SIGNAL TRANSDUCTION PATHWAYS IN PROGRESSIVE BREAST CANCER MODEL: EXAMINING CELLULAR STRESS (IRES) PATHWAYS

A Senior Scholars Thesis

by

DONIKA SHPATI

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Biomedical Sciences

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Approved by:

Research Advisor: Raj Venkatraj
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Signal Transduction Pathways in Progressive Breast Cancer Model:
Examining Cellular Stress (IRES) Pathways. (May 2012)

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Breast cancer affects a variety of individuals and encompasses a vast number of treatment plans. Two commonly used agents, Cyclophosphamide and Adriamycin, will be used at clinical dosage on a progressive breast cancer cell line model. By studying four cancer cell lines, it enables the examination of breast cancer pathways from precancerous to highly aggressive tumor growth. Preliminary results have shown that the hypoxia inducible factor pathway is one of the key pathways altered. Using Real Time PCR, we will study the gene expression changes in HIFa, as well as many additional stress related internal ribosomal entry site genes, which aid in tumor growth. Our findings highlight HIFa, RUNX1, VEGF and BIRC2 as integral components of breast cancer pathways. Our next step includes blocking one gene with RNA-i technology, to create possibilities of new cancer treatment and alternate pathways. Targeting these pathways can lead to inhibiting tumor growth and preventing its reoccurrence.

ACKNOWLEDGEMENTS

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CHAPTER I

INTRODUCTION

Breast cancer is the most common cancer in women in the developed world [1]. Recently, tremendous progress has been made using global gene expression technology (transcriptome) to understand the basis of breast cancer progression. This knowledge in gene expression patterns has not only furthered our understanding of the molecular basis of breast cancer progression, but also developed specific targeted therapy on alternate genetic pathways [2,3]. Breast cancer is a heterogeneous disease that is progressive both genetically and histologically. The three common types of breast cancer are atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCIS) and invasive ductal carcinomas (IDC) [3,4]. Subtyping of breast cancer cells using molecular profiling has resulted in at least 6 types of breast cancer, classified as Basal-Like, ERBB2+, Normal Breast-Like, Luminal Subtype A, B, and C [5]. Each subtype has a heterogeneous cell population and needs both global chemotherapeutic agents, such as C and A, and targeted chemotherapeutic agents, such as Trastuzumab, an antibody specifically against Her2/neu.

This thesis follows the style of Journal of Molecular Medicine.

Chemotherapy

Adriamycin, A, is a cytotoxic anthracycline that binds to DNA (more active in dividing cells) and inhibits nucleic acid synthesis, while cyclophosphamide, C, is a synthetic antineoplastic drug related to nitrogen mustard. In combination, they have been the work horse among chemotherapeutic agents for the past several years. Although they have been successful as chemotherapeutic agents, there have been a significant number of cases acquiring resistance to these drugs. From my initial studies using this breast cancer cell line model, I uncovered a key signal transduction pathway wherein HIF gene expression was altered.

Hypoxia inducible factor

HIF is key protein that has been indicated as a player in drug resistance and breast cancer metastasis [6, 7]. Cancer cells compete for oxygen and thrive on it by taking advantage and up regulating genes such as HIF A, which serve to mediate cell responses during a state of low oxygen concentration and cellular stress. Blocking the HIF pathway is suggested to be a useful therapy for cancer treatment; however, these cells continuously create new pathways to survive [8].

IRES genes are cap independent genes that are translated mostly under cellular stress and have received increased attention as recent evidence points to their significance in both physiological and pathological stress conditions [9]. Currently, there are about 54 IRES genes that are involved in various signal transduction pathways, such as HIF, MYC, IGF-

2, VEGF, NOTCH, and several others [10]. Comprehensive knowledge of IRES genes and their interplay in breast cancer specifically, is severely lacking. Since IRES genes are selected by evolutionary pressure over time to deal with cellular stress, they may be important during cancer and its treatment, especially in the time period of development of resistance during the clonal evolution of cancer cells, adapting to the chemotherapeutic agent.

CHAPTER II

METHODS

Tissue culture

Four progressive breast cancer cell lines from plain transformed to cell line with metastatic potential, (MCF 10A) NeoT, 1A, 1H and a) were obtained from ATCC. These cell lines were grown in DMEMF medium supplemented with 15% horse serum, 10mig/mL insulin, 0.5mig/mL hydrocortisone, 100 ng/mL cholera toxin, 20ng/mL EGF and 5mL pen/strep. 3.5 X 10^5 cells were plated in triplicates in 100mM petri dishes. After 24 hours, they were exposed to chemotherapeutic agents, Adriamycin (A) and Cyclophosphamide (C), at clinical doses of 60mg/m^2 and 600mg/m^2, respectfully. Each of the cell lines was treated individually with A, C, and also in combination (A+C). Cells were incubated for 4 hours and 24 hours before extracting RNA.

RNA extraction

Total RNA was extracted following the manufacturer's instruction (Stratagene Absolutely RNA Miniprep Kit catalog #400800) after exposure of 4 and 24 hours. Briefly, cells were thoroughly lysed using the lysis buffer provided. The homogenate underwent a micro centrifuge spin to retain the RNA in an RNA binding receptacle tube. The RNA was later collected into a micro centrifuge tube using appropriate buffers that

released RNA from the filter. RNA quality control was performed running 4μL of each sample through gel electrophoresis with a 1% agarose gel at 70V for 2 hours. The gel was evaluated and image capturing was done using Alpha Innotech system. Nano spectrometry evaluations for each RNA sample were quantified.

cDNA synthesis

cDNA was synthesized following manufacturer's instructions applying the AffinityScript QPCR cDNA Synthesis Kit. 5 μg of RNA was used to synthesize cDNA. The quality and quantity of cDNA were also calibrated using gel electrophoresis and Nano spectrometry.

Real time PCR

100 ng of each sample was amplified using specific primers for 40 cycles in Strata gene 3000px real time PCR machine. The annealing temperature was 60C for all primers.

Fold change calculation

The fold changes of gene expression were evaluated using the Comparative CT methodology, a relative quantification methodology, based upon calibration of relative gene expression using internal control [11]. Ingenuity Pathway Systems (IPA), commercially available software, was used to analyze the complex biological signaling network emanating from our data [12]. IPA provides insight into the causes of observed gene expression changes and into the predicted downstream biological effects of those changes using data from extensive literature survey and current data provided.

CHAPTER III

CONCLUSION

Results

Our results, as illustrated in the bar diagram below, pointed to a few critical pathways that are affected by Adriamycin and Cyclophosphamide, used in combination. Expressions showed to be especially high in categories include cell death, cell development, cell growth and proliferation and cell cycle. Evidence also exists for variations in gene expression pattern at the 4 and 24 hour, both between and within cell lines.

The two least aggressive cell lines, MCF-10A (10A) and MCF 10A-NeoT (NeoT), do not cause tumors in nude (immune compromised) mice when injected. The key pathways affected differ not only between cell lines, but also with exposure time. For example, in cell line 10A, the signaling involved in molecular transport is upregulated in the first four hours, yet after 24 hours, cell death genes have been activated.

There are clear differences in signaling between specific drug administration (A, C or A+C) and cell lines. For example, all three combinations are active in MCF 10-1H (1H) in both 4 and 24 hours treatments. Conversely, treatment with only C has little impact

when used as a monotherapy in cell line 10A, 24 hour. Interestingly, Neo T illustrates synergy between the drugs, however only in the 24 hour treatment. A+C combination is more active after longer exposure to chemotherapeutic agents, while it shows no expression at 4 hours for this cell line. As illustrated in Figure 1, in our most aggressive cell line, 1H, the combination treatment, A+C, consistently provides higher expression values when compared to A only or C only treatment.

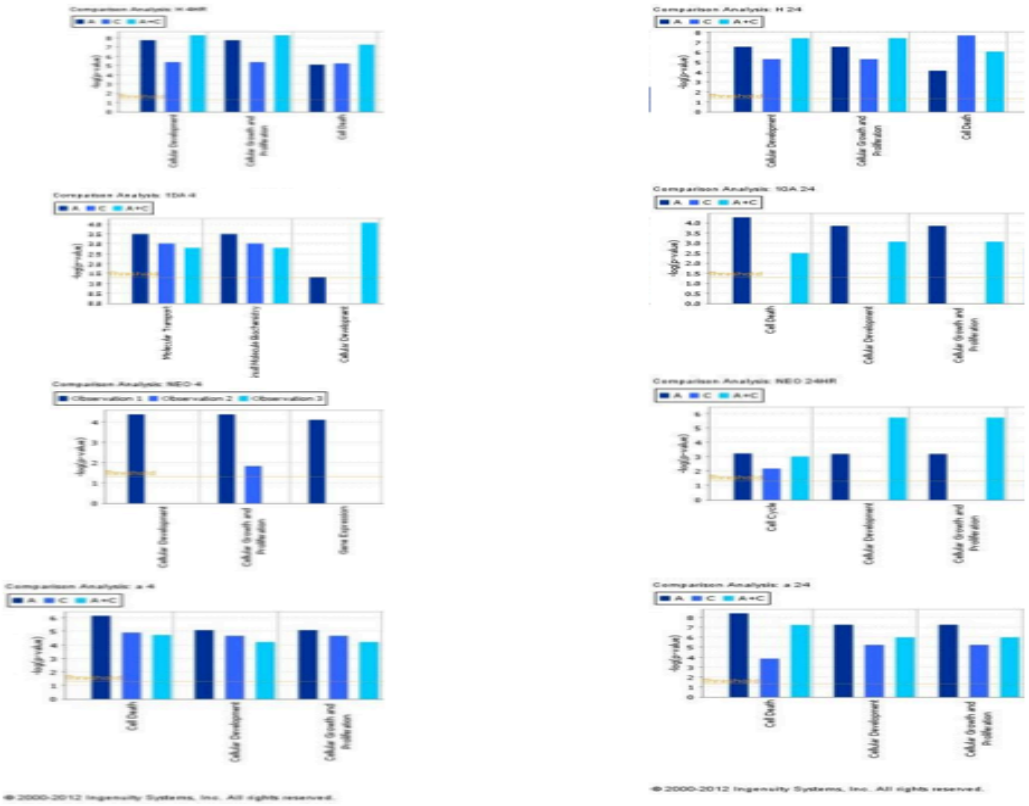

Figure 1 Functions of Most Expressed Genes. This chart illustrates the differences between functions of gene expression among the four progressive cell lines and between the three different treatments provided A, C, & A+C [12].

Networks

A noteworthy result from our study is the clear finding of upregulated genes involved in growth promotion, such as IGF2, FGF2 and CYR6, as well as genes in the anti-apoptotic pathway, including BIRC2.

The two key tumor suppressor genes, RB and TP53, are differentially expressed at the 4 and 24 hour treatments in 10A, reflecting a change in their significance over time of exposure. NAT1 (N-acetyltransferase), a gene whose product is critical in activation of xenobiotic and chemotherapeutic agents, is also highly upregulated as illustrated in figure 2. There is also a significant change in IGF2 expression between 4 and 24 hours, which may lead to interesting conclusions, as IGF1 regulation is well known in breast cancer.

As in the previous cell line, IGF2 is much more upregulated at 24 hours than 4 hours in NeoT cell line. However, ACTB (beta actin), which participates in both cell structure and motility, shows to be highly expressed in the 4 hour. The oscillations between TP53, and MDM2 (which regulates TP53), vary between the 4 and 24 hour, reflecting gene expression changes between them.

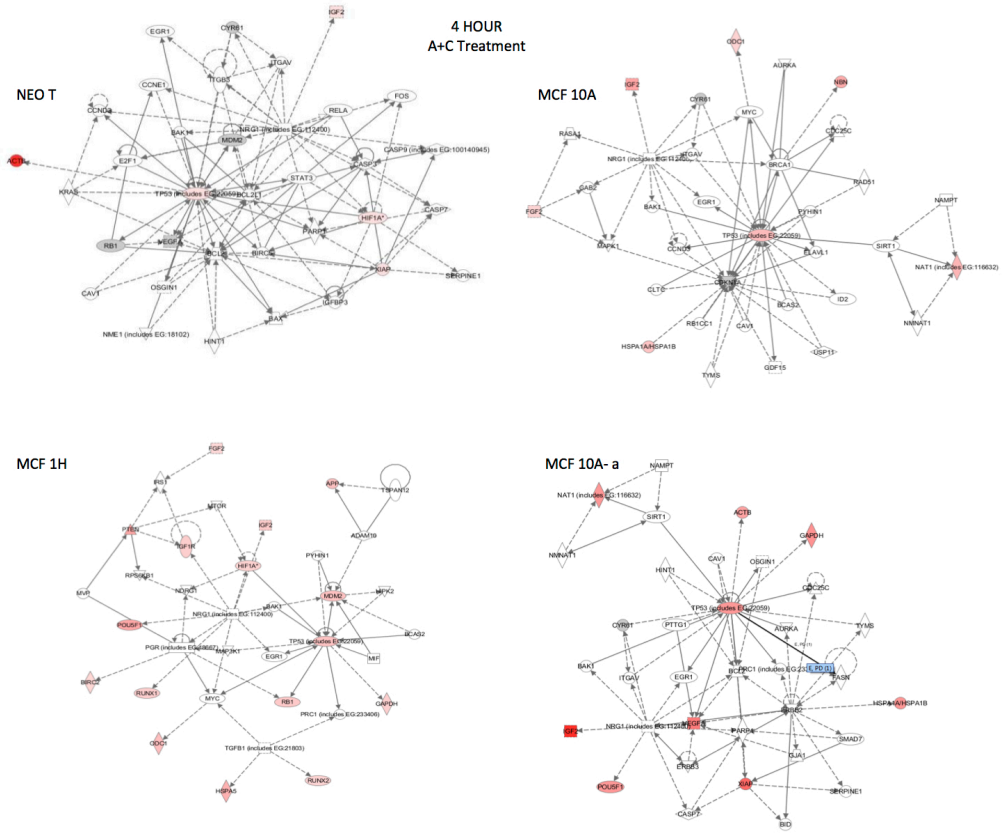

Figure 2 Treatment A+C, 4 Hours. These pathways show major up and downregulated genes when exposed to combination drugs for four hours. Red colored symbols identify those genes in which upregulation is evident [12].

For cell line MCF 10A-a (10A-a) there were common genes that were upregulated, as evidence of expression of IGF2, POU5F1 and VEGF in Figure 2. VEGF is a pro-angiogenic factor, increasing blood supply to tumors and is has high expression in 10A-a, which is one of the two most aggressive forms. There are also differences in gene expression pattern, such as NAT1, involved in oxidative metabolism, upregulated at 4 hour (early exposure) and RUNX2, a transcription factor involved in bone metabolism and development, which may cause bone metastasis.

MCF 1H cell line highlights many of the tumor suppressor genes, including RB1, TP53 and MDM2, which are upregulated in both 4 and 24 hours. Interestingly, HIFa, hypoxia inducible factor, is upregulated in the 4 hour, while Figure 3 does not depict HIF upregulated in the 24 hour exposure. Also, ODC1, a gene involve in polyamine biosynthesis and a progrowth factor, is seen in both time exposures, but has several folds higher in 24 hour. The RUNX 1 and 2 transcription factors were also upregulated in both 4 and 24 hour treatments.

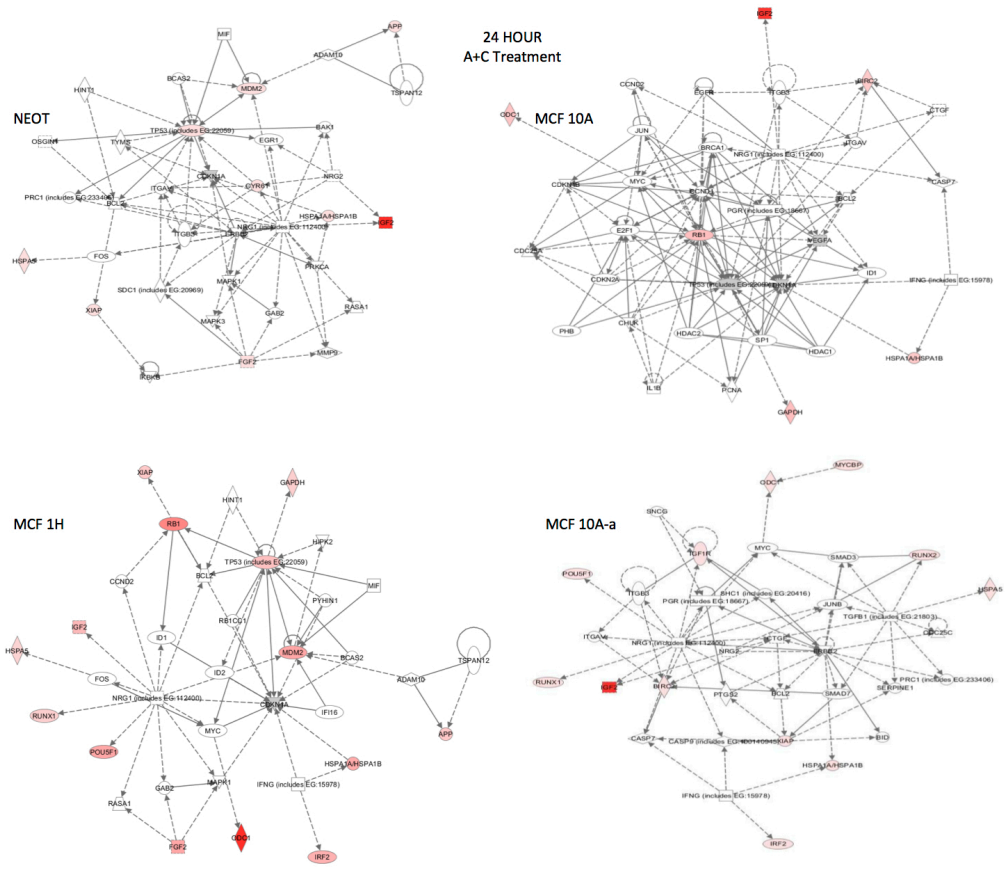

Figure 3 Treatment A+C, 24 Hours [11]

CHAPTER IV

SUMMARY AND DISCUSSION

In these studies, we found that less aggressive cell lines (Neo T and 1A) had fewer gene expression changes than our more aggressive cell lines (a and H). This reflects an increase in complexity of signal transduction pathways recruited as a response to chemotherapeutic agents by these cell lines. As the tumorigenesis advances, there is an increase in complexity of cellular interactions, inducing several genetic pathways in cell lines a and H that have a tendency to cross talk [13]. This is in agreement with DNA work using comparative genomic hybridization, which found consistently more complex changes in advanced tumors, as when compared to tumors in earlier stages [13].

Upregulated HIFa is considered to be the main cause of increased tumor growth, allowing for increased blood supply and metabolic changes, as well as giving rise to an angiogenesis and metabolic phenotype. There is increased glycolytic flux, also known as Warburg effect, wherein the glycolytic pathway is chosen over the oxidation phosphorylation pathway, producing lactic acid. This acid environment further provides growth advantage to the tumor cells, as when compared to the normal cells.

Future

Since HIFa signaling pathway is critical in tumor aggressiveness, as well as resistance, much work has been done using both RNA-I technology, as well as other inhibitors. MCF 10A-H cell line provides a good model to understand the basis of HIF inhibition in aggressive breast cancer. RNA-inhibition methods to block HIFa will significantly reduce the proliferation of tumor cells. Without HIFa as their main source of oxygen, we expect the tumor cells to adopt new genetic pathways, upregulating other genes to contribute to their growth. Identifying such genes will provide opportunities to further block the proliferation of cancer cells.

Additionally, this work brought forth several other target genes that play a critical role in breast tumorigensis. For example, RUNX1 and RUNX2 are pro-growth genes, which are highly expressed in cell lines a and H. The RUNX (Runt-related transcription factor) family of genes, also known as the acute myeloid leukaemia (AML), core-binding factor- (CBF) and polyoma enhancer-binding protein-2 (PEBP2) family, encode the DNA-binding -chain partners of the heterodimeric CBF complex [14]. These genes have the potential to be classified as both tumor suppressor and oncogenes due to their broad capabilities [14]. There are several genes that are targets of RUNX transcription factor, many of which are both differentiation and growth factors. This suggests that RUNX genes, which were primarily thought to be involved in hematological cancers may play a part in breast cancer, specifically at later stages, as pointed out by expression patterns of cell lines in our experiment. Opportunities to manipulate this pathway for future

endeavors in treating breast cancer may be highly beneficial. Other genes of interest include BIRC2 and VEGF. BIRC2, is a gene member of a family of proteins that inhibits apoptosis by binding to tumor necrosis factor receptor-associated factors TRAF1 and TRAF2 [15]. Meanwhile, VEGF is a growth factor involved in tumor angiogenesis. Both genes are upregulated in a hypoxic environment, which often results in fast growing breast cancer cells. Several drugs and clinical trials targeting both these tumor-driving proteins are present.

Recently, reports on cancer statistics published by CDC highlight encouraging drops in cancer deaths among several categories [16]. We hope our type of experiments will further help in reducing cancer death in breast cancer by allowing development of more tools that can be used in targeted therapy.

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