CLINICAL SIGNIFICANCE OF ANNEXIN A2 IN PREDICTING POOR PROGNOSIS IN AFRICAN AMERICAN WOMEN WITH TRIPLE-NEGATIVE BREAST CANCER

DISSERTATION

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By

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ABBREVIATIONS

AA	African American
AnxA1	Annexin A1
AnxA2	Annexin A2
AnxA3	Annexin A3
AnxA4	Annexin A4
AnxA5	Annexin A5
AnxA6	Annexin A6
AnxA7	Annexin A7
AnxA8	Annexin A8
BRCA	Breast Invasive Carcinoma
CA	Caucasian American
CISH	chromogenic in situ hybridization
DMFS	Distant Metastasis Free Survival
EA	European American
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
Exo-AnxA2	Exosomal Annexin A2
FISH	fluorescence in situ hybridization
GEO	Gene Expression Omnibus
GEP	Gene Expression Profiling
HA	Hispanic American
HER2	Human Epidermal Growth Factor Receptor/ ErBb2

Hematoxylin & Eosin	H&E
IHC	Immunohistochemistry
IlluminaHiSeq	Illumina HiSeq 2000
IRB	Institutional Review Board
LCKLSL	AnxA2 competitive inhibitory peptide
LGKLSL	AnxA2 control peptide
n	number
NCBI	National Center for Biotechnology Information
OS	Overall Survival
P	*P*-value
PR	Progesterone Receptor
RFS	Relapse Free Survival
RT	Room Temperature
TCGA	The Cancer Genome Atlas
TNBC	Triple-Negative Breast Cancer
TNM	T, Tumor Size; N, Nodal Status; M, Metastasis
+	Positive
-	Negative

CONTRIBUTION OF THE AUTHORS

Chapter II

Lee D. Gibbs, Sayantan Maji, and Jamboor K. Vishwanatha conceptualized the study. Lee D. Gibbs and Sayantan Maji standardized and performed ELISAs and serum isolation. Lee D. Gibbs performed *in vivo* matrigel plug assay and statistical analysis. Cheryl M. Lewis provided patient archived serum collection. Piyush Kumar measured the size of the exosomes. Lee D. Gibbs and Jamboor K. Vishwanatha interpreted the data.

Chapter III

Lee D. Gibbs and Jamboor K. Vishwanatha conceptualized the study. Lee D. Gibbs performed bioinformatics and statistical analyses, and in situ hybridization. Richard J. Hare and Rebecca A. Mantsch blindly scored tissue sections and provided useful inputs from a clinical perspective. Lee D. Gibbs and Jamboor K.Vishwanatha interpreted the data.

Chapter IV

Lee D. Gibbs and Jamboor K. Vishwanatha conceptualized the study. Lee D. Gibbs performed bioinformatic and statistical analyses. Lee D. Gibbs and Jamboor K. Vishwanatha interpreted the data.

CHAPTER I

INTRODUCTION

Breast Cancer

Everyone has had a relative, friend, neighbor, classmate or co-worker that has been diagnosed with cancer. One of the most feared cancers among women is breast cancer. The fear of breast cancer has left many woman with the belief that their breast is a ticking time bomb counting down to the explosion of uncontrolled cells that will one day lead to cancer. This fear has driven women that have a familial history of breast cancer to go as far as to remove their breast to prevent the possibility of suffering the same fate as their ancestors. Breast cancer research seeks to comprehensively understand breast cancer's biology to discover innovative and appropriate preventative, diagnostic, and prognostic methods to eradicate this fear forever.

The American Cancer Society (ACS) predicts that in 2017 252,710 new cases of invasive breast cancer and 63,410 new cases of carcinoma in situ (CIS) will be diagnosed in the United Sates (1). Further, a predicted 40,610 women will succumb to this disease. Based on a 2011-2013 report by the Surveillance, Epidemiology, and End Results (SEER) Program, approximately 12.4% of women will be diagnosed with breast cancer at some point during their lifetime (2). The yearly statistics from all major national health related organizations such as: The National Institute for Health (NIH), National Cancer Institute (NCI), Centers for Disease Control and Prevention (CDCP), World Health Organization (WHO), SEER, ACS and others have detailed the risk of breast cancer nationally and internationally. Although, breast cancer is the second leading cause of cancer death among women, the discoveries made in research have dropped breast cancer rates significantly since 1989 (3-5). This would suggest that continued strides in raising awareness of early detection, discovering innovative screening techniques, and

establishing appropriate clinical recommendations to adequately diagnose and prognosticate this disease will continue to decrease the overall number of lives affected by breast cancer.

Breast Cancer Molecular Subtypes

At a glance, we often think of breast cancer as a single homogenous disease. But, in fact it is a heterogeneous complex of diseases. This complex has been delineated into several molecular subtypes that have different treatment options, responses to therapy, and clinical outcomes. These advances in breast cancer classification have led to the identification of three molecular markers: Estrogen Receptor (ER), Progesterone Receptor (PR), and Human Epidermal Growth Factor Receptor (HER2) (6-10). These analysis separated breast cancer into three subtypes: ER+ and/ or PR+, HER2+, and Triple-Negative (lacks expression of all three markers) breast cancer subtype. The presentation of these markers is determined after breast lumpectomy and sent to a histology lab where a pathologist performs immunohistochemistry or *in situ* hybridization to determine the expression of these markers. Taken together, tumor size, tumor grade, and nodal involvement are conventionally used for prognosis and therapeutic management of a patient's disease (11-13). Unfortunately, some patients do not benefit from this standard of care and often have higher risk of recurrence, distant metastasis, and death.

Breast Cancer Intrinsic Subtypes

The development of microarrays and genomic sequencing has expanded our interpretation of breast cancer classification (14,15). Sorlie *et al.* utilized gene expression profiling (GEP) to create a distinctive molecular portrait of breast cancer using 456

cDNA clones, and reclassified tumors into five intrinsic clinical subtypes: Luminal A (ER+ and/or PR+/HER2-), Luminal B (ER+ and/or PR+/HER2+), Basal-like (ER- and/or PR-/HER2-), HER2+ (ER- and/or PR-/HER2+), and normal-like tumors (16,17). These analyses also revealed that the reclassification of these subtypes could also potentiate clinical outcomes and prognoses (18). Figure 1 demonstrates the association of each intrinsic subtype to histological grade, potential prognosis, and therapeutic regimen. Luminal A tumors have the more favorable prognoses and make up approximately 40% of all breast cancer cases. It is often diagnosed at lower grades (well differentiation of cells) and their morphology mimics the luminal epithelial component of the breast (16,19). Patients with Luminal A tumors are often given targeted endocrine therapies toward the expression of their receptors such as anti-estrogen or aromatase inhibitors (20). Luminal B tumors are very similar to Luminal A tumors as they express the ER receptor and have favorable prognoses (21). Additionally this subtype expresses HER2 receptor and has higher expression of proliferative genes in comparison to Luminal A (19). These tumors makes up approximately 20% of breast cancer cases and tend to be diagnosed at higher tumor grades than Luminal A tumors. HER2 is as a unique identifier of a subset of breast cancer patients that was found after the discoveries of ER and PR. Unlike, ER and PR, HER2 can be identified by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH). Although these two experimental techniques have been perfected throughout their use, all HER2 positive tumors do not show expression at the protein and transcriptional level. Thus, HER2 classification is also

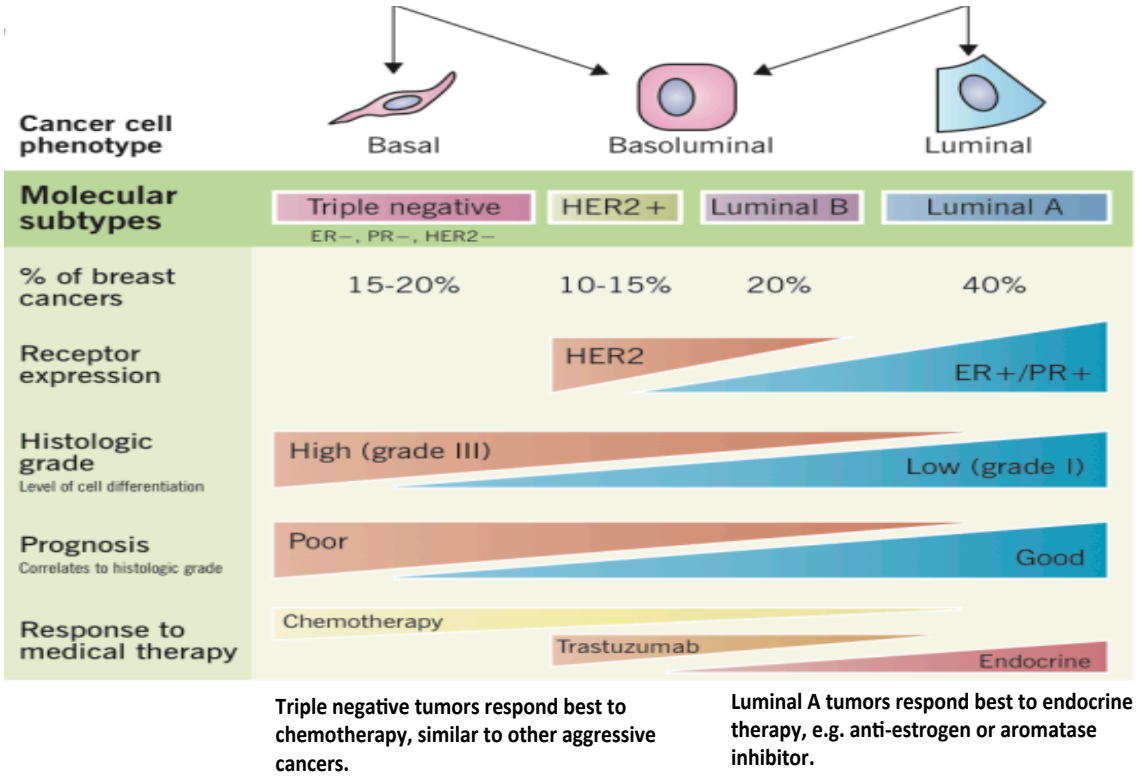

Figure 1. *Breast Cancer Intrinsic Subtypes. Modified from*

http://www.pathophys.org/breast-cancer/.

characterized by expression of other genes in the HER2 amplicon such as: Growth Factor Receptor Bound Protein 7 (GRB7), Post-GPI Attachment To Proteins 3 (PGAP3), and TP53 (Tumor Protein 53) mutation (17, 22). HER2 tumors are often aggressive and have poor prognoses. They are more likely to be diagnosed at higher grades and are usually treated by a well-known targeted therapy, Trastuzumb (HER2 antibody), coupled with radiation. Additionally these tumors are sensitive to anthracycline and taxane-based neoadjuvant chemotherapy (23). Triple-Negative breast cancer (TNBC) makes up approximately 15-20% of breast cancer diagnoses and consists of 60-90% basal like tumors, mimicking basal epithelial cells found in other parts of the body (17,24). TNBC has a high proliferative index and has high expression of basal markers (such as keratins 5, 6, 14, 17, Epidermal Growth Factor Receptor) (17,24). They are often presented as higher grade (poor differentiation of cells) and have the worst prognosis (25). TNBC is the most aggressive breast cancer subtype and is unresponsive to anti-hormonal and Her2-targeted therapies due to the absence of hormone receptors and Her2 expression. Similar to other aggressive breast cancers, TNBC tumors respond best to a combination of chemotherapy and radiation. Though characterization of these entities of breast cancer have advanced our understanding of clinical outcomes and therapeutic approaches, we must continue studying tumor heterogeneity to yield the best descriptive analysis of each patient's tumor.

Triple Negative Breast Cancer: A Health Disparity

There are several risk factors that have been associated with TNBC such as: earlier menarche, high waist-to-hip ratio, and a lack of breast-feeding together with high parity

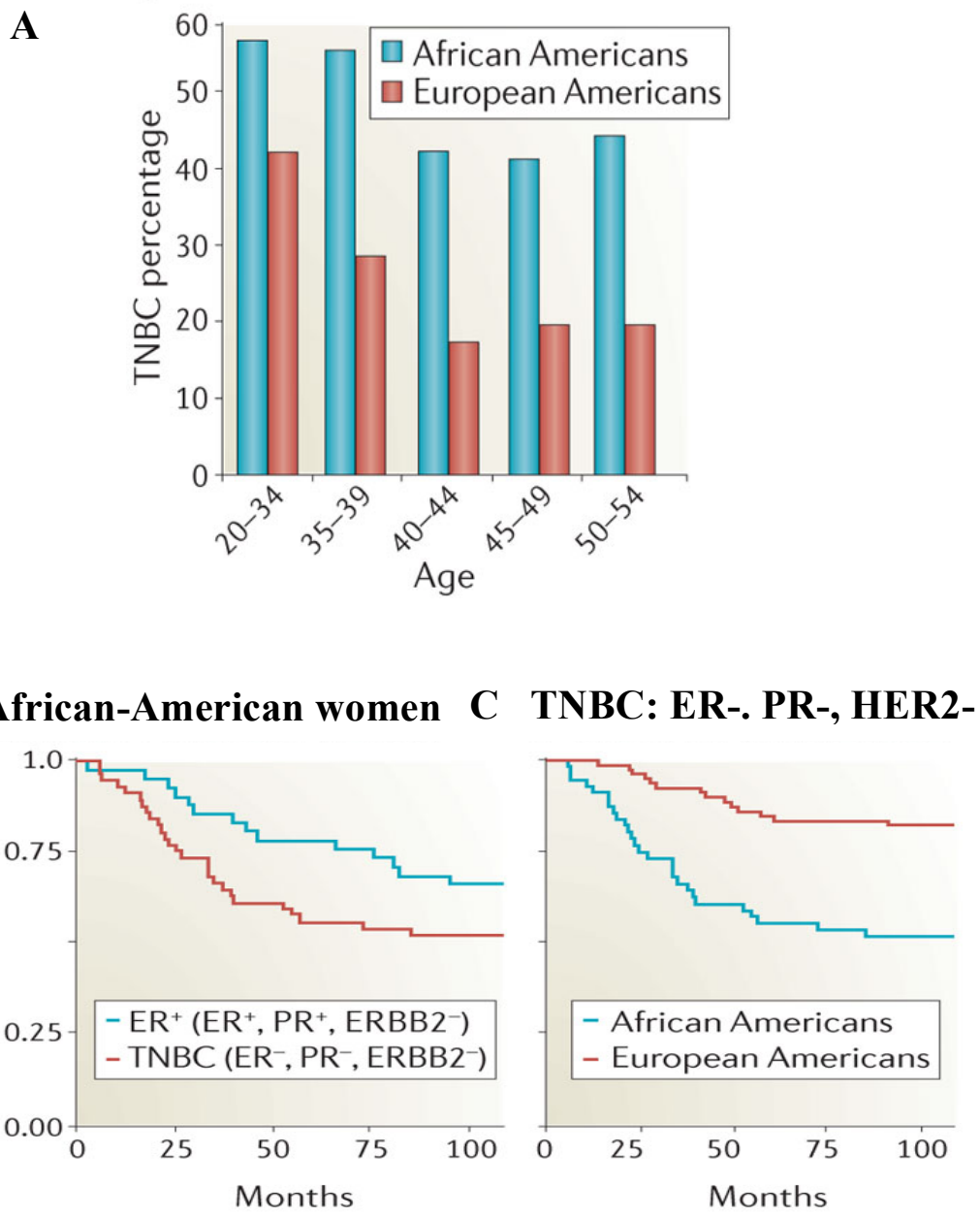

Figure 2. *TNBC: A Health Disparity (Modified from Dietze et. al. Nat. Rev. Can.)* (A-C). A) Overall breast cancer incidence of triple-negative breast cancer by race and age. B) Overall Survival of African American ER+ breast cancer patients and TNBC breast cancer patients. C) Overall Survival of TNBC patients by race (African American and European Americans).

(25). Additionally, a younger age at the first full-term pregnancy increases the incidence for TNBC tumors (25). Compared to other subtypes, TNBC is associated with younger patients and are commonly diagnosed in African-American women (27). Many studies have shown that the incidence of TNBC is much higher in premenopausal African American women and women of African descent in comparison to woman of European descent (28,29). The disparities in breast cancer seen in African American women may arise due to biological and environmental causes. Further, life style and genetic differences correlate with high incidence of basal breast carcinoma in African American women (29). Figure 2 shows TNBC in African American women has been associated with worst overall survival after controlling for socioeconomic factors (low family income, education, occupation and hereditary background), treatment latency, and tumor receptor expression (27). This suggests that the clinical outcome of TNBC in African American women may result from biological differences. Unfortunately, there are low numbers of African Americans enrolled in clinical trials, biobanks, and archived databases (30). There are a number of salient reason that would explain the underrepresentation of African Americans in biomedical research such as: misperceptions of biomedical research, mistrust of the medical community, lack of awareness of studies for which minorities are eligible, employment of unsuccessful and inconsistent recruitment strategies and lack of research studies that incorporate non-invasive methods (31-37). If recruitment strategies do not overcome these barriers we will negate the scientific community's ability to fully understand and eradicate this health disparity (30). As enrollment of ethically diverse populations increase through improved recruitment

strategies we will be able to identify new biological target(s), that can be used as diagnostic and prognostic tools, and targets for therapeutic intervention that would provide health equity for African American female TNBC patients. One potential biological target is Annexin A2 (AnxA2), a member of the human annexin family, which will be comprehensively discussed and analyzed throughout this study.

Annexins in Breast Cancer

Annexins were first identified in 1977 as intracellular proteins that were associated with intracellular membranes (37). Annexins are a family of calcium dependent phospholipid binding proteins that contain a conserved structural element, the so-called "annexin repeat", a segment of approximately seventy amino acid residues located in its carboxyl-terminus, and a divergent amino-terminus (38). Annexins, derived from the Greek word "Annex" means bring or hold together, are so named due to their ability to bind and possibly hold together certain biological components and complexes, particularly in the plasma membrane. The annexin family consists of 12 members (AnxA1–A13, AnxA12 is unassigned) that make up the human annexin family (38) Annexins have been investigated more than fifteen years to study their relationship with breast cancer. These studies have demonstrated that certain annexins are associated with proliferation, migration, invasion, angiogenesis and metastasis. Throughout the years of investigation Annexin A1 (AnxA1), Annexin A2 (AnxA2), Annexin A3 (AnxA3), Annexin A4 (AnxA4), Annexin A5 (AnxA5), Annexin A6 (AnxA6), and Annexin A8 (AnxA8) have all been identified as potential modulators of breast cancer progression. Evidence has shown AnxA1, AnxA2, AnxA8 are associated with the basal-like phenotype and potentiates poor prognosis of basal-like breast cancer (39,40,41).

Recent studies have shown AnxA3 potential as a serum biomarker and regulator of apoptosis (42). AnxA4 and AnxA5 are expressed in breast cancer tissues and upregulation of AnxA4 promotes chemo-resistance of breast cancer (43,44,45,46). AnxA6 expression is reduced in breast cancer cells and when expressed terminates EGFR signaling (47). AnxA2, the annexin protein that has been studied in detail in breast cancer, has been shown to promote TNBC progression, through angiogenesis and metastasis.

Gene of Interest: Annexin A2 in Breast Cancer

Our lab in the past has investigated the prevalence, functionality, and mechanistic properties of one of the members of the annexin family, AnxA2, a 36 kDa calcium-dependent phospholipid binding protein. AnxA2 amino terminal domain is the site for several post-translational modifications such as acetylation and phosphorylation. AnxA2 is a substrate for protein kinase C and tyrosine kinase src (48). Phosphorylation of the Tyr-23 site and calcium influx result in AnxA2 translocation to the membrane (49). AnxA2 is translocated form the inner leaflet of the membrane to the cell surface via the exocytosis pathway (50). AnxA2 is involved in diverse cellular processes including endocytosis, organization of exocytosis of intracellular proteins, cell motility, fibrinolysis, ion channel formation, linkage of membrane associated protein complexes to the actin cytoskeleton and has proven its classification as a pleiotropic protein. Reports have demonstrated that AnxA2 exists as a monomer in the cytosol and as a heterotetrameric complex with the plasminogen receptor protein, S100A10 (p11) at the cell surface. Together the AnxA2.p11 heterotetramer complex plays multiple roles in

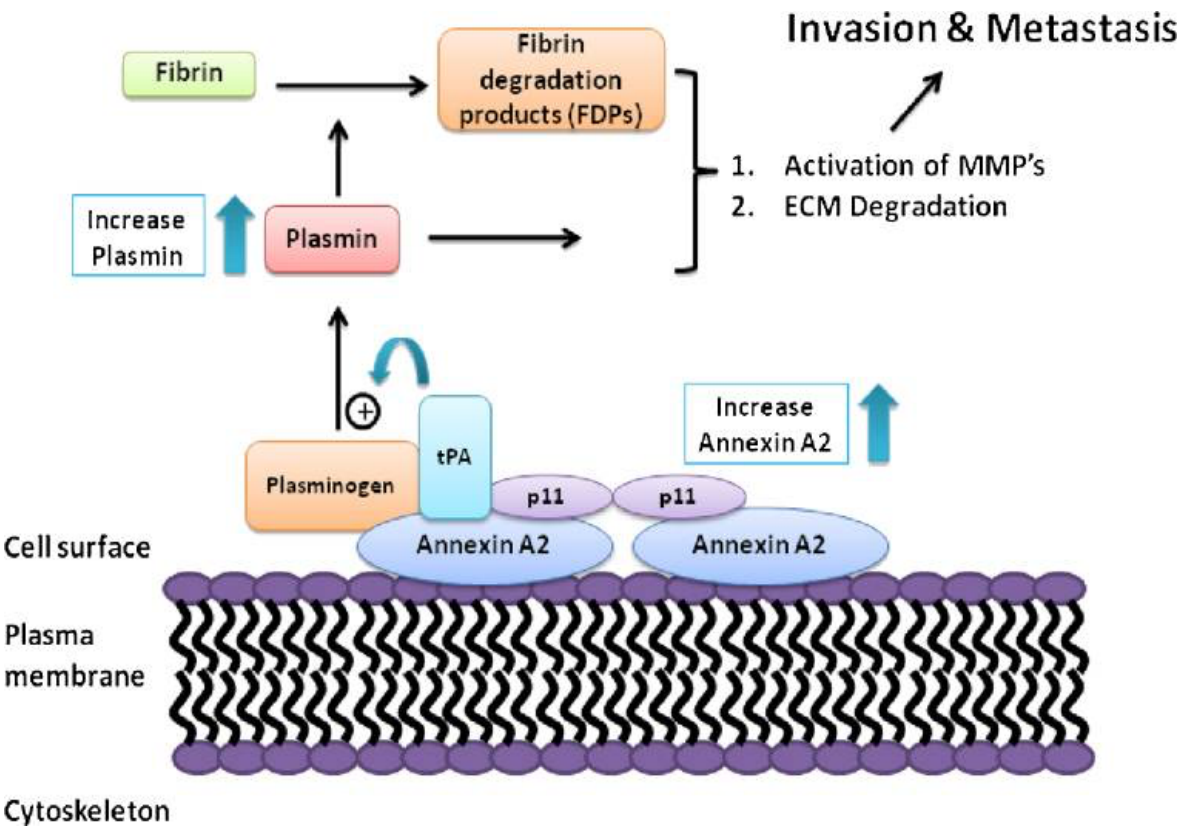

Figure 3. *AnxA2 promotes invasion and metastasis.* From *Lokman et. al. Cancer Microenvironment*. Lokman *et. al.* proposed mechanism of AnxA2 promoting invasion and cancer metastasis through its involvement in the plasminogen activation system. The formation of the heterotetramer by AnxA2 and p11 provides a binding dock for t-PA and facilitates the conversion of plasminogen to plasmin. This conversion results in plasmin activating MP9s and leads to ECM degradation. Overexpression of AnxA2 results in increased plasmin and enhances cancer invasion and metastasis.

regulating cellular functions, including proliferation, migration, invasion, angiogenesis, adhesion and ion channel conductance (Figure 3) (50-52). AnxA2 protein is aberrantly overexpressed in patients with both invasive ductal mammary carcinoma and ductal carcinoma in situ (DCIS) (53). In contrast, it is undetectable in normal and hyperplastic ductal epithelial cells and ductal complexes, suggesting a pivotal role of AnxA2 in breast tumor malignancy and invasiveness. In summary, our previous studies have demonstrated that AnxA2 protein is abundantly expressed in TNBC cell lines (HCC-1187, MDA-MB-231, HCC-38, HCC-1143, BT-549, HCC-1937, and HCC-70) and has a reciprocal relationship with HER2 (Human Epidermal Growth Factor Receptor 2/ErBb2) at mRNA and protein levels (53). Further, the high expression of cell-surface AnxA2 in TNBC has an important compensatory and regulatory role via the EGFR-mediated oncogenic processes by keeping EGFR signaling events in an activated state (54). Tissue microarray and paraffin-embedded tissue specimens were analyzed by immunohistochemistry (IHC) for Her-2 and AnxA2 expression. Her-2 amplified specimen showed weak staining of AnxA2 while Her-2 null/basal breast cancer specimen demonstrated strong membrane expression of AnxA2. We additionally found that AnxA2 expression increases with the progression of Her-2 negative breast cancer. TCGA-assembler was executed in R-3.2.2. (https://www.r-project.org) software environment for statistical computing and graphics, to download, assemble, and process Breast Invasive Carcinoma (BRCA) Illumina RNASeq (RNASeq) normalized gene expression data for the human annexin family gene expression levels (Figure 4).

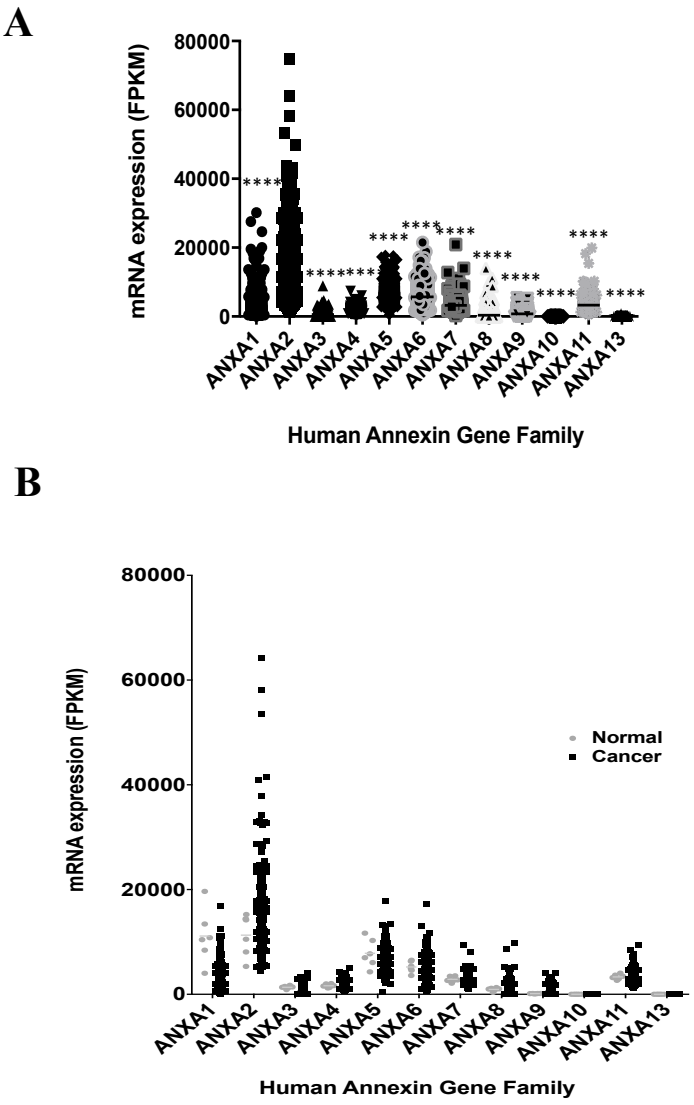

Figure 4. *Annexin Family gene expression in breast cancer subtypes and AA breast cancer and normal patients*. (A-B). A) Scatterplot graph of annexins RNA expression obtained from the TCGA RNAseq database for 1,080 breast cancer patients. B) Scatter plot analysis of annexins RNA expression obtained from the TCGA RNAseq database for analysis of AA normal (n=6) and breast cancer patients (n=171). Results are considered significant if *P*-value was at least ≤ 0.05. (*), $p \leq 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (***) $p < 0.0001$ for all figures.

AnxA2 is statistically significant ($p \leq 0.0001$) in comparison to the all genes of the human annexin family (Figure 4A). In AA patients, AnxA1 ($p \leq 0.0001$) and AnxA2 ($p \leq 0.0001$) are the only two genes in the human annexin family that show statistical significance between non-malignant (n=6) and breast cancer (n=171) (Figure 4B). The two genes demonstrate a reciprocal relationship in breast tissue patients with increased expression of AnxA1 in non-malignant samples and decreased expression in breast cancer; while AnxA2 gene expression is low in non-malignant samples and is increased in breast cancer samples of AA women.

Exosomal annexin A2 (exo-AnxA2) promotes angiogenesis and breast cancer metastasis.

Exosomes are small membrane vesicles of endosomal origin, containing various bioactive molecules (proteins, RNA, DNA, lipids). Exosomes were initially thought to be a means by which unwanted molecules are removed from cells. Subsequent studies, however, demonstrated that the cargo of exosomes remain biologically functional, and amazingly, can be transferred to distant recipient cells and affect their function (54). Nearly every cell type secretes exosomes, but transformed cells on average secrete more exosomes than healthy cells. Exosomes derived from tumor play an important role in pre-metastatic niche formation and subsequently attracting cancer cells to that site (55). Our recent studies have identified AnxA2 as one of the most highly expressed proteins in breast cancer exosomes (56). Exo-AnxA2 expression is significantly higher in malignant cells than normal and pre-metastatic breast cancer cells. Our studies in MCF10A breast cancer progression model (MCF10A, immortalized mammary epithelial cell line; MCF10AT, premalignant cell line generated by HRAS transformation of MCF10A; and MCF10CA1a, derived from poorly-differentiated malignant tumors from MCF10AT

xenografts) revealed that the expression levels of exo-AnxA2 are significantly correlated with the aggressiveness of the breast cancer cells, with lower levels in MCF10A, moderate levels in MCF10AT, and significantly higher levels in MCF10CA1a. Interestingly, the levels of other angiogenic markers, including Vascular Endothelial Growth Factor (VEGF), urokinase-type Plasminogen Activator (uPA), and matrix metalloproteinase 9 (MMP9), were relatively unchanged. Furthermore, our *in vivo* studies demonstrated that exo-AnxA2 derived from breast cancer cells promote angiogenesis. Analysis of the matrigel plug assay showed that incubation of the matrigel plug-exosome mixture with LCKLSL (AnxA2 competitive peptide inhibitor) resulted in a drastic decrease in angiogenesis compared to LGKLSL (control peptide) treatment. Hemoglobin content analysis from MCF10CA1a exosome-treated homogenized matrigel plugs confirmed these results, showing a decrease with LCKLSL treatment, which did not occur with LGKLSL or exosome treatment alone. Additionally we found metastatic TNBC exosomes create a favorable microenvironment for metastasis and exo-AnxA2 plays an important role in this process. This phenomenon was observed in our lung and brain metastasis model where exosomes collected from both control and shRNA transfected MDA-MB-231 cells were delivered by lateral tail vein injection in athymic nude mice. The mice were challenged with luciferase-positive MDA-MB-231-luc cells (tail vein) after one month of exosome priming and showed increased in lung and brain metastasis in 231-Control-Exo-primed animals compared to 231- shAnxA2-Exo-primed and PBS-treated animals. Collectively, our studies suggest that exo-AnxA2 plays an important role in angiogenesis and breast cancer metastasis, which can be exploited as a potential therapeutic target for the treatment of metastatic breast cancer (Figure 5).

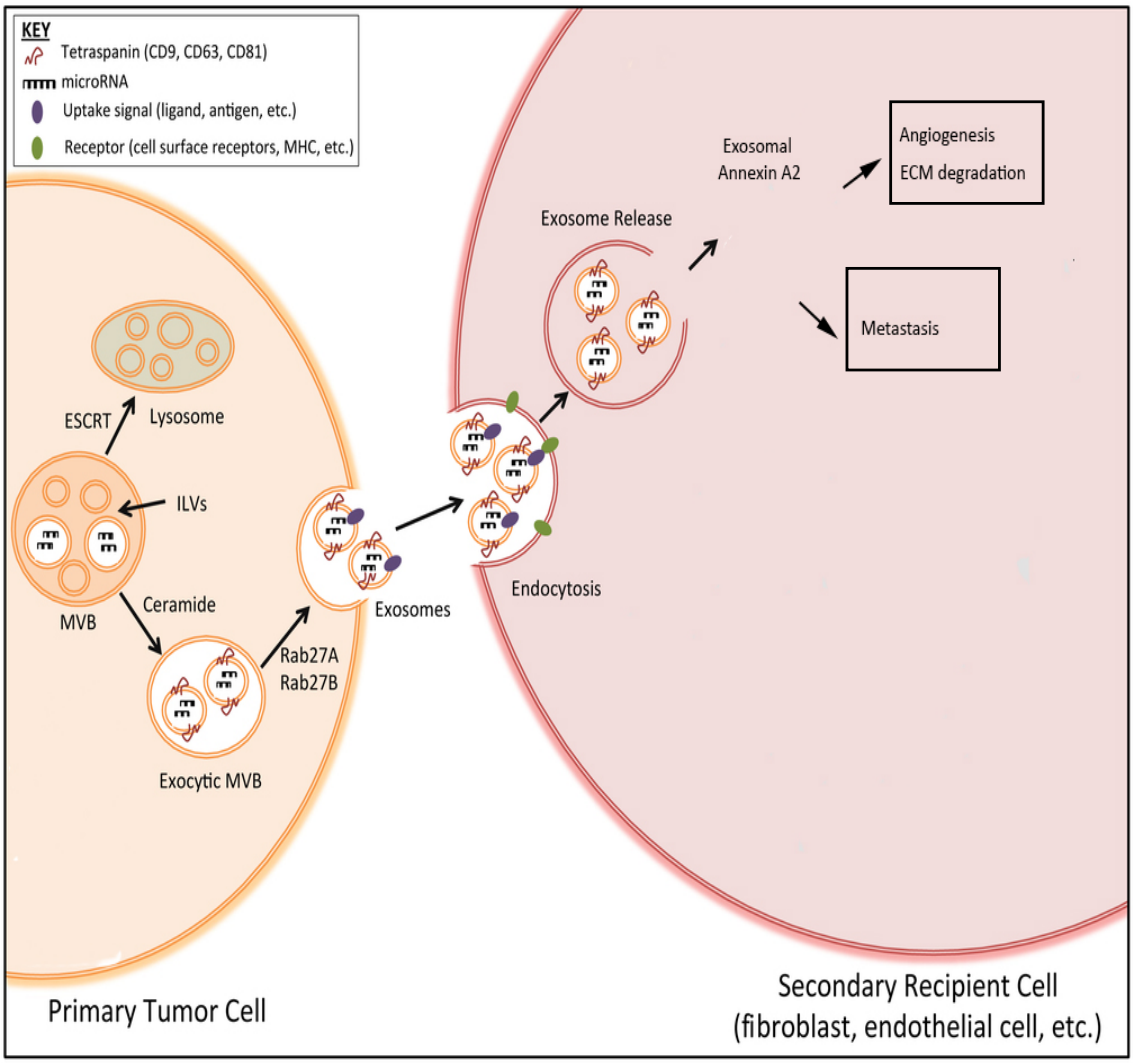

Figure 5: *Model for mechanism of action for Exosomal-AnxA2 (Exo-AnxA2). (Modified from Ding et al. IJMS, 2013).*

This indicates the capacity of AnxA2 associated with exosomes to be involved in tumorigenesis and it's potential to be a prognostic or diagnostic marker (57). Given the fact that breast cancer cells and tumors secrete significant amounts of exosomes, we hypothesize that exo-AnxA2 from AA TNBC patients will have elevated amounts of exo-AnxA2 secreted in their serum that contributes to the aggressiveness of their disease.

Objectives of this study

Hypothesis and Specific Aims

TNBC is the most aggressive breast cancer subtype and is unresponsive to anti-hormonal and Her2-targeted therapies due to the absence of hormone receptors and Her2 expression. Currently there are no tailored therapies available for triple-negative disease, and patients undergo surgery, chemotherapy, and radiation with limited success. This presents an urgent clinical need to identify new biological markers that can be used for prognosis, diagnosis, and as therapeutic targets. Utilizing a large archived breast cancer cohort of genome sequencing information and validation of these targets in patient's tissue and serum specimens can lead to recognition of reliable biological markers that have potential to enhance detection, treatment, and prognosis. Our previous studies have shown that Annexin A2 (AnxA2), a 36 kda calcium-dependent phospholipid binding protein, has a reciprocal relationship with HER2 and is abundantly expressed in TNBC (53,58). We have shown AnxA2 to play multiple roles in regulating cellular functions, including plasminogen activation, angiogenesis, proliferation, migration, invasion, and metastasis (6,7). AnxA2 is one of the most identified proteins expressed in exosomes (56), small vesicles that are secreted from tumors as metastatic regulators. We have previously demonstrated exosomal AnxA2 (exo-AnxA2) contribution to metastasis of

TNBC cells in vivo (57). The proposed study will determine the correlation of AnxA2 with poor prognosis in AA TNBC patients, and establish the clinical significance of exo-AnxA2 in contributing to the poor clinical outcomes seen in AA TNBC patients.

Our long-term goal is to establish AnxA2 as a prognostic indicator and a potential therapeutic target for TNBC in African American women. Our **hypothesis** for the proposed studies is that AnxA2 overexpression accounts for the disproportionate occurrence of TNBC and contributes to poor clinical outcomes in African American TNBC patients. The rationale for this hypothesis is based on published literature and our own data that shows an overexpression of AnxA2 in TNBC compared to other breast cancer subtypes and its ability to drive tumorigenesis. We will address our hypothesis by the three specific aims:

Specific Aim 1: Determine the association of secreted and exosomal AnxA2 with TNBC amongst AA and CA patients. The correlation of secreted and exo-AnxA2 expression levels in breast cancer patient serum will be determined by sandwich ELISA. The functional importance of exo-AnxA2, derived from patient serum, will be determined by blocking its function using competitive inhibitory peptide for AnxA2 in an in vivo matrigel plug assay.

Hypothesis: AA TNBC patients will have higher amounts of exo-AnxA2 secreted in their serum and contributes to the aggressiveness of their disease.

Specific Aim 2: Validate AnxA2 expression in TNBC amongst a clinical breast cancer patient cohort of various breast subtypes. *In Situ* hybridization will be performed on tissue microarrays and tissue sections among various breast cancer subtypes from AA and

Caucasian American (EA) patients to validate AnxA2 gene expression in TNBC observed in our *in silico* analysis of The Cancer Genome Atlas (TCGA).

Hypothesis: AnxA2 gene expression is associated with progression of various breast cancer subtypes AA TNBC tissue specimen.

Specific Aim 3: Determine the correlation of AnxA2 gene expression with poor pathological and prognostic variables in TNBC patients. The correlation of AnxA2 with ethnicity, molecular subtypes, intrinsic subtypes and prognostic variables will be determined by bioinformatic analysis.

Hypothesis: AnxA2 is overexpressed in TNBC cohorts and is associated with the disproportionate occurrence of TNBC in African American women and poor clinical outcomes.

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CHAPTER II

Exosomal Annexin A2 is associated with African American Triple-Negative Breast Cancer and promotes angiogenesis

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Abstract

Background

Currently, limited information is available on potential diagnostic and prognostic markers in Triple-Negative Breast Cancer (TNBC) that can address the higher incidence and aggressiveness of TNBC in African American versus other ethnicities. Our previous studies have demonstrated AnxA2 association with exosomes promotes angiogenesis and metastasis. Therefore, our goal was to correlate exosomal (exo-AnxA2) serum expression with AA TNBC and determine the functional role of AnxA2 within their exosomes.

Methods

We isolated exosomes from 161 breast cancer serum samples and 65 normal archived serum samples and analyzed exo-AnxA2 levels for each breast cancer subtype and ethnicity. We examined exo-AnxA2 ability to promote angiogenesis through an *in vivo* matrigel study in athymic nude mice using isolated exosomes and inhibiting AnxA2 function with competitive inhibitory peptide. Angioigenesis was quantified through hemoglobin estimation by Drabkin's method.

Results

Exo-AnxA2 expression was significantly elevated in TNBC (n=65, 106.3 ± 2.612 ng/mL) in comparison to ER+, n=50, 57.35 ± 1.545 ng/mL, $p < 0.0001$), HER2+ (n=54, 78.25 ± 1.086 ng/mL, $p < 0.0001$), and Normal (n=65, 34.21 ± 2.238 ng/mL, $p < 0.0001$) patient serum. Exo-AnxA2 expression was significantly elevated in AA TNBC (n=31, 99.23 ± 4.142 ng/mL) in comparison to ER+ (n=25, 50.01 ± 2.223 ng/mL, $p < 0.0001$), HER2+ (n=14, 71.81 ± 1.125 ng/mL, $p = 0.0001$), and Normal (n=23, 32.79 ± 4.034 ng/mL, $p < 0.0001$) patient serum. Further, we observe a significant association of exo-AnxA2 with

AA TNBC patients in comparison with CA TNBC patients. We observed a visible increase in angiogenesis from AA TNBC exosomes (~20.17± 1.49 fold change) in comparison to other subtypes and CA TNBC exosomes (~16.87± 1.21 fold change). We visually observed attenuation of angiogenesis in the presence of AnxA2 inhibitory peptide (~2.68± 0.34 fold change) in comparison to AA TNBC exosomes (~18.54± 0.91 fold change) in the presence of a control peptide.

Conclusion

In conclusion, exo-AnxA2 holds promise as a potential prognosticator that can ne analyzed in a non-invasive procedure in AA TNBC patients and may lead to an effective therapeutic option.

Introduction

In the past decade, tumor derived exosomes (50-150 nm extracellular microvesicles) have been heavily studied in cancer development, metastasis, and drug resistance. Nearly every cell type secretes exosomes, but transformed cells on average secrete more exosomes than healthy cells. Interestingly, tumor exosomes maintain proper compartmentalization of important micro and micro molecules that are regulators of many hallmarks of cancer (1-3). Tumor derived exosomes are secreted by tumors into the bloodstream and are known to manipulate the metastatic cascade through angiogenesis, signal transduction, chemo-resistance, genetic intercellular exchange, and pre-metastatic-niche formation (4-9). Additionally, circulating tumor-derived exosomes have been identified as having potential prognostic and diagnostic significance in cancer subtypes. The standard clinical recommendation to diagnose the presence of a malignant tumor is often procurement biopsy, but this invasive standard often has detrimental effects (10). Thus, the investigation of tumor exosomes as a diagnostic or prognostic marker may offer new opportunities for a minimally invasive procedure that would adequately prognosticate and diagnosis a patient's disease.

Triple-negative breast cancer (TNBC) lacks the three widely used diagnostic markers (Her-2, PR, and ER). Thus, women diagnosed with this disease are unable to benefit from the identification of the markers for early detection, targeted therapy, and prognosis. Overall, TNBC is associated with poor prognosis, high mortality rate, shorter median time to relapse (due to its aggressive tumor phenotype(s)), high recurrence rate, and visceral metastatic spread to the brain and lungs. The disparities in breast cancer seen in AA women may arise due to biological and environmental causes. Though, life style

and genetic differences are correlated with high incidence of basal breast carcinomas in AA women, after adjusting for socioeconomic factors the incidence and mortality rate remains higher that other ethnicities. This suggests that the clinical outcome of TNBC in AA women may result from biological differences. There is an urgent clinical need to identify new target(s) that can be used as diagnostic, prognostic tools, and targets for therapeutic intervention that would eradicate this health disparity and provide health equity for AA TNBC patients.

Our recent studies have identified AnxA2, a 36 kDa calcium-dependent phospholipid binding protein, in breast cancer as one of the most highly expressed proteins in breast cancer and breast cancer exosomes (11). Additionally, exosomal AnxA2 (Exo-AnxA2) expression is significantly higher in malignant cells than normal and pre-metastatic breast cancer cells. Our studies in MCF10A breast cancer progression model (MCF10A, immortalized mammary epithelial cell line; MCF10AT, premalignant cell line generated by HRAS transformation of MCF10A; and MCF10CA1a, derived from poorly-differentiated malignant tumors from MCF10AT xenografts) revealed that the expression levels of exo-AnxA2 are highly associated with the aggressiveness of breast cancer cells, with lower levels in MCF10A, moderate levels in MCF10AT, and significantly higher levels in MCF10CA1a; however, the whole cell lysate analysis of the progression model revealed no significant changes in the levels of AnxA2 in MCF10AT and MCF10CA1a (12). Interestingly, the levels of other angiogenic markers, including Vascular Endothelial Growth Factor (VEGF), urokinase-type Plasminogen Activator (uPA), and matrix metalloproteinase 9 (MMP9), were relatively unchanged. Furthermore, our i*n vitro* and *in vivo* studies demonstrated that exo-AnxA2 derived from breast cancer

cells promote angiogenesis. Additionally, our *in vivo* studies indicate that metastatic TNBC exosomes create a favorable microenvironment for metastasis and exo-AnxA2 plays an important role in establishing a pre-metastatic niche at the site of metastasis. This indicates AnxA2 association with exosomes is involved in tumorigenesis and have potential to be a prognostic or diagnostic marker. Given the fact that breast cancer cells and tumors secrete significant amounts of exosomes, we hypothesize that exo-AnxA2 from AA TNBC patients will have higher amounts of exo-AnxA2 secreted in their serum that contributes to the aggressiveness of their disease. Our efforts to establish AnxA2 as an important determinant of racial disparity and disease aggressiveness in TNBC are highly innovative as this is the first study in which AnxA2 is evaluated in a race-derived patient cohort. Currently, limited information is available on potential diagnostic and prognostic markers in TNBC that can address the higher incidence and aggressiveness of TNBC in African Americans in comparison to Caucasian American women. Thus, we aim is to correlate exo-AnxA2 serum expression with AA TNBC and determine AnxA2 functional role in their exosomes to determine AnxA2 potential as a prognostic marker.

Materials and Methods

Archived Serum Collection- Breast cancer serum samples (n=161) and normal archived serum samples (n=65) were collected from UT Southwestern Medical Center. The samples were stored at -140^{0}C and were thawed at room temperature (RT) and immediately placed on ice prior to use. All the archived serum samples were acquired under Institutional Review Board (IRB) approved protocols at the site of collection and UNT Health Science Center. The samples were analyzed in a double blind study where

the identity of the sample was not revealed to the investigator or the supplier of samples until after completion of analysis.

Exosome Isolation from Serum- Exosomes from breast cancer serum samples were isolated by using total exosome isolation reagent (Life technologies, catalog: 4478360) according to the manufacturer's protocol. Briefly, the serum samples were thawed at RT and centrifuged at 2000 × g for 30 minutes at 4°C to remove cells and debris. 100 µL of this clarified serum sample was mixed with 20 µL of the reagent and by vigorously vortexing and pipetting up and down until there was a homogenous solution. This mixture was incubated at 4°C for 30 minutes. After incubation, the sample was centrifuged at 10,000 × g for 10 minutes at room temperature. The supernatant was discarded and the exosomal pellet was resuspended in 100 µL of autoclaved 1X PBS and for analysis.

Serum AnxA2 and Exosomal AnxA2 analysis by ELISA (enzyme-linked immunosorbent assay) - AnxA2 levels in serum samples as well as serum exosomes were analyzed by an ELISA kit (R&D systems) according to manufacturer's protocol. Briefly, 96 well microplate was coated with capture antibody overnight at 4 degrees, washed three times and blocked with blocking buffer for 2 hours at room temperature (RT). Next, the plates were incubated with serum or exosomes from serum and diluted in buffer for 2 hours at RT. The plates were washed and coated with detection antibody for 2 hours at RT and washed again. The plates were incubated with Streptavidin-HRP for 20 mins at RT, washed and further incubated with TMB peroxidase substrate. The reaction was stopped using 2N H2SO4 and the optical density was read at 450 nm with wavelength correction at 540nm. Each samples/ standard was run in triplicates (n=3).

In vivo matrigel plug assay: The Matrigel plug assay was performed as described previously by Merchan *et al.* with slight modifications (13). Briefly, 500 µl of unpolymerized growth factor reduced high concentration Matrigel (BD Biosciences) (~20 mg/ml), either with PBS (negative control) or in the presence of different exosome treatments (pooled from 5 random patients to eliminate bias) for each breast subtype with or without LGKLSL (control peptide) / LCKLSL (AnxA2 inhibitory peptide) peptides were injected subcutaneously at the left or right lower abdominal wall of athymic nude mice (4- to 6-weeks-old) (Harlan Laboratories, Madison, WI). Three mice were injected for each control and experimental group. Mice were sacrificed 18-20 days after the matrigel injections, and the matrigel plugs were recovered and photographed. Matrigels were snap frozen in liquid nitrogen for hemoglobin estimation using Drabkin's reagent.

Hemoglobin estimation by Drabkin's reagent: Hemoglobin estimation from the matrigel was performed by Drabkin's method (14). To quantify the formation of functional vasculature in the Matrigel plug, the amount of hemoglobin was measured using a Drabkin reagent kit 525 (Sigma, St. Louis, MO) following the Drabkin and Austin method (15). Briefly, the matrigel plugs were homogenized in a Dounce homogenizer on ice in presence of 0.5 ml deionized water and allowed to stand overnight at 4^{o}C. The lysate was centrifuged at 5000 x g for 10 mins and the supernatant was collected. 0.3 ml of each sample was mixed with 0.5 ml of Drabkin's reagent and allowed to stand for 15 mins at room temperature. The absorbance was read at 540 nm by using Drabkin's reagent solution as blank. A standard curve was constructed by using known concentrations of hemoglobin and the concentrations of the samples were obtained from the standard curve.

Data analysis- Scatter plot analysis was used to plot the serum AnxA2 and serum exosomal AnxA2 levels and analyze the correlation of serum AnxA2 and serum exosomal AnxA2 levels with normal, HER2+, ER+ and TNBC samples. The *p-value* was calculated according to Student's t-test when comparing two groups. Results were considered significant if P-value was at least ≤ 0.05. (*), $p \leq 0.05$, (**) $p<0.01$, (***) $p<0.001$, (***) $p < 0.0001$ for all figures.

Size analysis of exosomes - Average sizes of the exosomes were determined by Malvern Zetasizer particle size analyzer (Malvern Instruments, Ltd., Malvern, UK). The exosomal pellet was resuspended in autoclaved 1X PBS and the size distribution was analyzed. The results were reported as the average of five runs with triplicates in each run.

Results

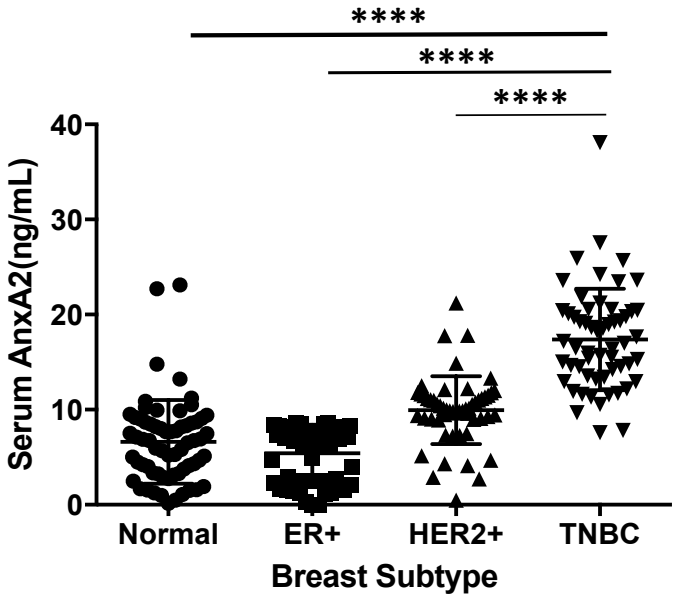
Serum AnxA2 and exosomal AnxA2 is associated with TNBC Patients

Serum samples from119 breast cancer serum samples and 65 normal were analyzed in a double-blind study were analyzed for AnxA2 and exosomal-AnxA2 protein expression (Figure 1). We first analyzed the size of our isolated exosomes from patient serum to confirm they are in the known size range (~50-150 nm in diameter) (15). Our characterization (Malvern Zetasizer) revealed an average size of 87.85 ± 21.30 nm (Supplemental Figure S1). Our ELISA analysis shows differential AnxA2 expression among breast cancer subtypes. Whole AnxA2 expression was significantly elevated in TNBC (n=57, 17.38 ± 0.7062 ng/mL) in comparison to ER+ (n=50, 5.421 ± 0.4221 ng/mL, $P < 0.0001$), HER2+ (n=54, 9.938 ± 0.4854 ng/mL, $P < 0.0001$), and Normal (n=65, 6.616 ± 0.5447 ng/mL, $P < 0.0001$) patient serum. Exo-AnxA2 expression was significantly elevated in TNBC (n=65, 106.3 ± 2.612 ng/mL) in comparison to ER+

(n=50, 57.35 ± 1.545 ng/mL, $P < 0.0001$), HER2+ (n=54, 78.25 ± 1.086 ng/mL, $P < 0.0001$), and Normal (n=65, 34.21 ± 2.238 ng/mL, $P < 0.0001$) patient serum. These observations show whole AnxA2 and exo-AnxA2 significant association with TNBC. Our observation of exo-AnxA2 preferential association with TNBC suggests a functional role of exo-AnxA2 in predicting TNBC progression.

FIGURE 1

A


****
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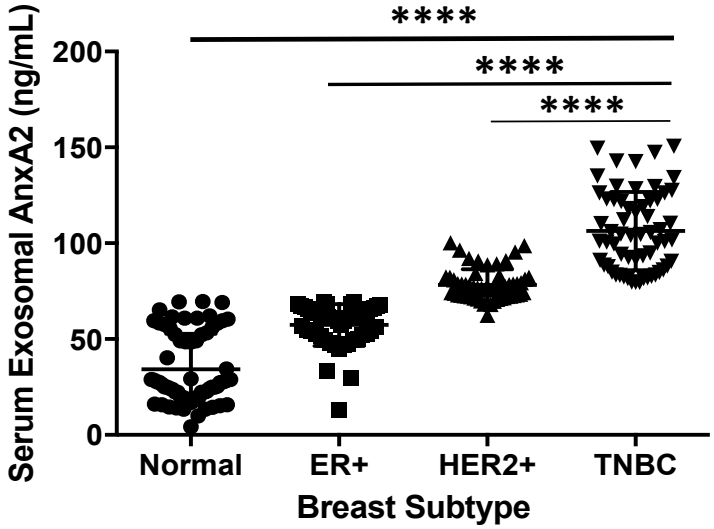
40
30
20
10
0
Serum AnxA2(ng/mL)
Normal
ER+
HER2+
TNBC
Breast Subtype

B


****
****
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200
150
100
50
0
Serum Exosomal AnxA2 (ng/mL)
Normal
ER+
HER2+
TNBC
Breast Subtype

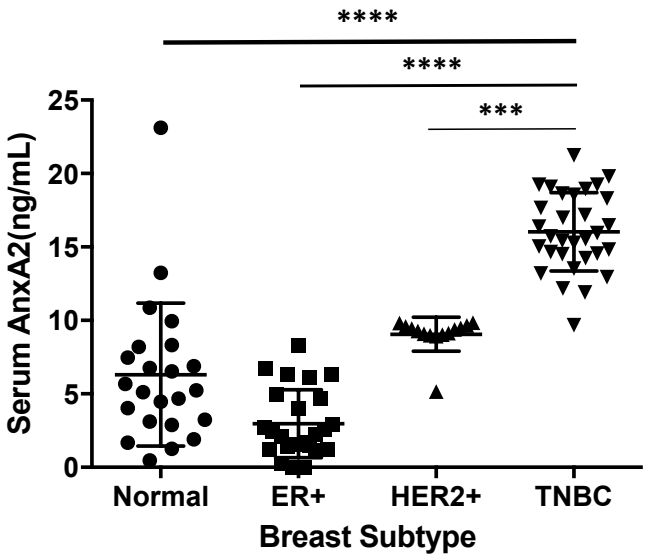
Figure 1: *Serum AnxA2 and Exosomal AnxA2 expression among breast cancer subtypes.* (A-B). A) AnxA2 and B) exosomal AnxA2 protein expression obtained through ELISA analysis from serum of Normal (n=65), ER+ (n=50), HER2+ (n=54), and TNBC (n=57) patients.

Serum AnxA2 and exosomal AnxA2 is associated with African American TNBC Patients

Previous studies have shown a disproportionate occurrence of TNBC in AA women and this occurrence in AA women is significantly more aggressive often leading to an increase of metastatic patients and mortality. Here, we examine serum AnxA2 and exo-AnxA2 association with African American TNBC patients (Figure 2). Our ELISA analysis reveals differential AnxA2 expression among AA breast cancer subtypes. Whole AnxA2 expression was significantly elevated in AA TNBC (n=31, 16.03 ± 0.4779 ng/mL) in comparison to AA ER+ (n=25, 2.965 ± 0.4637 ng/mL, $P < 0.0001$), HER2+ (n=14, 9.061 ± 0.3113 ng/mL, $P = 0.0001$), and Normal (n=23, 6.308 ± 0.5447 ng/mL, $P < 0.0001$) patient serum. Exo-AnxA2 expression was significantly elevated in AA TNBC (n=31, 99.23 ± 4.142 ng/mL) in comparison to ER+ (n=25, 50.01 ± 2.223 ng/mL, $P < 0.0001$), HER2+ (n=14, 71.81 ± 1.125 ng/mL, $P = 0.0002$), and Normal (n=23, 32.79 ± 4.034 ng/mL, $P < 0.0001$) patient serum. In addition to results detailed in Figure 2, these observations show whole AnxA2 and exo-AnxA2 significant association with AA TNBC. Further, we observe a significant association of exo-AnxA2 with AA TNBC patients in comparison with CA TNBC patients (Supplemental Figure S2). Our observation of exo-AnxA2 preferential association with AA TNBC patients and our previous studies suggests a potential role for exo-AnxA2 as a contributor to the aggressiveness of TNBC in AA women.

FIGURE 2

A


****
****
***
25
20
15
10
5
0
Serum AnxA2(ng/mL)
Normal
ER+
HER2+
TNBC
Breast Subtype

B

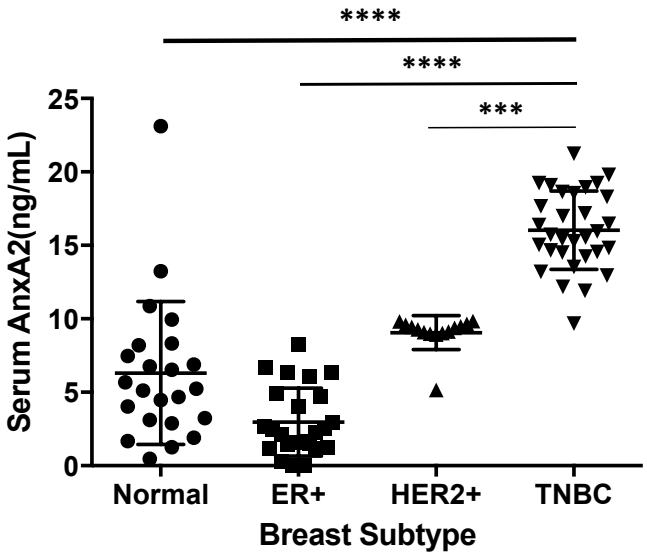

****
****
***
25
20
15
10
5
0
Serum AnxA2(ng/mL)
Normal
ER+
HER2+
TNBC
Breast Subtype

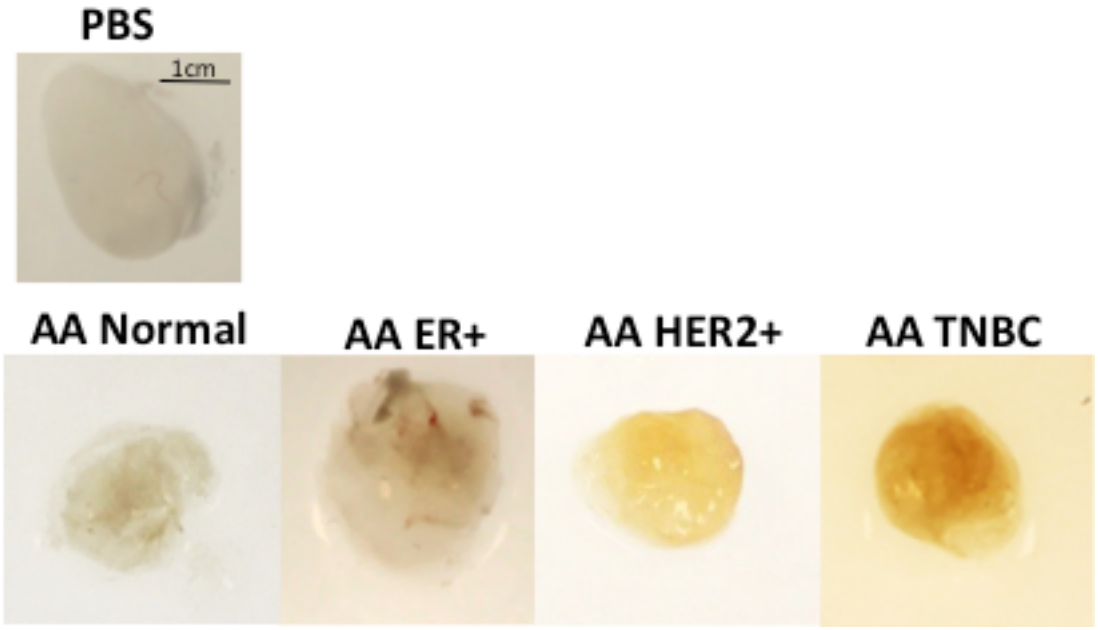
Figure 2: *Serum AnxA2 and Exosomal AnxA2 expression among AA breast cancer subtypes.* (A-B). A) AnxA2 and B) exosomal AnxA2 protein expression obtained through ELISA analysis from serum of AA Normal (n=23), AA ER+ (n=25), AA HER2+ (n=14), and AA TNBC (n=31) patients.

Exosomal AnxA2 promotes angiogenesis in AA TNBC patients

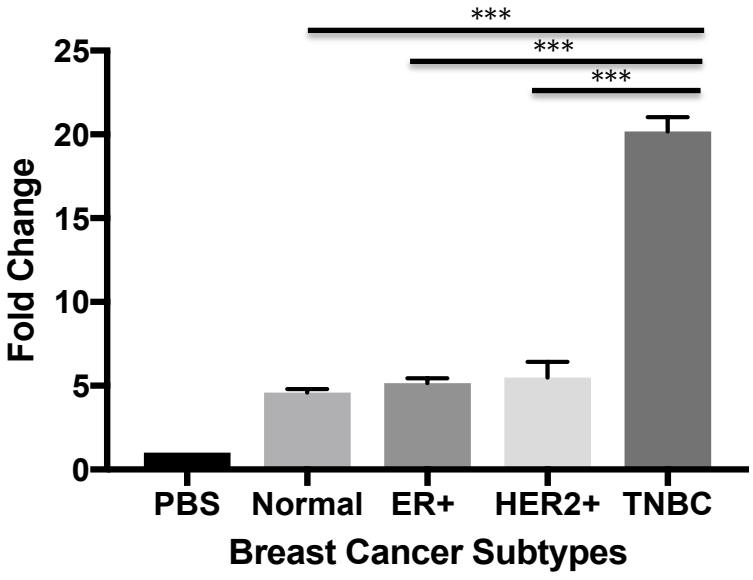
Exo-AnxA2 role as a promoter of angiogenesis and its association with AA TNBC patients led our investigation to examine the ability of exosomes from AA breast cancer patients' ability to induce angiogenesis in an *in vivo* matrigel plug assay. In Figure 3A, we observed a visible increase angiogenesis from AA TNBC exosomes in comparison to other subtypes. We confirmed our observation thorough quantification of new blood vessel formation within these matrigel plugs through hemoglobin estimation by Drabkin's method (Figure 3B). In addition, we analyzed AA TNBC exosomes and inhibited AnxA2 function through use of an inhibitory peptide (LCKLSL) to observe AnxA2 relationship to the promotion of angiogenesis by exosomes from these patients. Additionaly, we used CA TNBC exosomes to compare the promotion of angiogenesis to the relationship of exo-AnxA2 observed between AA TNBC and CA TNBC (Supplemental Figure S2). We visually observe attenuation of angiogenesis in the presence of LCKLSL and increased angiogenesis in exosomes alone and in the presence of control peptide (LGKLSL)(Figure 3C). Further, we observed a significant association of exo-AnxA2 with AA TNBC patients in comparison to CA TNBC patients (Figure 3D). Our observations demonstrate exo-AnxA2 functional role in AA TNBC patients and demonstrates its potential as a poor prognostic predictor.

FIGURE 3

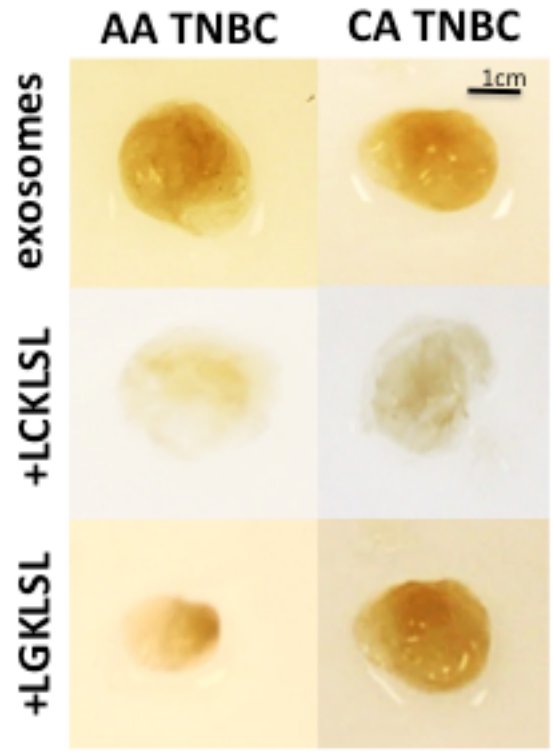
A


PBS
1cm
AA Normal
AA ER+
AA HER2+
AA TNBC

B


***
***
***
25
20
15
10
5
0
Fold Change
PBS
Normal
ER+
HER2+
TNBC
Breast Cancer Subtypes

C


AA TNBC
CA TNBC
1cm
exosomes
+LCKLSL
+LGKLSL

D

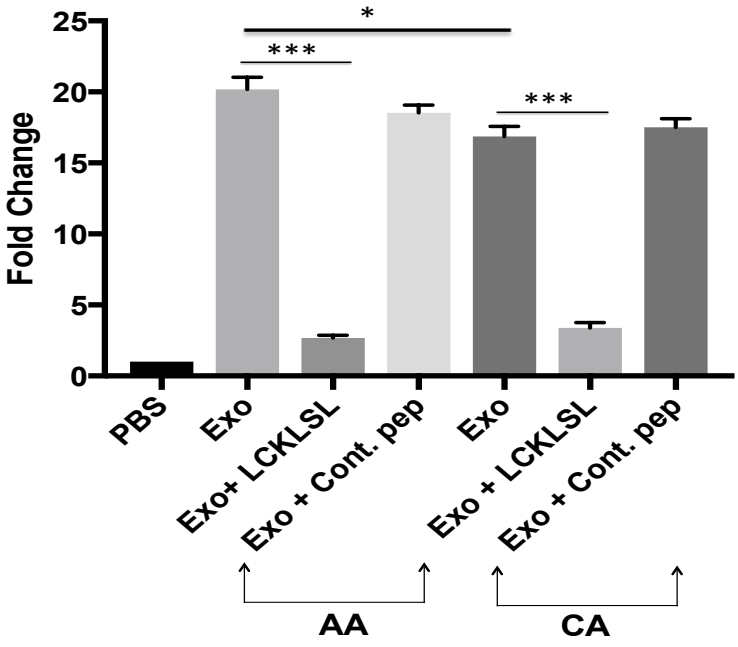

Fold Change
25
20
15
10
5
0
*
***
***
PBS
Exo
Exo+ LCKLSL
Exo + Cont. pep
Exo
Exo + LCKLSL
Exo + Cont. pep
AA
CA

Figure 3: *Exosomal AnxA2 is associated with AA TNBC and promotes angiogenesis*. (A-D) A) Representative images of matrigel plugs removed from Normal, ER+, HER2+, TNBC breast subtypes and their impact on angiogenesis. B) Quantification of angiogenesis formation through hemoglobin estimation by Drabkin's method. C) Representative images of matrigel plugs from AA TNBC and CA TNBC patients that show comparison of angiogenesis between AA and CA patients. LCKLSL (AnxA2 inhibitory peptide) and LGKLSL (AnxA2 control peptide) were used to demonstrate the functional role of AnxA2 in contributing to angiogenesis. D) Quantification of angiogenesis formation through hemoglobin estimation by Drabkin's method.

Discussion

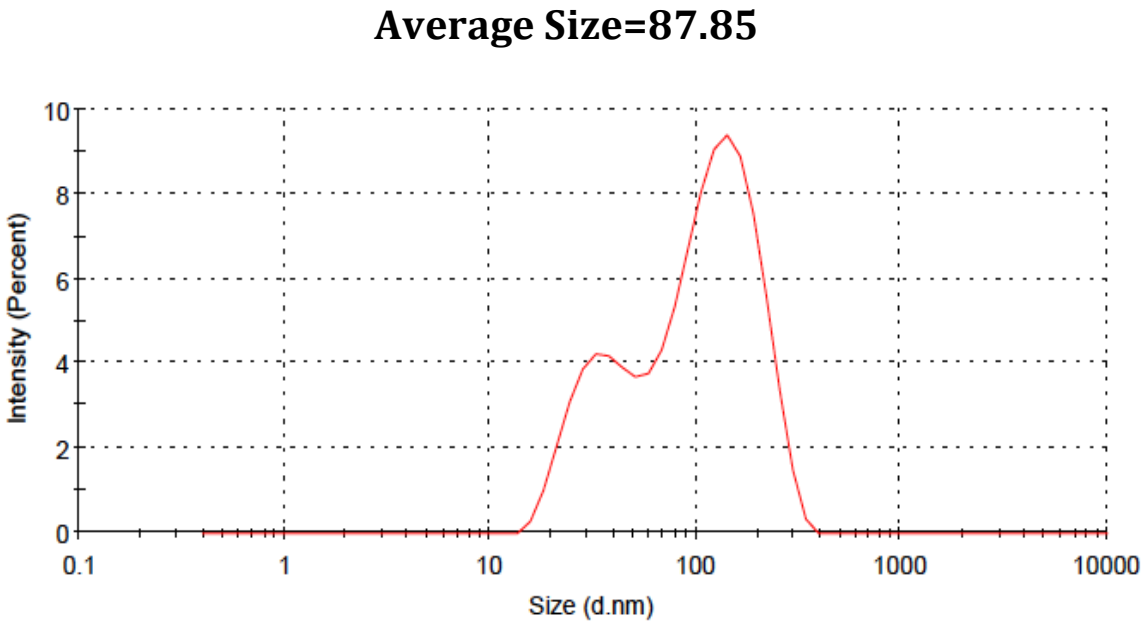
Success in the prognosis of cancer is largely dependent on a comprehensive understanding of cancer biology and its relationship to clinical outcomes. Exosomes are precursors of metastasis that have huge potential to broaden our ability to provide adequate prognoses (16-19). Exosomes secretion of diverse biological molecules enables a variety of markers that can be analyzed to assist diagnosis and prognosis of a patient's cancer (20-21). Here we have analyzed a race-derived patient cohort in a double-blind study and were able to link exo-AnxA2 to the most aggressive subtype of cancer, TNBC. We also found that exo-AnxA2 expression were higher in AA TNBC patients in comparison to CA TNBC patients. This unique phenomenon may explain the aggressiveness of TNBC observed in AA women.

Our previous determined functional analysis of AnxA2 correlates with our current studies as exo-AnxA2 contributes to the formation of new blood vessels in TNBC patients (12). These observations suggest that exo-AnxA2 facilitates neo-angiogenesis in TNBC and may contribute to the increase of distant metastasis seen in TNBC patients. Thus, exo-AnxA2 not only presents itself as potential prognostic and diagnostic marker, but also as a potential therapeutic target. Further, exo-AnxA2 presents a unique opportunity for use in a minimally invasive procedure for AA TNBC patients that are often diagnosed at later stages and have higher treatment latency. In several routine blood draws over the course of a patient's disease we can potentially monitor cancer progression, treatment response, and predict clinical outcomes.

This study was significant as it detailed exo-AnxA2 association with TNBC in AA women and its contribution to the aggression of their disease. Despite the relevance

and innovation of this study, there were several limitations. First, TNBC only makes up 10-15% of all breast cancer cases and is often difficult to acquire in large numbers, especially from AA women. Further, our low number of patients did allow for any significant correlation of exo-AnxA2 levels to clinical outcomes such as: stage, metastatic sites, relapse, age, menopausal status and mortality (22-24). Our full understanding of serum derived exo-AnxA2 and its association with metastasis would be a seminal discovery that would allow the clinician the opportunity to provide the appropriate therapeutic option. Additionally, we would like to understand exo-AnxA2 relationship with other ethnicities and ancestry to better understand its association with the disproportionate occurrences in incidence, mortality, metastasis, and relapse seen within these patients. In conclusion, exo-AnxA2 holds promise as potential prognostic predictor that can ne analyzed in a non-invasive procedure in in AA TNBC patients and may lead to an effective therapeutic option.

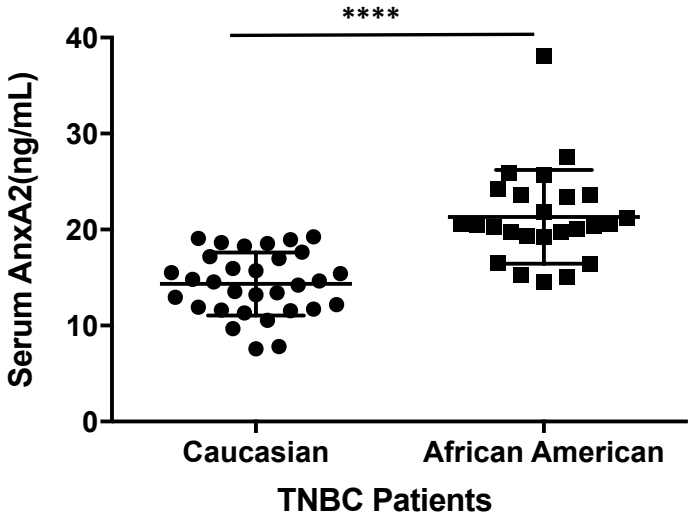
Supplemental Figure S1



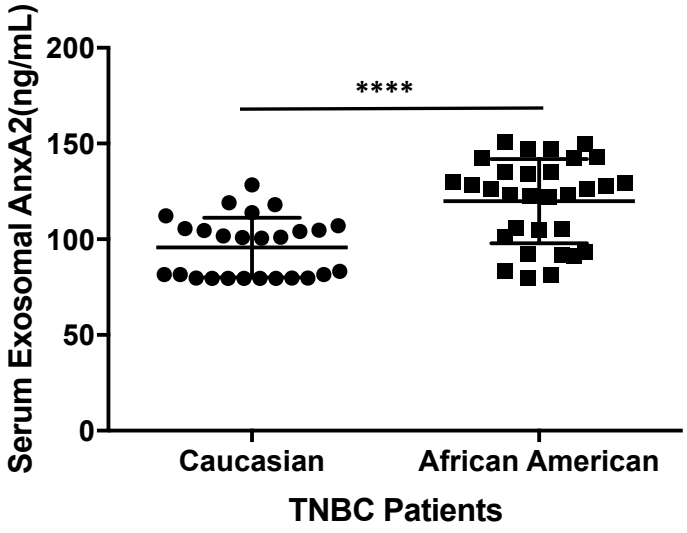
***Supplemental Figure S1*:** *Size analysis of exosomes.* Representative image of the average size of exosome isolated form patient's serum Malvern Zetasizer particle size analyzer. Studied exosomes range of size is 52.06-122.3 nm in diameter with an average size of 87.85 ± 21.30 nm.

Supplemental Figure S2

A



B



Supplemental Figure S2: *Serum AnxA2 and Exosomal AnxA2 are associated with AA TNBC patients in comparison to CA TNBC patients.* (A-B). A) AnxA2 and B) exosomal AnxA2 protein expression obtained through ELISA analysis from serum of AA TNBC (n=26) and CA TNBC patients (n=31).

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CHAPTER III

Annexin A2 is overexpressed in African American Triple-Negative Breast Cancer Patients and Predicts Patient Survival

Lee D. Gibbs, Richard J. Hare, Rebecca A. Mantsch, and Jamboor K. Vishwanatha

Abstract

Background

Triple-negative breast cancer (TNBC) has a disproportionate occurrence and poor prognosis in African American (AA) women. Our aim was to determine the role of Annexin A2 (*AnxA2*), a protein we have previously found to contribute to the aggressiveness of cancers, with AA TNBC patients and clinical outcome.

Methods

We analyzed the TCGA breast cancer database to observe AnxA2 gene association with breast cancer subtypes and overall survival in a 5 to 10 year follow-up study. We validated these findings in breast tissue specimens (n=119) through *chromogenic in situ hybridization* (CISH) and specimen were independently scored by two pathologists in a blinded study.

Results

In our TCGA analysis, high expression of AnxA2 is associated with poor survival (hazard =3.235; 95% confidence interval {CI} = 1.31 3- 7.97, *P* = 0.0232). AnxA2 gene expression was not associated with poor survival in other subtypes, indicating the specificity of AnxA2 in determining mortality in TNBC. AnxA2 average *CISH* intensity score (*CISH* score = 0, null expression to 3, high expression) for TNBC (CISH Average Score = 2.125 ± 0.2045) was significantly higher in comparison to Estrogen Receptor positive (ER+) and/or Progesterone Receptor Positive (PR+) (CISH Average Score = 1 ± 0.1805, *P* = 0.0063), Human Epidermal Growth Factor positive (HER2+) (CISH Average Score = 1.2 ± 0.3887, *P* = <0.0001) and Normal (CISH Average Score = 0.23±0.1216, *P* = 0.0001) breast tissues. Furthermore, AnxA2

average intensity score was significantly higher (P = 0.0493) in AA TNBC patients (CISH Average Score = 2.45 ± 0.3266) in comparison to Caucasian TNBC patients (CISH Average Score = 1.1 ± 0.4069).

Conclusion

AnxA2 overexpression is associated with TNBC in AA women and is a potential prognostic molecule.

Impact

AnxA2 has potential prognostic value, implicating a role for AnxA2 in the aggressive biology of TNBC in AA women.

Introduction

The American Cancer Society's (ACS) estimates that in 2017, approximately 316, 120 women will be diagnosed with new cases of invasive breast cancer and 40,610 will succumb to the disease (1). Breast Cancer is the most frequently diagnosed cancer and the leading cause of cancer death amongst women; accounting for 23% of the total cases and 14% of the cancer death (2). Triple-negative breast cancers (TNBC) are identified by the absence of three major receptors that drive most breast cancer: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2); and constitute 15 to 20% of diagnosed breast cancers (3). Racial variation resides in breast cancer presentation and clinical outcome, with African American (AA) women, especially pre-menopausal AA women, being diagnosed with more advanced breast cancer, which predominantly includes TNBC (4). TNBC in AA women has been associated with worst overall survival after controlling for socioeconomic factors, treatment latency, and tumor receptor expression. This suggests that the clinical outcomes of TNBC in AA women may not only result from the effects of lifestyle factors but may result from biological differences. Overall, TNBC is associated with poor prognosis, high mortality rate, shorter median time to relapse due to its aggressive tumor phenotype(s)), high recurrence rate, and visceral metastatic spread to the brain and lungs (5).

The heterogeneity of TNBC has become a challenge in today's clinical practice and significant research efforts have been deployed to better understand the molecular nature of TNBC (6-9). Clinically the heterogeneous nature of TNBC

has not been accounted for hence leading to resistance, metastasis, and relapse (10). Taken together, data suggest that a multifactorial approach is required to eradicate TNBC and prevent recurrence of this disease. This evidence presents an urgent clinical need to recognize molecular attributes that have potential to enhance detection, treatment, and prognosis of TNBC.

Previously, our lab has investigated the prevalence, functionality, and mechanistic properties of one of the members of the human annexin family, Annexin A2 (AnxA2), a 36 kDa calcium-dependent phospholipid binding protein in breast cancer. AnxA2 is involved in diverse cellular processes including endocytosis, organization of exocytosis of intracellular proteins, cell motility, fibrinolysis, ion channel formation, linkage of membrane associated protein complexes to the actin cytoskeleton and has proven its classification as a pleiotropic protein (11-15). Reports have demonstrated that AnxA2 exists as a monomer in the cytosol and as a heterotetrameric complex with the plasminogen receptor protein, S100A10 (p11) at the cell surface. Together the AnxA2.p11 heterotetramer complex plays multiple roles in regulating cellular functions, including proliferation, migration, invasion, angiogenesis, adhesion, and ion channel conductance (16). We have previously demonstrated that AnxA2 is abundantly expressed in TNBC cell lines and has a reciprocal relationship with HER2 (Human Epidermal Growth Factor Receptor 2/ErBb2) (17). In this study, we aim to investigate AnxA2 gene expression in AA breast tissues to determine AnxA2 association with clinical outcomes and implicate AnxA2 as a potential prognostic marker.

Materials and Methods

TCGA Expression Data-TCGA-Assembler was executed in R-3.2.2. (https://www.r-project.org), software environment for statistical computing and graphics, to download, assemble, process, and normalize public Breast Invasive Carcinoma (BRCA) Illumina RNASeq gene expression data. This platform allows publicly available data from Illumina HiSeq 2000 (IlluminaHiSeq) RNA sequencing data to determine gene expression levels for 1,098 BRCA patients (18).

Kaplan-Meier Plots—Overall survival (OS) of patient groups was based on BRCA RNASeq normalized gene expression data for 1,098 patients with integration of corresponding clinical information for each patient. OS was defined as the interval between the date of surgery and date of death from any cause or last contact. Survival probabilities were estimated for breast cancer patients and split into two groups based on the median of AnxA2 gene expression among breast cancer subtypes.

Chromogenic In Situ Hybridization- Paraffin embedded tissue sections from the University of Alabama Birmingham (UAB) Comprehensive Tissue Network (Birmingham, AL) (n=40) and Biomax breast cancer tissue array (US Biomax, Inc.,) (n=79) were used for *in situ* analysis. Samples were collected under the approval of the Institutional Review Board at the site and at UNT Health Science Center. The anatomic pathologists independently read the Hematoxylin (stains nuclei purple) & Eosin (stain acidophilic structures red or pink) (H&E) stained sections and hybridized sections to determine AnxA2 mRNA intensity scores (0, +1, +2, +3; 0 being null to very low intensity to a score of +3 with cells showing very high staining

intensity) for normal and breast tissue sections. A chromogenic assay based on DIG labeled probes detected by alkaline phosphatase conjugated anti-DIG and NBT-BCIP substrate was used for staining (identified by intensity of blue staining). Protocols were optimized to standardize and perform *in situ* hybridization, using scrambled mRNA and the 5′- and 3′-DIG double-labeled AnxA2 custom designed probe (Exiqon).

Statistical Analyses- The appropriate number of samples, as indicated in the figures, were used for the analysis of TCGA derived data with exemption of statistical outliers. The results were represented as mean ± S.E.M. The *p-value* was calculated according to Student's t-test when comparing two groups. The patient cohorts are compared by Kaplan-Meier survival analysis. The analysis provides hazard ratios with 95% confidence intervals and calculation of log-rank P values. Results were considered significant if *p*-value was ≤ 0.05. (*), $p \leq 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (***) $p < 0.0001$ for all figures.

Results

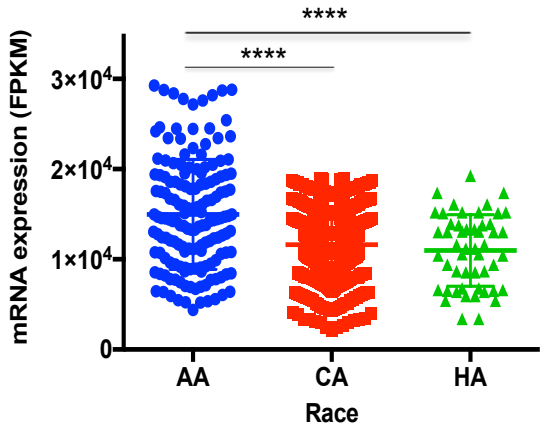
AnxA2 gene expression is associated with AA TNBC patients.

We analyzed the gene expression levels of AA (n=158), Hispanic American (HA; n=51), and Caucasian American (CA; n=654) women with breast cancer in the TCGA cohort (Figure 1). AnxA2 gene expression was significantly elevated in AA in comparison to CA ($p < 0.0001$, Figure 1A) and Hispanic ($p < 0.0001$, Figure 1A) breast cancer patients. We determined the hormonal classification of the patients by the clinical information provided for each patients immunohistochemical tumor staining. This provided three subtypes that we used for analysis: TNBC (ER-/PR-

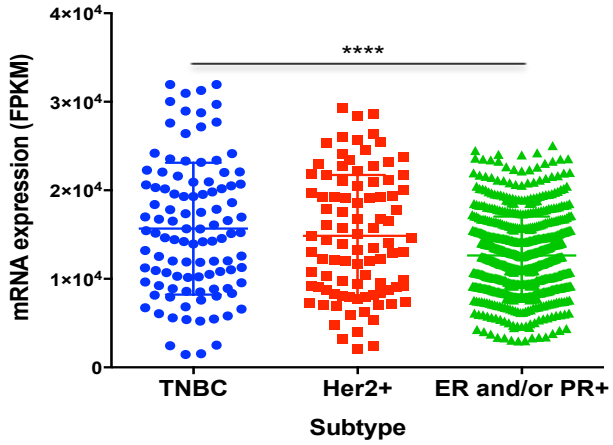
/HER2-; n=105), HER2+ (ER-/PR-/HER2+; n=93), ER+/and or PR+ breast cancer (ER+ and/or PR+; n=690). Our analysis demonstrated a significant elevation of AnxA2 in the TNBC subtype in comparison with ER+ ($p < 0.0001$, Figure 1B) subtype. AnxA2 gene expression was not significant when compared to HER2+ patients ($p = 0.4249$, Figure 1B). The significant elevation of AnxA2 gene expression observed in AA cohort led us to investigate AnxA2 gene expression in AA women among TNBC (n=40), HER2+ (n=20), ER+ (n=84), and Normal (n=6). AnxA2 gene expression was significantly elevated in AA TNBC patients in comparison to ER+ ($p < 0.0001$, Figure 1C) and normal samples ($p = 0.0323$, Figure 1C) from AA women. AnxA2 gene expression was not significant when compared to HER2+ ($p = 0.1177$, Figure 1C). These observations suggest that AnxA2 gene expression is significantly increased in aggressive tumor phenotypes and there is large sub-population of TNBC and AA TNBC patients whom disease is associated with high expression of AnxA2.

Figure 1

A


****
****
mRNA expression (FPKM)
3×10^4
2×10^4
1×10^4
0
AA
CA
HA
Race

B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mRNA expression (FPKM)
4×10^4
3×10^4
2×10^4
1×10^4
0
TNBC
Her2+
ER and/or PR+
Subtype

C

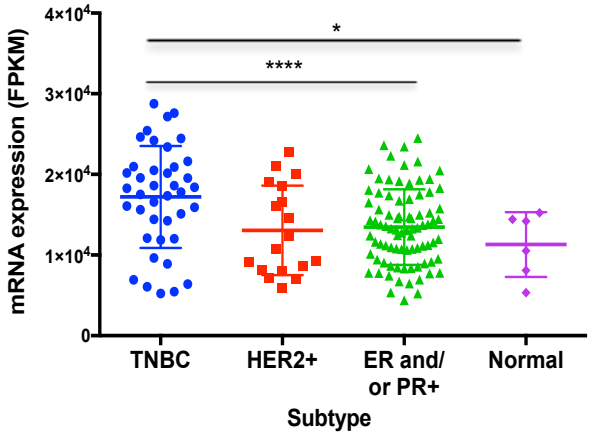

*
****
mRNA expression (FPKM)
4×10^4
3×10^4
2×10^4
1×10^4
0
TNBC
HER2+
ER and/
or PR+
Normal
Subtype

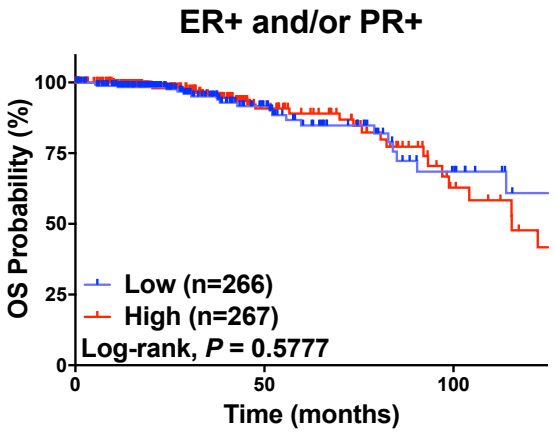
***Figure 1*:** *AnxA2 gene expression amongst breast cancer subtypes and race.* (A-C). A) AnxA2 RNA expression obtained from the TCGA RNAseq database for analysis of racial variation of gene expression for African American (AA; n=158), Caucasian (CA; n=654), and Hispanic American (HA; n=51) breast cancer patients. B) AnxA2 RNA expression obtained from the TCGA RNAseq database for TNBC (n=105), HER2+(n=93), and ER+(n=690) breast cancer subtypes. C) AnxA2 RNA expression amongst African American TNBC (n= 40), HER2+ (n=20), ER+(n=34), and Normal (n=6) patients.

High AnxA2 expression predicts poor survival preferentially in TNBC patients.

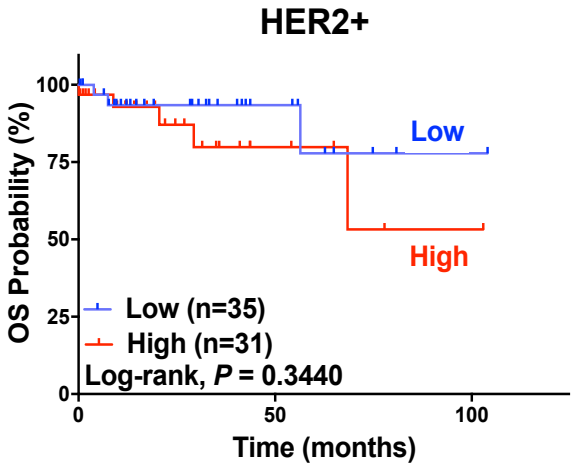
In Figure 2, we further utilized our TCGA cohort to analyze AnxA2 gene expression association with OS in breast cancer subtypes. AnxA2 expression was dichotomized into low and high, based on the median of logarithmized expression values. We observed a significant reduced lower survival of TNBC patients (hazard =3.235; 95% confidence interval {CI} = 1.31 3- 7.97, *P* = 0.0232, Figure 2C) in comparison with ER+ (hazard = 1.171, 95 % CI = 0.6715-2.042, *P* = 0.5777, Figure 1A) and HER2+ patients (hazard = 1.959, 95% CI = 0.4865-7.89, P= 0.3440, Figure 2B). Taken together, our survival analysis and univariate analysis not only confirms that high AnxA2 expression results in a poor survival in TNBC, but that this phenomenon is preferential for TNBC patients and suggests AnxA2 as a potential prognostic predictor.

Figure 2

A


ER+ and/or PR+
OS Probability (%)
100
75
50
25
0
0
50
100
Time (months)
Low (n=266)
High (n=267)
Log-rank, P = 0.5777

B


HER2+
OS Probability (%)
100
75
50
25
0
0
50
100
Time (months)
Low
High
Low (n=35)
High (n=31)
Log-rank, P = 0.3440

C

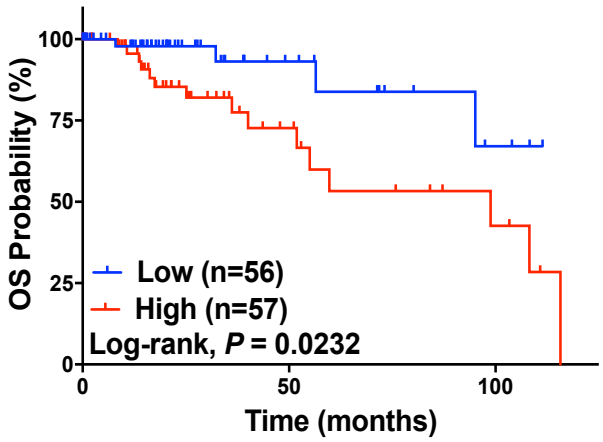

TNBC
OS Probability (%)
100
75
50
25
0
0
50
100
Time (months)
Low (n=56)
High (n=57)
Log-rank, P = 0.0232

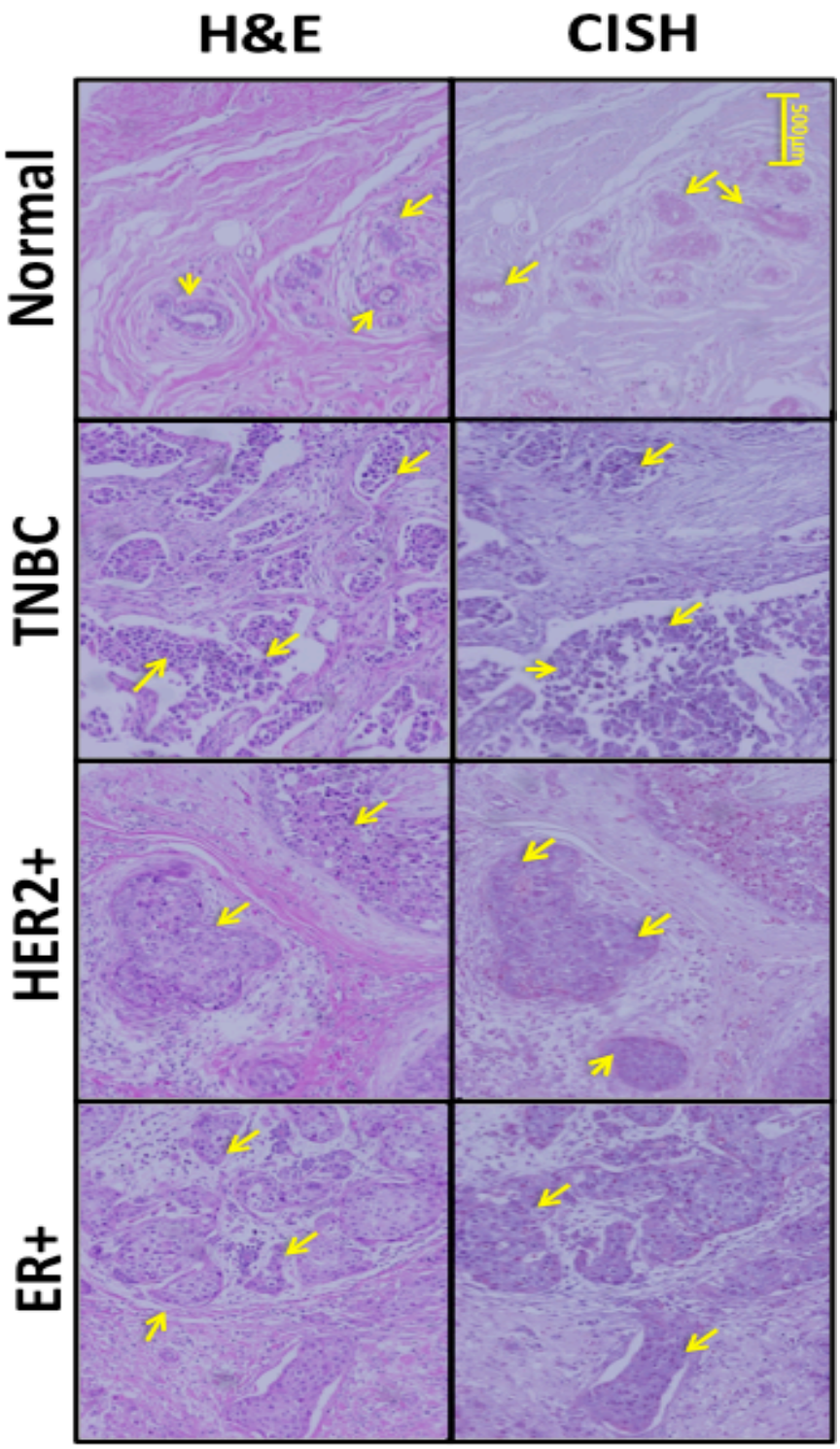
Figure 2. *AnxA2 Association with Overall Survival within Breast Cancer Subtypes.* A-C). Kaplan-Meier curves with univariate analyses (log-rank) for patients with low and high AnxA2 gene expression versus high AnxA2 expression from tumors in our TCGA cohort for A) ER+ and/or PR+, B) HER2+, and C) TNBC breast cancer subtype.

AnxA2 is overexpressed in TNBC tissue samples.

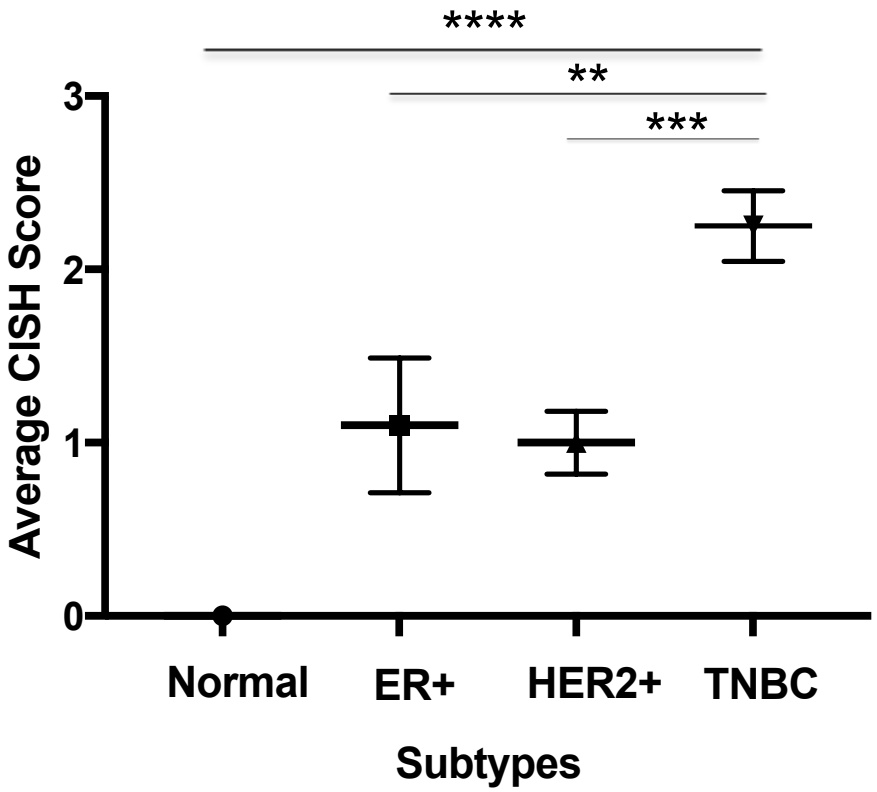
Tissue microarray specimens were analyzed by chromogenic in situ hybridization (CISH) for AnxA2 mRNA expression. Representative CISH images of AnxA2 in Normal, ER+, HER2+, and TNBC patients are shown in Figure 3A. Our two independent pathologist blinded scores were examined together and averaged between both reports within each breast cancer subtype. Furthermore, each patient had two sections of tumor analyzed to negate bias due to tumor heterogeneity. Normal tissues (n=12; Figure 3A) showed null staining of AnxA2 (CISH average score = 0.23 ± 0.1216, P = 0.0001, Figure 3B), ER+ specimen (CISH average score = 1 ± 0.1805, n=10, P = 0.0063, Figure 3B) and HER2+ (n=24, CISH average score = 1.2 ± 0.3887, n=24, P = <0.0001, Figure 3B) specimen showed very weak staining of AnxA2, while TNBC specimen (CISH average score = 2.125 ± 0.2045, n=33, Figure 3B) demonstrated strong AnxA2 cytosolic staining. Additionally, we found AnxA2 association with TNBC progression (Figure 3D). TNBC specimens were separated into the American Joint Committee on Cancer (AJCC) TNM (T, Tumor Size; N, Nodal Status; M, Metastasis) stages (Stage I, II, III, IV). AnxA2 average score intensity in more advanced stages of cancer, Stage III/IV (CISH average score = 2.45 ± 0.2111, n=11, Figure 3D), is significantly (P = 0.0381) higher in comparison to less advanced stages of cancer, Stage I/II (CISH average score = 1.55, n=20, Figure 3D), which often have favorable prognoses.

Figure 3

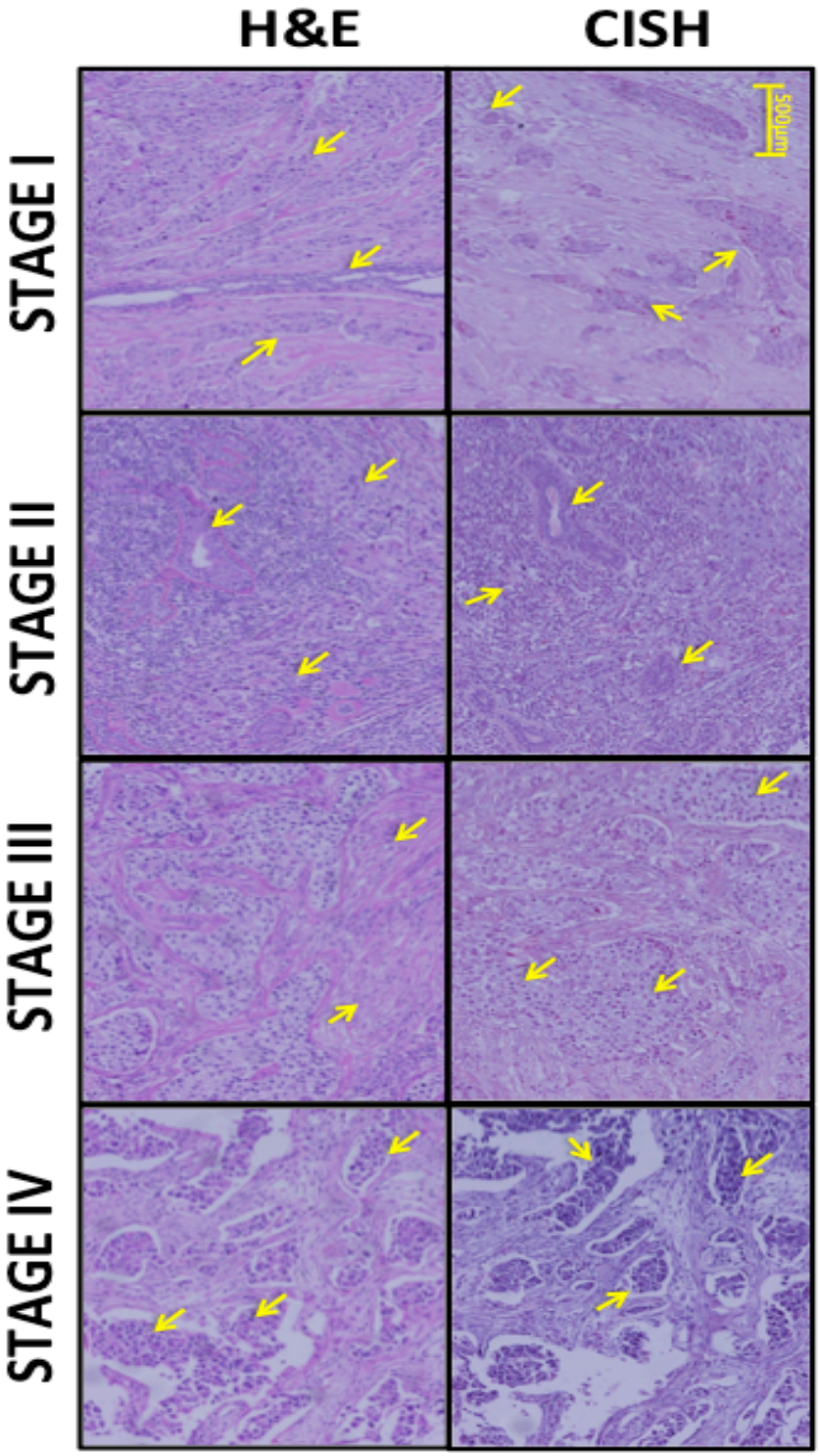
A



B



C



D

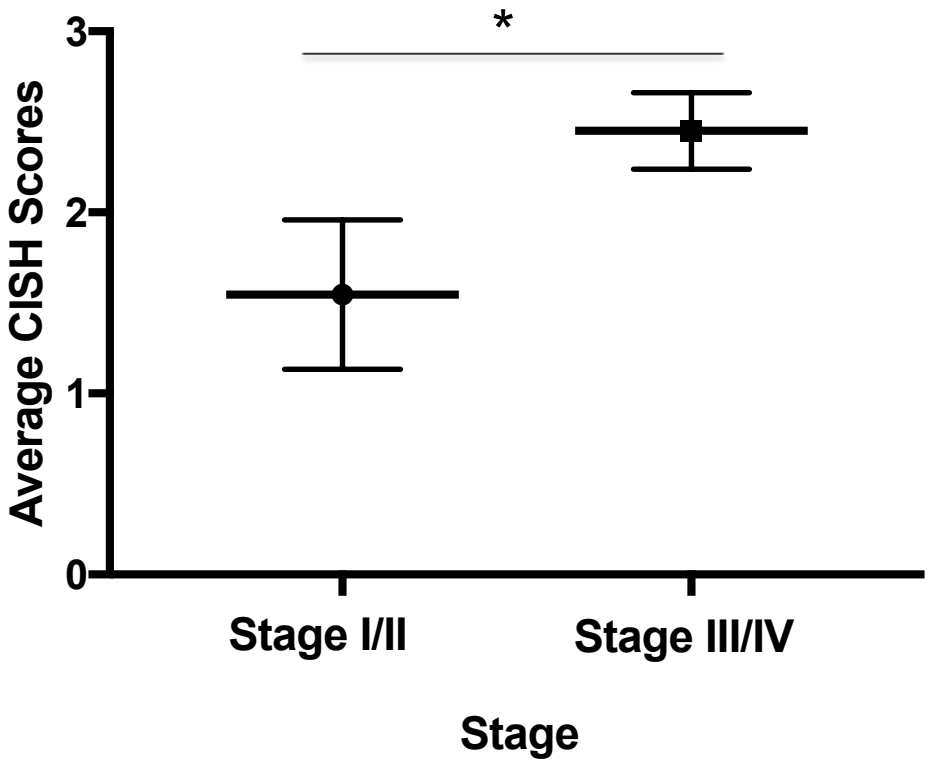


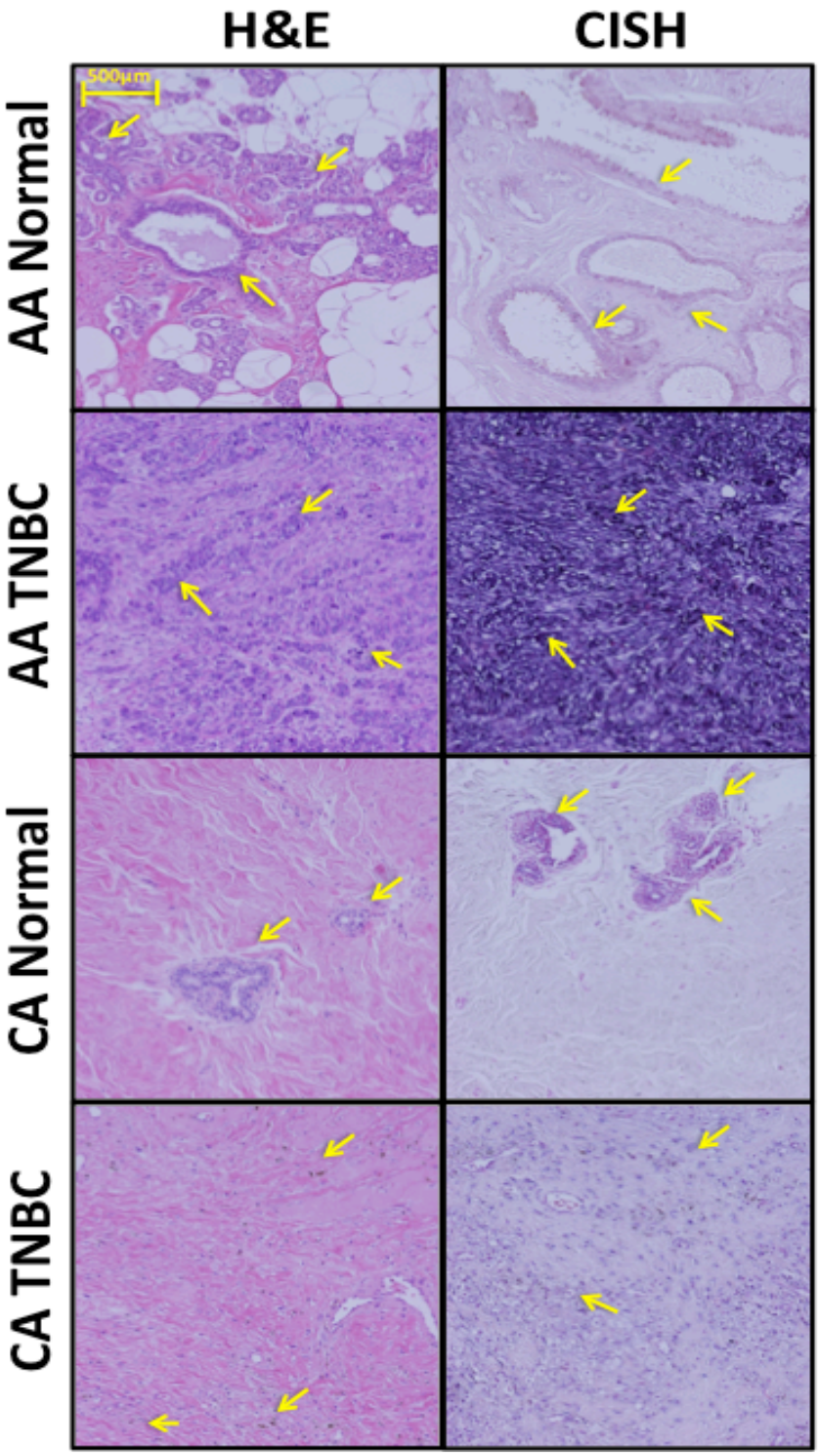
Figure 3. *AnxA2 expression in human breast cancer and normal tissues.* A) CISH and H&E Representative images of Normal (n=12), ER+ (n=10), HER2+ (n=24), TNBC (n=33) patient tissue specimen. B) CISH Average Score Analysis of patient tissue sections in within each subtype. C) CISH and H&E Representative images of STAGE I (n=1), STAGE II (n=10), STAGE III (n=2), and STAGE IV (n=18) TNBC patient tissue specimen. D) CISH Average Score comparison between STAGE I/II (low aggressive stages) and STAGE III/IV (high aggressive stages) among TNBC patient tissue specimen. Yellow arrows indicate malignant cells. 10X Magnification, scale bar = 500 μm.

AnxA2 is overexpressed in AA TNBC tissues in comparison to normal tissues and CA TNBC tissues.

We analyzed AnxA2 CISH intensity score of 10 AA TNBC Patients with matched normal tissue and10 CA TNBC patients with matched normal tissue (Figure 4). Representative CISH images of AnxA2 in AA Normal, AA TNBC, CA Normal, and CA TNBC patients are shown in Figure 4A. We observed AnxA2 CISH staining intensity to be significantly higher in AA TNBC patients in comparison to AA matched Normal, CA TNBC and CA matched normal patients. The average CISH intensity score in AA TNBC patients (CISH average score = 2.45 ± 0.3266, Figure 4B; ~100% of cell showed no staining, Figure S2) was significantly higher in comparison to AA matched normal (CISH average score = 0.3 ± 0.1527, P = <0.0001, Figure 4B; ~100% of cell showed no staining, Figure S2). Further, the average CISH score was statistically significant in AA TNBC patients in comparison to CA TNBC patients (CISH average score = 1.1 ± 0.4068, Figure 4B; ~100% of cell showed no staining, Figure S2). This observation potentiates a strong association of high AnxA2 expression with AA TNBC patients and may be a determinant in TNBC classification in AA women.

Figure 4

A



B

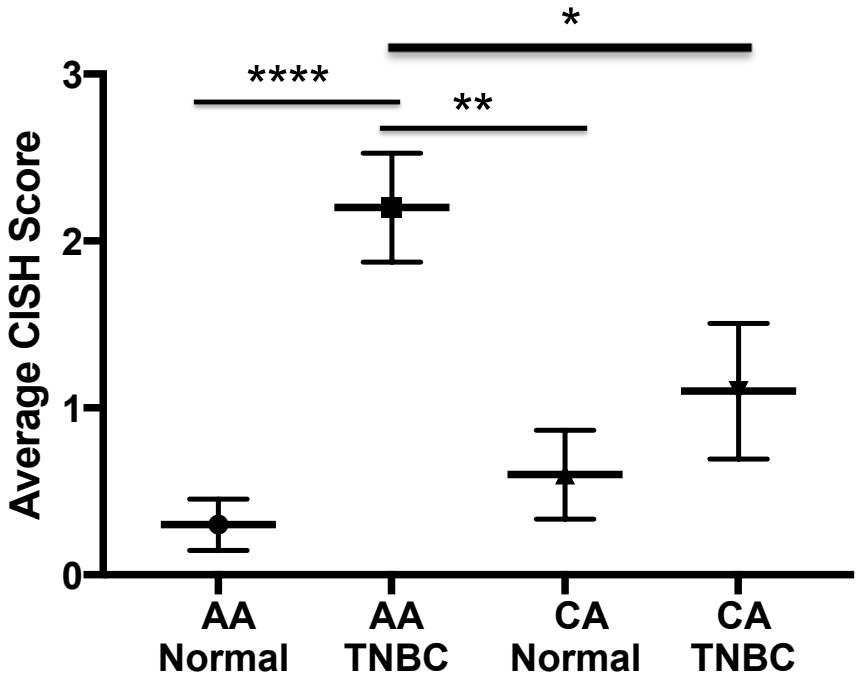


Figure 4. *AnxA2 expression in AA and CA TNBC tumor matched normal tissues.*

A) CISH and H&E Representative images of AA Matched Normal (n=10), AA TNBC (n=10), CA Matched Normal (n=10), CA TNBC (10) patient tissue specimen from an independent cohort obtained from UAB Comprehensive Tissue Network. B) CISH Average Score Analysis of patient tissue sections in within each cohort. Yellow arrows indicate malignant cells. 10X Magnification, scale bar = 500 μm.

Discussion

TNBC is an aggressive subtype of breast cancer and is often associated with a rapid progressive course (22). Elevated rates of triple-negative breast cancer have been observed in pre-menopausal African American women and women of African ancestry who are BRCA1/2 mutation carriers (23-24). Unfortunately, these women are at higher risk for metastasis to the lung and brain subjecting these women to a low survival probability. The most intriguing phenomenon is the disproportionate occurrence of TNBC amongst women of African descent in comparison with women of European (25-28). Further, TNBC has a significantly higher occurrence in patients of African descent than any other subtype of breast cancer in comparison to breast cancer patients of European descent (28). Although, our understanding of breast cancer subtypes, tumor heterogeneity, and their link to underlying determinants, such as genetics or lifestyle, the reasons for this disproportionate occurrence has remained unclear (31-38). We suggest here that distinct molecular differences in tumor biology may have significant impact in determining aggressiveness and poorer survival in African American women.

AnxA2 has been observed in breast cancer progression, and metastases. (19). Further, Jeon *et a*l. identified secretory AnxA2 as a potential prognostic marker for tumor malignancy and metastatic recurrence of breast cancer (21). Our study indicates a strong association of AnxA2 with TNBC and AA TNBC patients. Although AnxA2 expression was not significant in our TCGA cohort between TNBC and HER2+ subtypes, its preferential prognostic power of survival in TNBC patients when compared to HER2+ and ER+ patients emphasizes the potential significance of

AnxA2 expression in AA TNBC patients' mortality. Anatomical pathologists validated the significance of AnxA2 in TNBC progression and linked strong AnxA2 staining to less differentiated cells and advanced stages of TNBC. We have shown previously that AnxA2 is strongly associated with TNBC exosomes and contributes to cancer progression by forming a pre-metastatic niche at the site of metastasis that provides a favorable tumor microenvironment for disseminating cells. Further, we have recently found a significant link of exosomal AnxA2 in AA TNBC patients (unpublished data). Our results here and our previous work provides a strong case for AnxA2 as a potential prognostic predictor and demonstrates the importance of tumor biology in discerning clinical outcome in patients of different ethnic backgrounds.

The importance of tumor heterogeneity in ancestry and race in determining poor prognosis in underrepresented populations and the medically underserved remains ambiguous due to a number of limitations that we have experienced throughout the course of our study. Although we commend TCGA for their valiant effort in enrolling minorities in this seminal study, the small populations within ethnic groups does not allow robust studies to determine significant genetic differences (39). Additional studies with a larger number of patients of African and Hispanic ancestry need to be conducted to determine AnxA2 as a prognostic marker. Further, our blind scoring of tissue specimens validates and promotes AnxA2 significance in TNBC and progression, but the lack of clinical information of our specimen does not allow the study of clinicopathological differences such as age, menopause status, stage, tumor grade, and race/ethnicity for many of our patients.

Although this information was not available for our study, we were able to identify AnxA2 as a potential prognostic candidate for AA TNBC patients and demonstrate evidence for AnxA2 as a predictor of advanced stage and mortality. The results of this study highlight the biological difference in the presentation of a patient's disease in various ethnic backgrounds and the potential of using these biological differences to provide an adequate prognosis to ensure personalized treatment and care. These biological differences may provide a better understanding of prognosis, treatment options, as well as a definitive diagnosis. In conclusion, AnxA2 is associated with AA TNBC patients and is a potential prognostic predictor of TNBC progression and poor survival.

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CHAPTER IV

Prognostic impact of Annexin A1 and Annexin A2 gene expression in Triple-Negative Breast Cancer

Lee D. Gibbs and Jamboor K. Vishwanatha

Abstract

Background

Previous studies have shown Annexin A1 (AnxA1) and Annexin A2 (AnxA2) association with the aggressive behavior of Triple Negative Breast Cancer (TNBC). Our aim was to determine the correlation of AnxA1 and AnxA2 with poor prognosis of Triple Negative Breast Cancer (TNBC).

Methods

We analyzed the gene expression of the human annexin family from microarray datasets and correlated with clinical outcomes thorugh Cox regression analysis and univariate analysis to determine their ability to predict prognosis.

Results

Within a mean follow-up time of 57.2 months, our analyses of TNBC patients with both high AnxA1 and AnxA2 demonstrate a significant decrease in overall survival ($p = 0.0017$) and relapse free survival ($p = 0.0002$) when compared to the expression of these genes independently. Furthermore, AnxA1 prognostic impact relies on AnxA2 expression and both are preferential for TNBC when compared to other breast cancer subtypes.

Conclusion

Together these findings indicate that AnxA1 and AnxA2 are preferential dual prognostic predictors among TNBC patients.

Introduction

Breast cancer ranks second amongst malignancies and mortality in women in the United States of America, with 1 in 8 women diagnosed in their lifetime (1-3). Advances in breast cancer research have led to the identification of four molecular subtypes; Luminal A, Luminal B, Triple negative/basal-like, and HER2 (Human Epidermal Growth Factor [Erbb2]) type. Triple-negative breast cancers (TNBC) are estrogen receptor negative (ER-), progesterone receptor-negative (PR-), and HER2 receptor-negative (HER2-) and constitutes 15 to 20% of diagnosed breast cancers (4-8). TNBC is associated with poor prognosis, high mortality rate, shorter median time to relapse and visceral metastatic spread to the brain and lungs due to its aggressive tumor phenotype. Despite significant advances in diagnosis, treatment, and care, the prognosis of TNBC has not improved satisfactorily.

Previous studies have demonstrated that certain human annexins [12 members (AnxA1–A13, AnxA12 is unassigned)] are associated with proliferation, migration, invasion, angiogenesis and metastasis with Annexin A1 (AnxA1) and Annexin A2 (AnxA2) as stimulators for these hallmarks of cancer (10,11). Our lab has reported on the prevalence, functionality, and mechanistic properties of AnxA2 and demonstrated AnxA2 aberrant expression in patients with both invasive ductal mammary carcinoma and ductal carcinoma in situ (DCIS) (11). In contrast, it is undetectable in normal and hyperplastic ductal epithelial cells and ductal complexes, suggesting a pivotal role of AnxA2 in breast tumor malignancy and invasiveness (11). Furthermore, our studies have demonstrated that AnxA2 is abundantly expressed in TNBC patients and has a reciprocal relationship with HER2

at mRNA and protein levels (11,12). We recently published that exosomes derived from TNBC contains a large concentration of AnxA2 and has a functional role in distant metastasis to the brain and lungs *in vivo* by preparing a favorable pre-metastatic niche for disseminating cells (13).

In our analyses of our breast cancer cohort we additionally discovered AnxA1 significance and preferential association with clinical outcomes of TNBC. Regarding clinical significance, AnxA1 mRNA expression has been previously reported as a poor prognostic indicator of survival in TNBC (14). Despite the reported associations with aggressiveness, metastasis, and higher grade no data is available on the prognostic power of AnxA2 or the dual prognostic impact of AnxA1 and AnxA2 in TNBC patients. Thus, we set out to investigate AnxA1 and AnxA2 as preferential and independent indicators of poor clinical outcomes in TNBC in a study of 4,142 breast cancer patients of various subtypes with clinical follow-up data (mean observation time = 75.5 months, median = 72.4 months) and compare the individual prediction power of these genes to their dual prognostic impact.

Materials and Methods

Gene expression of solid breast tumors was obtained from combining 23 Gene Expression Omnibus (GEO) microarray datasets from Affymetrix HG-U133A, HG-U133 Plus 2.0 and HG-U133A 2.0 arrays only and aggregation of datasets are described in detail by Gyorffy *et al* (15). The cutoff values for AnxA1 and AnxA2 expression for "low" and "high" were determined using the median of their individual gene expression range. Overall Survival (OS) was defined as the interval between the date of surgery and date of death from any cause or last contact.

Relapse Free Survival (RFS) was defined as the interval from the date of surgery to the date of recurrence diagnosis or last contact. Distant Metastasis Free Survival (DMFS) was defined as the interval from the date of surgery to the date of metastasis diagnosis to brain, lungs, bone or last contact. Survival analyses were based on Kaplan–Meier estimations and the log-rank test was used to analyze differences in survival durations. Cox proportional hazard regression models were fitted to determine the impacts of the annexins on OS, RFS, and DMFS. All statistical tests were two-sided, and *P* values <0.05 were considered statistically significant.

Results

Annexins are associated with breast cancer subtypes and potentiates poor clinical outcomes.

In our TNBC cohort (mean observation time = 57.2 months, median = 45.5 months), 51 deaths of any cause, 220 recurrences, and 56 metastatic events were reported. All annexins gene expression was analyzed to determine their individual association with TNBC. AnxA1, AnxA2, and AnxA6 were the only annexins identified to be significantly associated with clinical outcomes of TNBC patients in comparison with all other breast cancer subtypes (Table 1). Significantly worse OS ($P = 0.007$, Figure 1A) and RFS ($P < 0.0001$ Figure 1B) was observed among patients with high AnxA1 expression compared to low expression and is independently associated with poor OS prognosis [hazard ratio (HR), 2.14; 95% confidence interval (CI), 1.22-3.78] and poor RFS prognosis [HR, 1.66; 95% CI, 1.28-2.17] (Table 1). High AnxA1 expression was not significantly associated with DMFS or poor prognosis [$P < 0.27$, HR, 1.33; 95% CI, 0.79-2.24, Table 1, Figure 1C]. Similar to AnxA1, AnxA2 is

associated with unfavorable clinical outcomes and poor prognosis. Significantly worse OS, RFS, and DMFS (p =0.019, p = 0.0051, p = 0.0021, Figure 1D, Figure 1E, Figure 1F, respectively) were observed among patients with high AnxA2 expression compared to low expression. High AnxA2 is independently associated with poor OS [HR, 2.14; 95% CI, 1.22-3.78, Table 1], RFS [HR, 1.45; 95% CI, 1.12-1.89, Table 1], and poor DMFS prognosis [HR, 1.5; 95% CI, 1.16-1.95, Table 1]. AnxA6 analysis shows conflicting results as high AnxA6 expression significantly correlated to unfavorable (RFS, $p < 0.028$, Figure 1H) and favorable prognosis (OS, p = 0.003, Figure 1G; DMFS, p = 0.019, Figure 1I). These results could not determine AnxA6 as a potential reliable prognostic predictor.

TABLE 1.

Table 1. Cox Regression analysis of clinical outcome in patients with breast cancer (intrinsic subtypes).

Variables	Overall Survival		Relapse Free Survival		Distant Metastasis Free Survival	
	HR [a] (95% CI) [b]	P-value [c]	HR [a] (95% CI) [b]	P-value [c]	HR [a] (95% CI) [b]	P-value [c]
All Breast Cancer Subtypes						
AnxA1 (high vs. low)	1.02 (0.8-1.29)	0.89	1.07 (0.96-1.2)	0.22	0.95 (0.78-1.16)	0.62
AnxA2 (high vs. low)	1.04 (0.82-1.32)	0.74	1.11 (0.99-1.25)	0.067	1.05 (0.86-1.29)	0.63
AnxA6 (high vs. low)	0.8 (0.63-1.02)	0.067	0.96 (0.86-1.08)	0.52	0.87 (0.71-1.07)	0.18
Luminal A Breast Cancer						
AnxA1 (high vs. low)	0.93 (0.64-1.36)	0.7	0.95 (0.8-1.14)	0.58	0.92 (0.69-1.24)	0.58
AnxA2 (high vs. low)	0.71 (0.48-1.04)	0.08	0.94 (0.79-1.12)	0.48	1 (0.74-1.35)	1
AnxA6 (high vs. low)	1.19 (0.81-1.73)	0.38	0.89 (0.75-1.06)	0.2	1.07 (0.79-1.44)	0.66
Luminal B Breast Cancer						
AnxA1 (high vs. low)	0.86 (0.57-1.31)	0.49	0.92 (0.75-1.12)	0.41	0.78 (0.53-1.13)	0.19
AnxA2 (high vs. low)	1.33 (0.88-2.01)	0.18	1.12 (0.91-1.37)	0.28	1.05 (0.72-1.52)	0.81
AnxA6 (high vs. low)	0.85 (0.56-1.28)	0.43	1.07 (0.87-1.31)	0.52	0.76 (0.52-1.11)	0.16
HER2+ Breast Cancer						
AnxA1 (high vs. low)	0.61 (0.28-1.33)	0.21	0.72 (0.47-1.09)	0.12	1.36 (0.71-2.59)	0.35
AnxA2 (high vs. low)	0.77 (0.36-1.65)	0.5	1.01 (0.66-1.52)	0.98	1.27 (0.66-2.42)	0.47
AnxA6 (high vs. low)	1.36 (0.63-2.94)	0.43	1.41 (0.93-2.15)	0.11	1.41 (0.74-2.71)	0.3
Triple-negative/Basal Breast Cancer						
AnxA1 (high vs. low) *	**2.14 (1.22-3.78)**	**0.007**	**1.66 (1.28-2.17)**	**0.00014**	**1.33 (0.79-2.24)**	**0.27**
AnxA2 (high vs. low) *	**2.66 (1.14-6.25)**	**0.019**	**1.45 (1.12-1.89)**	**0.0051**	**1.5 (1.16-1.95)**	**0.0021**
AnxA6 (high vs. low) *	**0.43 (0.24-0.76)**	**0.003**	**1.34(1.03-1.74)**	**0.028**	**0.54 (0.32-0.91)**	**0.019**

Table 1. *Cox Regression analysis of AnxA1 and AnxA2 with clinical outcomes in patients with breast cancer (intrinsic subtypes).*

[a] HR=Hazard Ratio

[b] CI= Confidence Interval

[c] p-value = $p \leq 0.05$ considered significant.

[*] = Significant genes (Genes and subtypes in bold to indicate preference for subtype).

FIGURE 1

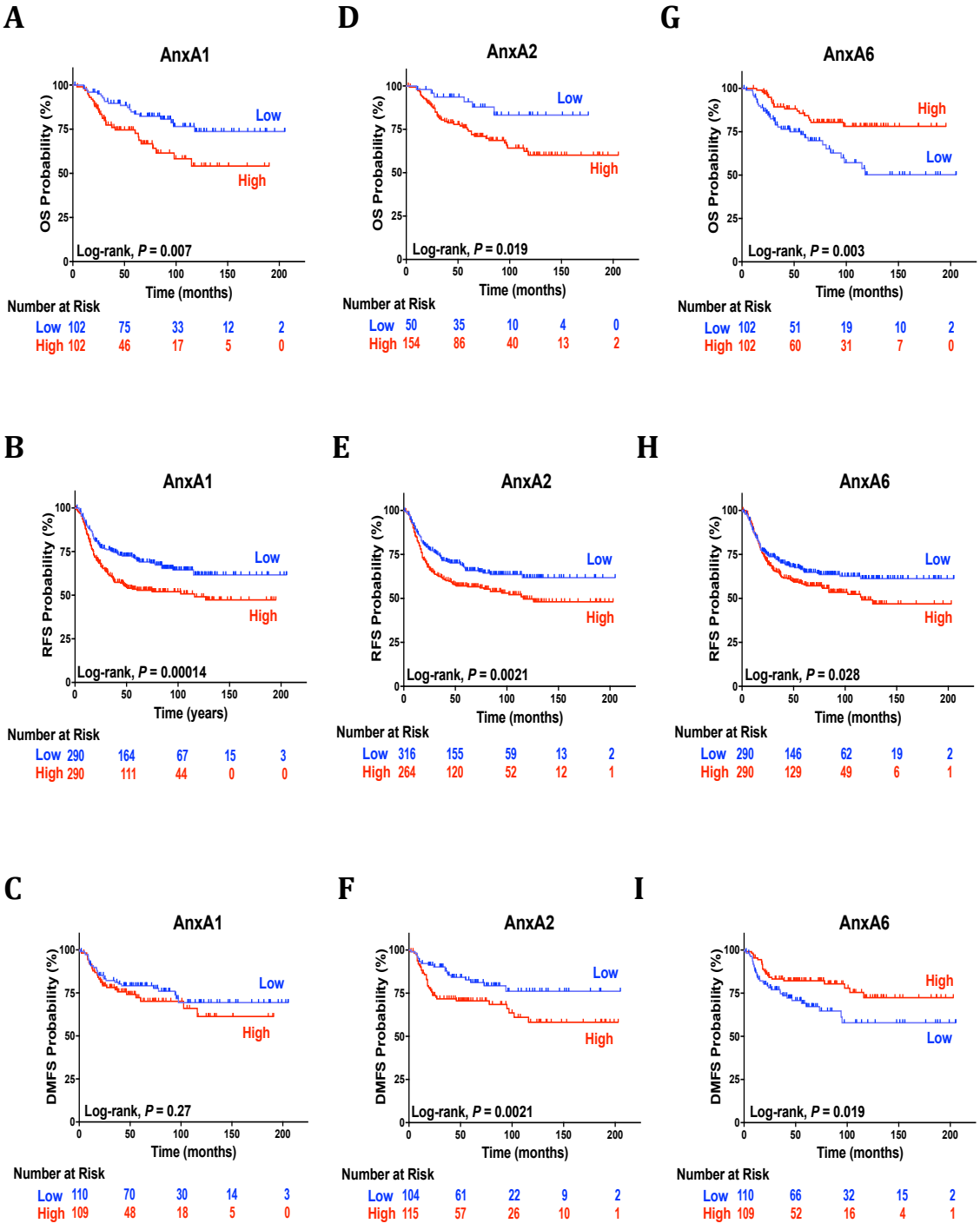

A
AnxA1
OS Probability (%)
Low
High
Log-rank, P = 0.007
Time (months)
Number at Risk
Low 102 75 33 12 2
High 102 46 17 5 0
D
AnxA2
OS Probability (%)
Low
High
Log-rank, P = 0.019
Time (months)
Number at Risk
Low 50 35 10 4 0
High 154 86 40 13 2
G
AnxA6
OS Probability (%)
High
Low
Log-rank, P = 0.003
Time (months)
Number at Risk
Low 102 51 19 10 2
High 102 60 31 7 0
B
AnxA1
RFS Probability (%)
Low
High
Log-rank, P = 0.00014
Time (years)
Number at Risk
Low 290 164 67 15 3
High 290 111 44 0 0
E
AnxA2
RFS Probability (%)
Low
High
Log-rank, P = 0.0021
Time (months)
Number at Risk
Low 316 155 59 13 2
High 264 120 52 12 1
H
AnxA6
RFS Probability (%)
Low
High
Log-rank, P = 0.028
Time (months)
Number at Risk
Low 290 146 62 19 2
High 290 129 49 6 1
C
AnxA1
DMFS Probability (%)
Low
High
Log-rank, P = 0.27
Time (months)
Number at Risk
Low 110 70 30 14 3
High 109 48 18 5 0
F
AnxA2
DMFS Probability (%)
Low
High
Log-rank, P = 0.0021
Time (months)
Number at Risk
Low 104 61 22 9 2
High 115 57 26 10 1
I
AnxA6
DMFS Probability (%)
High
Low
Log-rank, P = 0.019
Time (months)
Number at Risk
Low 110 66 32 15 2
High 109 52 16 4 1

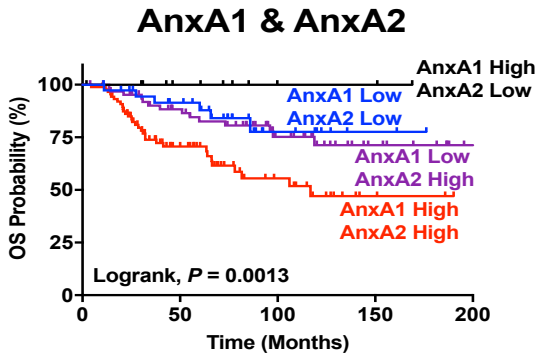
Figure 1. *AnxA1, AnxA2, and AnxA6 independent association with clinical outcomes.* (A-I). A) Kaplan-Meier curves with univariate analyses (log-rank) for patients with low AnxA1 gene expression versus high AnxA1 expression from tumors in triple negative breast cancer for overall survival B) relapse free survival and C) distant metastasis free survival. D) Kaplan-Meier curves with univariate analyses (log-rank) for patients with low AnxA2 gene expression versus high AnxA2 expression from tumors in triple negative breast cancer for overall survival E) relapse free survival and F) distant metastasis free survival. G) Kaplan-Meier curves with univariate analyses (log-rank) for patients with low AnxA2 gene expression versus high AnxA2 expression from tumors in triple negative breast cancer for overall survival H) relapse free survival and I) distant metastasis free survival.

AnxA1 and Anx2 have dual association with TNBC and predict poor clinical outcomes.

Our analysis of AnxA1 and AnxA2 dual association with clinical outcomes reveals extremely poor OS and RFS in TNBC patients with high AnxA1/AnxA2 expression. High AnxA1/low AnxA2 and low AnxA1/AnxA2 expression showed the most favorable OS and RFS respectively (P = 0.0013, Figure 2A; P = 0.0002, Figure 2B). Although our analysis of AnxA1 and AnxA2 dual association with DMFS was not significant (P = 0.0591, Figure 2C), we observed an interesting trend of unfavorable DMFS in patients with low AnxA1/high AnxA2 and a more favorable outcome in patients with low AnxA1/AnxA2. Thus, our evidence concludes AnxA1 prognostic prediction power in mortality and recurrence relies on high AnxA2 expression.

FIGURE 2

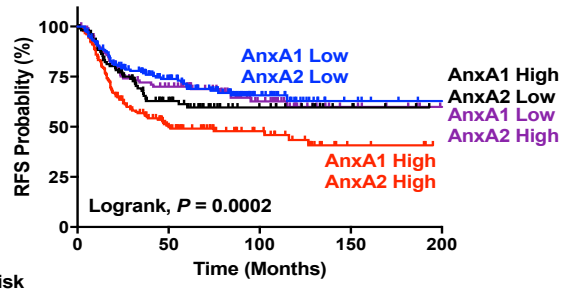
A



Number at Risk

AnxA1/AnxA2					
Low/Low	38	29	9	3	1
High/Low	12	8	3	2	1
Low/High	64	48	26	11	1
High/High	89	39	15	3	1

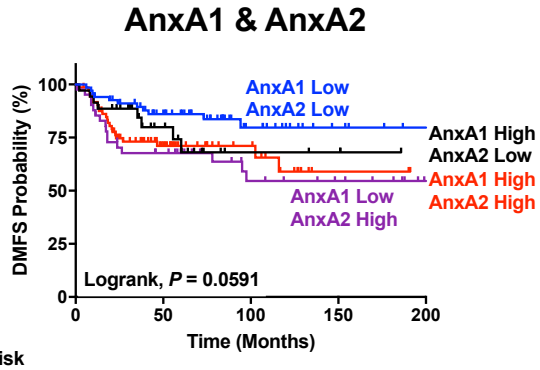
B



Number at Risk

AnxA1/AnxA2					
Low/Low	181	98	36	36	1
High/Low	105	45	45	19	1
Low/High	106	66	66	32	1
High/High	180	67	67	27	1

C



Number at Risk

AnxA1/AnxA2					
Low/Low	68	46	19	7	1
High/Low	35	16	5	3	1
Low/High	42	25	13	19	1
High/High	73	33	14	3	1

Figure 2. *AnxA1 and AnxA2 dual association with clinical outcomes.* (A-C). Survival estimations of TNBC patients stratified by combined tumor AnxA1 and AnxA2 gene expression status are shown for A) overall survival B), relapse free survival C) and distant metastasis free survival.

Discussion

Previous studies striving for more reliable predictors of TNBC progression focus on immunohistochemical analysis and expression profiles (4,5,6,7,8,14). However, none of the proposals have been implemented as clinical recommendations to adequately determine prognosis. The data here comprehensively demonstrates that AnxA1 and AnxA2 may be translated into novel markers of prognostic power. Application of such markers will assist in overcoming the current limitations of histological classification and prognostic evaluation. Information on AnxA1 *and* AnxA2 gene expression will also allow the clinician to identify patients that disease may be at higher risk than patients that may have favorable outcomes. Additionally, our lab's previous and current studies evaluating the functional role of AnxA2 in establishing a favorable tumor microenvironment for migrating TNBC cells provides additional support for AnxA2 as an independent and reliable prognostic predictor for DMFS (14). Furthermore, our results demonstrate AnxA1 prognostic predictive power is driven by high expression of AnxA2 and significantly increases a patient risk for death and relapse. Although AnxA6 expression had significant correlation with clinical outcomes of TNBC, ambiguous results and lack of supporting literature on its role in TNBC did not suggest further investigation. Although this study was informative, the present study had several limitations. First, the retrospective nature of this study should be noted. To decrease potential biases, however, we analyzed large numbers of patients from multiple institutions and several investigators. Further, the number of cases and lack of detailed clinical information does not allow for robust biological conclusions on the

effect of age, menopause status, stage, tumor grade, and race/ethnicity to adequately assess the association of AnxA1 and AnxA2 with the disparity of TNBC in pre-menopausal and women of African descent (15). In conclusion, AnxA1 and AnxA2 are dually associated with unfavorable clinical outcomes and may be useful tools in predicting poor prognosis in TNBC patients.

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CHAPTER V

Summary and Future Directions

Summary

This study demonstrates many applications of AnxA2 as a prognosticator for TNBC patients and its significant association with AA TNBC patients. This study was innovative as it investigated the relationship of AnxA2 with poor prognosis in AA TNBC patients. Furthermore, our study demonstrated the potential of AnxA2 to address the disproportionate occurrence and poor clinical outcomes of TNBC in AA women (1,2). The evaluation of AnxA2 gene expression and protein expression in solid tumors and serum from TNBC patients will provide new valuable information as a prognosticator of aggression, metastases, mortality and relapse. Altogether, these studies suggest the potential development of AnxA2 as a prognostic and therapeutic target for an effective prognosis and therapeutic response for AA TNBC patients.

In ***Chapter 2***, we demonstrated overexpression of exo-AnxA2 has a preferential association with AA TNBC patients in comparison to other breast cancer subtypes and Caucasian Americans. This unique association also has a functional role as a facilitator of angiogenesis. Our *in vivo* matrigel plug assay revealed increased angiogenesis from least aggressive subtypes, such as normal-like and ER+, to the most aggressive subtype, TNBC. Since, TNBC is often associated with unfavorable outcomes in comparison to other breast cancer subtypes, we believe exo-AnxA2 contributes to this aggressiveness through angiogenesis. Our inhibition of exo-AnxA2 through use of an inhibitory peptide unveiled exo-AnxA2 as a potential therapeutic target and contributes to the aggressive biology seen in TNBC patients. This also provides a minimally invasive procedure that may increase the effectiveness of recruitment strategies of minority patients (3,4). ***Chapter 3*** shows the relationship of AnxA2 with the disproportionate occurrence in AA

TNBC patients. The use of publically available data from TCGA database gave us access to genomic sequencing information for more that one thousand breast cancer patients. The power of this analysis was only restricted by low minority numbers, but did unveil AnxA2 high expression in AA TNBC patients and its ability to predict poor survival. Further, our evidence gave us cause to validate the discovery we made in our *in silico* analysis through *in situ* hybridization in breast cancer tissue specimen. The pathologists' blind scores demonstrates that AnxA2 can be clinically applicable as a diagnostic and prognostic tool. This coupled with our previous finding of EGFR and AnxA2 interaction in TNBC patients provides a strong implication for AnxA2 use as a characterization marker for basal-like breast cancer (5,6,7,8). Additionally, we unveiled AnxA2 preferential association with TNBC patients and its specific ability as a prognostic predictor of poor survival in TNBC patients. Our analysis of AnxA2 as a potential prognostic predictor continued in ***Chapter IV*** as we examined approximately four thousand patients and correlated each member of the human annexin family with favorable or unfavorable clinical outcomes. Our analysis not only shows AnxA2 preferrential association with TNBC, but also its close relative AnxA1. They both have the ability to predict poor clinical outcomes, but when analyzed together we observe AnxA1 prediction power is driven by high expression of AnxA2. Further, our current observation of AnxA2 as prognostic predictor of distant metastasis is justified by the studies of Maji. *et. al.* as it demonstrated that AnxA2 promotes angiogenesis and metastasis *in vivo* (9). These studies suggest that AA TNBC patients have the potential to be appropriately prognosticated through serum analysis of exo-AnxA2 in combination with AnxA2 analysis from solid tumor tissue sections (**Figure 1**.)

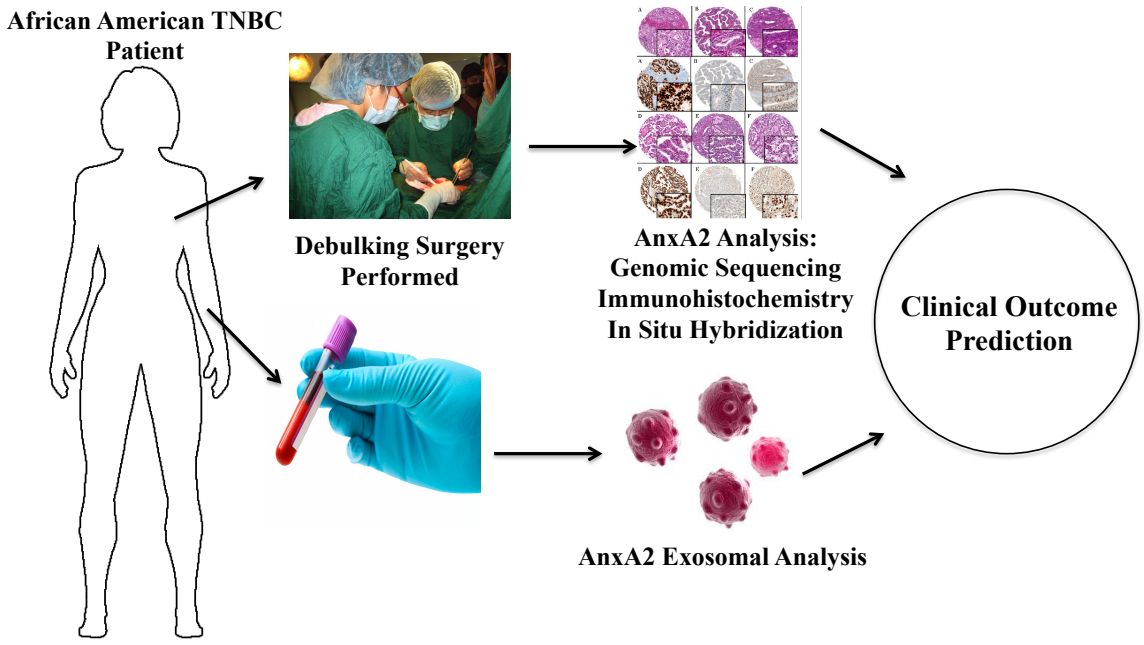

Figure 1. *Proposed model for the use of AnxA2 as a prognostic tool for AA TNBC patients.*

Future Directions

In the future we would like to overcome the barriers of the underrepresentation of minorities seen in databases with our continued collaborations in ongoing studies with nearby hospitals in the Dallas-Fort Worth metroplex in an attempt to reach a reliable minimal patient size of 2500 subjects (10). This would allow us to use evolved recruitment strategies to analyze AnxA2 within African American, Hispanic and other underrepresented populations. Additionally we would like to analyze ancestry informative markers of these patients to understand how ancestry compared to race may be driving the aggressive presentation of TNBC. We will also be able to recurrently retrieve specimen from a patient and monitor their exo-AnxA2 levels over a period of time to further understand its potential to predict metastasis. Further, increased enrollment would also allow us to statistically determine AnxA2 potential as a prognostic marker. In addition, we would like to further examine TNBC exosomes with and without expression of AnxA2 and determine a metastatic proteomic profile that may increase the precision of our metastatic prediction. The output of these recruitment strategies and experiments will provide essential information to establish an early detector of prognosis for an aggressive disease that prognosis is often determined in late stages of cancer.

In conclusion, this study provides information that is not only useful for AA TNBC patients, but can benefit all TNBC patients that express high levels of AnxA2. Our interest in AnxA2 functional properties in TNBC led our investigation to evaluate its association with AA TNBC patients and provide a rationale for the disproportionate number of AA TNBC patients with poor clinical outcomes in comparison to other ethnicities. The evolution of personalized and precise strategies to evaluate the patient

and not just the disease has increased exponentially in the past decade. Thus, the study of an individual protein, such as AnxA2, and their functional roles in disease may remove the ambiguity found in disease presentation and provide the patient the appropriate personalized standard of care.

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