Breast Cancer Research and Treatment (2019) 174:113–120
https://doi.org/10.1007/s10549-018-5030-5

PRECLINICAL STUDY

ANXA2 expression in African American triple-negative breast cancer patients

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Received: 10 July 2018 / Accepted: 27 October 2018 / Published online: 26 November 2018

Abstract

Purpose Our aim was to determine the role of Annexin A2 (*AnxA2*), which we have previously found to contribute to the aggressiveness of TNBC, with AA TNBC patients and clinical outcome.

Methods We analyzed TCGA breast cancer database ($n=1098$) to observe AnxA2 expression within breast cancer subtypes and is correlation with overall survival. Further, we examined breast tissue specimens ($n=119$) through chromogenic in situ hybridization (CISH) and specimen were scored independently by two pathologists in a blinded study.

Results In our TCGA analysis, high expression of AnxA2 was correlated with poor survival in patients with TNBC. AnxA2 gene expression was not correlated with poor survival in other breast cancer subtypes. AnxA2 average CISH intensity score (CISH score = 0, null expression to 3, high expression) for TNBC was significantly higher in comparison to estrogen receptor and/or progesterone receptor positive, human epidermal growth factor positive, and non-malignant tissues. Furthermore, AnxA2 average score was significantly higher 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 is overexpressed in TNBC, implicating AnxA2 as a contributor to the aggressive biology of TNBC in AA women.

Keywords Triple-negative breast cancer · African American · Annexin A2 · Prognosis · Biomarker

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

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10549-018-5030-5) contains supplementary material, which is available to authorized users.

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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 15–20% 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, 6].

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 [7–10]. Clinically the molecular heterogeneity 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 identify the appropriate molecular targets 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, adhesion, chemoresistance, plasmin generation, angiogenesis and ion channel conductance [16–22]. 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) [23]. Further, we have shown AnxA2 as a potential prognostic predictor in TNBC patients [24]. In this study, we aim to investigate AnxA2 gene expression in AA breast tissues to determine AnxA2 association with AA patients with TNBC 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. Navigating through these files manually would be next to impossible without data mining tools that can retrieve, process and normalize these files that are accessible through the open-access HTTP directory on the servers of TCGA's Data Coordinating Center [25]. This platform retrieves publicly and processes gene expression levels for 1098 BRCA patients [26]. 194 Patients were excluded from breast subtype analysis due to unavailable clinical information to determine breast cancer subtype. 386 patients were excluded from survival analysis due to unavailable clinical follow-up information. Breast cancer-specific mortality was not reported.

Kaplan–Meier plots

Overall survival (OS) of patient groups was based on BRCA RNASeq normalized gene expression data for 1098 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. Media follow-up time was 27.7 months, median time to an event, 41.8 months, and median time to censorship, 25.0 months.

Chromogenic in situ hybridization (CISH)

CISH, recently introduced tissue staining method, although it makes use of the in situ hybridization technology of FISH, it also takes advantage of the chromogenic signal detection of immunohistochemistry (IHC). that can be detected with a light microscope. Paraffin embedded tissue sections from the Cooperative Human Tissue Network (CHTN) Southern Division at the University of Alabama at Birmingham (UAB; 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 (IRB) at the site and at UNT Health Science Center. The anatomic pathologists independently read the Hematoxylin (stains nuclei purple) and Eosin (stain acidophilic structures red or pink) (H&E) stained sections and hybridized sections to determine AnxA2 mRNA intensity scores for normal and breast tissue sections. Scoring is performed very similar to IHC [27]. Immunoreactivity was defined as negative with a score of 0 (no staining in all cells or very weak staining in less than 10% of the tumor cells) or 1+ (weak partial staining in more than 10% of the tumor cells), and was defined as positive with a score of 2+ (weak to moderate complete staining in more than 10% of the tumor cells) or 3+ (strong complete staining in more than 10% of the tumor cells). 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 ± SEM. 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 hazard ratios were estimated using a cox proportional unadjusted model. 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 ($n = 160$), Hispanic American (HA; $n = 51$), and Caucasian American (CA; $n = 654$) women with breast cancer in the TCGA cohort (Fig. 1). AnxA2 gene expression was significantly elevated in AA in comparison to CA ($P < 0.0001$, Fig. 1a) and Hispanic ($P < 0.0001$, Fig. 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−; $n = 121$), 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+ ($P < 0.0001$, Fig. 1b) subtype. AnxA2 gene expression was not significant when compared to HER2+ patients ($P = 0.4249$, Fig. 1b). The

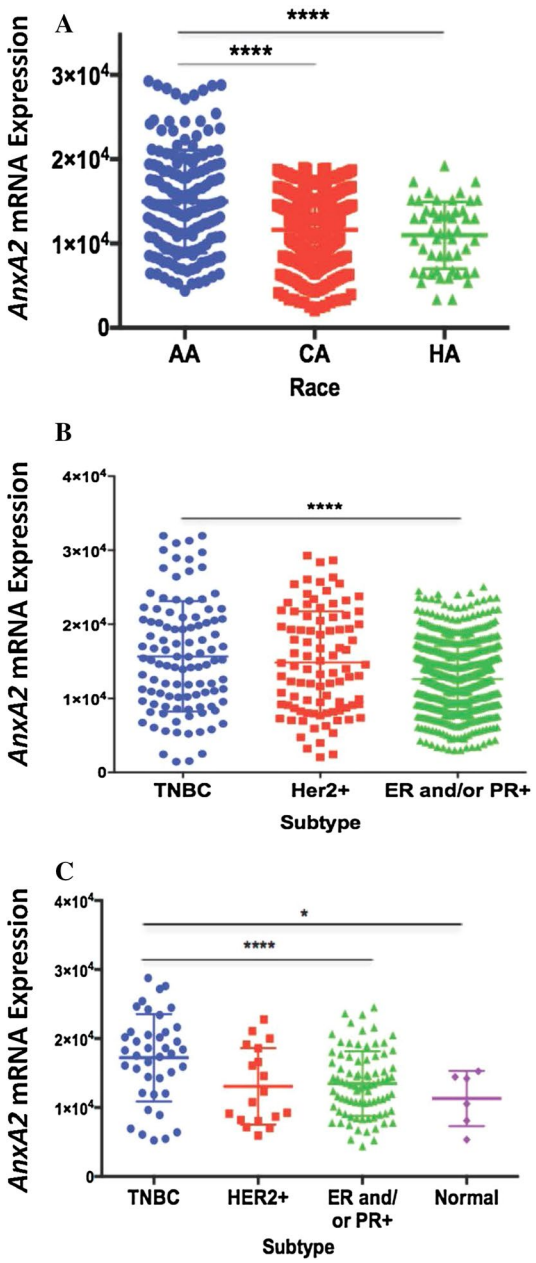

Fig. 1 AnxA2 gene expression amongst breast cancer subtypes and race. **a** AnxA2 RNA expression obtained from the TCGA RNAseq database for analysis of racial variation of gene expression for African American (AA; $n = 158$), Caucasian (CA; $n = 654$), and Hispanic American (HA; $n = 51$) breast cancer patients. **b** AnxA2 RNA expression obtained from the TCGA RNAseq database for TNBC ($n = 121$), 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

significant elevation of AnxA2 gene expression observed in AA cohort led us to investigate AnxA2 gene expression among TNBC ($n=31$), HER2+ ($n=16$), ER+ ($n=107$), and Normal ($n=6$) patients. AnxA2 gene expression was significantly elevated in AA TNBC patients in comparison to ER+ ($P<0.0001$, Fig. 1c) and normal samples ($P=0.0323$, Fig. 1c) from AA women. AnxA2 gene expression was not significant when compared to HER2+ ($P=0.1177$, Fig. 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.

High AnxA2 expression is correlated with poor survival in TNBC patients

In Fig. 2, we further utilized our TCGA cohort to analyze AnxA2 gene expression association with OS in ER and/or PR+ ($n=533$), HER2+ ($n=66$) TNBC ($n=113$) patients with clinical follow-up data. AnxA2 expression was dichotomized into low and high, based on the median of logarithmized expression values. We observed a significant lower survival in TNBC patients with high AnxA2 gene expression [hazard = 3.235; 95% confidence interval (CI) = 1.313–7.97, $P=0.0232$, Fig. 2c] in comparison with ER+ (hazard = 1.171, 95% CI 0.6715–2.042, $P=0.5777$, Fig. 2a) and HER2+ patients (hazard = 1.959, 95% CI 0.4865–7.89, $P=0.3440$, Fig. 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.

AnxA2 is overexpressed in TNBC tissue samples

Tissue microarray specimens were analyzed by chromogenic in situ hybridization (CISH) for AnxA2 mRNA expression. The two pathologists agreed on 108/119 scores with a percent agreement of 90.7%. Representative CISH images of AnxA2 in Normal, ER+, HER2+, and TNBC patients are shown in Fig. 3a. The CISH 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 ($n=12$; Fig. 3a) showed null staining of AnxA2 (CISH average score = 0.23 ± 0.1216, $P=0.0001$, Fig. 3b), ER+ specimen ($n=10$, CISH average score = 1 ± 0.1805, $P=0.0063$, Fig. 3b) and HER2+ ($n=24$, CISH average score = 1.2 ± 0.3887, $n=24$, $P=<0.0001$, Fig. 3b) specimen showed very weak staining of AnxA2, while TNBC specimen (CISH average score = 2.125 ± 0.2045, $n=33$,

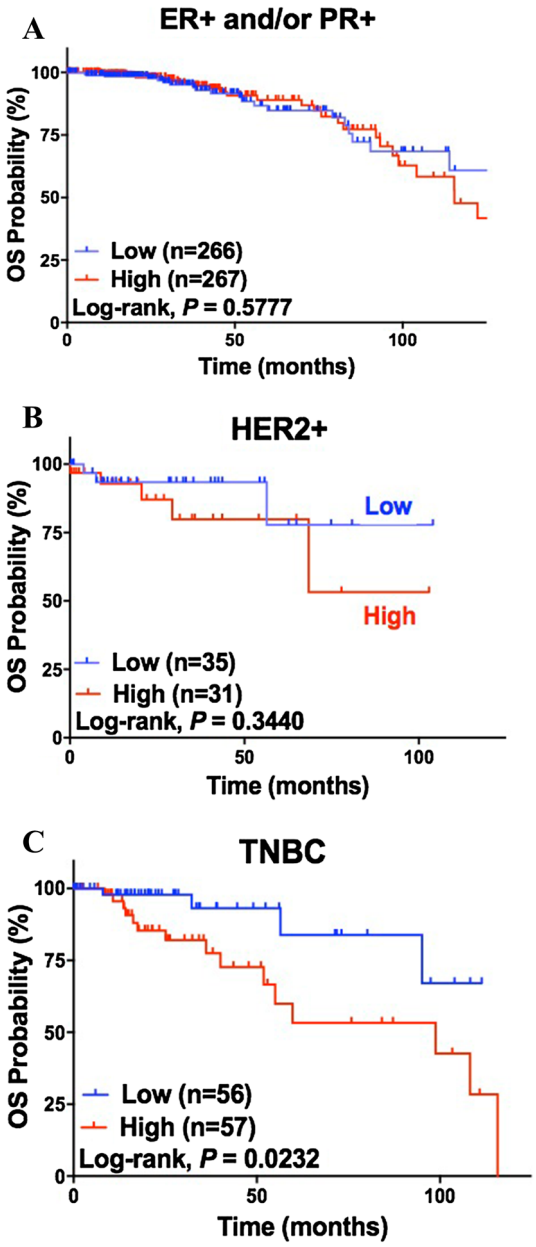

Fig. 2 AnxA2 association with overall survival within breast cancer subtypes. Kaplan–Meier curves with univariate analyses (log-rank) for patients with low and high AnxA2 gene expression versus high AnxA2 expression from tumors in our TCGA cohort for **a** ER+ and/or PR+, **b** HER2+, and **c** TNBC breast cancer subtype

Fig. 3b) demonstrated strong AnxA2 cytosolic staining. Additionally, we found AnxA2 association with TNBC progression (Fig. 3d). TNBC specimens were separated into the American Joint Committee on Cancer (AJCC) TNM (T, Tumor Size; N, Nodal Status; M, Metastasis) stages (Stages I, II, III, IV). AnxA2 average score intensity in more advanced stages of cancer, Stage III/IV ($n=11$, CISH average score = 2.45 ± 0.2111, Fig. 3d), is significantly ($P=0.0381$) higher in comparison to less

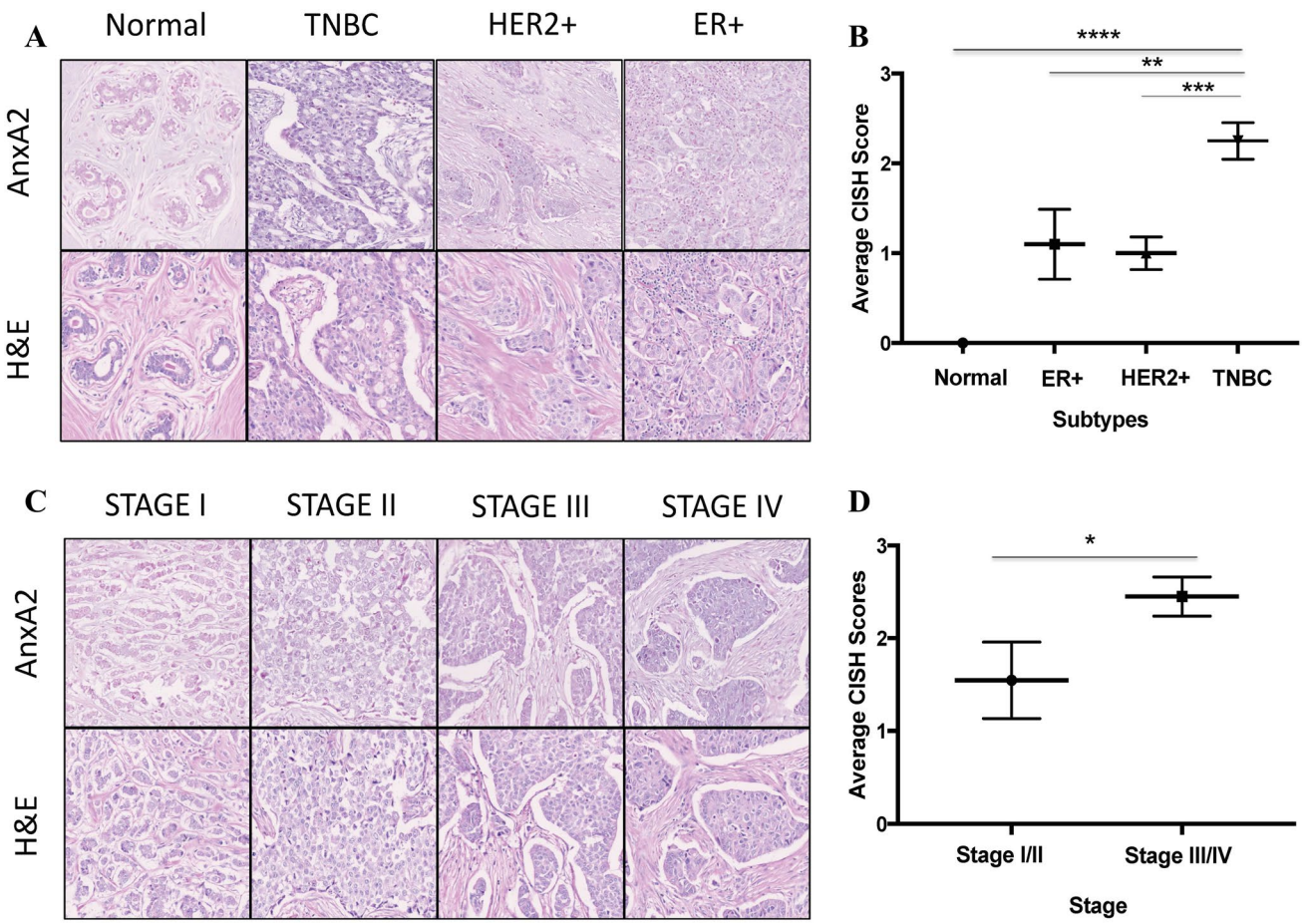

Fig. 3 AnxA2 expression in human breast cancer and normal tissues. **a** CISH and H&E representative images of normal ($n=12$), ER+ ($n=10$), HER2+ ($n=24$), TNBC ($n=33$) patient tissue specimen. **b** CISH Average Score Analysis of patient tissue sections in within each subtype. **c** CISH and H&E 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. ×10 Magnification, scale bar = 500 µm. Images were generated using Axio Scan.Z1 slide scan

advanced stages of cancer, Stage I/II ($n=20$, CISH average score = 1.55, Fig. 3d), which often have favorable prognoses.

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, 10 AA benign, 10 CA TNBC, and 10 CA benign breast tissues samples (Fig. 4). Representative CISH images of AnxA2 in AA Normal, AA TNBC, CA Normal, and CA TNBC patients are shown in Fig. 4a. We observed AnxA2 CISH staining intensity to be significantly higher in AA TNBC in comparison to AA benign breast tissue samples, CA TNBC and CA benign breast tissue samples. The average CISH intensity score in AA TNBC patients (CISH average score = 2.45 ± 0.3266, Fig. 4b) was significantly higher in comparison to AA benign breast tissue samples (CISH average score = 0.3 ± 0.1527, $P \leq 0.0001$, Fig. 4b). 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, Fig. 4b). 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.

Discussion

TNBC is an aggressive subtype of breast cancer and is often associated with a rapid progressive course [28]. Disproportionate rates of triple-negative breast cancer have been observed in pre-menopausal African American women

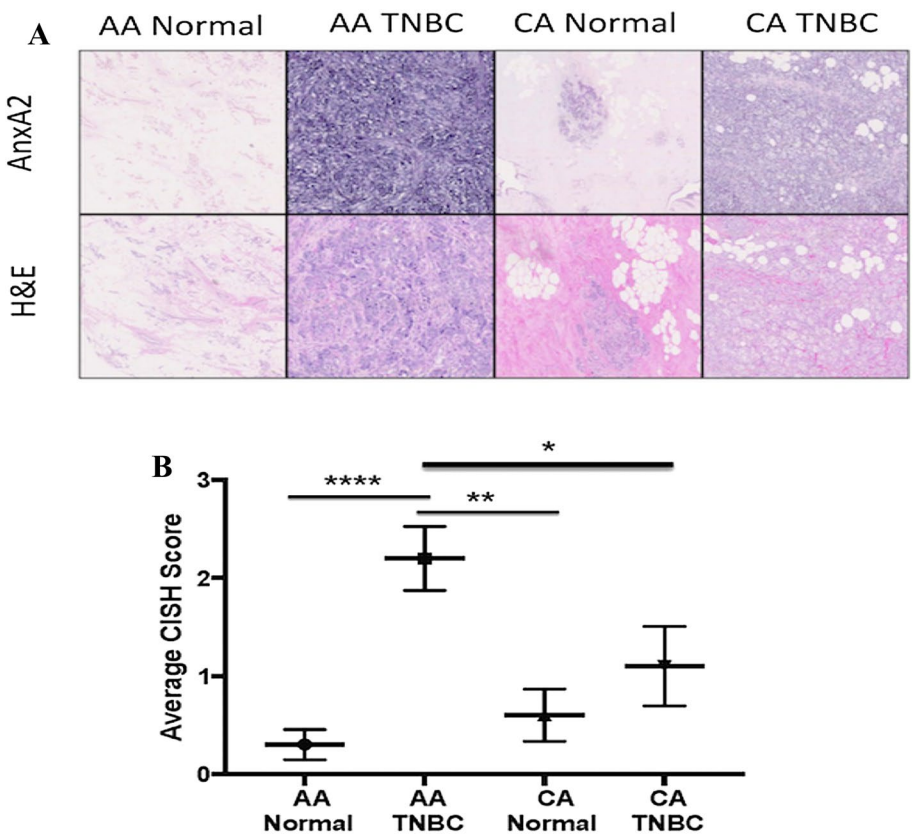

Fig. 4 AnxA2 expression in AA and CA TNBC tumor and benign tissues. **a** CISH and H&E Representative images of AA benign breast ($n=10$), AA TNBC ($n=10$), CA benign breast ($n=10$), CA TNBC (10) tissue specimen from an independent cohort obtained from UAB Comprehensive Tissue Network. **b** CISH Average Score Analysis of patient tissue sections in within each cohort. ×10 Magnification, scale bar = 500 µm. Axio Scan.Z1 slide scan

and women of African ancestry who are BRCA1 mutation carriers in comparison of women of European ancestry [29–34]. Unfortunately, these women are at higher risk for metastasis to the lung and brain subjecting these women to a low survival probability. 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 [35–42]. 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 [18]. Further, Jeon et al. identified secretory AnxA2 as a potential prognostic marker for tumor malignancy and metastatic recurrence of breast cancer [19]. 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 [43]. 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 [44]. 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. 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.

Author contributions LDG, PC and JKV conceived and designed the experiments. LDG, KM, RJH, RAM, and JKV performed the research and analyzed the data. LDG, PC, and JKV interpreted the data. LDG, PC, and JKV contributed to IRB approval and procurement of breast tissues. LDG wrote the paper. All authors read and approved the final manuscript.

Funding Research reported in this publication was supported by of the National Institutes of Health under the National Cancer Institute Award Number R01CA220273 and National Institute on Minority Health and Health Disparities Award Number P20MD006882. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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