Caveolin-1: A Potential Biomarker of Aggressive Triple-Negative Breast Cancer in African American Women

by

Julie Getz

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved June 2015 by the
Graduate Supervisory Committee:

Douglas F. Lake, Co-Chair
Lisa Baumbach-Reardon, Co-Chair
Kenro Kusumi
Kimberly Bussey

ARIZONA STATE UNIVERSITY

December 2015

ABSTRACT

In the U.S., breast cancer (BC) incidences among African American (AA) and CA (CA) women are similar, yet AA women have a significantly higher mortality rate. In addition, AA women often present with tumors at a younger age, with a higher tumor grade/stage and are more likely to be diagnosed with the highly aggressive triple-negative breast cancer (TNBC) subtype. Even within the TNBC subtype, AA women have a worse clinical outcome compared to CA. Although multiple socio-economic and lifestyle factors may contribute to these observed health disparities, it is essential that the underlying biological differences between CA and AA TNBC are identified. In this study, gene expression profiling was performed on archived FFPE samples, obtained from CA and AA women diagnosed with early stage TNBC. Initial analysis revealed a pattern of differential expression in the AA cohort compared to CA. Further molecular characterization results showed that the AA cohort segregated into 3-TNBC molecular subtypes; Basal-like (BL2), Immunomodulatory (IM) and Mesenchymal (M). Gene expression analyses resulted in 190 differentially expressed genes between the AA and CA cohorts. Pathway enrichment analysis demonstrated that differentially expressed genes were over-represented in cytoskeletal remodeling, cell adhesion, tight junctions, and immune response in the AA TNBC -cohort. Furthermore, genes in the Wnt/β-catenin pathway were over-expressed. These results were validated using RT-qPCR on an independent cohort of FFPE samples from AA and CA women with early stage TNBC, and identified Caveolin-1 (CAV1) as being significantly expressed in the AA-TNBC cohort. Furthermore, CAV1 was shown to be highly expressed in a cell line panel of TNBC, in particular, those of the mesenchymal and basal-like molecular subtype. Finally,

silencing of CAV1 expression by siRNA resulted in a significant decrease in proliferation in each of the TNBC cell lines. These observations suggest that CAV1 expression may contribute to the more aggressive phenotype observed in AA women diagnosed with TNBC.

DEDICATION

This dissertation is dedicated to my friends and family who have supported me unconditionally throughout this process.

I would like to thank my mother, who showed me that it was never too late to pursue your dreams and for never letting me give up.

Special thanks to my boys, TJ and Jordan, for making sure that the house wasn't on fire and that there was food in the fridge, especially during my late nights and long weekends in the lab.

To my sister, Heather and her family, for sending me love and support.

To my awesome friends Shuk Mei, Kaitlyn and Mary Ellen for their guidance and endless hours of support on and off the lab bench.

To my exceptional interns; Layla, Roberto, Carlie and Davyd who have all worked tirelessly and provided hours of enjoyment at the lab bench.

Thank you to Wendi, the SOLS Graduate Programs Coordinator, for helping me to get through the maze of iPOS and graduate paperwork.

To my very best friend Heidi, and her family, for the love and support only a very best friend can offer.

To my committee members, Dr. Douglas Lake, Dr. Kenro Kusumi and Dr. Kimberly Bussey, for their mentorship and guidance.

Dr. Jeanne Wilson-Rawls for her guidance and support through the more difficult times of my gradate career.

To the late Dr. Richard Shand, who taught me to love research and showed me how to find a research project that 'spoke to me'.

I would like to express my deepest gratitude to my mentor, Dr. Lisa Baumbach-Reardon "LBR", who took a chance on one last graduate student.

To Dr. John Carpten, for allowing LBR to take on one last student and for finding the necessary funds and providing essential guidance to complete this project.

Finally, to my late father, Edward Frances Getz. He was my biggest fan and supported me even through his battle with Alzheimer's.

ACKNOWLEDGMENTS

This project was generously supported by the Translational Genomics Research Institute (TGen), the Virginia C. Piper endowment for cancer research, Saks 5th Avenue Key to the Cure Donation for Breast Cancer Research and greatly benefited by a strong collaboration with Almac Diagnostics. The following grants supported the collection of the original samples and the work with Almac Diagnostics:

- Genome Wide Expression Analysis of Breast Cancer in African-Americans. Department of Defense Concept Awards, 2002 (L. Baumbach, P.I.)
- Evaluation of Genome Wide Changes in Hispanic Breast Cancer Univ of Miami/ Woman's Cancer Association, 2007(L. Baumbach, P.I.)
- Genome Wide Expression Analysis as a Basis of Prognosis in African-American Breast Cancer Patients. Susan G. Komen Foundation for Breast Cancer Research, 2008 (L. Baumbach, P.I.)
- Defining Genomic Changes in Triple Negative Breast Cancer in Women of African Descent. Dept of Defense Breast Cancer Research Program Synergy Grant, 2009 (M. Pegram and L. Baumbach, Co-PI's)
- Defining Ethnic-Specific Transcriptional Differences in Breast Tissue. Bankhead-Coley (State of FL), 2010(L. Baumbach, P.I.)
- Molecular Analysis of Neoadjuvant Platinum in Triple Negative Breast Cancer. NIH-NCI, 2011 (M. Pegram, P.I.; L Baumbach- co-investigator)

I thank all of our very important collaborators that helped move this project from the drawing board to the lab bench:

- Macromolecular Analysis & Processing Center (MAPC)
- Drs. John Carpten, Janine LoBello
- Drs. Sara Nasser, Drs. Venkata 'Teja'Yellapantula, Waibhav Tembe and Christophe Legendre
- University of Miami Hospital & Clinics/Jackson Memorial Hospital
- Drs. Gomez & Dr. Jorda (UM Pathology) and Dr. Mark Pegram, UM Oncology

Finally, thank you to the courageous women who donated tissue samples for research, without whom this work would not be possible.

TABLE OF CONTENTS

Page

LIST OF TABLES vii

LIST OF FIGURES viii

CHAPTER

1 INTRODUCTION 1

2 COMPARISON OF TRANSCRIPTIONAL SIGNATURES FROM U.S. AFRICAN AMERICAN AND CAUCASIAN WOMEN DIAGNOSED WITH EARLY STAGE TNBC IDENTIFIES DIFFERENTIAL EXPRESSION IN KEY ONCOGENIC PATHWAYS

Abstract 13

Material and Methods 14

Results 19

Conclusion 34

3 CAVEOLIN-1 IS EXPRESSED IN AN INDEPENDENT COHORT OF AA-TNBC SAMPLES AND MESENCHYMAL/BASAL-LIKE TNBC CELL LINES

Abstract 37

Material and Methods 37

Results 41

Conclusion 47

4 SILENCING OF CAVEOLIN-1 INHIBITS CELL PROLIFERATION IN TNBC CELL LINES

CHAPTER Page

Abstract 49

Material and Methods 49

Results 53

Conclusion 59

5 SUMMARY AND CONCLUSIONS 60

REFERENCES 65

LIST OF TABLES

Table Page

1. Molecular Subtypes of Breast Cancer: Clinical, Pathological and Molecular Characteristics 3
2. FFPE Tumor Sample Characteristics 20
3. List of Differentially Expressed Genes, AA vs CA 26
4. Clinicopathological Characteristics of the Breast Cancer Cell Line Panel....................42
5. TNBC Node 0, Independent Cohort Clinical Annotation 45
6. CAV1-siRNA Significantly Decreased TNBC Cell Line Proliferation....................58

LIST OF FIGURES

Figure Page

1. Age-Adjusted Breast Cancer Incidence and Death Rates in the U.S. by Race and Ethnicity....11
2. PCA Analysis: No Batch Effect Observed....23
3. Heatmap of TNBC -Node 0, AA and CA Cohorts....25
4. Functionally Enriched Pathways in TNBC -Node 0, AA vs. CA Cohorts....29
5. Representative Wnt Pathway: AA vs CA....30
6. Molecular Subtyping of the TNBC -Node 0, AA and CA Cohorts....32
7. Technical Validation of Relative mRNA Expression of Candidate Genes....34
8. Cell Line Panel Expression of CAV1....44
9. Cav1 Expression is Higher in an Independent Cohort of the AA TNBC....47
10. Overview of Experimental Design....55
11. Validation of CAV1 Silencing of mRNA Expression....56
12. Validation of Cav1 Silencing of Protein Expression....57
13. CAV1 Silencing Inhibits Cell Proliferation in TNBC Cell Lines....58

Chapter 1

INTRODUCTION

Over 235,000 U.S. women will be diagnosed with invasive breast cancer (BC) each year, and it is estimated that over 40,000 women will die from the disease in 2015 (1). Although BC incidences have increased over the past decade, BC mortality has shown a steady decline, most likely due to the availability of screening, earlier detection of breast masses, and targeted therapy. Responses to chemotherapy, hormone and targeted therapy, as well as overall survivorship are highly correlated with the stage at time of diagnosis. The earlier the stage at diagnosis, the better the overall response rate and 5 year survival rate (1,2). Therefore, investigations to improve the understanding of underlying tumor biology of early stage breast cancer and the identification of biomarkers to provide diagnostic and prognostic indicators are imperative.

Advances in understanding breast cancer through molecular subtyping

BC is no longer thought of as a single disease. Instead, it is considered a complex and molecularly heterogeneous entity, varying in biology, presentation and response to treatment. BC classifications divide BC into categories according to different schemes, each based on different criteria and serving a different purpose. The major categories are the histopathological type, tumor grade, tumor stage and the expression patterns of proteins and genes. As knowledge of cancer cell biology increases, these classifications are revised and demonstrate a need for more effective prognostic and predictive tools beyond purely clinicopathological characteristics. Even before newer classification tools were developed, it was known that hormone receptor status, - estrogen receptor positive (ER^+) or negative (ER^-) progesterone receptor positive (PR^+) or negative (PR^-) - and later

Her2 receptor status (HER2), was correlated with significant clinical outcomes and therapies (2-5). ER^+ tumors consistently have a better 5-year overall survival outcomes, as well as better response to hormonal therapies like tamoxifen and aromatase inhibitors (3-7). Thus, the ability to discriminate between tumors even at the “crude level” of receptor-type has significant treatment and prognostic value. Over a decade ago, Perou, Sorlie and colleagues at Stanford sought to more precisely classify breast cancer samples through a then unique approach termed “gene expression profiling” (8). Using a large scale genomic survey of breast tumors, these groups reported in a number of successive publications, five intrinsic subtypes that share molecular characteristics and similarities in tumor biology; Luminal A (Luminal A), Luminal B (Luminal B), Her2-Enriched (HER2), Basal-like (BLBC), and Normal-Like (8-12). This set of publications dramatically changed our understanding of breast cancer and led to development of the PAM50 Breast Cancer Intrinsic Classifier that uses a 50-gene set to classify breast tumors into one of the five -recognized molecular subtypes (11). Current statistics quote that approximately 40% of all breast cancers are molecularly classified as Luminal A; 10-20% Luminal B; 10-20% “normal-like”; 10 % Her2 and 10-20% are BLBC (Table 1). These molecular subtypes have been repeatedly shown to be independent predictors of prognosis, survival, and response to therapy (7-13).

Table 1. Molecular Subtypes of Breast Cancer: Clinical, Pathological and Molecular Characteristics.

	Luminal A (LumA)	Luminal B (LumB)	Her2-Enriched (Her2)	Basal-Like (BLBC)
Prevalence	40%	10-20%	10%	10-20%
Clincial	ER+ and/or PR+, HER2-, low Ki67	ER+ and/or PR+, HER2+ (or HER2- with high Ki67)	ER-/PR-/HER2	75%-ER-/PR-/HER2-, EGFR+, CK 5/6, high Ki-67
Prognosis	Good	Intermediate	Poor	Poor
Hormone Therapy	Adjuvant Hormone Therapy: aromatase inhibitor (Anastrozole, Letrozole) or Tamoxifen	Adjuvant Hormone Therapy: aromatase inhibitor (Anastrozole, Letrozole) or Tamoxifen	None	None
Chemotherapy	Adjuvant Chemotherapy Doxorubicin/Cyclophosphamide	Adjuvant Chemotherapy Doxorubicin/Cyclophosphamide or Docetaxel	Adjuvant Chemotherapy Doxorubicin/Cyclophosphamide or Paclitaxel	Doxorubicin or Epirubicin w/Cyclophosphamide
Targeted Therapy	None	Trastuzumab +/- Pertuzumab	Trastuzumab +/- Pertuzumab	None

NCCN Clincial Practice Guidelines in Oncology v3.20.14, Rosen's Breast Pathology 2014

The “Luminal” molecular subtypes of BC express genes and protein of keratins 8/18, commonly associated with luminal epithelial cells. Emerging data suggests that Luminal A and Luminal B tumors may be distinct entities (14). Luminal A tumors account for 40% of all diagnosed BC and are receptor positive for estrogen (ER^+) and/or progesterone (PR^+), and negative for human epidermal growth factor receptor 2 ($HER2^-$), as determined by immunohistochemistry or molecular profiling (8). Luminal A tumors have a low Ki_{67}, a protein that increases in cells as they prepare to divide, indicating slow growth and they tend to be far less aggressive compared to other molecular subtypes. Therapy often includes adjuvant endocrine therapy such as an aromatase inhibitor (anastrozole, letrozole) or tamoxifen with or without adjuvant chemotherapy such as doxorubicin/cyclophosphamide followed by paclitaxel (5). As a result of their slow growth response to hormone therapy, and far less aggressive nature, Luminal A tumors have a very favorable 5 year survival prognosis.

Luminal B tumors are characterized by ER^+ and/or PR^+ and $HER2^+$ (luminal-HER2 group) or ER^+ and/or PR^+, $HER2^−$, and high Ki67 (>14%), indicating cell growth and division. Similar to Luminal A tumors, the typical treatment options include adjuvant chemotherapy followed by docetaxel. In addition, due to the Her2 protein over-expression a typical treatment course might include of trastuzumab (Herceptin) or lapatinib with or without pertuzumab (Tykerb), which are often used in combination with the standard chemotherapy and an aromatase inhibitor such as letrozole (Femara). Compared to Luminal A, these tumors are diagnosed at a much younger age (>50 yrs) (13-15), higher tumor grade (Grade 3) (14-17). Often they fail to respond to hormone based therapy, probably due to the lower levels of ER-related genes relative to Luminal A

tumors. A study by Creighton et. al, found that Luminal B (50%) patients had a 30% decrease in distant metastasis-free survival compared to Luminal A (80%) patients at 12.5 years (14). Additionally, Sorlie et al determined that Luminal A patients had an overall survival that was 71 months higher than in Luminal B patients (14, 10-12). These studies support the lower relapse-free and overall survival differences observed in the Luminal molecular subtypes.

The HER2-enriched subtype ($HER2^{+}/ER^{-}/PR^{-}$) is less common (10%), and is characterized by high-grade/node positive tumors. Treatment options include trastuzumab (Herceptin) and anthracycline-based chemotherapy. Tumors with the HER2 gene amplification and/or overexpression HER2 protein have a poor prognosis that includes a greater risk of relapse and shortened overall survival (17-20).

BLBC accounts for 10-20% of all diagnosed breast cancers and is identified by the lack of clinically significant levels of protein receptors for estrogen (ER), progesterone (PR), or human epidermal growth factor receptor 2 (Her2) and expression of one or more high-molecular-weight/basal cytokeratins (CK5/6, CK14, and CK17). BLBC also has a high rate of metastasis that often involves brain and lung and although treatment options are available, overall, patients diagnosed with BLBC have a lower overall survival (<48 months) compared to all other molecular subtypes of breast cancer (7-23).

Characteristics of TNBC

Several studies have shown that ~70% of TNBC tumors fall into the BLBC molecular subtype, however, it is important to note that not all BLBC are TNBC (8-10). TNBC accounts for ~ 10-15% of all diagnosed breast cancers and is identified by the lack of

clinically significant levels of estrogen, progesterone receptors and HER2 (8-10). Additional characteristics of TNBC include an early age of onset (≤50), higher tumor grade (>3), and larger primary tumor (> 2.0 cm) (24-30). TNBC often have markers for basal cytokeratins, ck5/6, CK14 and CK17, as well as a myoepithelial marker P-cadherin, a mesenchymal marker vimentin and lower levels of E-cadherin, all suggesting characteristics of epithelial-mesenchymal transition (EMT), a critical event during metastasis (24-27). Common mutations in include genes associated with DNA-repair mechanisms (BRCA1) and tumor suppression (p53/RB1). TNBC also has a high rate of recurrence that often involves metastasis to the brain and lung (24-30). There is a lower detection rate of TNBC/BLBC on mammography and combined with enhanced tumor cell proliferation in TNBC, this may explain how TNBC can develop between yearly mammograms, and has been described as an "interval cancer" (25,28). Although treatment options are available (surgery+/- radiation; combination of platinum-based chemotherapy), the lack of cell surface hormone receptors often make TNBC unresponsive to conventional hormone or targeted therapy (24-27). Overall, patients diagnosed with TNBC have a much lower disease-free survival interval, lower overall survival, and a much worse overall prognosis and have a much higher incidence in minority women, African and Hispanic American women and those of African Descent (29,30).

Wnt/β-catenin dependent signaling pathway (Canonical Wnt signaling) and the normal breast development

The Wnt/β-catenin signaling pathway is known to play an important role in mammary gland development and deregulation of this pathway and associated genes have been

implicated in cancer initiation and progression (31). Wnt/β-catenin signaling regulates distinct stages of remodeling during development and the reproductive lifetime of the mammary gland, including involution cycles back to adult stage during pregnancy and lactation (31). During the normal development of the breast, mechanisms such as proliferation and terminal end bud branching occur in response to Wnt/β-catenin signaling (31). In the absence of Wnt ligands, accumulated β-catenin is phosphorylated resulting in ubiquitination and degradation. Wnt ligands bind to low-density lipoprotein receptor –related proteins 5/6 (LRP5/6) and Frizzled (FZD) and activate the Wnt/β-catenin signaling pathway. This binding/activation signals the recruitment of Disheveled (Dsh), which inactivates the β-catenin destruction complex. Stabilized β-catenin translocates to the nucleus and the signaling effects of β-catenin are mediated through transcription factor 4 (TCF4) and lymphoid enhancer factor (LEF-1). The TCF4/LEF-1 complex binds Wnt response elements, providing docking sites for β-catenin at the nucleus, and induces the expression of Wnt target genes that regulate the cell cycle, cell growth and proliferation.

Dysregulation of Wnt signaling in Breast Cancer

Wnt signaling is necessary for maintaining the proliferation- differentiation balance and dysregulation of this pathway has been shown to play a role in tumor development and progression in cancer and in particular, TNBC. For example, the overexpression of TCF4 in rectal cancer has been shown to confer resistant to therapy and was associated with a shorter overall survival (32). Caveolin-1 (CAV1), an integral plasma membrane protein associated with β-catenin signaling, has been shown to be overexpressed in BLBC and TNBC, leading to epithelial mesenchymal transition (EMT)

(33,34). Tenascin-C (TNC) has been shown to significantly down-regulate the Wnt inhibitor dickkopf, promoting Wnt signaling in glioblastoma cells (35). Finally, forkhead box 3A (FOXO3A) has been shown to be a pro-apoptotic transcription factor and Wnt signaling activator (35,36). Recently, whole genomic and transcriptome sequencing (RNA-seq) was performed on 14 TNBC patients, including six AA and eight CA. Unique homozygous deletions were seen in in two tumors (TNBC -001, TNBC -006) that involved α-catenin and the Wnt/β-catenin signaling pathway (adjacent CTNNA1 and SIL1 loci at 5q31.2)(38). Interestingly, the two tumors that exhibited this α-catenin homozygous deletion were both from AA breast cancer patients. Downregulation of α-catenin, a tumor repressor that associates directly with β-catenin/cadherin complex and the actin skeleton, has been correlated with tumor progression and cell growth, (38). Recent studies have observed deregulation of the Wnt/β-catenin signaling pathway in TNBC and BLBC, associated with high grade, poor prognosis and metastatic disease (39). However, these studies did not specifically investigate differences between CA and women of African descent (39,40).

Health Disparities in Triple-negative Breast Cancer

The Carolina Breast Cancer Study (CBCS), a population based control study which sought to investigate breast cancer in AA women, has improved our understanding of tumor molecular subtype, menopause status, tumor characteristics and survival in a large cohort (41,42). The CBCS statistics show that CA women actually have a higher BC incidence rate than AAs, followed by Hispanic, Asian/Pacific Islander, and American Indian/Alaska Native women (2,41-43). Additionally, AA women have a lower lifetime risk of developing breast cancer than CA women, yet, from 1999–2011, the CDC and

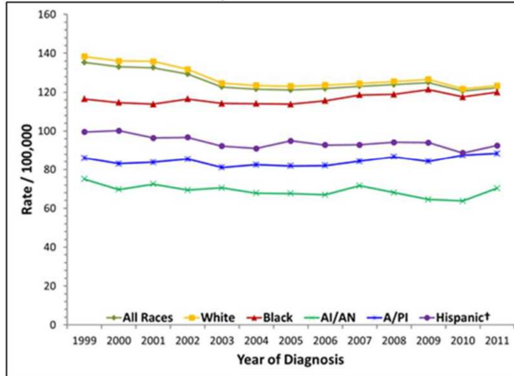
CBCS reported that the mortality rate for AA women (36 per 100,000) was significantly higher than any other group including CA (28 per 100,000) (28,30). The CBCS study found that young, premenopausal AA women had a high frequency of TNBC (77%) and lower survival (10% less) than that of CA women with TNBC. Finally, the study found that even after adjusting for age, race and stage TNBC was higher in AA women compared to CA women. As depicted in the pie chart in figure 1B, Africa American women and those of African descent have a larger distribution of TNBC diagnosis compared to women of European/Caucasian decent (29). AA women are 3-fold more likely to develop TNBC, often at a younger age (premenopausal) and more likely to be diagnosed with a higher grade tumor (>3) (28-30,41-43). Emerging evidence demonstrates a correlation between African ancestry and TNBC, indicating that there are intrinsic genes or mutations that predispose women of African descent to this more aggressive subtype (44). A study investigating African Ancestry and TNBC, found that the prevalence of TNBC diagnosis highest in Ghanaian women (82%) followed by AA women (26%) and CA women (16%) (45). These data suggests the possibility that increasing extent of African Ancestry may 'predispose' a woman to early onset or high-risk breast cancer such as TNBC. The relapse-free and overall survival period is significantly lower in AA TNBC than in CA cohorts and AA are more likely to have a BRCA1 mutation (44). As illustration, a study by Mefford et al identified an inherited BRCA1 founder mutation, BRCA1 943ins10, in families of African ancestry (44).

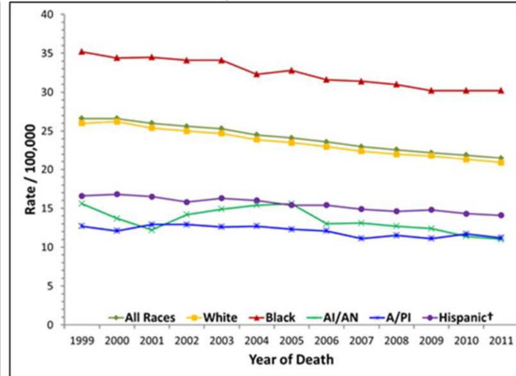
A combination of factors, including socio-economic, lifestyle, access to healthcare and differences in treatment protocols likely contribute to the health disparities that exist between AA and CA diagnosed with TNBC (44-46), but they do not fully

explain them. Social factors, such as poverty and restricted access to quality healthcare, often underestimate the 'hidden' factors such as lack of transportation or the inability to undergo therapy due to work, which can significantly minority patients (27,41,43). In addition to socio-economic factors, underlying differences in tumor biology and intrinsic factors lend support to the hypothesis that TNBC in AA is biologically different from that in CA. Differences in presentation or treatment (chemotherapy and/or radiation) alone do not account for the health disparities observe between AA and CA with TNBC (41). Overall, reducing a women's lifetime exposure to estrogens, such as a higher frequency and duration of breast feeding and higher parity beginning at a younger age, has shown to be protective but only in hormone receptor positive breast cancer (2). Conversely, regardless of race, women with TNBC are often younger when they begin menarche (<12.58 yrs) and at first pregnancy (22 yrs) (46). Risk and protective factors in the development of TNBC have been shown to overlap between AA and CA; however there are some distinct differences. For example, increased parity is still considered protective in CA and considered a risk factor in AA women. Additionally, AA women have a significantly lower frequency of breastfeeding (31%) compared to CA women (58%) (36,45,46). This combination of risk factors, higher parity and a decreased duration of breast feeding in AA women, may contribute to the health disparity observed in AA with TNBC. A study by Stead et al. investigated TNBC and associated risk factors and found that the 3-fold higher prevalence of TNBC in AA women was regardless of other risk factors such as age or weight (29). These observations guide our research into the biological patterns of gene expression between AA women with TNBC and a cohort of CA women with this disease.

In this study, we performed gene expression profiling on a cohort of early stage (Node 0) TNBC samples from AA and CA women. Our analysis revealed a distinct pattern of gene expression, mostly upregulated, in the AA cohort. Using molecular profiling of the gene expression data, we determined that the AA TNBC cohort was of the BLBC molecular subtype (64%) and segregated into three of the TNBC molecular subtypes: Basal-like (BL2), Immunomodulatory (IM) and Mesenchymal (M). In contrast, the CA cohort was distributed among the six TNBC-subtypes. This suggests there are distinct differences between AA TNBC and CA TNBC, even in early stages of tumor development. Differential gene expression analysis of the expression array data resulted in 190 differentially expressed genes between the AA and CA cohort. Using pathway enrichment (GeneGo) analysis, we found that the majority of differentially expressed genes were over-represented in pathways such as cell adhesion, tight junctions, immune response and the Wnt/β-catenin pathway in the AA TNBC cohort. After validation experiments, we identified Caveolin-1 (CAV1) as being significantly expressed in the AA-TNBC cohort. In an independent cohort of TNBC Node 0, FFPE samples from AAs and CAs we found that the AA cohort had a significantly higher level of Cav1 protein, compared to the CA cohort. Using a cell line panel, we determined that CAV1, mRNA and protein, was higher in the TNBC cell lines than in luminal cell lines. Finally, a panel of TNBC cell lines was used to demonstrate that CAV1 silencing by siRNA resulted in a significant decrease in proliferation in each of the TNBC cell lines, while there was no affect observed in the luminal breast cancer cell lines. These observations suggest that CAV1 expression may contribute to the more aggressive phenotype observed in AA women diagnosed with TNBC and may be a potential biomarker and therapeutic target.

A





B

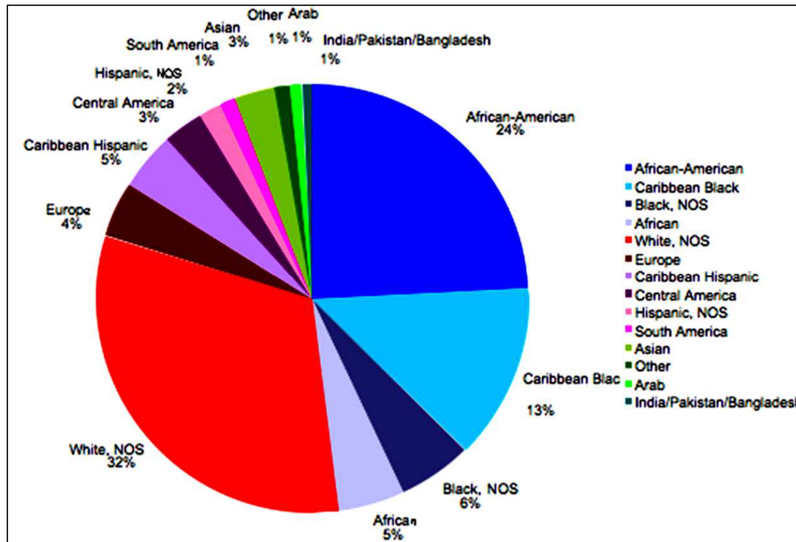


Figure 1. A) Age-Adjusted Breast Cancer Incidence and Death Rates in the U.S. by Race and Ethnicity. The BC incidence and mortality rate is grouped by race and ethnicity, using combined data from the National Program of Cancer Registries as submitted to CDC and from the Surveillance, Epidemiology and End Results program as submitted to the National Cancer Institute in November 2013. Data shows that CA women had the highest incidence, followed by African American, Hispanic, Asian/Pacific Islander, and American Indian/Alaska Native women. From 1999–2011, the mortality rate for BC varied, depending on their race and ethnicity, however, for each year. AA women were significantly more likely to die of BC than any other racial group. (http://www.cdc.gov/cancer/breast/statistics/race.htm). B) Racial distribution of TNBC. As depicted in the pie chart, AA women and those of African descent accounted for a much larger proportion of TNBC compared to those of European/Caucasian descent. (Stead LA, et al, BCR 11:R18, 2009)

COMPARISON OF TRANSCRIPTIONAL SIGNATURES FROM U.S. AFRICAN AMERICAN AND CAUCASIAN WOMEN DIAGNOSED WITH EARLY STAGE TNBC IDENTIFIES DIFFERENTIAL EXPRESSION IN KEY ONCOGENIC PATHWAYS

Abstract

Disparities in breast cancer stage of presentation and survival rates exist in patients of different ethnicities. Although a women's race alone is not considered a risk factor in developing BC, strong epidemiological data supports BC as the second leading cause of cancer death among U.S. AA women, with a 20% greater mortality rate than that of CA women. BC incidence rates for AA and CA women have become comparable, but AA women still have a higher mortality rate; AA women with BC often present with tumors that are of higher grade and later stage and their relapse-free and overall survival period is significantly lower than CA. Additionally, the more aggressive TNBC phenotype has a higher prevalence in AA women. Combined observations have led to a much poorer prognosis for AA BC patients. These differences are undoubtedly a result of a combination of factors; including socio-economic, lifestyle, access to health care, tumor characteristics and inherent factors, such as genetic composition. In order to begin to understand the biological differences in AA patients diagnosed with TNBC, we analyzed tumor and self-matched normal tissue samples from AA and CA patients from south Florida who were diagnosed with early stage (Node 0), TNBC . Comparisons of transcriptional differences between AA and CA TNBC using GeneGo pathway enrichment analysis, suggest expression alterations in several key pathways, including cytoskeletal remodeling, cell adhesion, Wnt-signaling, cell adhesion, tight junctions, and

immune response. This suggests that inherent gene expression differences exist between AA and CA TNBC samples in pathways previously-recognized as important in onocogenesis. Finally, the analysis revealed novel and significantly deregulated genes associated with the Wnt/β-catenin pathway in the AA cohort, as compared to the CA, suggesting this pathway may contribute to the more aggressive phenotype in AA women diagnosed with TNBC .

Materials and Methods

Tumor Material for Gene Expression Microarray Analysis

Matched tumor and adjacent normal breast tissue FFPE samples AA and CA patients were obtained from the University of Miami (UM) /Sylvester Breast Tissue Bank (UM/S BTB) under an IRB-approved protocol, in collaboration with the Drs. Carmen Gomez, Merce Jorda (UM Pathology) and Mark Pegram, (UM Oncology), between 2006-2012. Samples were chosen based on TNBC status, lack of lymph node involvement, ethnicity, age, lack of exposure to adjuvant and/or chemotherapy and the availability of matched adjacent control. Each of the samples was evaluated using immunohistochemical staining to confirm ER/PR/Her2 receptor status. A total of 23 samples from AA and CA patients diagnosed with Lymph Node 0 TNBC, along with a pool of adjacent matched controls, were used in the study.

RNA isolation and Hybridization

Total RNA extraction was performed on tumor and matching adjacent normal FFPE sections, in collaboration with Almac Diagnostics, using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Life Technologies). The RNA extracted was evaluated for concentration and purity using the Nanodrop 1000 and overall RNA integrity using the

Agilent Bioanalyzer. Total RNA samples that passed QC and had sufficient concentration were amplified with the NuGen WT-Ovation FFPE Amplification Kit v2 (NuGEN). The resulting cDNA product was assessed for concentration (>3.5 ug). To achieve optimum concentration for array processing it was necessary to amplify, pool and vacuum concentrate the RNA samples. The cDNA samples were fragmented and labeled with the NuGen Encore Biotin Module (NuGEN), then hybridized to the Almac Diagnostics Breast Cancer DSA (http://www.almacgroup.com/biomarker-discovery-development/proprietary-discovery-arrays/dsa/). The BC DSA research tool is a research platform for the study of breast cancer that includes >60,000 biologically relevant transcripts, many of which are not available on traditional array, including transcripts expressed in normal breast tissue and unique transcripts related to cancer initiation and development. Almac Quality Control (QC) included the evaluation of the gene chip for hybridization performance and a data integrity assessment to ensure there are no underlying variables that may cause an unexpected pattern in the data. As these samples were processed from FFPE, Almac lowered the percent call from 25% to 20%. Almac provided raw data files, gene-to-chip annotation file and QC analysis documents to demonstrate that 23 of the 23 samples along with matched adjacent control data passed final QC and were used for downstream analysis.

Microarray Analysis

Gene expression analysis was conducted using GeneSpring 12.3® analytical software. Signal intensity value above the 20th percentile in 100% of the samples of at least 1 experimental condition was used to filter the raw data (60,856 probe sets). 41,802 probe sets were normalized using the Robust MultiArray Average (RMA) technique, which

briefly, provides non-linear background correction on a per-chip basis, and log transformed to the baseline median of all samples. The normalized data set was used for all downstream analyses. Principal Component Analysis tool (PCA) was used to detect outliers and/or batch effects. After quality control assessment of array data, 23/23 samples were retained. Prior to further analysis, low intensity signals in the normalized data was filtered by expression; Step one, remove low-intensity signals of genes that are not expressed, Lower cut-off: 20.0/Upper cut-off: 100.0. Step two; retain entities within the cut-off limits. This resulted in a final total of 18,296 probes. In our first level of analysis, hierarchical clustering of the normalized data was examined for gene clusters between the AA and CA cohort. Unsupervised cluster analysis was performed using the hierarchical cluster algorithm, based on ethnicity and gene probes (p-value < .05, fold change >2.5) and Pearson's uncentered similarity metric with centroid linkage rule. Differentially gene expression between the two groups were identified using the 'Biological Significance Workflow' in GeneSpring 12.3® using the Benjamani-Hochberg multiple test correction method to reduce the false discovery rate (FDR) with a significance cutoff of adjusted p-value < 0.05. This method assumes independence of p-values across genes.

GeneGoEnrichment Pathway Analysis

The list of differentially expressed genes with a threshold value of 1.50 (Fold Change) and adjusted p-value of ≤ 0.05 was imported into GeneGo for enriched pathway analysis; GeneGo Pathways Software (MetaCore™) (https://portal.genego.com/).

TNBC Type

Using the normalized and filtered raw data, the AA and CA TNBC Node 0 cohort was classified by TNBC subtype using the "TNBC type: A Subtyping Tool for TNBC", http://cbc.mc.vanderbilt.edu/TNBC /

Technical Validation of FOXO3A, TNC, TCF4 and CAV1

If total RNA used in the gene expression array was depleted then the corresponding FFPE block was used to create three10 uM scrolls for RNA extraction using RNeasy FFPE Kit (Qiagen) per manufacturer's protocol. RNA integrity and purity was measured using a 2100 Bioanalyzer (Agilent Technologies) and concentration was determined using the Nanodrop ND-1000 Spectrophotometer (Nanodrop). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA in a 20 µl reaction volume using the SuperScript III First-Strand Synthesis kit, per the manufacturer's protocol (Life Technologies). Preamplification of cDNA was done using the TaqMan PreAmp Master Mix Kit, which allows use of very small of amounts of cDNA to increase the quantity of specific cDNA targets, without introducing amplification bias. The kit has been optimized to work specifically with TaqMan Gene Expression Arrays. Briefly, TaqMan GEA (Lifetechnologies) for FOXO3A, TNC, TCF4 and CAV1 were pooled with the PreAmp Master Mix (Lifetechnologies) and then added to the cDNA correlating to the samples in the gene expression microarray. Preamplification was setup (mixing the pooled assays with cDNA sample and TaqMan® PreAmp Master Mix) then run in a 96 well plate on an ABI 7900HT using the following program; consisting of enzyme activation, 95°C for 10 min; Denature, 95°C for 15 sec, Anneal/Extend 60 sec for 14 cycles.

Breast Cancer Cell Lines

Breast cancer cell lines, MDAMB231 and MCF7, were used as controls in the technical validation. Total RNA was extracted using Trizol (phenol, guanidine isothiocyanate) followed by sequential precipitation with chloroform/isopropanol. The precipitated RNA was purified with the RNeasy Micro Kit (Qiagen), per the manufacturer's instructions and RNA integrity and purity was measured using a 2100 Bioanalyzer (Agilent Technologies) and concentration was determined using the Nanodrop ND-1000 Spectrophotometer (Nanodrop). Complementary DNA (cDNA) was synthesized from 1 µg of total BC cell line RNA in a 20 µl reaction volume using the SuperScript III First-Strand Synthesis kit, per the manufacturer's protocol (Life Technologies).

Reverse Transcriptase Quantitative Real-time PCR Analysis

Relative expression levels of mRNAs were measured using the TaqMan® Gene Expression Assays (GEA, Life Technologies), which consist of a pair of unlabeled PCR primers and a TaqMan® probe with a FAM™ on the 5' end and minor groove binder (MGB) and non-fluorescent quencher (NFQ) on the 3' end. The primer probeset IDs for quantitative real-time PCR were as follows: CAV1 (Hs00971716_m1), FOXO3 (Hs04194415_s1) TCF4 (Hs00162613_m1), TNC (Hs01115665_m1) and Human ACTB (B-actin) Endogenous Control. The KAPA Probe Fast qPCR Master Mix Kit designed for ABI Prism (KAPA Biosystems) and 100 ng of cell line control or 5 ul of diluted (1:20) preamplified FFPE cDNA product was used per reaction and run in triplicate. The following reaction protocol was used for all TaqMan RT-qPCR and analyzed on an ABI Prism 7900HT (Applied BioSystems): Enzyme Activation, 95°C for 10 min; Denature, 95°C for 15 sec, Anneal/Extend 60 sec for 40 cycles. Relative mRNA expression was

calculated relative to β-actin amplification using the comparative C_T method, also known as the $\Delta\Delta C_T$ method. First, the mean and standard deviation values of the replicates are calculated. Next, the C_T values of both the calibrator and the samples of interest are normalized to B-actin. Finally, fold-change is calculated using the ΔΔCT relative to a calibrator, typically the sample with the highest ΔCt. This is achieved by using the formula below, where Gene x could be CAV1, FOXO3A, TNC, or TCF4. Significance was determined using an unpaired, two-tailed t-test.

$$\mathbf{2^{-\Delta\Delta C_T} = [\Delta\Delta C_T\ \text{Gene X} = \Delta C_T,\ \text{Target Gene} - \Delta C_T\ \text{B-actin}] - \Delta\Delta C_T\ \text{Calibrator}}$$

Results

FFPE samples used the study were obtained from AA and CA patients with matched tumor and adjacent normal breast tissue from the University of Miami /Sylvester Breast Tissue Bank (UM/S BTB), in collaboration with the Dr. Carmen Gomez, Dr. Merce Jorda (UM Pathology) and Dr. Mark Pegram, (UM Oncology), between 2006-2012. As shown in table 2, the samples were matched for TNBC status, lymph node involvement (Node 0), ethnicity, age and exposure to adjuvant and/or chemotherapy. In addition, each of the samples was evaluated using immunohistochemical staining to confirm ER/PR/Her2 receptor status. A total of 23 samples from AA and CA patients diagnosed with Lymph Node 0 TNBC were used in further studies.

Table 2. FFPE Tumor Sample Characteristics

	Hispanic American	African American	Caucasian American
Tumor Samples	10	10	13
Matched, Adjacent Controls	8	10	10
Lymh Node Status	Node 0	Node 0	Node 0
ER	Neg	Neg	Neg
PR	Neg	Neg	Neg
Her2	Neg	Neg	Neg
Phenotype	TNBC	TNBC	TNBC
Chemotherapy Adjuvant Therapy	None/No Exposure	None/No Exposure	None/No Exposure

Neg: Negative; TNBC: Triple-Negative Breast Cancer.

Gene expression profiling was performed on each sample using the breast cancer enriched gene expression array Affymetrix Platform, Breast Cancer DSA Research Tool (BC DSA). The BC DSA research tool has a 3′ focus providing an optimized platform for the analysis of samples from FFPE, laser capture microdissection and fine needle aspirates. This research tool includes > 60,000 biologically relevant transcripts, many of which are not available on traditional array, including transcripts expressed in normal breast tissue and unique transcripts related to cancer initiation and development.
A total of 60,856 gene/probes were checked for quality control of the gene chip and gene probes. Gene expression analysis was conducted using GeneSpring 12.3® analytical software. The combined samples were hybridized on different dates and geographical locations; therefore our first step was to determine if there was any batch effect, which could cause non-biological variations in the gene expression data. A PCA analysis was performed, based on batch date, and it was determined that there were no non-biologically relevant variations (Fig. 2).

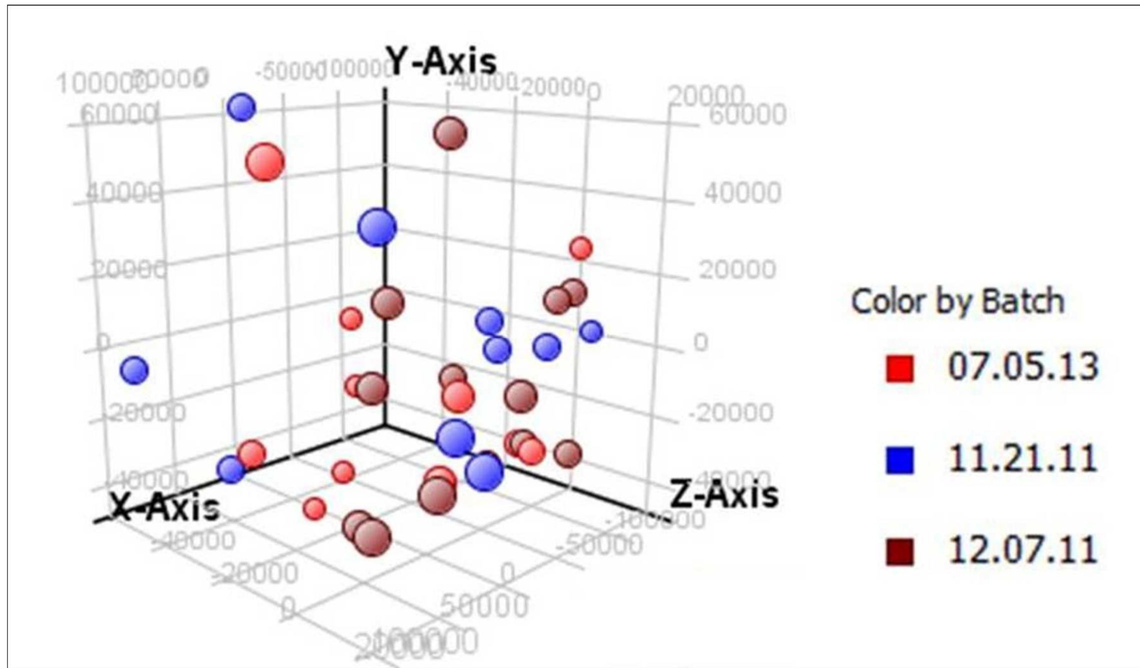


Figure 2. PCA Analysis: No Batch Effect Observed. Total RNA was isolated from FFPE samples, amplified and hybridized to the Almac Breast Cancer DSA™ (Affymetrix Platform), which includes over 60,000 probes for normal breast and BC. Gene Expression analysis was conducted using GeneSpring 12.3 analytical software, briefly; Robust Multi-Array Average (RMA) for background correction was performed to normalize baseline to median and Log2 transform the raw data. Principal component analysis (PCA) was performed to determine if Batch Effect occurred. Although the samples were processed in multiple batches, the PCA data demonstrates that a ‘Batch Effect’ did not occur and 23/23 samples were retained.

The data was normalized and filtered by expression to remove any unreliable expression values, including probe sets representing genes that are not expressed in any of the samples or if they had values too high or too low to be considered biologically relevant. This pre-filtering step created a revised list of 14,802 gene/probes for further analysis. Using matched adjacent normal tissue as controls, unsupervised hierarchical cluster analysis was performed (p-value $<.05$, fold change >2.0) and Pearson's un-centered similarity metric with centroid linkage rule. Results are depicted in a heatmap (Fig. 3), and revealed a pattern of differential expression in the AA Tumor cohort compared to CA. All subsequent experiments will be comparing the AA and CA TNBC, Node 0, cohort.

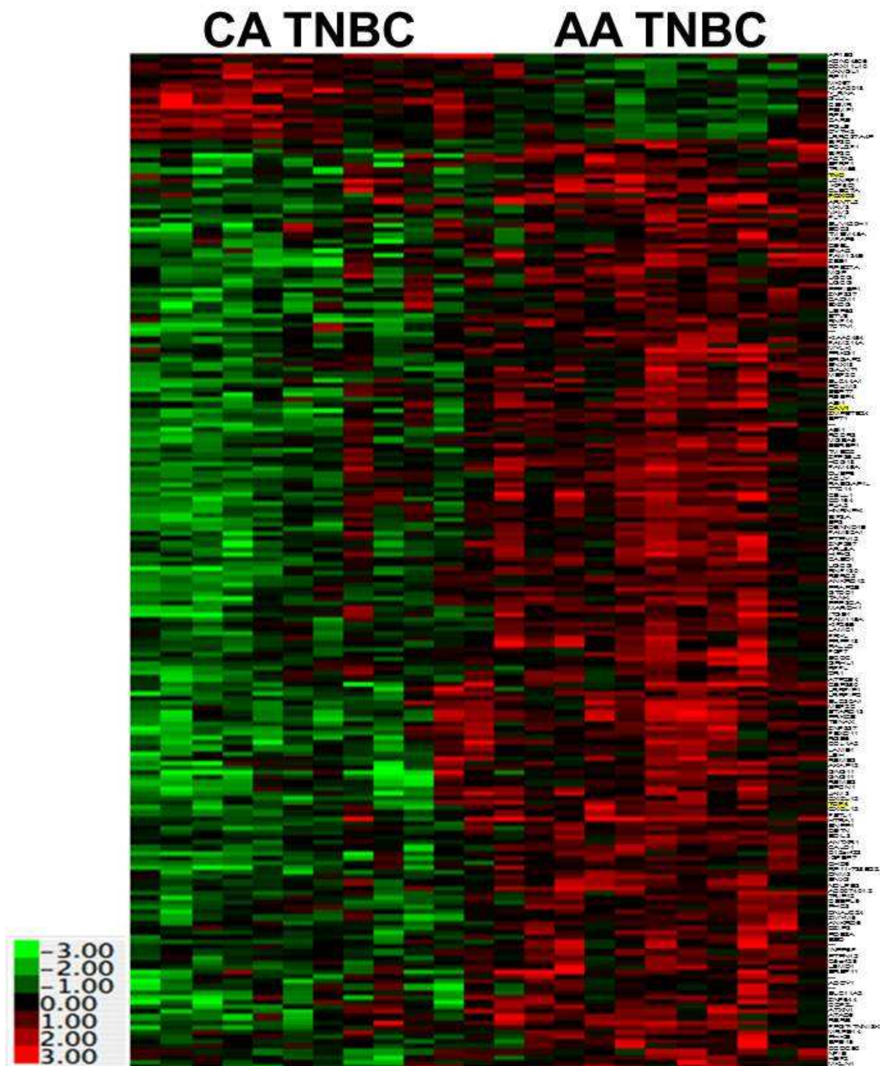


Figure 3. Heatmap of TNBC-Node 0, AA and CA Cohort. Unsupervised hierarchical clustering was performed on each tumor cohort using the gene list derived from the pre-filtered probe list. Using a pool of matched adjacent normal tissue as controls, unsupervised hierarchical cluster analysis was performed (p-value <.05, fold change >2.0) using the Pearson's un-centered similarity metric with centroid linkage rule. Results revealed a majority of down-regulated genes (shown in red) found in the AA Tumor cohort compared to CA.

To identify the genes that were differentially expressed between the AA TNBC and CA TNBC cohorts the filtered list of 14,802 gene/probes was used. Using the ‘Biological Significance Workflow’ in GeneSpring 12.3®; the gene list was analyzed by an unpaired t- test by Benjamani-Hochberg Multiple Test Correction Method to reduce False Discovery Rates (FDR) with a significance cutoff of adjusted p-value < 0.05. After filtering for duplicate genes a total of 190 differentially expressed genes (DEGs), including 173 up-regulated and 17 down-regulated, were identified in the AA TNBC Node 0 cohort (Table 3). The list of DEG was then imported into GeneGo Pathways Software (MetaCore™) to identify biological processes or enriched pathways that consistently display differential expression between the AA and CA cohort.

The AA TNBC gene expression profile was enriched for pathways in cytoskeletal remodeling, cell adhesion, Wnt, cell adhesion, tight junctions, and immune response (Fig. 4). Additionally, we observed significantly upregulated genes associated with the Wnt/β-catenin pathway in the top six pathways; CAV1, FOXO3A, TNC and TCF4 (Fig. 5). Based on the results from the gene enrichment pathways, AA compared to CA, and current literature we chose to investigate the following genes and there role in the more aggressive TNBC; CAV1, FOXO3A, TNC and TCF4.

Table 3. List of Differentially Expressed Genes, AA vs CA.

Gene Symbol	Regulation (Up/Down)	Fold Change
EDIL3	up	5.45
HBB	up	4.76
FAM92A1	up	3.48
MIR4668	up	3.35
GNG11	up	3.28
DNM3	up	3.17
NFIB	up	3.15
RGS5	up	3.09
MGP	up	2.96
ANKRD6	up	2.95
ZFP36L2	up	2.90
RBBP4P1	up	2.89
RBMS3	up	2.85
MGEA5	up	2.82
SPON1	up	2.77
IGFBP7_ SUCP2	up	2.75
RERE_PALLD	up	2.74
CAV1	**up**	**2.74**
SERTAD4	up	2.73
ENPP1	up	2.72
LSMD1	up	2.71
NDUFS3	up	2.68
SLC11A2	up	2.67
SRGAP2	up	2.65
PPAP2B	up	2.64
CALD1	up	2.63
FAM214A	up	2.62
TANK	up	2.59
JAM3	up	2.58
ATXN1_ZEB1	up	2.56
FGF7	up	2.55
HIPK3_FAM115A_EIF3C_PTPN12	up	2.54
VAV3	up	2.52
MEF2C_SRSF11_ACTA2	up	2.50
TNC	**up**	**2.48**
GRHL1	up	2.48
PDLIM3	up	2.45
LAMB1_ACLY_FLT1_FPGT-TNNI3K	up	2.44
TCF4	**up**	**2.42**
STARD13	up	2.42
C12orf23_TCTN1_RSRC2_MYLK	up	2.38
PPFIBP1	up	2.37
ZMYM5	up	2.35
PJA2_SFRP1_ABI1	up	2.34

GTDC1_DAAM1_HCG18	up	2.32
C5orf25_RNF130_RCOR3_ANTXR1_UGCG_CLEC7A_RABGAP1L	up	2.30
CTSK_EXOG_DZIP3_FRYL	up	2.28
DUSP6	up	2.27
KIF26B	up	2.26
PPP3CA_ITGB1	up	2.25
AKAP12_ADCY1_RNF14_CXCL12_CADM1_FAM46A	up	2.23
FOXO3	**up**	**2.23**
ZMPSTE24_DSEL_FBXO11_CCDC50	up	2.22
PRKCE_KIAA0494_WWP1	up	2.21
CHD9_TMEM45A_SERBP1_KIF5C_ANKRD12_HSF2	up	2.20
RFFL_ZNF267_ZNF267_USP53	up	2.19
TRIM56	up	2.18
SNX18_MARCH1_SEP7_DENND1B_MFAP5_CASD1	up	2.17
HNRNPK	up	2.16
ARL5A	up	2.15
DNAJC24_BTF3L4_LRRFIP1_SDC3_CEP350	up	2.14
ZNF337_INPP5F	up	2.13
ATAD5_FAM134B	up	2.12
PDE8A_SUCLA2	up	2.11
TC2N_ADAM22_SLC2A13_ZNF644	up	2.10
EPT1_CD164	up	2.08
COL1A2_EIF3A_NAV1_TTC14	up	2.07
SNAI2_NCOA2_SP3_LBH_TRIP12_ESD_PHC1	up	2.06
PRPF18_SLC30A1_ODF2L_DSTN	up	2.05
CBLL1_HTRA1_RPS27A_OSBPL9_ETV5_SLC44A1	up	2.04
MKLN1_LAMC1_ZNF562_POU2F1_PHC3	up	2.03
TMED2_ARNTL2_FSTL1_PHKB	up	2.02
TYMS_DR1_MRPS14_EPS15_LONRF1_SNX3	up	2.01
TSNAX_OLFML3_GALNT1_SERINC3_ATP2B4	up	2.00
PGLS_CARS_SMR3B	down	-2.01
CYTH2	down	-2.04
EIF3D	down	-2.05
MKI67	down	-2.08
LRRC37A4P	down	-2.15
GLUL	down	-2.16
KIAA2018	down	-2.18
Y-RNA	down	-2.22
VANGL1_AP1S3	down	-2.24
LZTS3	down	-2.31
MUC7	down	-2.40
DDX11L2	down	-2.46
OSMR	down	-2.89
PSMF1	down	-2.91

Gene Enrichment Pathway Analysis

	Enrichment by Pathway Maps	p-value	Genes Active In Data
1	Cytoskeleton remodeling: TGF, WNT	6.225E-05	TCF4, FOXO3A, MYLK1, Tcf(Lef), Destrin, CAV1, Actin, MLCK
2	Cell adhesion/Tight junctions	2.154E-04	ITGB1, Tcf(Lef), CAV1, Actin, JAM3
3	Immune response: Function of MEF2 in T lymphocytes	2.557E-04	PKC, MEF2C, Calcineurin A (catalytic), MEF2, NCOA2 (GRIP1/TIF2)
4	Development: TGF-beta-dependent induction of EMT via	3.156E-04	SLUG, ACTA2, Caldesmon, CAV1, Actin
5	Immune response: IL-13 signaling via PI3K-ERK	1.109E-03	Tenascin-C, PKC-epsilon, Adenylate cyclase type I, COL1A2
6	Signal transduction: Activation of PKC via G-Protein coupled	1.194E-03	PKC-epsilon, Calcineurin A (catalytic), MEF2, MLCK
7	Development: WNT signaling pathway. Part 2	2.100E-03	TCF4, SLUG, Tcf(Lef), ITF2
8	Development: Role of HDAC and calcium/calmodulin-	3.818E-03	MEF2C, Calcineurin A (catalytic), MEF2, NCOA2 (GRIP1/TIF2)
9	Muscle contraction: ACM regulation of smooth muscle	1.024E-02	PKC, LPP3, PKC-epsilon, MLCK
10	Cytoskeleton remodeling	1.309E-02	MYLK1, Tcf(Lef), Destrin, CAV1, MLCK

Gene Symbol	Gene Name	Gene Function	pValue	FC	Log FC
TCF4	Transcription Factor 4	Binds to Wnt response elements to provide docking sites for β-catenin	0.001	3.3	1.74
CAV1	Caveolin-1	Wnt/β-catenin signaling and EMT associated signalling	0.006	3.3	1.72
FOXO3A	Forhead Box 3A	β-catenin binds FOXO and enhances FOXO transcriptional activity	0.014	2.6	1.4
TNC	Tenascin -C	Down-regulation of the Wnt inhibitor Dickkopf	0.011	2.6	1.4

Figure 4. Functionally Enriched Pathways in TNBC -Node 0, AA vs. CA Cohorts. The DEG list from table 2, was imported into Metacore GeneGo to identify functionally enriched pathways. The AA Cohort was enriched for pathways in cytoskeletal remodeling, cell adhesion, Wnt, cell adhesion, tight junctions, and immune response. Additionally, we observed significantly upregulated genes associated with the Wnt/β-catenin pathway in the top 10 pathways.

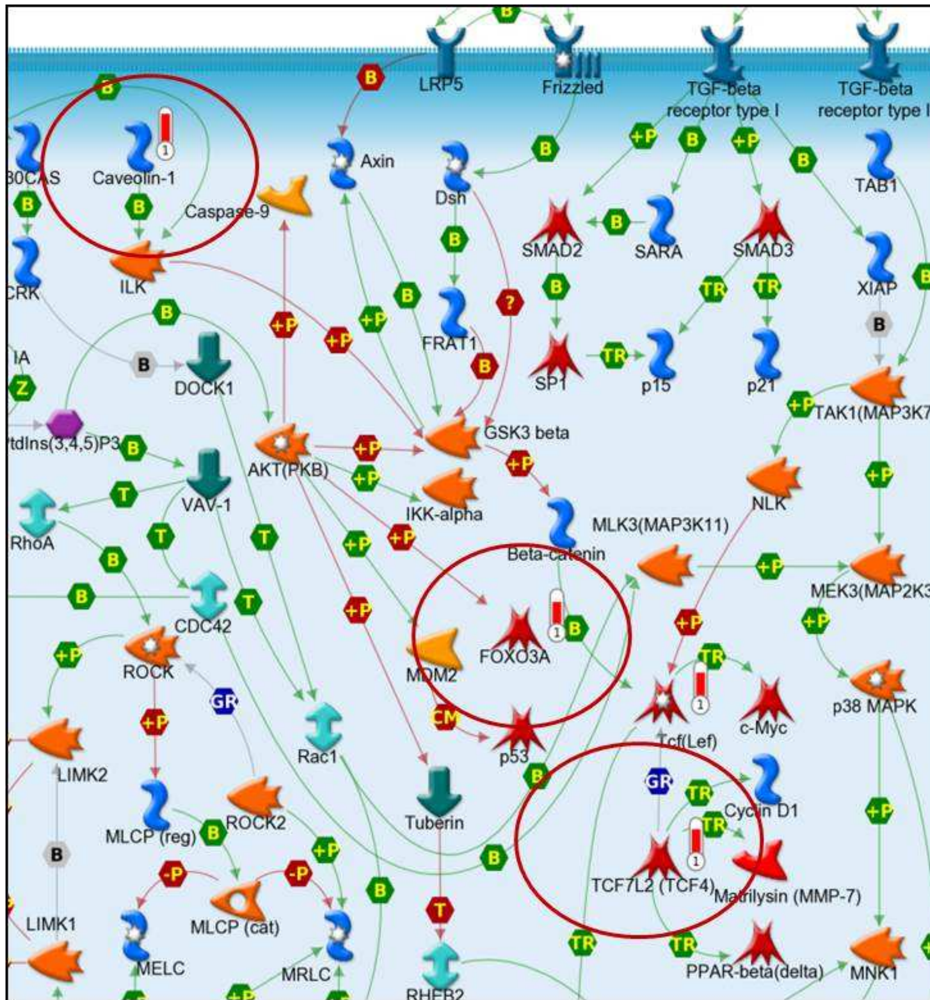


Figure 5. Representative GeneGo Enrichment for of the Wnt-β-catenin Pathway, AA TNBC compared to CA TNBC. The red thermometer indicates that the associated gene is significantly upregulated in the AA cohort compared to the CA cohort. Significant genes of interest was enriched for the Wnt/β-catenin pathway are circled in red.

Large scale genomic surveys of breast tumors using microarray-based technologies reveal at least five predominating molecular subtypes. The predominating subtypes (Basal-like, Luminal B, Her2-enriched, Luminal B, Luminal A and Normal Breast-like) share molecular characteristics and similarities underlying tumor biology and have contributed to improved prognostication and clinical decision-making (8-13). In addition, there are at least six TNBC subtypes including 2 basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem–like (MSL), and a luminal androgen receptor (LAR) subtype (48-49). It is also known that AA TNBC patients are enriched in the basal and immunomodulatory subtypes. In order to further characterize the molecular subtype of the AA and CA patients used in the Node 0 TNBC cohort, two accepted subtype predictor algorithms - the PAM50-defined subtype predictor and the "TNBC type: A Subtyping Tool for TNBC" were applied to each cohort (9, 48-49).
The PAM50-Defined Subtype Predictor algorithm revealed that the majority of the AA-cohort classified into Basal-Like (BLBC) Molecular Subtype (64%), which includes the TNBC -phenotype and that the CA Cohort also included the BLBC -subtype, however, at a lower percentage than AA (46%). Additionally, the CA Cohort contained larger percentages of the HER2 (23%) and Luminal B (23%) subtypes (Fig. 6A). Further characterization using the TNBC type Tool, found that the CA-TNBC Cohort have a common distributed among the six of the TNBC molecular subtypes using the TNBC type Tool (48,49). In contrast, the AA Cohort was comprised primarily of 3-TNBC molecular subtypes; 14% Basal-like (BL2), 43% Immunomodulatory (IM) and 43% Mesenchymal (M) (Fig. 6B).

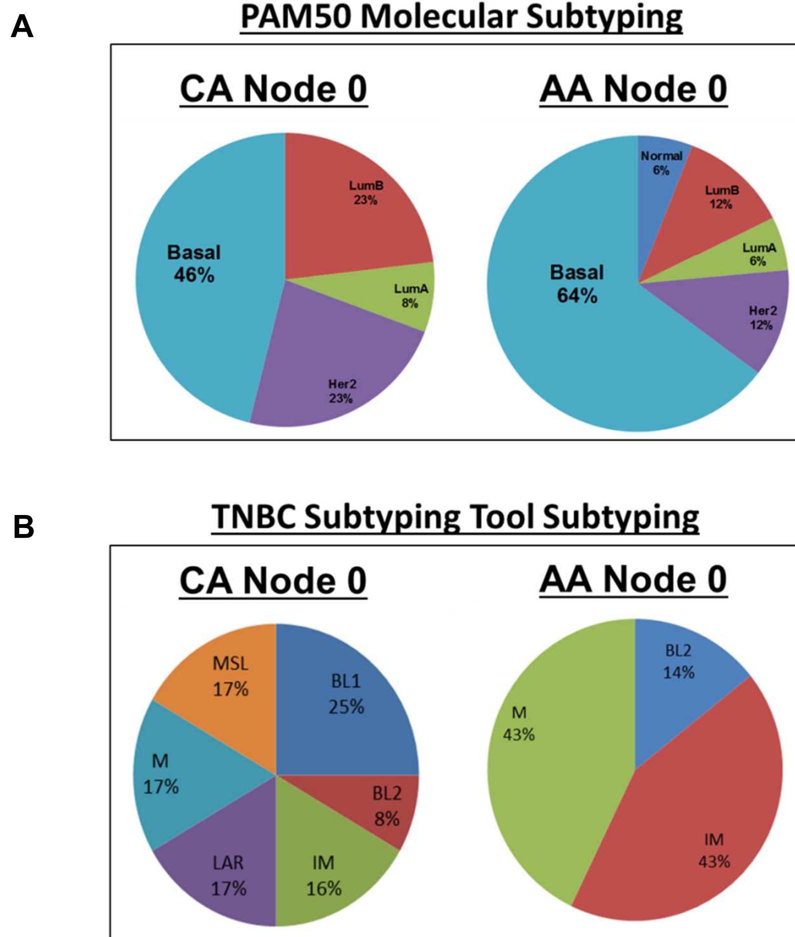


Figure 6. Molecular Subtyping of the TNBC -Node 0, AA and CA Cohorts. A) Using the PAM50-Defined Subtype Predictor algorithm, the cohorts were classified into one of the predominating molecular subtype(s). The AA Cohort (64%) and CA Cohort (46%) comprised of mainly the Basal-Like Molecular Subtype that includes the TN-phenotype. Additionally, the CA Cohort included 23% of the HER2 and Luminal B subtypes. B) TNBC Subtyping of TNBC -Node 0, AA and CA Cohorts. TNBC subtype was performed using a web-based prediction tool; TNBC type: A Subtyping Tool for TNBC, http://cbc.mc.vanderbilt.edu/TNBC/. The AA Cohort comprised primarily of 3-TNBC molecular subtypes; 14% Basal-like (BL2), 43% Immunomodulatory (IM) and 43% Mesenchymal (M). The CA TNBC Cohort was distributed among all six of the TNBC molecular subtypes.

Microarray results showed significantly upregulated genes associated with the Wnt/β-catenin pathway in the top six pathways; CAV1, FOXO3A, TNC and TCF4 (Fig. 4). Gene expression microarrays provide a snapshot of the transcriptional activity in a sample and within a cohort, however, there is sample variability, technical and user variations associated with these types of experiments. In addition, use of RNA isolated from archived FFPE samples is difficult process and requires careful QC at each process. Our experimental design was created with these difficulties in mind; however, it was still necessary to validate our findings. Using Life Technologies Taqman GEA created for each of the candidate genes we performed RT-qPCR using the remaining RNA; from 7-AA Tumor, 7-AA controls from adjacent normal tissue, 8-CA Tumor and 3-CA Controls. Significance was determined using an unpaired, two-tailed t-test. CAV1 expression was significantly higher ($p = 1.22 \times 10^{-05}$) in the AA TNBC cohort compared to the TNBC CA cohort (Fig. 7).

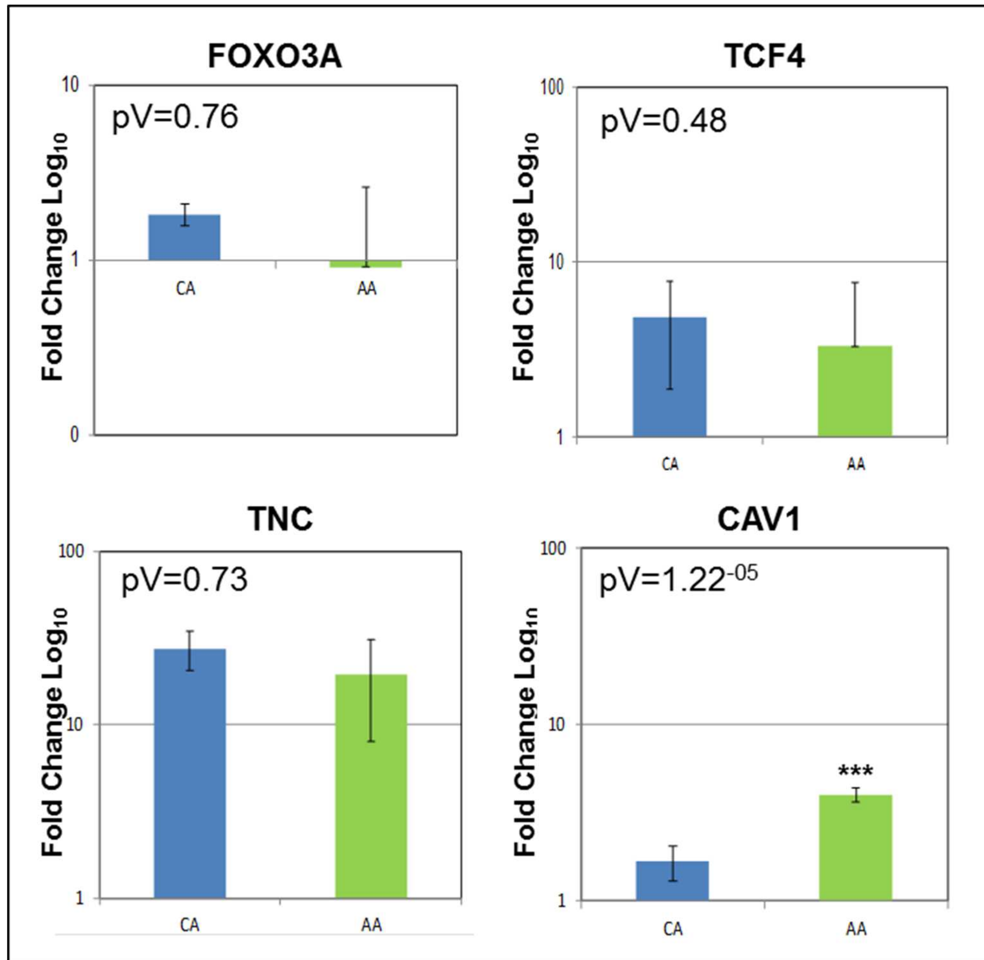


Figure 7. Technical Validation of Candidate Genes. RNA from the Affymetrix BC DSA was pre-amplified using the TaqMan PreAmp Master Mix Kit prior to cDNA synthesis. If total RNA used in the gene expression array was depleted then the corresponding FFPE block was used extract RNA. TaqMan Gene Expression Assays for FOXO3A, TNC, TCF4 and CAV1 were used to measure relative expression levels of mRNA, calculated relative to β-actin amplification using the comparative C_T method. Significance was determined using an unpaired, two-tailed t-test. CAV1 expression was significantly higher (p-Value 1.22^{-05}) in the AA TNBC cohort compared to the CA cohort.

Conclusion

Although multiple factors may contribute to the observed health disparities in TNBC, it is essential that we identify the molecular characteristics and any underlying biological differences between CA and AA TNBC. In collaboration with the University of Miami /Sylvester Breast Tissue Bank (UM/S BTB), we obtained FFPE samples from AA and CA patients women diagnosed with early stage, Node 0 TNBC. Gene expression profiling was performed on each sample, matched tumor and adjacent normal breast tissue, using Affymetrix Breast Cancer DSA Research Tool (BC DSA). Unsupervised hierarchical cluster analysis of the gene expression data revealed a pattern of differential expression in the AA Tumor cohort compared to CA, with mostly upregulated genes in the AA. Differential gene expression analysis identified 190 genes, 173 up-regulated and 17 down-regulated, in the AA cohort pathway enrichment analysis identified that the AA TNBC gene expression profile was enriched for pathways in cytoskeletal remodeling, cell adhesion, Wnt-β-catenin, cell adhesion, tight junctions, and immune response (Fig. 4). Interestingly, we observed significantly upregulated genes associated with the Wnt/β-catenin pathway in the top six pathways; CAV1, FOXO3A, TNC and TCF4 (Fig. 5). These results were validated using the Taqman Gene Expression Assay for each of the candidate genes with remaining RNA from each cohort. We determined that CAV1 expression was significantly higher (p-Value = 1.22 x 10-05) in the AA TNBC cohort compared to the TNBC CA cohort (Fig. 7).

We also found that the basal-like breast cancer subtype was the predominate molecular subtype in the AA (64%) cohort, and that the AA Cohort was comprised primarily of 3-TNBC molecular subtypes; 14% Basal-like (BL2), 43% Immunomodulatory (IM) and

43% Mesenchymal (M) (Fig. 6B). These data suggest that the even at the earliest stage of TNBC, there are significant differences among AA and CA tumors and suggest that in this cohort CAV1 expression may contribute to the more aggressive phenotype observed in AA women diagnosed with early stage TNBC.

Caveolin-1 (CAV1) gene is located on chromosome 7 (locus 7q31.1), near the fragile site, FRA7G locus. This gene includes three exons (30, 165 and 342 bp) and two introns (1.5 and 32 kb). CAV1 is a scaffolding protein and the main component of the caveolae that make up the plasma membrane and involved cell signaling and molecular transport, cell adhesion, intracellular trafficking of lipid and cholesterol (50). Although CAV1 is ubiquitously expressed, it is highest in adipocytes, endothelial, epithelial and myoepithelial cells and less in luminal cells (51,52). CAV1 in cancer appears to be tumor/stage and even subtype specific and although there have been published reports of CAV1 as a tumor suppressor in cancer (colon, ovary, lung), data is increasing suggesting an oncogenic role for CAV1 (53-55). Increased CAV1 levels induced EMT and cell survival and showed that serum levels could predict risk of relapse in pancreatic cancer (55-58). Similarly in hepatocellular carcinoma (HCC), CAV1 expression induced Epithelial–mesenchymal transition (EMT) through the Wnt/β-/β-catenin-TCF/LEF pathway, and was a potential biomarker for worse prognosis (59). In prostate cancer, CAV1 has been shown to be a biomarker to identify patients at high risk of recurrence (60). High CAV1 expression in tumor and loss in stromal cells has been shown to be predictive of poor survival in BC (61). CAV1 and CAV2 have been shown to associate with BLBC and TNBC (61-62) and shown to correlate with increased cell proliferation, migration and invasion in the same subtype (63). CAV1 gene amplification in metaplastic

and BLBC and associated with lower overall survival and is preferentially expressed in basal-like BC and cell lines (51). However, the role of CAV1 in health disparities of TNBC has not yet been addressed.

CHAPTER 3

CAV1 IS EXPRESSED IN AN INDEPENDENT COHORT OF AA-TNBC SAMPLES AND MESENCHYMAL/BASAL-LIKE TNBC CELL LINES

Abstract

In order to validate the CAV1 results (Fig. 7), an independent cohort of TNBC Node 0 FFPE samples from patients matched for race/ethnicity, age, prior treatment, pathological stage and confirmed TNBC status was used to construct a tissue microarray (TMA). In addition, a panel of triple-negative and ER+ breast cancer cell lines were used to characterize endogenous CAV1 expression *in vitro*. Results demonstrated that CAV1 expression was differentially expressed among molecular subtypes of TNBC and ER^+ BC. The TNBC -Group 1, consisting of mesenchymal and basal-like TNBC cell lines, expressed high levels of CAV1 mRNA and endogenous protein. TNBC -Group 2, consisting of epithelial-like TNBC cell lines, expressed lower levels of CAV1 mRNA and protein. Data suggests that CAV1 expression may be a candidate biomarker of TNBC with mesenchymal characteristics in AA women.

Materials and Methods

Cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in RPMI-1640 (MDA-MB-231, MDA-MB-157, T47D, MCF7),or RPMI-1640 with a supplement of 0.01 mg/ml insulin (HCC70, HCC1806) medium. Cells were maintained with 10% fetal bovine serum (FBS) and 1x Penicillin-Streptomycin, in constant humidity at 37°C in 5% CO_2. All media and supplements were obtained from Life Technologies (Carlsbad, CA).

SDS-PAGE and Western blotting

Whole cell protein lysates were prepared using a RIPA lysis buffer kit that contained a protease inhibitor cocktail in DMSO and phosphatase inhibitors (odium sodium orthovandate, phenylmethylsulfonyl fluoride) per the manufacturer instructions (Santa Cruz). Protein samples from wild-type BC cell lines were collected when cell lines were ~75% confluent to minimize variability in expression due to cell density and the protein concentration was determined using Pierce BCA Protein Assay Kit (Life Technologies). Approximately 30 μg of Cav1/lane was separated using 4-12% Bis-Tris precast gels in MES buffer in the NuPage electrophoresis system (Invitrogen). Protein was transferred onto Invitrolon PDF membranes using the iBlot Dry Blotting System (Life Technologies) for 6 min, per the manufacturer instructions. Each membrane was incubated in nonfat dry milk (Walmart) blocking solution consisting of 5% nonfat dry in tris-buffered saline with 0.1% Tween (TBST; Sigma). Western blotting was performed using rabbit monoclonal antibody to Cav1 and (ab52938) and loading control, alpha-tubulin, a rabbit polyclonal antibody (ab52866) was purchased from Abcam. The Horseradish peroxidase-conjugated secondary antibody was purchased from GE Healthcare (anti-rabbit; Pittsburgh, PA) and each were used at a dilution of 1:12,000. All antibodies (primary and secondary) were incubated for 1 hr, at room temperature on a rocking platform. SuperSignal West Femto Maximum Sensitivity Substrate was used to develop the western blots followed by chemiluminescence detection high sensitivity film for autoradiography (USA Scientific).

Independent Cohort of TNBC Node 0 FFPE Samples

An independent cohort of Node 0 TNBC FFPE tissue samples from AA (13) and CA (10) patients were obtained from three vendors; Asterand Bioscience, Advanced Tissue

Services (ATS) and National Disease Research Interchange (NDRI). Samples were chosen based on similar characteristics from our original samples in chapter 2; TNBC status, Stage 0-Stage IIB (AJCC Staging), < 60 years of age and no prior adjuvant or chemotherapy. The FFPE blocks were used to construct a multi-ethnic TNBC Tissue MicroArray (TMA) and for independent validation of CAV1 mRNA expression using RT-qPCR.

Total RNA Isolation from FFPE Samples and cDNA Synthesis

Total RNA isolation from the independent cohort of TNBC FFPE samples was extracted using the RNeasy FFPE Kit (Qiagen) per manufacturer's protocol. Total RNA from the TNBC Cell Line Panel was extracted, as described in chapter2, following the Qiagen RNeasy Kit protocol. RNA integrity and purity was measured using a 2100 Bioanalyzer (Agilent Technologies) and concentration was determined using the Nanodrop ND-1000 Spectrophotometer (Nanodrop). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA in a 20 μl reaction volume using the SuperScript III First-Strand Synthesis kit, per the manufacturer's protocol (Life Technologies).

Quantitative Real-time PCR Analysis

Relative expression levels of mRNAs were measured using the TaqMan® GEA, as described in chapter 2. The primer probeset ids for RT-qPCR were as follows: CAV1 (Hs00971716_m1) and Human ACTB (B-actin) Endogenous Control. The KAPA Probe Fast qPCR Master Mix Kit designed for ABI Prism (KAPA Biosystems) was used for all PCR amplifications and analyzed on an ABI Prism 7900HT. The 20 ul reaction was prepared with 5ul of cDNA, 10 ul of 2X Kapa Master Mix, 1 ul 20x TaqMan® GEA and 4 ul H_2O, and run in triplicate. The following reaction protocol was used for all TaqMan

RT-qPCR and analyzed on an ABI Prism 7900HT (Applied BioSystems): Enzyme Activation, 95°C for 10 min; Denature, 95°C for 15 sec, Anneal/Extend 60 sec for 40 cycles. Expression levels of mRNA were calculated using the comparative C_T method, as described in chapter 2. Significance was determined using an unpaired, two-tailed t-test.

TMA Construction staining and scoring

The TMA was constructed in the Macromolecular Analysis & Processing Center (MAPC) at TGen. Each FFPE block used in the construction of the TMA block is represented as double-punched 0.6 mm diameter core biopsy on the array along with positive (uterus) and negative (testis) internal controls. The slides made from the TMA block were subjected to heat induced epitope retrieval using a proprietary citrate based retrieval solution for 20 minutes. Endogenous peroxidase was blocked and all slides were incubated for 30 minutes with a rabbit monoclonal antibody to Cav1 (ab52938) at a dilution of 1:1000 obtained from Millipore (Abcam). The sections were visualized using the Bond™ Polymer Refine Detection kit (Leica) using diaminobenzidine chromogen as substrate and then counterstained with hematoxylin. Once dry the TMAs were coverslipped with permamount and reviewed by the MAPC resident pathologist. Each individual core was scored for Cav1 location (stromal cell or nuclear) along with matching stain intensity (0-3). In addition, an HScore was derived from a semi-quantitative assessment of both staining intensity (scale 0–3) and the percentage of positive cells (0–100%), which, when multiplied, generated a score ranging from 0 to 300. Statistical analysis was performed using a student's t-test.

Results

To characterize the expression levels of CAV1, nine breast cancer cell lines were used. The nine cell lines and their characteristics including ER, PR, and HER2 expression status are shown in Table 4. The TNBC cell lines consisted of three ER-negative mesenchymal BC cell lines (BT549, MDAMB-231, MDAMB-157), three ER-negative, epithelial-like BC cell lines (BT20, HCC70, MDAMB468), a basal-like ER-negative (HCC1806) and two ER-positive, luminal A BC cell lines (MCF7, T47D). CAV1 mRNA expression was determined using RT-qPCR. The TNBC -Group 1 of high CAV1 mRNA expression included the three ER-negative mesenchymal BC cell lines (BT549, MDAMB-231, MDAMB-157) and the basal-like ER-negative (HCC1806). The TNBC -Group 2, included the three ER-negative, epithelial-like BC cell lines (BT20, HCC70, MDAMB468). MCF7, the ER-positive, luminal A BC cell line had expression levels similar to TNBC -Group 1 while T47D, consistently lacked CAV1 mRNA expression (Fig 8A). Similar to mRNA expression, TNBC -Group 1 had a higher level of endogenous Cav1 protein expression compared to TNBC -Group 2. MCF7 had Cav1 protein expression comparable to the TNBC -Group 1 and T47D, again lacked evidence of Cav1 expression (Fig 8B).

Table 4. Clinicopathological Characteristics of the Breast Cancer Cell Line Panel.

BC Cell Line	Histological Grade/Phenotype	Molecular Subtype	Tumor Type	Cell Type
MDA-MB-231	$ER^-/PR^-/Her2^-$; TNBC	Basal B	Metastatic Adenocarcinoma	Mesenchymal
BT549	$ER^-/PR^-/Her2^-$; TNBC	Basal B	Primary Ductal Carcinoma	Mesenchymal
MDA-MB-157	$ER^-/PR^-/Her2^-$; TNBC	Basal B	Metastatic Medulallary Carcinoma	Mesenchymal
HCC1806	$ER^-/PR^-/Her2^-$; TNBC	Basal B	Squamous Cell Carcinoma	Basal-like
MDA-MB-468	$ER^-/PR^-/Her2^-$; TNBC	Basal A	Metastatic Adenocarcinoma	Epithelial-like
HCC70	$ER^-/PR^-/Her2^-$; TNBC	Basal A	Primary Ductal Carcinoma	Epithelial-like
BT20	$ER^-/PR^-/Her2^-$; TNBC	Basal A	Primary Mammary Gland Carcinoma	Epithelial-like
MCF7	$ER^+/PR^+/Her2^-$	Luminal A	Metastatic Adenocarcinoma	Epithelial-like
T47D	$ER^+/PR^+/Her2^-$	Luminal A	Metastatic Ductal Carcinoma	Epithelial-like

All data on the cell lines was obtained from ATCC and
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2730521/

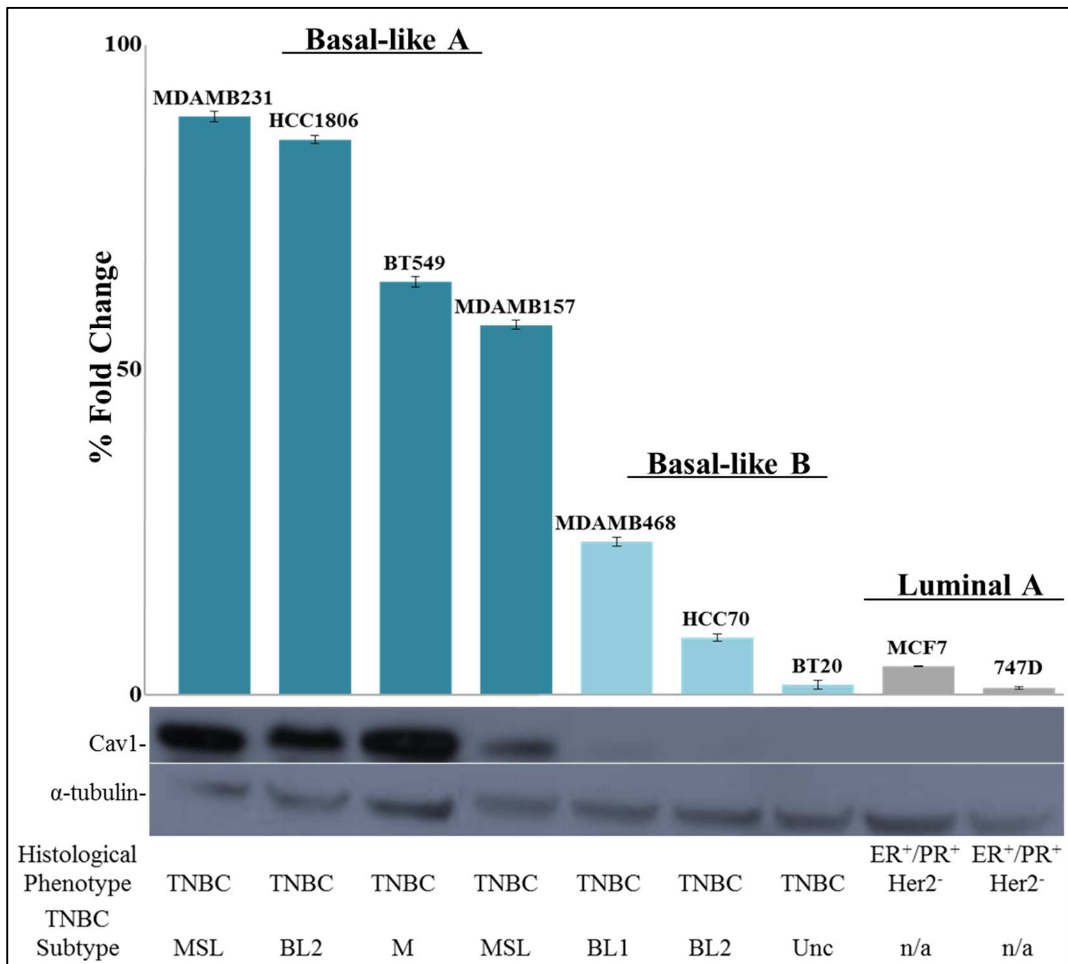


Figure 8. Cell Line Panel of CAV1 Expression. A) cDNA was prepared from total RNA from each of the cell lines and the TaqMan Gene Expression Assay for CAV1 and β-actin was performed. Relative expression levels of CAV1 mRNA, calculated relative to β-actin amplification was determined using the comparative C_T method. B) Cav1 Endogenous Protein Expression. Western blot analysis, using cell lysates from each of the cell lines, revealed differential expression of endogenous Cav1.

Using archived FFPE samples from 13-AA and 10-CA women diagnosed with TNBC, an independent cohort of TNBC Node 0 TMA was constructed. Patient clinical annotation is listed in Table 5. Immunohistochemistry was performed to determine the location of Cav1 (nucleus, cytoplasm or membrane) along with stain intensity (0-3) in the AA and CA TNBC cohort. In addition, an H-Score was derived from a semi-quantitative assessment of both staining intensity (scale 0–3) and the percentage of positive cells (0–100%), which, when multiplied, generated a score ranging from 0 to 300. Cav1 Expression is higher in the AA TNBC Cohort. The Cav1 stain intensity showed no difference in Cav1 location between the AA and CA cohort of TNBC (Fig 9A-B). The AA cohort of TNBC had a significantly higher HScore for Cav1 compared to the CA cohort (Fig 9C).

Table 5. TNBC Node 0, Independent Cohort Clinical Annotation.

ID number	Ethnicity	Gender	AJCC Stage	Diagnosis	Sample Source
SM08-4907	AA	Female	I	IDC	ATS
SM08-5190	AA	Female	I	IDC	ATS
312966A1	AA	Female	IA	MC	Asterand
312996A2	AA	Female	IA	IFDC	Asterand
313004A3	AA	Female	IA	IFDC	Asterand
S11-1838	AA	Female	IA	IDC	ATS
SM08-1816	AA	Female	IA	IDC	ATS
313005A3	AA	Female	IIA	IFDC	Asterand
343307A1	AA	Female	IIA	IFDC	Asterand
SM08-4047	AA	Female	IIA	IDC	ATS
SM10-1587	AA	Female	IIA	IDC	ATS
SM10-1637	AA	Female	IIA	IDC	ATS
SM11-2677	AA	Female	IIA	IDC	ATS
ND03627	CA	Female	IA	IDC	NRDI
ND03628	CA	Female	IA	IDC	NRDI
313450B2	CA	Female	IA	IFDC	Asterand
313497A1	CA	Female	IA	IFDC	Asterand
ND03625	CA	Female	IIA	IDC	NRDI
ND03626	CA	Female	IIA	IDC	NRDI
ND03629	CA	Female	IIA	IDC	NRDI
313500A3	CA	Female	IIA	IFDC	Asterand
313514A2	CA	Female	IIA	IFDC	Asterand
343294A2	CA	Female	IIA	MC	Asterand

ATS, Advance Tissue Services; NDRI, National Disease Research Interchange; IDC, Invasive Ductal Carcinoma; IFDC, Infiltrating Ductal Carcinoma; MC, Metastatic Carcinoma

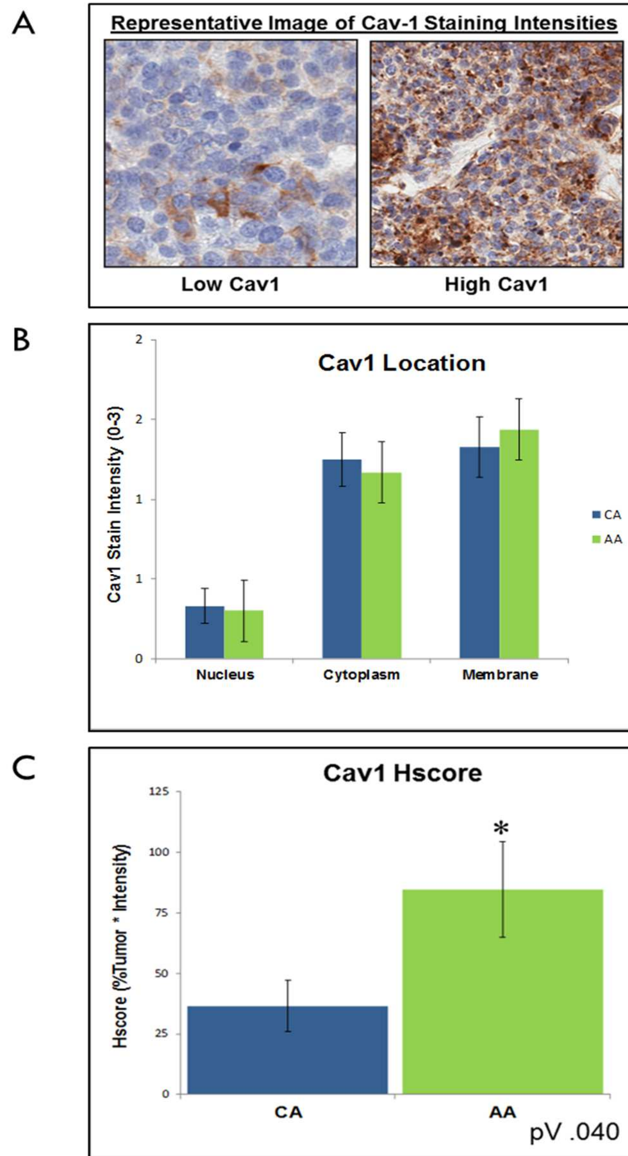


Fig 9. Cav1 Expression is higher in an Independent Cohort of the AA TNBC. A) Immunohistochemistry of high (right) and low (left) in TNBC tissue sections stained with the anti-Cav1 antibody. B) Cav1 stain intensity (0-3). There is no difference between location of Cav1 between the AA and CA cohort of TNBC. C) The percent tumor and stain intensity were used to calculate an Hscore. Results show that the AA cohort of TNBC had a significantly higher HScore for Cav1 compared to the CA cohort.

Conclusion

A cell line panel of triple-negative and ER+ breast cancer cell lines were used to characterize endogenous CAV1 expression *in vitro*. Results demonstrated that CAV1 was differentially expressed among molecular subtypes of TNBC and ER^+ BC. The TNBC -Group 1, consisting of mesenchymal and basal-like TNBC cell lines, expressed high levels of CAV1 mRNA and endogenous protein. TNBC -Group 2, consisting of epithelial-like TNBC cell lines, expressed lower levels of CAV1 mRNA and protein (Fig 8).

In order to validate the CAV1 results (Fig. 7), an independent cohort of TNBC Node 0 FFPE samples from patients with matched for race/ethnicity, age, prior treatment, pathological stage and confirmed TNBC status was used to construct a tissue microarray (TMA). In addition, using the same archived FFPE samples from 13-AA and 10-CA women diagnosed with TNBC, tissue microarray (TMA) was constructed. Patient clinical annotation is listed in Table 5. Immunohistochemistry was performed to determine the location of Cav1 (nucleus, cytoplasm or membrane) along with stain intensity (0-3) in the AA and CA TNBC cohort. In addition, an H-Score was derived from a semi-quantitative assessment of both staining intensity (scale 0–3) and the percentage of positive cells (0–100%), which, when multiplied, generated a score ranging from 0 to 300. Cav1 Expression is higher in the AA TNBC Cohort. The Cav1 stain intensity showed no difference in Cav1 location between the AA and CA cohort of TNBC (Fig 9A-B). The AA cohort of TNBC had a significantly higher HScore for Cav1 compared to the CA cohort (Fig 9C).

Data suggests that CAV1 expression may be a candidate biomarker of TNBC with mesenchymal characteristics in AA women.

CHAPTER 4

SILENCING OF CAVEOLIN-1 INHIBITS CELL PROLIFERATION IN TNBC CELL LINES

Abstract

CAV1 has been implicated as an oncogene in BC, a biomarker of aggressive disease and been shown to promote proliferation and invasion. In this study, small interfering RNA (siRNA) technology was used to inhibit the expression of CAV1 in TNBC cell lines followed by suppression of CAV1 to determine the role of CAV1 on proliferation. The TNBC , ER-negative mesenchymal BC cell lines, MDAMB-231, MDAMB-157, and basal-like ER-negative line, HCC1806, that expressed high levels of endogenous Cav1 along with the ER-negative, epithelial-like BC cell lines HCC70, were used to assess the functional role of Cav1 in TNBC . Additionally, the two ER-positive, luminal A BC cell lines, MCF7, and T47D were used. TNBC cell lines, mesenchymal and epithelial-like, exhibited a decrease in cell proliferation compared to the luminal A BC cell lines, MCF7, T47D.

Materials and Methods

Cell lines used were obtained from the ATCC (Manassas, VA) and were maintained in RPMI-1640 (MDA-MB-231, MDA-MB-157, T47D, MCF7), or RPMI-1640 with a supplement of 0.01 mg/ml insulin (HCC70, HCC1806) medium. Cells were maintained with 10% fetal bovine serum (FBS) and 1x Penicillin-Streptomycin, in constant humidity at 37°C in 5% CO_2. All media and supplements were obtained from Life Technologies (Carlsbad, CA).

Transfection of siRNA

Three Silencer® Select pre-designed and validated siRNAs to CAV1 (id: s2446, s2447, s2448) were purchased from Life Technologies. Optimal transfection conditions were determined using Lipofectamine® RNAiMAX Transfection Reagent, GAPDH positive and scrambled negative control #1 siRNAs (AM4605) and KDalert GAPDH Assay Kit (Life Technologies). Optimal siRNA transfection parameters; 0.60 ul Lipofectamine® RNAiMAX Transfection Reagent, 30 nM siRNA, and the reverse transfection method, all prepared in Opti-MEM® I Reduced Serum Medium (Life Technologies) were used to determine which siRNA had the best reduction in target mRNA levels. Each of the three CAV1 siRNAs were used with the MDAMB-157 cell line and measured by RT-qPCR as described above. Although each of the CAV1-siRNAs reduced mRNA and protein levels, CAV1 s2446 had the highest level of transfection efficiency and was used for all subsequent transfection of siRNA. For downstream siRNA isolations, 5×10^4 cells per well were used in reverse transfection method and seeded into clear, 96-well plates triplicate with a total volume of 200 ul. Opaque walled, 96-well plates seeded at 5×10^4 cells per well were used for downstream proliferation assays. Protein validation plates were seeded in duplicate at 5×10^5 cells per well into 6-well plates with a final volume of 2000 ul.

SDS-PAGE and Western blotting

Whole cell protein lysates were prepared as described in Chapter 2. Protein was isolated at; 24, 48, 72, and 120 –hrs post siRNA and were performed using 2-wells of a 6-well plate. The concentration of protein was low, as determined using the Pierce BCA Protein Assay Kit (Life Technologies) and was concentrated approximately 30-fold with Amicon

Ultra Centrifugal Filter Devices (10,000 nominal molecular weight limit; Millipore). Approximately 10 μg Cav1/lane was separated using 4-12% Bis-Tris precast gels in MES buffer in the NuPage electrophoresis system (Invitrogen). Protein was transferred onto Invitrolon PDF membranes using the iBlot Dry Blotting System (Life Technologies) for 6 min, per the manufacturer's instructions. Western blot analysis was performed, described in Chapter 3, using a rabbit monoclonal antibody to Cav1 and (ab52938) and alpha-tubulin.

Cell Proliferation

Cell proliferation rates were determined by measuring ATP levels with the CellTiter-Glo Luminescent Cell Viability Assay (Promega), which uses a proprietary thermostable luciferase reaction. 100 ul of supernatant was removed from the 96 well plate and 100 ul of room temperature CellTiter-Glo buffer was added, gently mixed and incubated at room temperature for 2 hrs. ATP, which is indicative of metabolically active cells, was measured at 24, 48, 72, 96 and 120 hrs post siRNA transfection, using the Cytation Multi-Mode Reader and collected in the Gen5 Data Analysis Software (BioTek). Each cell line has a different doubling-time; therefore, we choose to run the assay to 120 hrs, instead of the typical 96 hrs, in order to capture any changes in cell proliferation based on the loss of CAV1. GraphPad Prism Software (GraphPad Software Inc.) was used to obtain the proliferation rate, using nonlinear regression and the exponential growth equation (doubling time; (k is ln(2)/K)). Next, the proliferation rate of the CAV1 siRNA transfected cell lines were compared to the proliferation rate of the scrambled negative control, using an unpaired t-test.

RNA Isolation from TNBC Cell Lines and cDNA Synthesis

For samples collected post-siRNA transfection the following procedure was used to isolate RNA and synthesize cDNA. Each of the six breast cancer cell lines was seeded into 96-well plates at 5 x 104 cells/well. RNA was isolated at 72, 96 and 120 hrs post siRNA using the Cells-to-cDNA II Kit (Life Technologies). Each plate was washed 2x in cold PBS, followed by addition of 100 ul of the Cell Lysis Buffer, heating the samples to 75°C for 15 min, to simultaneously lyses the cells and inactivate RNases. DNase digestion is then performed by adding 2 ul of DNase and incubating for 15 min at 37oC, to remove any genomic DNA followed by an inactivation step of 75oC for 5 min. The crude RNA lysate (10 ul) was immediately used in complementary DNA (cDNA) synthesis in a 40 µl reaction volume using the SuperScript III First-Strand Synthesis kit, per the manufacturer's protocol (Life Technologies) followed by RNase H incubation to remove the RNA strand of an RNA-DNA hybrid.

Reverse-Transcriptase Quantitative Real-time PCR Analysis

Relative expression levels of mRNAs were measured using the TaqMan® GEA, as described in chapter 2, and Probe Fast qPCR Master Mix Kit designed for ABI Prism (KAPA Biosystems) was used for all PCR amplifications and analyzed on an ABI Prism 7900HT. The primer probeset ids for quantitative real-time PCR were as follows: CAV1 (Hs00971716_m1) and Human ACTB (B-actin) Endogenous Control. The following reaction protocol was used for all TaqMan RT-qPCR and analyzed on an ABI Prism 7900HT (Applied BioSystems): Enzyme Activation, 95°C for 10 min; Denature, 95°C for 15 sec, Anneal/Extend 60 sec for 40 cycles. Relative mRNA expression was using the

comparative C_T method as described in chapter 2. Significance was determined using an unpaired, two-tailed t-test. The CAV1 percent knockdown (%KD) for each cell line was determined using the results of the comparative C_T method and the calculation below.

% KD Calculation

- $[\Delta][\Delta]\ C_T = [\Delta]$ Control - $[\Delta]\ C_T$ siRNA-CAV1
- $(1 - [\Delta][\Delta]\ C_T)*100$ = % KD of CAV1

Results

CAV1 siRNA-directed gene knockdown was performed using the reverse transfection method. The cell lines were seeded in equal number for CAV1-siRNA or Scrambled-negative control and cell proliferation was determined at 24, 48, 72, 96 and 120 hrs post-siRNA using the Cell TiterGlo assay. An overview of the experimental design is shown in Figure 10. The TaqMan Gene Expression Array was used to determine the efficiency of transcript knockdown. The TNBC , ER-negative mesenchymal BC cell line, MDAMB-157, the ER-negative, epithelial-like BC cell lines, and the MCF7 and T47D showed transcripts that were successfully knocked down (> 94%) (Fig. 11). The TNBC ER-negative mesenchymal lines, MDAMB-231, and the basal-like ER-negative line, HCC1806 achieved >60% knockdown (Fig. 11). Cav1 protein expression to monitor siRNA efficiency is depicted at 120 hrs post siRNA transfection (Fig. 12). Finally, TNBC cell lines, mesenchymal and epithelial-like, exhibited a decrease in cell proliferation compared to siRNA-scrambled control and the luminal A breast cancer cell lines, MCF7, T47D (Fig. 13).

	Proliferation	mRNA Expression	Protein Expression
Lipofectamine	RNAiMax - 0.60 µL	RNAiMax - 0.60 µL	RNAiMax - 0.60 µL
siRNA	30 nM - CAV1 - Neg-Scramb/Control - Untreated	30 nM - CAV1 - Neg-Scramb/Control - Untreated	30 nM - CAV1 - Neg-Scramb/Control - Untreated
Cell Lines	5×10^4 cells/well - G1: 231, HCC1806, 157 - G2: HCC70 - LumA: MCF7, T47D	5×10^4 cells/well - G1: 231, HCC1806, 157 - G2: HCC70 - LumA: MCF7, T47D	5×10^5 cells/well - G1: 231, HCC1806, 157 - G2: HCC70 - LumA: MCF7, T47D
Plate	- 96 Well Opaque	- TC Treated - 96 Well Clear	- TC Treated - 6 Well Clear
Time Points	- 24, 48, 72, 96, 120 - Hrs Post Transf.	- 72, 96, 120 - Hrs Post Transf.	- 48, 72, 96, 120 - Hrs Post transf.
Method	- CellTiter-Glo® - Luminescent - Cell Viability Assay	1) Cells-to-cDNA 2) cDNA Synthesis 3) TaqMan Assay - CAV1/β-actin)	1) Collect Protein Lysate 2) Gel Electrophoresis 3) Western Blot - CAV1/α-tubulin

Figure 10. Overview of Experimental Design. The reverse transfection method was used with the Silencer® Select pre-designed and validated siRNAs to CAV1 and Lipofectamine® RNAiMAX Transfection Reagent. A panel of breast cancer cell lines were used to perform the a proliferation assay and validation of CAV1 silencing for each breast cancer cell line was determined by western blot and RT-qPCR technologies.

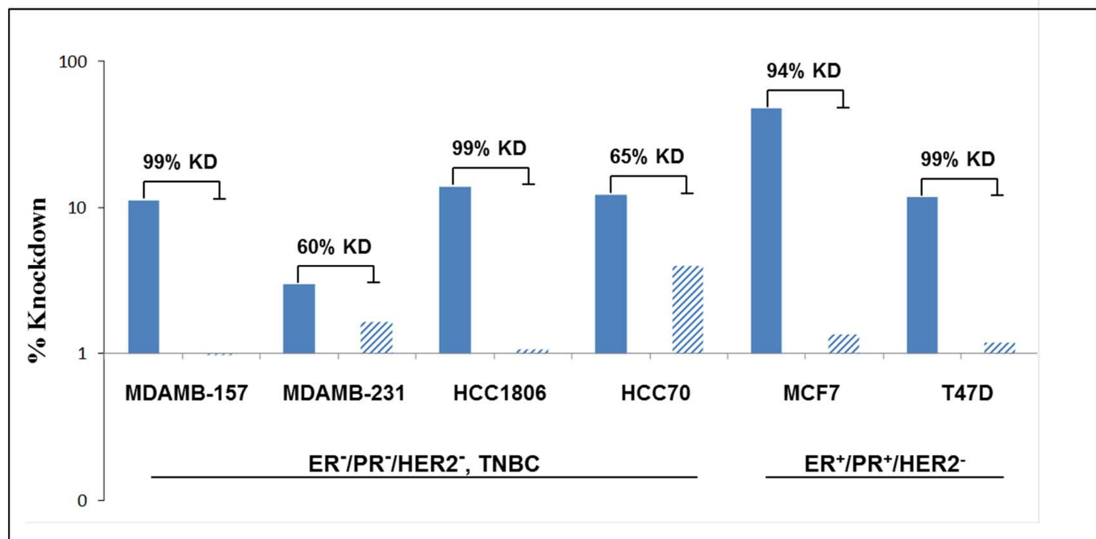


Figure 11. Validation of CAV1 Silencing of mRNA Expression. TaqMan Gene Expression Assays for CAV1 and β-actin was performed using cDNA prepared from total RNA isolated each of the cell lines. The comparative C_T method was used to calculate the relative expression levels of CAV1 mRNA (solid blue bar). The CAV1 percent knockdown (% KD) (striped blue bar) for each cell line was determined by using the results of the comparative C_T method.

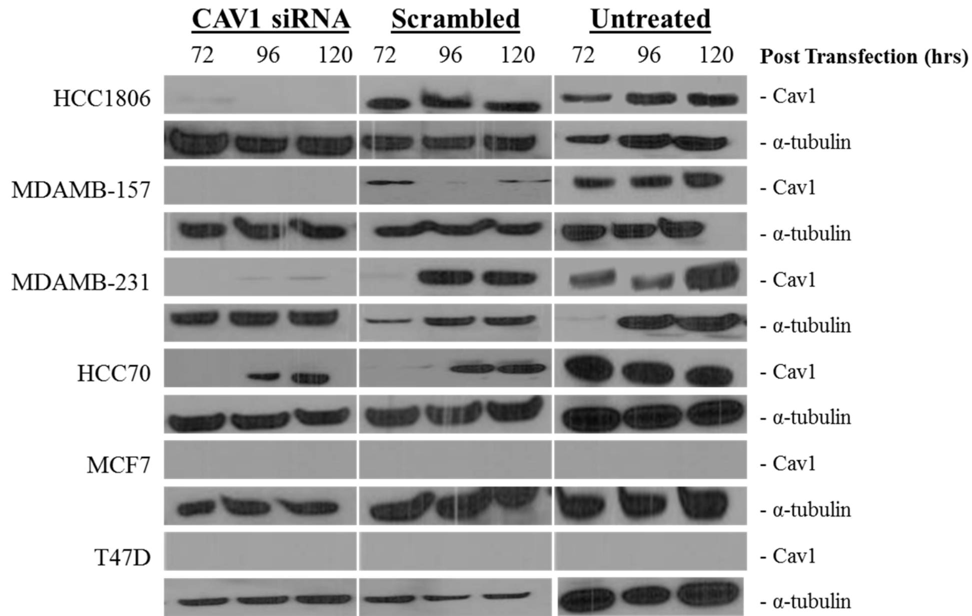


Figure 12. Validation of Cav1 Silencing of Protein Expression. Western blot analysis between 72, 96 and 120-hrs post transfection. Cell lysates from each of the cell lines were probed using Cav1and α-tubulin antibodies. Results are representative of three separate experiments and demonstrate a decrease in endogenous Cav1 expression in response to CAV1 siRNA treatment, when compared to scrambled and negative control.

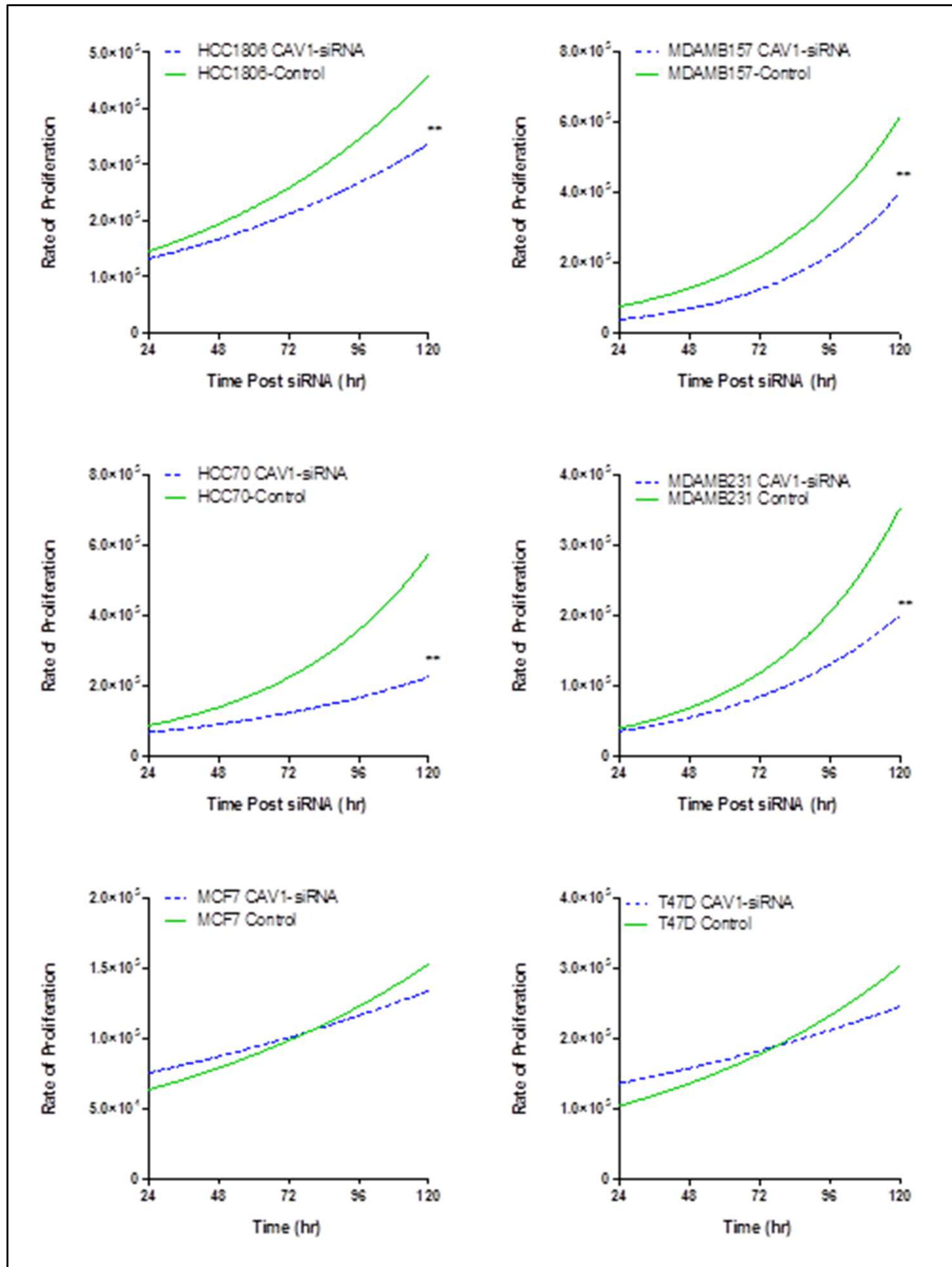


Figure 13. CAV1 Silencing Inhibits Cell Proliferation in TNBC Cell Lines. Cell proliferation rates were determined by measuring ATP levels with the CellTiter-Glo luminescent cell viability assay, at 24, 48, 72, 96 and 120 hrs post siRNA transfection. The proliferation rate was determined using the nonlinear regression and the exponential growth equation in GraphPad Prism. First, scrambled negative-control and CAV1-siRNA results were adjusted for background by using the results of the negative control and depicted in the graphs above. Then the adjusted proliferation rate (doubling time; (k is ln(2)/K)) of siRNA treated and scrambled negative control were compared using unpaired t- test, ** p<.0001 (table 6).

Table 6. CAV1-siRNA Significantly Decreased TNBC Cell Line Proliferation.

CAV1 siRNA vs. Scrambled Negative Control					
	Unpaired t-test			F test	
Cell Line	P value	Significance	R square	P Value	Significance
HC1806	<0.0001	Yes	0.06498	<0.0001	Yes
MDA-MB-157	<0.0001	Yes	0.09989	<0.0001	Yes
HCC70	<0.0001	Yes	0.2155	<0.0001	Yes
MDA-MB-231	<0.0001	Yes	0.05330	<0.0001	Yes
MCF7	0.3174	No	0.003355	<0.0001	Yes
T47D	0.3089	No	0.003474	<0.0001	Yes

Conclusion

CAV1 has been implicated as an oncogene in BC, as a biomarker of aggressive disease and has been shown to promote proliferation and invasion. In this study, we used siRNA technology to silence the expression of CAV1 mRNA in a panel of four-TNBC and two-luminal cell lines. We used RT-qPCR and western blot technology to confirm CAV1 mRNA and protein silencing throughout the experiment. The majority of the TNBC cell lines and both luminal cell lines displayed >90% knockdown efficiency (Fig, 11). Two of the highest endogenous expressers of CAV1, the TNBC mesenchymal line, MDAMB-231, and the basal-like HCC1806 achieved > 60% knockdown (Fig. 11). As depicted in figure 12, the Cav1 protein decreased in the CAV1-siRNA treated and increased in the corresponding scrambled negative control for each of the four TNBC cell lines. We found that the TNBC cell lines that expressed both high and moderate levels of endogenous CAV1 exhibited a decrease in cell proliferation, compared to scrambled negative controls while the luminal cell lines showed no effect (Fig 13, Table 6).

CHAPTER 5

SUMMARY AND CONCLUSIONS

This study presents a potential biological contributor to the observed health disparities between AA and CA women diagnosed with TNBC. First, a distinct pattern of gene expression was identified by unsupervised hierarchical clustering of gene expression array data derived from a cohort of TNBC from AA and CA women diagnosed with lymph node 0 diseases. The CA cohort segregated into all six of the TNBC subtypes while the AA cohort was comprised of mainly the Basal-like 2 (14%), Immunomodulatory (43%) and Mesenchymal (43%) TNBC subtypes. Next, comparative marker selection revealed 190 differentially expressed genes between the AA cohort and CA cohort; most of which were downregulated in the AA cohort. These observations demonstrate that even at the earliest stage of this disease, underlying differences in tumor biology exist between these cohorts.

Aberrant Wnt signaling has been shown to play a role in tumor development and progression in cancer and in particular, TNBC. Recent studies have observed deregulation of the Wnt/β-catenin signaling pathway in TNBC and BLBC, associated with high grade, poor prognosis and metastatic disease (47). However, these studies did not specifically investigate differences between CA and women of African descent (46-47). The data generated in this study, revealed that genes that were differentially expressed between the AA and CA cohort were over-represented in pathways associated with cytoskeletal remodeling, cell adhesion, tight junctions, and Wnt-β-catenin. Additionally, candidate genes investigated in this study (FOX03A, TNC, TCF4, CAV1) were shown to be over-expressed in the top 10-enrichment pathways and preferentially

involved in the Wnt-β-catenin pathway. Although technical validation of the original Almac samples identified CAV1 as being significantly expressed in the AA-TNBC cohort, the other candidate genes may deserve further investigation. It is possible that increasing the size of each cohort may reveal a significant correlation between any or all of the candidate genes. For instance, FOX03a has been shown to bind to the cav-1 promoter in idiopathic pulmonary fibrosis, which leads to an increase in endogenous levels of cav-1 mRNA and protein expression (64).

Technical validation of CAV1 overexpression in AA women with early stage TNBC was performed using an independent cohort of FFPE samples. First, IHC results confirmed Cav1 expression in both cohorts, although preferentially localized in the cytoplasm and plasma membrane. These results were expected considering the role of CAV1 at the plasma membrane. Although CAV1 is necessary in the normal development of the breast, more so in the myoepithelial cells/less in luminal cells, it was of great interest that Cav1 protein and mRNA expression was significantly higher in the AA cohort compared to the CA (51,53). Using cell line models of TNBC and RNAi technology we demonstrated that loss of endogenous CAV1 expression decreased the overall rate of cell proliferation in the TNBC cell lines, but had no effect on the luminal breast cancer cell lines, MCF7 and T47D. This would suggest a functional role of CAV1 in TNBC, even at the earliest stage of disease.

CAV1 has been termed a ‘Molecular Hub’, due to its role at the plasma membrane where it is involved in numerous signaling pathways such as Ras/Mek/Erk and Rock/Src (50-52). CAV1 has been shown to be a tumor suppressor in the colon, ovary, lung and in ER+ breast cancer (54-56). A study by Wiechen et al, found that CAV1

expression was lower in ovarian tumor samples and when over expressed in an ovarian cancer cell line it resulted in suppression of cell survival, suggesting a tumor suppressor role in ovarian cancer (55). There is controversy regarding the role of CAV1 in cancer, however these differences in seem to be dependent on the tumor grade, location and even the subtype of cancer. For example, within the human lung cancer types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), CAV1 seems to have very different roles; acing as a tumor suppressor in in SCLC and an oncogene in NSCLC (56). Additionally, CAV1 expression has been shown to be higher in metastatic versus primary cancers (53,57). Strong evidence seems to support CAV1 as an oncogene in bladder, thyroid, prostate, renal, and pancreatic cancer (50, 51, 58, 59). CAV1 has been shown to be an oncogene and possible biomarker of aggressive disease and predictive of relapse in prostate cancer (57,59,60). Of particular interest to this study, CAV1 overexpression has been shown to play a role in proliferation, migration and invasion and to be increased in BLBC/TNBC (51,53,61,62). Increased CAV1 expression in tumors has been associated with EMT, metastasis and resistance to therapy (50,51, 53,58). Location of CAV1 in BC has also been investigated and data suggest that high expression in tumor and low expression in stroma may be associated with poor prognosis (63).

TNBC, regardless of race, lack the common hormone receptors/targets required for conventional hormone or targeted therapy and have a lower relapse free and lower overall survival period compared to other subtypes of breast cancer. Although race is not considered a risk factor in the development of breast cancer, health disparities do exist. There is a high prevalence of TNBC in African American women and women with African Ancestry and this cohort has been shown to have an overall worse clinical

outcome compared to Caucasian women with the same disease. CAV1 may promote tumor progression, metastasis, and invasion and is highly expressed in ER- breast cancer cell lines, and the overexpression in our African American TNBC cohort may confer the unfavorable outcomes in AA women with TNBC. The combined study results suggest that CAV1 over -expression may be a biological contributor to the observed health disparity between AA women and CA diagnosed with early stage TNBC.

Future Directions

The observations from this study are striking considering a small initial sample size; additional studies involving TNBC from both AA and CA women would need to include an increased sample size. Additionally, there needs to be further investigations into all the differentially expressed genes between these cohorts. These studies should include exploring the interaction between the genes from the initial DEG list, which may lead to novel TNBC-specific pathways.

CAV1 overexpression has been shown to play a role not only in proliferation but also in migration and invasion and is associated with EMT, metastasis and resistance to therapy (50,51,53, 58,61,62). In this study, only the rate of proliferation was examined and additional studies will be necessary to further elucidate the functional role of CAV1 expression in TNBC. These studies would include the use of siRNA technologies to evaluate potential effects on migration, invasion, apoptosis, and EMT. Additionally, using patient derived xenograft models (PDX) of TNBC, investigations involving the levels of tumor CAV1 expression and chemosensitiviy to currently available treatment regimens could determine if CAV1 is a potential indicator of response to therapy.

It may be possible to exploit the high expression of CAV1 in tumors, based on the role of Cav1 at the plasma membrane by using nanoparticle albumin-bound (Nab) drug(s) to treat women with early stage TNBC. Nab technology uses the albumin receptor and CAV1 pathway to achieve targeted drug therapy and accumulation (65). A recent investigation into nab-paclitaxel (Nab-P) and combination therapy with carboplatin has shown some very promising results. They found that higher Cav1 expression in tumor-associated stroma was significantly associated with improved relapse-free survival and overall survival in advanced NSCLC patients (65). Finally, in a resent clinical trial (NCT00777673) using weekly Nab-P combination therapy (carboplatin followed by doxorubicin plus cyclophosphamide with concurrent bevacizumab) it was found that the majority of the TNBC patients achieved pathological complete response (66).

REFERENCES

1. Andre, F., Michiels, S., Dessen, P., Scott, V., Suciu, V., Uzan, C., Lazar, V., Lacroix, L., Vassal, G., Spielmann, M., et al. (2009). Exonic expression profiling of breast cancer and benign lesions: a retrospective analysis. Lancet Oncol 10, 381-390.
2. Breast Cancer Facts & Figures 2013-2014(1-25).
3. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA: A Cancer Journal for Clinicians 2014: n/a-n/a. Available at: http://dx.doi.org/10.3322/caac.21208.
4. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Science. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Jan 9;235(4785):177-82, 1987.
5. NCCN Clincial Practice Guidelines in Oncology v3.2014.
6. Rosen's Breast Pathology 2014 Early Breast Cancer Trialists' Collaborative Group. Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomized trials. Lancet. 365(9472):1687-717, 2005.
7. Davies C, Godwin J, Gray R, et al. for the Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet. 378(9793):771-84, 2011.
8. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, et al.: Molecular portraits of human breast tumours. Nature 2000, 406(6797):747-75, 2000.
9. Parker JS, Mullins M, Cheang MC, Leung S, Voduc D, Vickery T, Davies S, Fauron C, He X, Hu Z, et al.: Supervised risk predictor of breast cancer based on intrinsic subtypes. J Clin Oncol, 27(8):1160-1167, 2009.
10. Sorlie, T. et. al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Sep 11;98(19):10869-74, 2001. PNAS.
11. van de Vijver et al: A gene- expression signature as a predictor of survival in breast cancer. N Engl J Med 347: 1999-2009, 2002.
12. Sorlie T et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. PNAS, 100(14):8418-8423, 2003.
13. Rosen's Breast Pathology Syed A. Hoda, Edi Brogi, Fred Koerner, Paul Peter Rosen. Publisher: Lippincott Williams & Wilkins, Publication date: 3/6/2014, Pages: 1400.
14. Creighton CJ. The molecular profile of luminal B breast cancer. 2012. Biologics. 2012; 6: 289–297. PMCID: PMC3430090.
15. Blows FM, Driver KE, Schmidt MK, Broeks A, van Leeuwen FE, et al. (2010) Subtyping of breast cancer by immunohistochemistry to investigate a relationship between subtype and short and long term survival: a collaborative analysis of data for 10,159 cases from 12 studies. PLoS Med 7: e1000279.

16. Metzger-Filho O, Sun Z, Viale G, et al. Patterns of recurrence and outcome according to breast cancer subtypes in lymph node-negative disease: results from international breast cancer study group trials VIII and IX. J Clin Oncol. 31(25):3083-90, 2013.
17. Lund MJ, Butler EN, Hair BY, et al. Age/race differences in HER2 testing and in incidence rates for breast cancer triple subtypes: a population-based study and first report. Cancer. 116(11):2549-59, 2010.
18. Koboldt DC, Fulton RS, McLellan MD, et al. for the Cancer Genome Atlas Network. Comprehensive molecular portraits of human breast tumours. Nature. 490(7418):61-70, 2012.
19. Haque R, Ahmed SA, Inzhakova G, et al. Impact of breast cancer subtypes and treatment on survival: an analysis spanning two decades. Cancer Epidemiol Biomarkers Prev. 21(10):1848-55, 2012.
20. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987;235:177–182.
21. Carey LA, Perou CM, Livasy CA, et al. Race, breast cancer subtypes, and survival in the Carolina Breast cancer Study. JAMA. 295(21):2492-2502, 2006.
22. Voduc KD, Cheang MC, Tyldesley S, Gelmon K, Nielsen TO, Kennecke H. Breast cancer subtypes and the risk of local and regional relapse. J Clin Oncol. 28(10):1684-91, 2010.
23. Carey LA. Molecular intrinsic subtypes of breast cancer. In: UpToDate. Hayes DF, Dizon DS (eds.). Waltham, MA: UpToDate, 2013.
24. Carey, L. Winer, E. Viale, G. Cameron, D. and Gianni, L. 2010. TNBC : Disease Entity or Title of Convenience? Nat.Rev.Clin.Oncol 7: 683-692.
25. Rakha EA, Chan S: Metastatic TNBC. Clinical oncology 2011, 22(9):587-600.
26. Boyle, P. 2012. TNBC: Epidemiological Considerations and Recommendations. Annals of Onco 23(Supplement 6) vi7-vi12.
27. Brawley, O. 2013. Health Disparities in Breast Cancer. Obstet Gynecol Clin N Am 40:513-523.
28. Amirikia, K.C. Mills, P. Bush, J. and Newman, L.A. 2011. Higher Population-based Incidence Rates of TNBC Among Young African-American Women. Cancer 117(12): 2747-2753.
29. Stead, L. et al. 2008. TNBC s are Increased in Black Women Regardless of Age or Body Mass Index. Breast Cancer Res 11(2):R18.
30. Stark A, Kleer CG, Martin I, Awuah B, Nsiah-Asare A et al. 2010. African ancestry and higher prevalence of TNBC: findings from an international study. Cancer 116: 4926-4932.
31. Gjorevski, N. and Nelson, C.M. 2011. Integrated Morphodynamic Signalling of the Mammary Gland. Nat. Rev. Mole. Cell Bio 12: 581-593.

32. Kendziorra E, Ahlborn K, Spitzner M, Rave-Fra¨nk, M, Emons G, Gaedcke J, Kramer F, Wolff HA, Becker H, Beissbarth T, Ebner R, Ghadimi BM, Pukrop T, Ried T, and Grade M. 2011. Silencing of the Wnt transcription factor TCF4 sensitizes colorectal cancer cells to (chemo-) radiotherapy. Carcinogenesis 32(12):1824–1831.

33. Witkiewicz, A. K. et al. 2010. Loss of Stromal Caveolin-1 Expression Predicts Poor Clinical Outcome in Triple-Negative and BLBC s. Cancer Biol & Therapy 10(2): 135-143.

34. Van den Eynden GG. Van Laere SJ, Van der Auwera I, Merajver SD, Van MArick EA, van Dam P, Vermeulen PB, Dirix LY, van Golen KL. Overexpression of caveolin-1 and -2 in cell lines and in human samples of inflammatory breast cancer. Breast Cancer Research Treatment. 2006, 95-219-228.

35. Ruiz C, Huang W, Hegi ME, Lange K, Hamou MF, Fluri E, Oakeley EJ, Chiquet-Ehrismann R, Orend G. Growth promoting signaling by tenascin-C. Cancer Res. 2004 Oct 15;64(20):7377-85.

36. Karadedou CT, Gomes AR, Chen J, Petkovic M, Ho K-K, Zwolinska AK, Feltes A, Wong SY, Chan KYK, Cheung Y-N, Tsang JWH, Brosens JJ, Khoo U-S, Lam WW-F: FOXO3a represses VEGF expression through FOXM1-dependent and –independent mechanisms in breast cancer. Oncogene 2012, 31: 1845-1858.

37. Sunters A, Fernandez de Mattos S, Stahl M, Brosens JJ, Zoumpoulidou G, et al. (2003) FoxO3a transcriptional regulation of Bim controls apoptosis in paclitaxel-treated breast cancer cell lines. J Biol Chem 278: 49795–49805.

38. Craig DW, O'Shaughnessy JA, Kiefer JA, Aldrich J, Sinari S, Moses TM, Wong S, Dinh J, Christoforides A, Blum JL, Aitelli CL, Osborne CR, Izatt T, Kurdoglu A, Baker A, Koeman J, Barbacioru C, Sakarya O, De La Vega FM, Siddiqui A, Hoang L, Billings PR, Salhia B, Tolcher AW, Trent JM, Mousses S, Von Hoff D, Carpten JD.. Genome and transcriptome sequencing in prospective metastatic TNBC uncovers therapeutic vulnerabilities. Mol. Cancer. Ther. 12, 104–116 (2013).

39. Bilir B. Kucuk, O. and Moreno, C.S. 2013. Wnt Signaling Blockage Inhibits Cell Proliferation and Migration, and Induces Apoptosis in TNBC Cells. J Trans Med 11: 280.

40. Dey, N. et al. 2013. Wnt Signaling in Triple Negative Breast Cancer is Associated with Metastasis. BMC Cancer 13: 537.

41. Sachdev, J. Ahmed, S. Mirza, M.M. Farooq, A. Kronish, L. and Jahanzeb, M. 2010. Does Race Affect Outcomes in Triple Negative Breast Cancer? Breast Cancer : Basic and Clin Res 4: 23-33.

42. Carey, L.A. et al. 2006. Race, Breast Cancer Subtypes, and Survival in the Caroline Breast Cancer Study. JAMA 295(21): 2492-2502.

43. Wheeler, S.B. Reeder-Hayes, K.E. and Carey, L.A. 2013. Disparities in Breast Cancer Treatment and Outcomes: Biological, Social, and Health System Determinants and Opportunities for Research. The Onco 18:986-993.

44. H C Mefford, L Baumbach, R C Panguluri, C Whitfield-Broome, C Szabo, S Smith, M C King, G Dunston, D Stoppa-Lyonnet, F Arena. Evidence for a BRCA1 founder mutation in families of West African ancestry. 1999. Am J Hum Genet 65: 575-578.

45. Palmer, J.R. Boggs, D.A. Wise, L.A. Ambrosone, C.B. Adams-Campbell, L.L. and Rosenberg, L. 2011. Parity and Lactation in Relation to Estrogen Receptor Negative Breast Cancer in African American Women. Cancer Epidemiol Biomarkers Prev 20(9: 1883-91.

46. Shinde, S.S. et al. 2010. Higher Parity and Shorter Breastfeeding Duration. Cancer 116:4933-4943.

47. Dietze EC, Sistrunk C, Miranda-Carboni G, O'Regan R, Seewaldt VL.Triple-negative breast cancer in African-American women: disparities versus biology2015. Nature Reviews Cancer 15, 248–254.

48. Lehmann, B.D., Bauer, J.A., Chen, X., Sanders, M.E., Chakravarthy, A.B., Shyr, Y., and Pietenpol, J.A. (2011). Identification of human TNBC subtypes and preclinical models for selection of targeted therapies. J Clin Invest. 121: 2750–67.

49. Chen, X., Li, J., Gray, W.H., Lehmann, B.D., Bauer, J.A., Shyr, Y., and Pietenpol, J.A. (2012). TNBC type: A Subtyping Tool for TNBC. Cancer informatics 11, 147–156.

50. Yu H, Shen H, Zhang Y, et al. CAV1 promotes HCC cell progression and metastasis through Wnt/β-catenin pathway. PLoS One. 9(9):e106451, 2014.

51. Senetta, R., Stella, G., Pozzi, E., Sturli, N., Massi, D., & Cassoni, P. (2013). Caveolin-1 as a promoter of tumour spreading: when, how, where and why. Journal of Cellular and Molecular Medicine, 17(3), 325–336, 2013.

52. Rao, X., Evans, J., Chae, H., Pilrose, J., Kim, S., Yan, P., Nephew, K. P. (2013). CpG island shore methylation regulates caveolin-1 expression in breast cancer. Oncogene, 32(38), 4519–4528. doi:10.1038/onc.2012.474

53. Savage K, Lambros M, Robertson D, et al. Caveolin 1 is overexpressed and amplified in a subset of basal-like and metaplastic breast carcinomas: a morphologic, ultrastructural, immunohistochemical, and in situ hybridization analysis. Clin Cancer Res. 13(1):90-101, 2007.

54. Bender FC, Reymond MA, Bron C, Quest AFG. Caveolin-1 Levels Are Down-Regulated in Human Colon Tumors, and Ectopic Expression of Caveolin-1 in Colon Carcinoma Cell Lines Reduces Cell Tumorigenicity. Cancer Res. 60:5870-587, 2000.

55. Wiechen, K., Diatchenko, L., Agoulnik, A., Scharff, K. M., Schober, H., Arlt, K., Sers, C. Caveolin-1 Is Down-Regulated in Human Ovarian Carcinoma and Acts as a Candidate Tumor Suppressor Gene. The American Journal of Pathology, 159(5), 1635–1643, 2001.

56. Sunaga N, Miyajima K, Suzuki M, Sato M, White MA, et al. Different roles for caveolin-1 in the development of non-small cell lung cancer versus small cell lung cancer. Cancer Res 64: 4277–4285, 2004

57. Corn P, Thompson T. Identification of a novel prostate cancer biomarker, caveolin-1: Implications and potential clinical benefit. Cancer Manag Res. 2 111-22, 2010.
58. Huang C, Qiu A, Wang L, et al. A novel FoxM1-caveolin signaling pathway promotes pancreatic cancer invasion and metastasis. Cancer Res. 72(3):655-65, 2012.
59. Thompson T, Tahir A, Li L, et al. The role of caveolin-1 in prostate cancer: clinical implications. Prostate Cancer Prostatic Dis. 13(1):6-11, 2010.
60. Nassar ZD, Hill MM, Parton RG, Parat MO. Caveola-forming proteins caveolin-1 and PTRF in prostate cancer. Nat Rev Urol. 2013;10:529-536, 2013.
61. Pinilla S, Honrado E, Hardisson D, et al. Caveolin-1 expression is associated with a basal like phenotype in sporadic and hereditary breast cancer. Breast Cancer Res Treat. 99(1):85-90, 2006.
62. Elsheikh S, Green A, Rakha E, et al. Caveolin 1 and Caveolin 2 are associated with breast cancer basil-like and triple negative immunophenotype. Br J Cancer. 99(2):327-34, 2008.
63. Qian N, Ueno T, Kawaguchi-Sakita N, et al. Prognostic significance of tumor/stromal caveolin-1 expression in breast cancer patients. Cancer Sci. 102(8):1590-6, 2011.
64. Nho RS, Peterson M, Hergert P, Henke CA. FoxO3a (Forkhead Box O3a) Deficiency Protects Idiopathic Pulmonary Fibrosis (IPF) Fibroblasts from Type I Polymerized Collagen Matrix-Induced Apoptosis via Caveolin-1 (cav-1) and Fas. PLoS ONE 8(4): e61017, 2013.
65. Bertino EM, Williams TM, Nana-Sinkam SP, Shilo K, Chatterjee MC, Mo X, Rahmani M, Phillips GS, Villalona-Calero MA, Otterson GA. Stromal Caveolin-1 Is Associated With Response and Survival in a Phase II Trial of nab-Paclitaxel With Carboplatin for Advanced NSCLC Patients. Clin Lung Cancer S1525-7304(15)00122-9, 2015.
66. Snider JN, Sachdev JC, Allen JW, Schwartzberg LW, Young RR, Javed AY, Yunus F, Verrier CS, Jahanzeb M. Pathologic complete response (pCR) with weekly nanoparticle albumin bound (nab-P) plus carboplatin (C) followed by doxorubicin plus cyclophosphamide (AC) with concurrent bevacizumab (B) for triple-negative breast cancer (TNBC). J Clin Oncol 31, 2013 (suppl; abstr 1068)