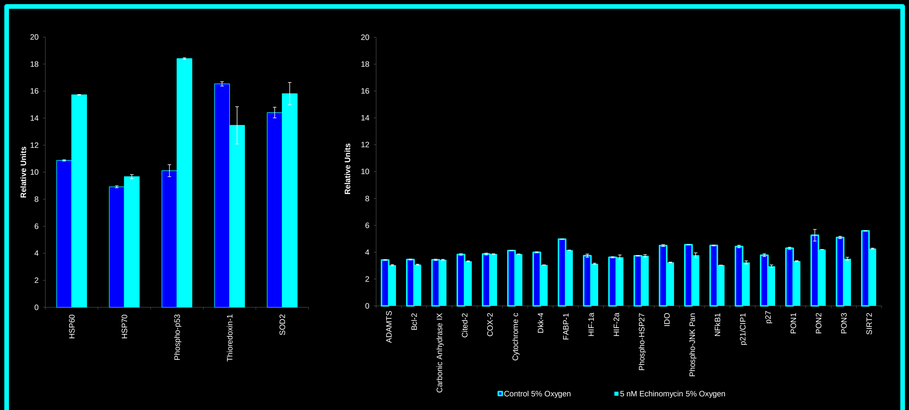


**Figure 5. Human cell stress array.**
Proteins harvested from both untreated S2VP10 control cells placed in hypoxic conditions and S2VP10 cells treated with 5 nM echinomycin for 24 hours in hypoxic conditions were incubated on nitrocellulose membranes containing binding sites for twenty-six cell stress related proteins. Relative levels of proteins were compared to standard reference spots on the membranes.

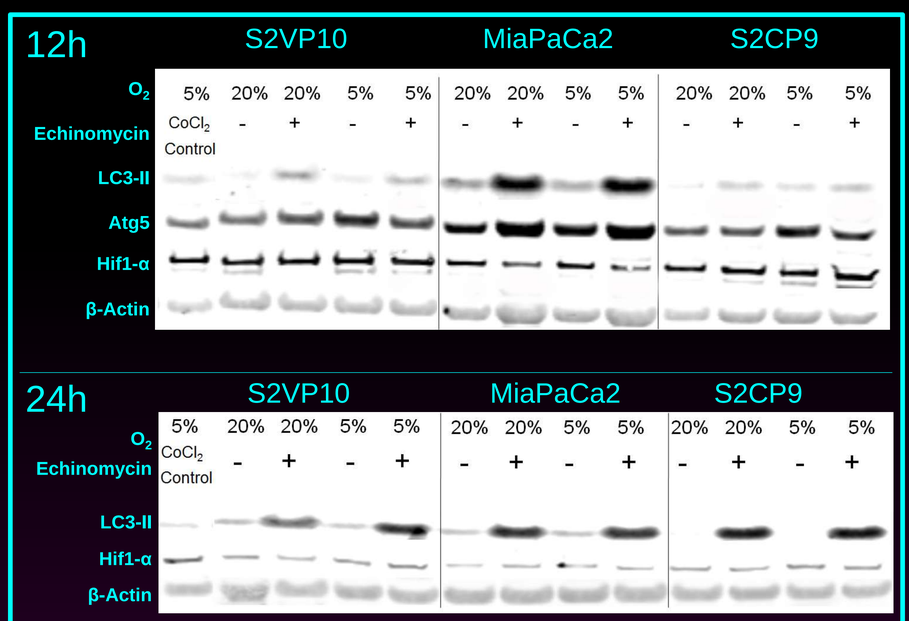


**Figure 6. Western blot analysis of S2VP10, MiaPaCa2, and S2CP9 proteins from untreated control cells or cells treated with 10 nM echinomycin for 12 and 24 hours.**
Treated cells showed a greater increase in the autophagy marker proteins LC3-II in comparison to untreated control cells. Oxygen level did not alter expression of LC3-II. Hif1-α levels were reduced with Echinomycin treatment in MiaPaCa2 cells at 12 h, but not significantly altered at 24h. ATG5 was altered in less aggressive MiaPaCa2 cells at 12 h in accordance with the increase in LC3-II.

## CONCLUSIONS

- Echinomycin treatment of pancreatic adenocarcinoma cells resulted in microscopic vesicles.
- Echinomycin treatment decreases pancreatic adenocarcinoma cell viability through inhibition of autophagy, as demonstrated by LC3-II protein analysis, rather than through apoptosis.
- Echinomycin treatment causes a greater decrease in cell viability in hypoxic conditions in comparison to normoxic conditions.

## Future Directions

- Evaluate in vivo therapeutic efficacy of Echinomycin for pancreatic adenocarcinoma.
- Further evaluate mechanism of action for Echinomycin

## ACKNOWLEGEMENTS

This project was supported by (NCI grant R00-CA139050) and the R25 Cancer Education Program at the University of Louisville (NIH/NCI grant R25-CA134283).

# Examination of Combined Effects of Well-done meat, and Rapid *N*-acetyltransferase 1 and 2 on Breast Cancer Risk among Participants of the Iowa's Women's Health Study

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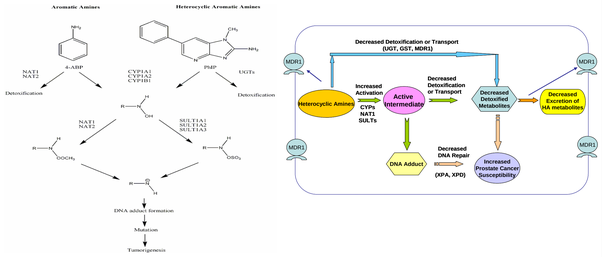
[1]Department of Pharmacology & Toxicology, School of Medicine, University of Louisville (UofL), Louisville, KY.

## INTRODUCTION

***Meat and Cigarette Derived Pro- carcinogens and Their Role In Breast cancer (BrCa)***

- Potent meat- or cigarette-derived pro-carcinogens have been implicated in BrCa development.
  - e.g., polycyclic aromatic hydrocarbons (PAH), heterocyclic amines (HCA) and aromatic amines
- Following metabolic activation, resulting genotoxic species may lead to DNA adduct formation and cause tumors in rat mammary glands.







***Epidemiological Evidence of the Role of Cooked Meat, Meat Doneness & Variant Carcinogen Metabolism Genes on BrCA***

| Allele/Genotype | Exposure | OR (95%CI) | Reference |
|---|---|---|---|
| --- | Fried/broiled Meat | | Zheng et al., 1998 |
| | Fish | 4.7 (1.9-12.2) | |
| | Poultry | 16.2 (9.5-27.1) | |
| | Beef | 2.21 (1.30-3.77) | |
| | Bacon | 1.64 (0.92-2.93) | |
| | Combined Meat | 5.1 (1.3-14.2) | |
| --- | Well done meat | | Zheng et al., 2009 |
| --- | IQ | 3.3 (1.8-6.0) | DeStefani et al., 1997 |
| | MeIQx | 2.1 (1.3-3.6) | |
| | PhIP | 2.6 (1.4-4.7) | |
| CYP1A2*F CC | --- | 2.75 (1.47-5.14) | Sangrajrang et al., 2009 |
| NAT1*11 | --- | 3.9 (1.5-10.5) | Zheng et al., 1999 |
| NAT1*11 | Smoking | 13.2 (1.5-116.0) | Zheng et al., 1999 |
| NAT2*Slow | --- | 1.5 (1.2-1.8) | Collishaw et al., 2009 |
| NAT2 Rapid/intermed. | Smoking | 5.0 (1.5-16.8) | Dietz et al., 2000 |
| NAT2 Rapid/intermed. | Well done meat | 3.3 (1.6-6.8) | Dietz et al., 2000 |

## RESEARCH OBJECTIVE

- We evaluated individual effects as well as complex interactions among three variant carcinogen metabolism genes, tobacco smoking, and preferences towards meats prepared well done as modifiers of breast cancer

## HYPOTHESIS

- Individuals who inherited two or more high-risk carcinogen metabolism genes (*CYP1A2*F, NAT1*10/11,* slow *NAT2*5,*) would have an increased risk of developing breast cancer.
  - These effects may be exasperated by exposure to direct or indirect exposure to cigarette or meat-derived carcinogens

## CLINICAL RELEVANCE

- Individual susceptibility toward cancer depends on whether a person's genetic makeup interacts with environmental exposures to hazardous chemicals.
- Ultimately, we hope to predict in individuals:
  - with a high likelihood to develop BrCa based on their genetic makeup;
  - who would benefit from preventative intervention strategies.

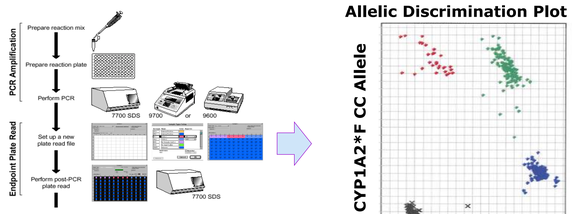
## METHODS

***Study Design***

- Using a nested case-control study design, we evaluated the independent and joint modifying effects of carcinogen metabolism related SNPs and lifestyle factors in relation to BrCa risk.
- Two hundred seventy-three BrCa cases and 657 disease-free female participants of the Iowa Women's Health Study (IWHS) cohort were used in the current study.

***Genetic Analysis***

- CYP1A2 and NAT SNPs were detected and evaluated in germ-line DNA using RFLP-PCR and RT-PCR Taqman assays, respectively.




***Statistical Analysis***

***Logistic Regression Modeling***

$\text{Logit} = \beta_0 + \beta_{1*}\text{environment}$

$\text{Logit} = \beta_0 + \beta_{1*}\text{gene}_1$

$\text{Logit} = \beta_0 + \beta_1\text{gene} + \beta_2\text{gene}_2 + \beta_3\text{gene}_1{*}\text{gene}_2$

$\text{Logit} = \beta_0 + \beta_1\text{gene} + \beta_2\text{environment} + \beta_3\text{gene*environment}$

***Figure 1. Multi-factor Dimensionality Reduction (MDR)***

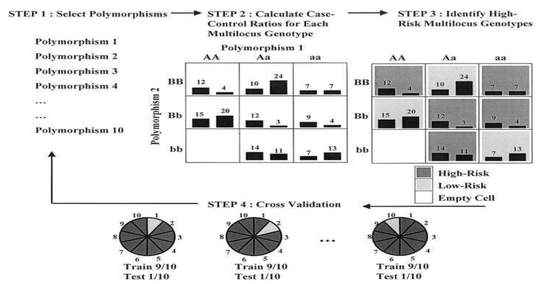
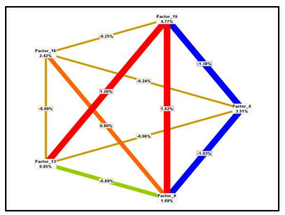



**Figure 2. Entropy Graph**



Red, gold, green, and blue lines indicate strong synergy, moderate synergy, Neutral, moderate redundancy and strong redundancy, respectively.

**Table 1. Demographic and lifestyle Characteristics among Participants of the IWHS**

| Demographic and major risk factors | Cases (n = 273) | Controls (n = 657) | P-value (P-trend) | P-trend |
|---|---|---|---|---|
| Age, median (range) | 61 (55-69) | 61 (55-70) | 0.120 | |
| WHR, median (range) | 0.83 (0.65-2.05) | 0.82 (0.55-1.64) | 0.028 | |
| Family History, n (%) | | | | |
| No | 226 (82.8) | 589 (89.6) | 0.004 | |
| Yes | 47 (17.2) | 68 (10.4) | | |
| BMI† | | | 0.088 | 0.032 |
| Underweight or Normal, n (%) | 97 (35.5) | 271 (41.2) | | |
| Overweight, n (%) | 105 (38.5) | 255 (38.8) | 0.398 | |
| Obese, n (%) | 71 (26.0) | 131 (19.9) | 0.028 | |
| Age at menarche, n (%) | | | 0.381 | |
| > 13 years of age | 70 (25.6) | 187 (28.5) | | |
| ≤ 13 years of age | 203 (74.4) | 470 (71.5) | | |
| Age of last menstruation, n (%) | | | 0.824 | |
| ≤ 50 yrs | 170 (62.3) | 404 (61.5) | | |
| >50 yrs | 103 (37.7) | 253 (38.5) | | |
| # of live births, n (%) | | | 0.152 | |
| < 3 | 166 (60.8) | 432 (65.8) | | |
| ≥ 3 | 107 (39.2) | 225 (34.2) | | |
| Age at 1st live birth, n (%) | | | 0.697 | |
| <25 yrs | 185 (72.6) | 436 (71.2) | | |
| ≥25 yrs | 70 (27.4) | 176 (28.8) | | |
| No live births | 18 (6.6) | 45 (6.7) | | |
| Alcohol intake, n (%) | | | 0.550 | |
| < 30 g/day | 264 (96.7) | 640 (97.4) | | |
| ≥ 30 g/day | 9 (3.3) | 17 (2.6) | | |
| Smoking Status | | | 0.405 | |
| Never smoker | 179 (66.5) | 448 (69.3) | | |
| Ever smoker | 90 (33.5) | 198 (30.7) | | |
| Missing | 4 (1.5) | 11 (1.7) | | |
| Red Meat Consumption, n (%) | | | 0.181 | 0.321 |
| 1st quartile | 63 (23.1) | 170 (25.9) | | |
| 2nd quartile | 62 (22.7) | 170 (25.9) | 0.939 | |
| 3rd quartile | 67 (24.5) | 166 (25.2) | 0.680 | |
| 4th quartile | 81 (29.7) | 151 (23.0) | 0.067 | |
| Doneness Preference, n (%) | | | 0.0008 | 0.003 |
| Rare or medium | 60 (24.6) | 224 (37.9) | | |
| Well done | 71 (29.1) | 155 (26.2) | 0.0086 | |
| Very well done | 113 (46.3) | 212 (35.9) | 0.0002 | |
| Well and very well done | 184 (75.4) | 367 (62.1) | 0.0002 | |

**Table 2. Relationship between Carcinogen Metabolism Genes and Breast Cancer Risk among WHS Participants.**

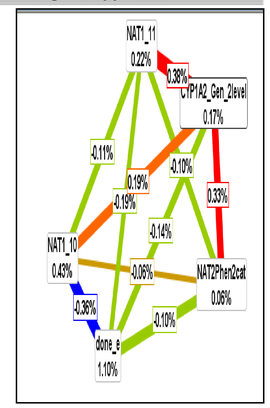
| Genetic Variants | Cases (n=273) | Controls (n = 657) | $OR_{adj}$$ | p-value |
|---|---|---|---|---|
| CYP1A2*F genotypes (phenotypes) | | | | 0.368 |
| CC (low inducibility) | 10 (5.9) | 37 (9.4) | 1.00 (referent) | |
| AC (intermediate inducibility) | 70 (41.4) | 151 (38.2) | 1.52 (0.70-3.27) | 0.161 |
| AA (high inducibility) | 89 (52.7) | 207 (52.4) | 1.49 (0.70-3.17) | 0.220 |
| AA vs. (CC/AC) | 89 (52.7) | 207 (52.4) | 1.05 (0.73-1.52) | 0.955 |
| Missing | 104 (38.0) | 262 (39.9) | | |
| NAT1*alleles | | | | 0.045 |
| *3/*4 alleles | 107 (53.5) | 300 (64.1) | 1.00 (referent) | |
| *10 allele(s) | 67 (33.5) | 121 (25.9) | 1.54 (1.08-2.18) | 0.020 |
| *11 allele(s) | 13 (6.5) | 7 (3.6) | 2.26 (1.06-4.80) | 0.053 |
| *14, *15, *17 or *22 allele(s) | 13 (6.5) | 30 (6.4) | 1.26 (0.64-2.50) | 0.635 |
| Missing | 73 (26.7) | 189 (28.8) | | |
| NAT2 Phenotype | | | | |
| Slow or very slow acetylators | 109 (53.7) | 282 (56.4) | 1.00 (referent) | |
| Intermediate acetylators | 81 (39.9) | 187 (37.4) | 1.15 (0.81-1.64) | 0.514 |
| Rapid acetylators | 13 (6.4) | 31 (6.2) | 1.00 (0.50-2.01) | 0.815 |
| Intermediate and rapid acetylators | 94 (46.3) | 218 (43.6) | 1.13 (0.81-1.58) | 0.513 |
| Missing | 70 (25.6) | 157 (23.9) | | |

**Table 3. Joint Modifying Effects among Variant Carcinogen Metabolism Genes and Well Done Meat in Relation to Breast Cancer Risk among IWHS Participants.**

| Reference Group | Risk Genotype | Exposure | ORadj (95%CI) |
|---|---|---|---|
| CYP1A2*slow/inter NAT1*3/*4 | CYP1A2*slow/inter NAT1*10 | --- | 2.43 (1.42-4.17) |
| | CYP1A2*slow/inter NAT1*11 | --- | 10.54 (2.74-40.52) |
| | CYP1A2*rapid NAT1*10 | | 1.91 (1.14-3.19) |
| | CYP1A2*rapid NAT1*11 | | 2.74 (1.10-6.84) |
| NAT2*slow rare/medium meat intake | NAT2*slow | Well / Very Well Done Meat | 2.05 (1.15-3.65) |
| | NAT2*rapid/inter | | 2.47 (1.37-4.44) |
| NAT1*3/*4 | NAT1*3/4 | | 1.82 (1.10-3.00 |
| | NAT1*10/11 CYP1A2*slow/inter | | 2.61 (1.52-4.48) |
| | NAT1*10 CYP1A2*slow/ inter | | 2.43 (1.42-4.17) |
| | NAT1*11 CYP1A2*slow/ inter | | 10.54 (2.74-40.52) |
| | NAT1*14,15,17,22 | | 1.91 (1.14-3.19) |

**Table 4. Evaluation of Main Effects and Interactions among Carcinogen Metabolism SNPs as Predictors of BrCa using Entropy Based MDR**

| Best Model | CVC | ATA | MDRpt P-value |
|---|---|---|---|
| One Factor doneness | 10/10 | 0.559 | 0.035 |
| Two Factor Doneness NAT1_10 | 8/10 | 0.556 | 0.048 |
| Three Factor Doneness, NAT1_10, CYP1A2 | 9/10 | 0.582 | 0.002 |
| Four Factor NAT2Phen, doneness, NAT1_10, CYP1A2 | 10/10 | 0.590 | 0.002 |




Abbreviations: CVC = Cross validation consistency;
ATA = average testing accuracy; MDRpt P-value = MDR permutation testing p-value

## CONCLUSIONS

- Consumption of well/very well done meat was related to a 1.8-1.9 fold increase in BrCa risk using LR modeling.
- Inheritance of at least one NAT1*10 or NAT1*11 alleles was linked to a 1.5-2.3 fold increase in BrCa risk.
- In an exploratory analysis, complex interactions among 3 carcinogen metabolism genes (CYP1A2, NAT1*10, and NAT2) combined with consumption of meats prepared well done may influence BrCa risk.

## FUTURE DIRECTIONS

- Upcoming studies evaluate complex interactions among biotransformation genes and environmental exposures and their joint effects in predicting individual predisposition toward BrCa Risk
  - This will require a larger sample size and sophisticated visualization tools to evaluate complex interactions.
- Our approach may be used to identify women who would benefit from intervention strategies based on their genetic and lifestyle preferences.

## ACKNOWLEDGEMENTS

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