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14. ABSTRACT: In the 2nd annual progress report, we report the following results: *Firstly,* we identified the corresponding tumor blocks pre- and post-NACT for the automatic IHC staining, clinical histology, and pathology analysis of a valuable cohort of 577 TNBC patients who have a natural partition of 50% African Americans (AA) and 50% Caucasian patients at stage 2-3-4 from Hampton Roads Virginia. In this cohort, AA TNBC patients have a high incidence rate of TNBC. KM survival curves showed that our local stage III-IV TNBC patients have a much reduced survival rate as compared to that of the national average of stage III-IV TNBC survival using the SEER database, indicative of a pressing need to identify chemo-resistant residual tumors, detect cancer disparities, forecast early tumor relapse, and predict patient survival in our local TNBC cohort as early as possible (**Figures 3 – 4**, and **Table 1**). *Secondly*, we reported that SIAH is a reliable prognostic biomarker in predicting patient survival in a 10-year study in NACT-treated high-risk breast cancer patients (**Figures 5** - **8**). *Thirdly,* we conducted reverse phase protein array (RPPA)-based kinomic analysis to delineate how the major cancer signaling pathways and network are rewired in response to anti-SIAH2 targeted therapy in TNBC cells lines (**Figures 10 - 15**). Due to the COVID-19 social distance restrictions at Sentara Hospitals, we have encountered repeated delays in carrying out the IHC staining of this TNBC project. With the state of Virginia re-opening on May 28, 2021, we are expected to catch up on this project rapidly.

15. SUBJECT TERMS
Early detection of chemo-resistant malignant TNBC tumor cells and tumor relapse in lymph-node positive TNBC mammary tumors post-neoadjuvant chemotherapies (NACT), TNBC disparity, and poor outcome in the Hampton Roads Virginia

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TABLE OF CONTENTS

1. INTRODUCTION

Breast cancer is the most commonly diagnosed cancer in women worldwide (Bray *et al*, 2018, 2020; Ferlay *et al*, 2019; Sung *et al*, 2021). More than 3.5 million women have been diagnosed with breast cancer in the United States alone (DeSantis *et al*, 2019a; Siegel *et al*, 2021). Breast cancer is still the 2nd leading cause of cancer-related deaths in American women (DeSantis *et al.*, 2019a; DeSantis *et al*, 2019b; Siegel *et al.*, 2021). While improvements in local and systemic therapies have resulted in significantly improved survival, an estimated 43,600 women are expected to succumb to their disease in the U.S. in 2021 alone (Siegel *et al.*, 2021). Currently, the average 5-year survival rate for female breast cancer stagnated at 90% (DeSantis *et al.*, 2019a; DeSantis *et al.*, 2019b; Graham *et al*, 2014; Siegel *et al.*, 2021). The remaining 10% (breast cancer death) represents a clear unmet need to treat locally advanced, high-stage, metastatic, and recurrent diseases (Gupta *et al*, 2020a; Gupta *et al*, 2020b).

TNBC is the most aggressive phenotypic subtype of breast cancer known for early relapse rate, chemo-resistance, and worst overall survival in breast cancer (Carey *et al*, 2010; Foulkes *et al*, 2010). TNBC represents 15% of all breast cancers, that is defined by the lack of expression of these three receptors, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Boyle, 2012; Cancer Genome Atlas, 2012; Ciriello *et al*, 2015; Dent *et al*, 2009; Gupta *et al.*, 2020b). Among all the major breast cancer molecular subtypes, TNBC has the highest relapse rate at 30%, which typically occurs within the first 3-years post initial diagnosis (Gabani *et al*, 2019; Kennedy *et al*, 2020; Steward *et al*, 2014). TNBC patients who are recurrence-free at 5-years post diagnosis/treatment have a very low tumor relapse rate of 3% at 10-years and 5% at 15-years (Reddy *et al*, 2018). TNBC has the worst outcomes of all breast cancer subtypes with a 5-year overall survival (OS) at 78.5%, i.e., 21.5% of TNBC patients will succumb from their diseases in 5-years (Carey *et al.*, 2010; Foulkes *et al.*, 2010; Waks & Winer, 2019). Thus, accurately predicting which TNBC partial responders will relapse and which ones will stay in remission post standard of care (SOC) therapies in the first 3-years remains an outstanding problem in clinical oncology.

TNBC is nearly twice as common in African American women than in Caucasian women, and it is more common in premenopausal women and *BRCA1/2* mutation carriers (DeSantis *et al*, 2016; Dietze *et al*, 2015; Huo *et al*, 2017; Iqbal *et al*, 2015; Newman *et al*, 2019; Newman & Kaljee, 2017; O'Keefe *et al*, 2015; Troester *et al*, 2018). Standard chemotherapy remains a mainstay of treatment for TNBC (Andreopoulou *et al*, 2015; Cancer Genome Atlas, 2012; Ciriello *et al.*, 2015). Patients diagnosed with TNBC tend to be younger, premenopausal, and African American (Anders & Carey, 2008; Howlader *et al*, 2014; Howlader *et al*, 2018; Lin *et al*, 2012). TNBC malignancy is a genetically diverse, highly heterogeneous, and rapidly evolving disease that challenges our ability to personalize medicine and tailor therapy for each unique TNBC patient in the clinic (Andreopoulou *et al.*, 2015; Bianchini *et al*, 2016; Cancer Genome Atlas, 2012; Ciriello *et al.*, 2015; Hanahan & Weinberg, 2011; Vogelstein *et al*, 2013).

List of Abbreviations:

ACT – Adriamycin, Cytoxan, and Taxotere, **DRFS** – distant recurrence-free survival, **EGFR** – epidermal growth factor receptor, **ER** – estrogen receptor, **HER2** – human epidermal growth factor receptor 2, **H&E** – hematoxylin and eosin staining, **IHC** – immunohistochemistry, **K-RAS** – Kirsten rat sarcoma viral oncogene homolog**, LN** – lymph node, **MRI** – m*agnetic resonance imaging,* **NACT** – neoadjuvant chemotherapy, **NSG** – NOD/SCID/IL2Rγ^{null}, **OS** – overall survival, **pCR** – pathological complete responder, **PFS** – progression-free survival, **pIR** – pathologic incomplete responder, **pNR** – pathologic non-responder, **pPR** – partial responder**, PR** – *progesterone receptor,* **RCB** – residual cancer burden**, SEER –** Surveillance, Epidemiology and End Results Program, **SIAH** – human homologues of *Drosophila* Seven-In-Absentia (**SINA**), **SOC** – standard of care, **TIL** – tumor infiltrating lymphocytes, **TME** – tumor microenvironment, **TNBC** – triple-negative breast cancer, **TNM** – tumor size, lymph node status, metastasis.

Neoadjuvant chemotherapy (NACT) is a standard regimen to treat high-risk TNBC to reduce tumor burden prior to surgical resection (Arteaga *et al*, 2012; DeMichele *et al*, 2015; King & Morrow, 2015; Nagayama *et al*, 2014; Prat *et al*, 2015; Tevaarwerk *et al*, 2013; Thompson & Moulder-Thompson, 2012; Zardavas *et al*, 2013). High-resolution imaging and clinicopathological parameters can help risk-stratify TNBC patients into two

groups: those with pathologic complete response (pCR) and/or pathologic incomplete response (pIR) (Baselga *et al*, 2012a; Baselga *et al*, 2012b; Bevers *et al*, 2009; Gass *et al*, 2018; Redden & Fuhrman, 2013; Tolaney *et al*, 2015). Radiologic assessments, however, are imperfect predictors of pathologic response at surgery. The outcomes of TNBC patients with residual tumors post-NACT may vary widely. Many TNBC patients with similar clinical and pathological presentations often respond very differently to standard chemotherapies (DeSantis *et al.*, 2016; Haddad & Goetz, 2015; Siegel *et al*, 2016). Predicting which incomplete responders post-NACT will relapse and which ones will stay in remission is an unmet need currently. As a result, developing new, precise, and high-resolution prognostic molecular biomarker(s) are needed to accurately differentiate high-risk from low-risk residual TNBC tumors. Such prognostic biomarkers would provide real-time quantitative and interactive tumor information that would be valuable in aiding and assisting oncologists in selecting evidence-based effective 2nd-line treatment in hopes of eradicating chemo-resistant residual TNBC tumors (Hutchinson, 2014; Prat *et al.*, 2015; Tevaarwerk *et al.*, 2013).

Finally, the survival rates for chemo-resistant, relapsed, and metastatic TNBC patients have not improved significantly over the past 30 years (Tevaarwerk *et al.*, 2013). Malignant TNBC has consistently challenged our ability to design effective targeted therapies to save more patients with progressive and relapsed and metastatic diseases (Alizadeh *et al*, 2015; Almendro *et al*, 2013; Paoletti *et al*, 2015; Parker & Perou, 2015; Polyak, 2011; Swain *et al*, 2015; Yap *et al*, 2012; Zardavas *et al*, 2015). Designing new and better strategies to control multidrug-resistant, relapsed, and incurable TNBC are urgently needed clinically (Konner, 2020).

2. KEYWORDS

- Tumor-driving EGFR/K-RAS/SIAH signaling pathway activation in high-risk and locally advanced TNBC
- Understanding SIAH biology and K-RAS/SIAH signaling pathway activation in TNBC maligancy
- Validating and verifying the clinical utility of a tumor-specific, therapy-responsive, and prognostic biomarker, **SIAH**, for future FDA approval and rapid clinical application in the future.
- Stratifying high-risk pIR patients, identifying chemo-resistant residual tumor clones, forecasting early tumor relapse, and predicting patient survival with high precision and increased accuracy in TNBC.
- Validating a new tumor vulnerability, **SIAH**, in relapsed, chemo-resistant and metastatic TNBC tumors
- Developing a novel and potent anti-SIAH-based anti-EGFR/K-RAS **targeted therapy** for preclinical testing.

1a. Addressing these pressing unmet needs in TNBC

- Distinguishing deadly from non-deadly TNBC. *By focusing on the tumor-driving K-RAS/SIAH pathway and assigning a SIAH$^{ON/OFF}$ binary expression in residual tumors,* we *will stratify high and low-risk partial responders by assessing SIAH$^{ON/OFF}$ expression in residual TNBC tumors post-NACT*.
- The EGFR/K-RAS/SIAH activation pathway is a major driving force in chemo-resistant TNBC. *We will utilize an in vivo strategy to demonstrate the therapeutic efficacy of anti-SIAH-based anti-K-RAS/EGFR strategies using PDX models derived from multidrug-resistant, late-stage, and relapsed TNBC tumors.*
- Reduce the cancer mortality associated with multidrug-resistant TNBC. *The stratification of high-risk and low-risk pIR TNBC patients will empower us to validate and verify SIAH as a key tumor vulnerability to develop anti-SIAH-based targeted therapy in TNBC. We have deployed cancer signaling pathway mapping in vivo using RPPA technology to identify major tumor driving pathways, key tumor vulnerabilities, and actionable targets in multidrug-resistant, metastatic and incurable TNBC.*

In this DOD-funded TNBC study, we aim to demonstrate the clinical utility of the **SIAH$^{ON/OFF}$ binary code** to stratify high-risk TNBC patients, identify chemo-resistant residual diseases, measure tumor response, quantify chemo-efficacy, forecast early tumor relapse, and predict patient survival TNBC. We will use two independent and separate TNBC cohorts (one training set – **Aim 1** and one validating set – **Aim 2**) to determine an optimized SIAH cut point to maximize AUC and ROC curve (sensitivity and specificity) in this study. Secondly, we will demonstrate the efficacy of a new anti-SIAH-based anti-EGFR/K-RAS-targeted therapy to control and eradicate multidrug-resistant, late-stage, and metastatic TNBC tumors resected from non-responders and/or RCB III tumors by conducting 2D and 3D assays using TNBC cell lines and PDX models (**Aim 3**).

1b. K-RAS/SIAH is a major tumor-driven signaling pathway in TNBC

Supported by strong evidence in developmental, evolutionary and cancer biology, we hypothesize that: (1) K-RAS/SIAH/EGFR pathway activation is a major tumor-driving force, (2) SIAH represents a potential therapeutic target in chemo-resistant TNBC, and (3) SIAH is a therapy-responsive, tumor-specific, and prognostic biomarker in TNBC (**Figure 1**). Our hypothesis was formulated based on our preliminary findings: *Firstly*, in a completed retrospective study of NACT-treated breast cancer cohort of mixed molecular subtypes, we found that SIAH$^{ON/OFF}$ binary code expression in residual tumors post-NACT correlated with tumor relapse/remission, chemo-resistance/sensitivity, and decreased/increased patient survival in a 5-year and a 10-year study. *Secondly*, we have gathered preliminary results demonstrating that anti-SIAH targeted therapy shuts down K-RAS/EGFR pathway activation and completely abolished exponential tumor growth of TNBC. This was found to be true in human TNBC cell lines MDA-MB-231 and MDA-MB-468, in 2D and 3D cell line models and xenograft mice. *Thirdly*, we completed a RPPA-kinomic study to identify cancer signaling rewiring mechanisms, new tumor vulnerabilities, and compensatory pathway activation/inhibition in response to SIAH inhibition in these TNBC cell lines where eradication is possible.

Assisted by multi-institutional experts on this TNBC team, we aim to develop and validate a EGFR/K-RAS/SIAH-centered precision oncology platform to identify the high-risk TNBC pIR patients with chemo-resistant and progressive diseases who are most likely to develop early tumor relapse, and who are most likely to benefit from additional rounds of evidence-based effective adjuvant therapies post-NACT.

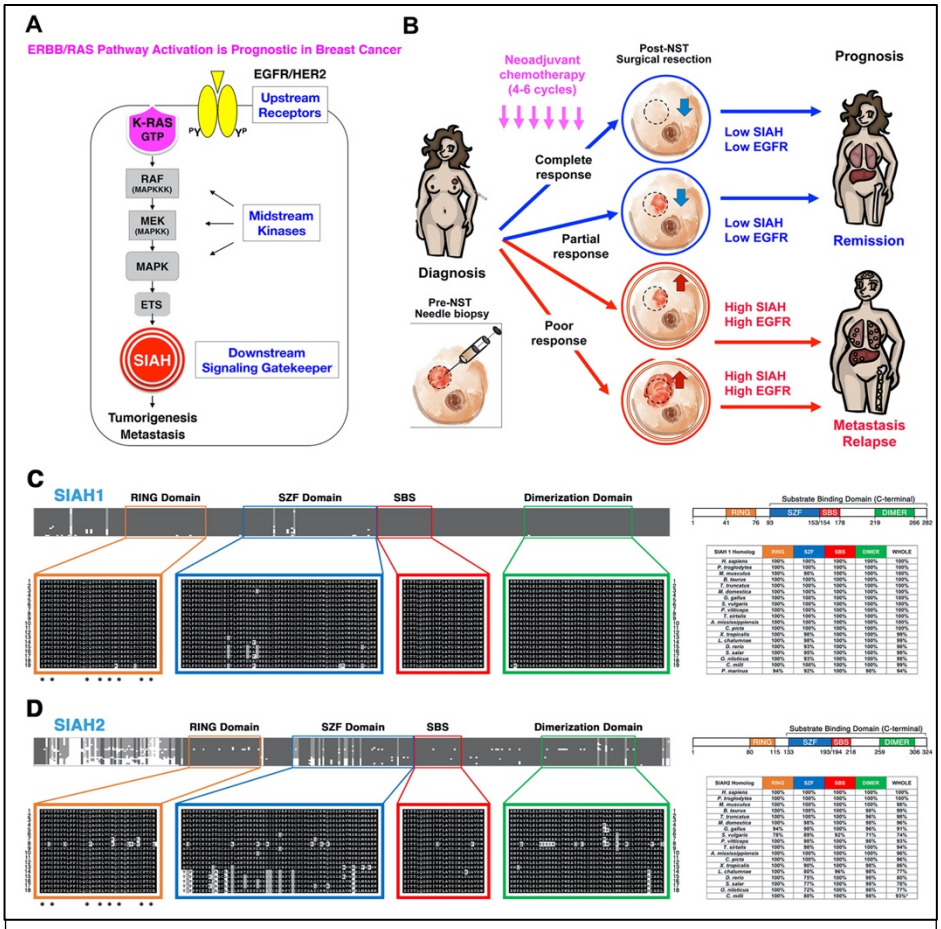


Figure 1: **The highly conserved tumor-driving EGFR/K-RAS/SIAH pathway activation in highly prevalent in high-risk and locally advanced TNBC**. (**A**) SIAH is the most downstream "gatekeeper" signaling module identified in the canonical K-RAS signal transduction pathway in human cancer cells. (**B**) Loss of SIAH expression post neoadjuvant chemotherapies (NACT) is correlated with K-RAS pathway inactivation and tumor regression, whereas persistent SIAH expression is correlated with K-RAS/EGFR pathway activation and tumor progression and relapse in TNBC. (**C**) Sequence alignment of the vertebrate SIAH1 subfamily reveals its extraordinarily degree of evolutionary conservation in its four conserved structural motifs. (**D**) Sequence alignment of the vertebrate SIAH2 subfamily reveals its extraordinarily degree of evolutionary conservation in its four conserved structural motifs. **Conclusion**: SIAH is one of the evolutionarily most conserved signaling modules identified in the EGFR/K-RAS signaling pathway. SIAH$^{ON/OFF}$ expression is a binary code that reflects K-RAS/EGFR pathway activation/inactivation. As such, SIAH is ideally positioned to become a new drug target and therapy-responsive prognostic biomarker in TNBC.

1c. SIAH's gatekeeper role is indispensable for this tumor-driven EGFR/K-RAS signaling pathway activation

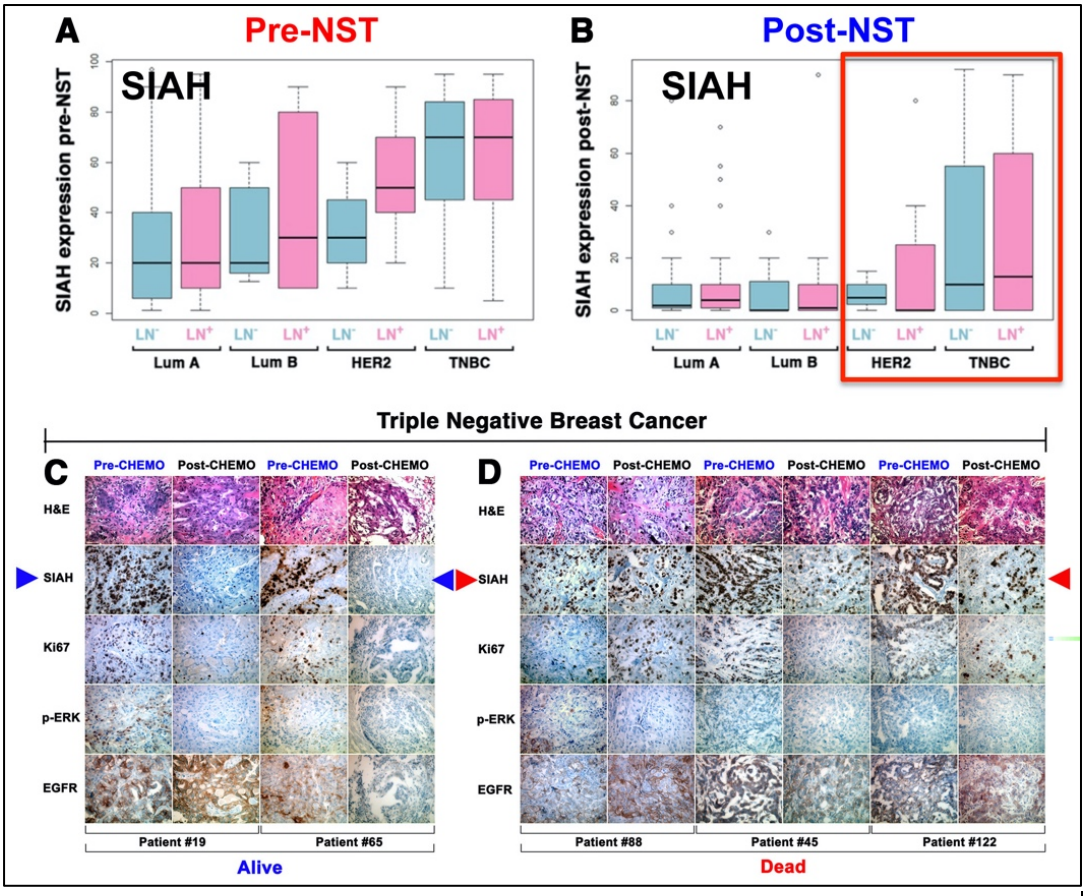
Normal K-RAS/SIAH/EGFR signaling pathway activation is indispensable for proper cellular communication, cell proliferation and tissue homeostasis in multicellular organisms. However, abnormal K-RAS/SIAH/EGFR pathway activation is highly prevalent in chemo-resistant, recurrent, and metastatic TNBC (Cox *et al*, 2014; Downward, 2003; Jiang *et al*, 2020; Pylayeva-Gupta *et al*, 2011; Tebbutt *et al*, 2013; Van Sciver *et al*, 2018; Van Sciver *et al*, 2016; Wright *et al*, 2015). Seven in absentia homologue (SIAH) RING-domain E3 ligase is the most downstream signaling gatekeeper and the most evolutionarily conserved signaling module in the EGFR/HER2/K-RAS signaling pathway (Ahmed *et al*, 2008; Gupta *et al.*, 2020a; Schmidt *et al*, 2007; Van Sciver *et al.*, 2018; Van Sciver *et al.*, 2016). Based on its extraordinary evolutionary conservation and significance as the most downstream signaling module required for proper K-RAS/EGFR signal transduction, $SIAH^{ON/OFF}$ expression is a reliable readout of K-RAS/EGFR pathway activation/inactivation in TNBC. Supported by strong evidence in developmental, evolutionary and cancer biology, we hypothesize that K-RAS-SIAH pathway activation is a major tumor driver, and SIAH represents a potential therapeutic target in chemo-resistant TNBC.

1d. K-RAS/SIAH/EGFR pathway is commonly activated in TNBC, and SIAH is a therapy-responsive and prognostic biomarker in TNBC

Genomic landscape studies have indicated that activation of the tumor-driving K-RAS–EGFR pathway is highly prevalent in high-grade, locally advanced, relapsed, and chemo-refractory TNBC (Clark & Der, 1995; Eckert *et al*, 2004; Loi *et al*, 2016; Malaney & Daly, 2001; McGlynn *et al*, 2009; Saini *et al*, 2013; Steelman *et al*, 2008; von Lintig *et al*, 2000). Furthermore, we and others have shown that K-RAS/SIAH pathway activation is associated with progression of DCIS to invasive ductal cancer, and reduced survival of luminal-type breast cancer (Behling *et al*, 2010; Wright *et al.*, 2015). Hence, studying activation/inactivation of the tumor-driving K-RAS/SIAH/EGFR pathway represents an opportunity to define therapy-responsive and prognostic K-RAS/SIAH-centered biomarkers in TNBC. This may provide a basis for us to stratify TNBC partial responders, identify chemo-refractory tumors, predict survival, and decide whether to add adjuvant therapies to control chemo-resistant TNBC.

$SIAH^{ON}$ expression indicates persistent EGFR/K-RAS pathway activation and cancer cell proliferation. $SIAH^{ON}$ predicts tumor progression, whereas $SIAH^{OFF}$ expression indicates EGFR/K-RAS pathway inactivation, diminished cell proliferation and tumor regression (van Reesema *et al*, 2016; Van Sciver *et al.*, 2016). As a binary code ($SIAH^{ON/OFF}$) to predict tumor progression/regression directly post-NACT, SIAH is a promising prognostic biomarker in TNBC (Ahmed *et al.*, 2008; Gupta *et al.*, 2020a; Schmidt *et al.*, 2007; van Reesema *et al.*, 2016; Van Sciver *et al.*, 2016) (**Figure 2**). We found that persistent expression of SIAH in residual tumors after NACT reflects activation of the "tumor-driving" K-RAS/SIAH/EGFR pathway in NACT-treated breast cancer (**Figure 2B**) (van Reesema *et al.*, 2016). Currently, there are no reliable prognostic molecular biomarkers that can be used to risk stratify pIR patients, identify chemo-resistant tumor clones, quantify tumor response, forecast early tumor relapse, and predict patient survival after surgical tumor resection post-NACT in TNBC. We propose that SIAH might serve as a new biomarker whose ON/OFF expression, particularly after NACT, predicts TNBC recurrence/remission. By comparing the percentage reduction (%) of SIAH expression in primary mammary tumors pre- and post-NACT, we aim to quantify the efficacy of chemotherapy, identify chemo-resistant residual tumors, forecast early tumor relapse, and predict patient survival in TNBC. Most importantly, the $SIAH^{ON/OFF}$ binary classification in each individual tumor cell will permit risk stratification and treatment separation of those low-risk pIR patients who are likely to stay in remission from high-risk pIR patients who are destined to relapse post-NACT and will benefit from additional adjuvant treatments.

Figure 2. SIAH$^{ON/OFF}$ binary expression in residual tumors post-NACT can be used to stratify pIR patients and predict decreased or increased patient survival in high-risk TNBC at 5 years.



(**A**–**B**) The box-and-whisker plots were used to graphically illustrate the population distribution of median SIAH expression levels in both node-positive (as marked by purple color bar graphs) and node-negative (as marked by teal color bar graphs) in breast cancer. (**A**) The median SIAH expression levels in the 4 molecular subtypes pre-NST were shown as follows: Luminal A (LN-negative LumA at 20% and LN-positive LumA at 20%), Luminal B (LN-negative LumB at 20% and LN-positive LumB at 30%), HER2-positive breast cancer (LN-negative HER2-positive breast cancer at 30% and LN-negative HER2-positive breast cancer 50%, and TNBC (LN-negative TNBC at 70% and LN-positive TNBC at 70%). (**B**) The median SIAH expression levels in the 4 molecular subtypes post-NST were shown as follows: Luminal A (LN-negative LumA at 2% and LN-positive LumA at 3%), Luminal B (LN-negative LumB at 0.5% and LN-positive LumB at 1%), HER2-positive breast cancer (LN-negative HER2-positive breast cancer at 5% and LN-negative HER2-positive breast cancer 0.5%, and TNBC (LN-negative TNBC at 8% and LN-positive TNBC at 15%). The pIR outliers with high SIAH expression in node-negative and node-positive residual tumors post-NACT are correlated with reduced survival. The error bars or whiskers in the histogram and bar charts represent the 95% CI, and in the box plots, they represent the upper (top) and lower quartiles (bottom) data distribution – with points beyond representing outliers. (**C**–**D**) Serial micro-sections were cut from the representative TNBC tumor paraffin blocks and the tissue slides were stained with H&E, SIAH, Ki67, phospho-ERK, and EGFR monoclonal antibody. (**C**) Representative IHC images of SIAH, EGFR, Ki67 staining in TNBC patients who were alive at 5-year post-NACT. We found that loss of SIAH expression post-NACT correlates with increased patient survival (**Alive**). (**D**) Representative IHC images of SIAH, EGFR, Ki67 staining in TNBC patients who were dead at 5-year post-NACT were shown. We found that persistent high SIAH expression post-NACT is associated with decreased patient survival (**Dead**). For both node-negative and node-positive TNBC, pIR patients who succumbed to their progressive diseases in less than 5 years have high SIAH expression in the residual tumors. SIAH^{ON} identified chemo-resistant TNBC tumor cells that are still growing post-NACT, predicting poor survival (**D**). In contrast, pIR patients who survived for longer than 5 years have no or low SIAH expression in the residual tumors post-NACT. SIAH^{OFF} marked chemo-sensitive TNBC tumor cells that have stopped growing in response to NACT, predicting increased survival (**C**).

3. ACCOMPLISHMENTS

3a. What were the major goals of the project (SOW)?

Specific Aim 1/2: *Validating the prognostic power of SIAH as a new biomarker that allows stratification of patients, quantification of tumor response, and prediction of tumor relapse and survival among TNBC partial responders following standard neoadjuvant chemotherapies (NACT)*

Major Task 1: Identifying and de-identifying 577 TNBC patients **– Completed**

Subtask 1: Identified 400 high-risk TNBC patients (**Aim 1**) who have or will receive neoadjuvant chemotherapies at 7x Sentara Breast Centers. **– Completed**

Subtask 2: Established a retrospective TNBC database, extracted and validated clinical information, including TNBC diagnosis, pathology and histology, MRI imaging, neoadjuvant chemotherapies, surgery, lymph node status, tumor size, pathological grades, molecular subtypes, and survival. **– Completed**

Subtask 3: De-identified 400 high-risk TNBC patients who received (**Aim 1**) neoadjuvant chemotherapies at Sentara cancer network and Sentara Breast Center **– Completed**

Milestone(s) Achieved: Establishing an interactive and de-identified TNBC database so that we can tally clinical performance, generate KM survival curves, and track tumor relapse status in support of Aim 1.

- Local IRB Approval **– Completed**
- Milestone Achieved: HRPO/ACURO Approval **– Completed**

Major Task 2: IHC staining of RAS pathway biomarkers SIAH and EGFR in 1400 paired matched TNBC tumor biospecimens pre- and post-NACT **– Ongoing**

Subtask 1: Pulling H&E histology slides and paraffin blocks from 700 TNBC patients to identify the representative tumor slides that accurately reflect each TNBC patient's molecular disease **– Ongoing**

Subtask 2: Pulling the representative TNBC tumor paraffin blocks from 7x Sentara Hospitals, and Iron Mountain (the senator tumor tissues and paraffin blocks storage units). **– Ongoing**

Subtask 3: The Sentara pathology unit will start cutting the 1,400 TNBC tumor paraffin blocks identified, and conduct the IHC staining using SIAH, EGFR, Ki67, and H&E staining. **– Ongoing**

Milestone(s) Achieved: The IHC staining will be scored, reviewed and tallied. The data will be digitalized and entered into a de-identified database. **– Ongoing**

Specific Aim 3: *Examining the efficacy of anti-SIAH strategy against multidrug-resistant and incurable TNBC tumors using patient-derived xenograft (PDX) models in vivo.*

Major Task 1: 40 Stage III and IV MBC tumors resected from non-responders will be processed and implanted into NSG mice **– COVID Delayed**

Subtask 1: Freshly resected and large TNBC tumors will be collected from non-responders post-NCT. **– COVID Delayed**

Subtask 2: Dr. Tang and her research team at EVMS will conduct biochemical, 2D/3D tumor assays *in vivo* and *in vitro* in parallel. **– COVID Delayed**

Milestone(s) Achieved: The anti-TNBC effect of anti-SIAH and/or anti-RAS/MEK/EGFR/PI3K/mTOR therapies will be determined *in vivo* and *in vitro.* **– COVID Delayed**

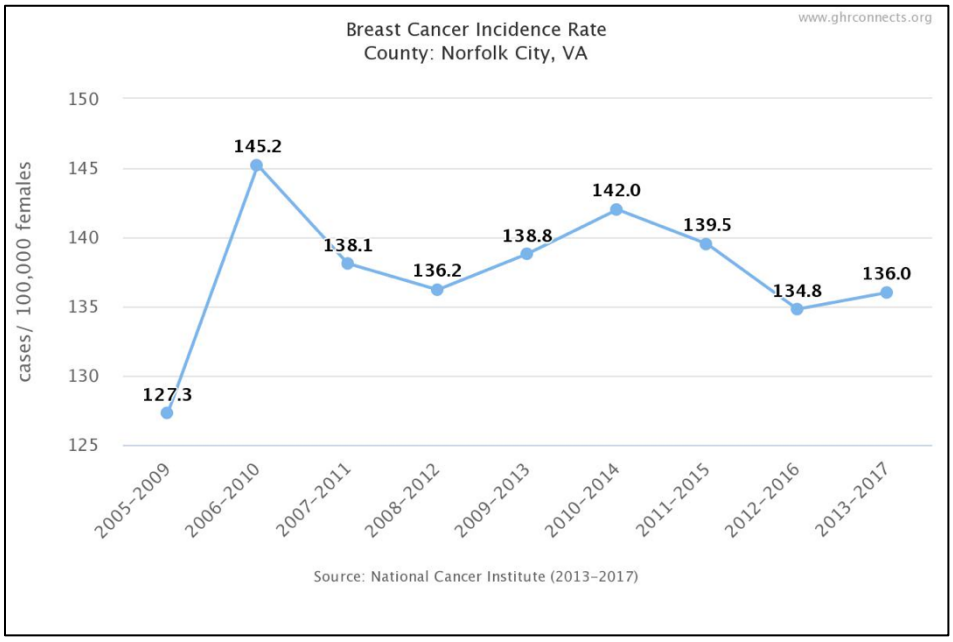
3b. What was accomplished under these goals?

3b1. Comparing patient survival of our local TNBC cohort with that of national SEER TNBC database

In the 2nd-year of this DOD-BCRP breakthrough study, we established, authenticated and validated this TNBC clinical database, extracted SOC treatment data (chemo- and radiation therapies), identified surgical paraffin tumor blocks for carrying out the IHC staining in this local cohort of 577 TNBC patients. We conducted KM survival analysis, and compared the 5-year survival of our local TNBC cohort with that of the national

SEER TNBC database. We found: (1) our local stage III and IV TNBC patients have much worse survival than their TNBC counterparts with stage III and IV diseases nationally according to the SEER TNBC database (**Figure 3**). (2) We detect cancer disparity in our local TNBC cohort, and African American TNBC over-representation in high-grade TNBC patients. African Americans composed only 30% of the local population, but they were 50% of the Sentara TNBC cohort with locally advanced and metastatic diseases (stage II, III and IV TNBC) (**Table 1**).

The reality is that the *breast cancer mortality rate in Portsmouth (1st), Suffolk and Southampton (3rd) and Norfolk (6th) remains the highest in the nation.* As such, As such, a synergistic research initiative centered on an innovative concept is urgently needed to control and eradicate multidrug-resistant, high-grade and intractable mammary tumors in the future. By leveraging our DOD Breakthrough Award (BC190807), we aim to stratify high-risk TNBC patients and reduce TNBC mortality in the Commonwealth of Virginia (**Figure 4**).



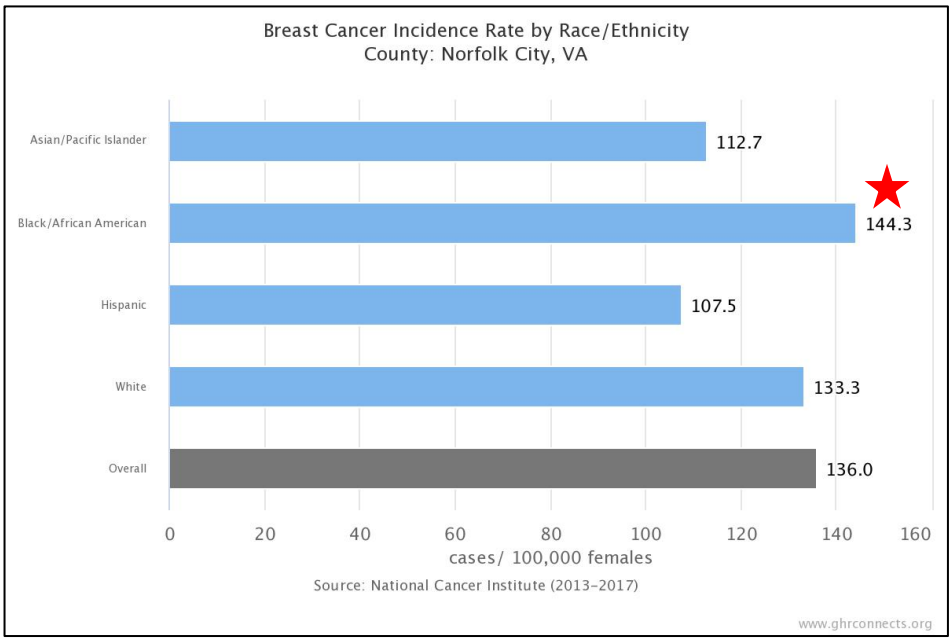


Figure 3. Incidence of breast cancer in Norfolk, VA (136 per 100,000 females) are higher than the VA average (127.4 per 100,000 females) and the U.S. average (125.9 per 100,000 females) (Measurement period 2013-2017). The incidence of breast cancer in the African American community in Norfolk, VA is particularly high at **144.3** per 100,000 females http://www.ghrconnects.org/indicators/index/view?indicatorId=180&localeId=2991

Firstly, cancer disparity was identified in this local TNBC cohort at Hampton Roads Virginia shown in **Table 1**.

Table 1. **Two de-identified clinical databases, TNBC and HER2+ breast cancer, were established.**
We stratified these two distinct patient cohorts based on AJCC pathological grades and the major race groups. Based on US census data, African Americans (AA) represents approximately 30% of the general population in the Hampton Roads Virginia. However, AA TNBC patients were found to be composed of roughly 45-51% of high-grade TNBC (stage II-III-IV), that was 15-21% higher than the local AA population at 30%.Even in the early-stage and low-grade TNBC (stage I TNBC), AA patients seemed to be over-represented at 38%, that was 8% higher above its 30% AA population. To confirm the AA over-representation in our local TNBC cohort, we extracted and tallied another highly aggressive breast cancer subtype, a local HER2-positive cohort collected from an identical time period in parallel, as an independent internal control and an endogenous comparison group for our local TNBC cohort. The data showed that the AA patient distribution was closely resembled that of its AA general population distribution in both the early-stage and low-grade (stage I) and high-grade (stage II-III-IV) of HER2-positive breast cancer.

TNBC	Stage I	Stage II	Stage III	Stage IV
African American	83 (38.1%)	114 (48.5%)	42 (51.2%)	9 (45%)
Caucasian	117 (53.7%)	113 (48.5%)	39 (47.6%)	9 (45%)
Hispanic/Asian	18 (8.3%)	6 (2.6%)	1 (1.2%)	2 (10%)
Total	218 (100%)	233 (100%)	82 (100%)	20 (100%)

HER2+	Stage I	Stage II	Stage III	Stage IV
African American	49 (24.6%)	66 (32.8%)	17 (30.3%)	9 (36%)
Caucasian	137 (68.8%)	128 (63.6%)	36 (64.2%)	15 (60%)
Hispanic/Asian	13 (6.5%)	7 (3.4%)	3 (5.3%)	1 (4%)
Total	199 (100%)	201 (100%)	56 (100%)	25 (100%)

This patient distribution study suggested a clear cancer disparity in the TNBC cohort when compared to the HER2-positive cohort, suggesting that Hampton Roads African American (AA) patients were significantly over-represented in high-grade TNBC (stage II-III-IV), when compared to their Caucasian counterparts locally.

Using this de-identified TNBC cohort, we conducted a KM survival study (**Figure 4**). We found our local stage III and IV TNBC patients experienced a much worse survival than their TNBC counterparts based on the national SEER TNBC database (the dotted versus solid orange and red lines as shown in **Figure 4**). Since AA TNBC patients are over-represented in late-stage TNBC, the TNBC survival data further corroborated and confirmed cancer disparity and increased TNBC mortality in our stage III and IV TNBC patients as compared to the national SEER TNBC dataset (**Figures 4 and 5**).

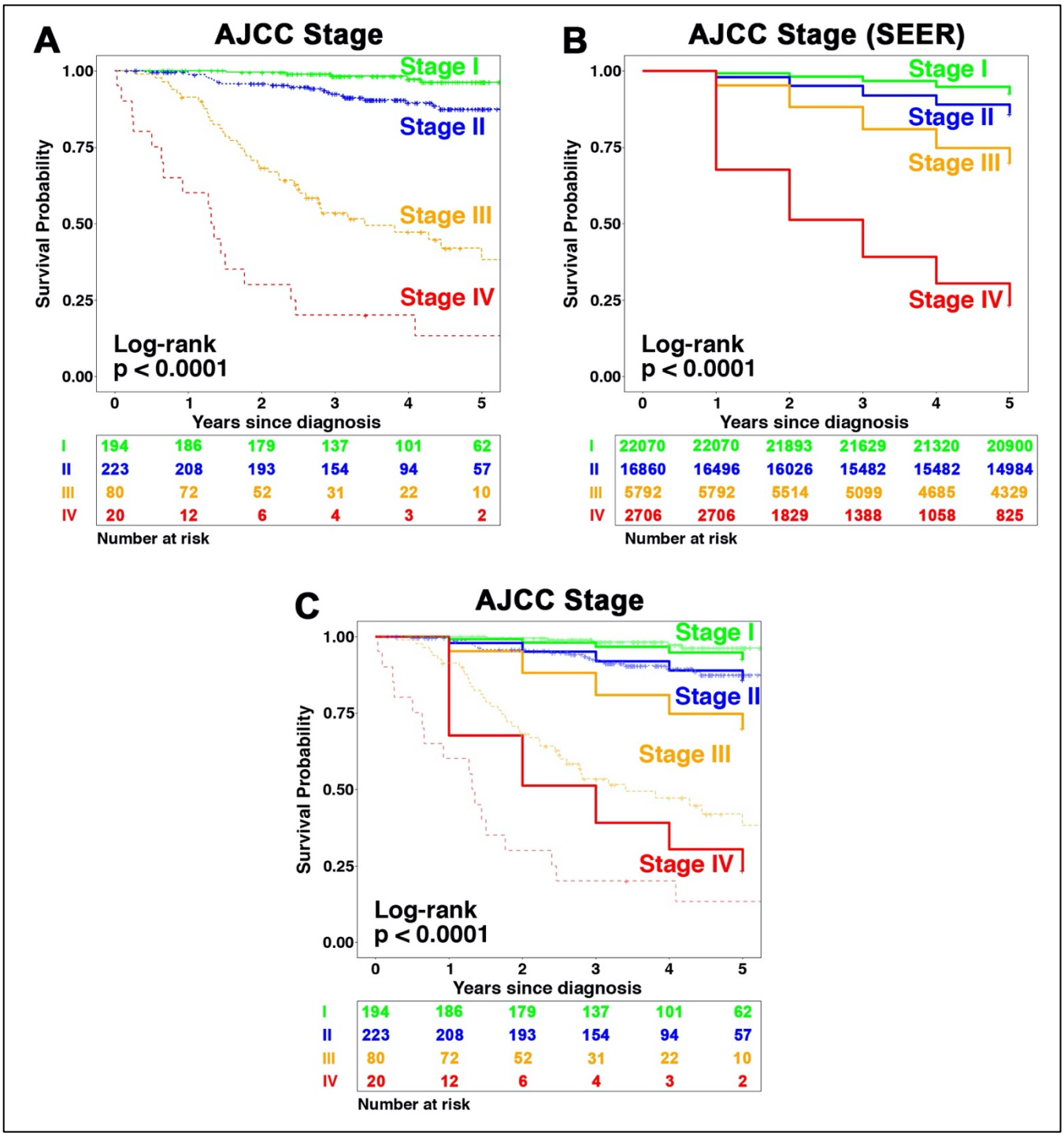


Figure 4. Our local TNBC cohort has a much reduced 5-year survival in stage III and IV TNBC as compared to the national SEER TNBC data.

Kaplan-Meier survival curves are shown in a 5-year study. (**A**) A local cohort of 577 TNBC patients was stratified based on AJCC staging: TNBC stage I (green dotted line), TNBC stage II (blue dotted line), TNBC stage III (orange dotted line), and TNBC stage IV (red dotted line). (**B**) The national NCI SEER TNBC database (a large TNBC cohort) was stratified based on AJCC staging: TNBC stage I (green solid line), TNBC stage II (blue solid line), TNBC stage III (orange solid line) ,and TNBC stage IV (red solid line). (**C**) The superimposed direct comparison of the 5-year survivals in our local TNBC cohort with the national NCI SEER TNBC database. The results showed that our early-stage (stages 1 and 2) TNBC patients have a similar 5-year survival that is comparable to national SEER average, whereas our late-stage (stages 3 and 4) TNBC patients have far worst survival statistics as compared to the national SEER data based on AJCC staging. **Conclusion:** Our local TNBC cohort is an ideally positioned and highly valuable cohort to study cancer disparity and treatment disparity in TNBC.

We analyzed the survival of our local TNBC cohort based on TNM staging.

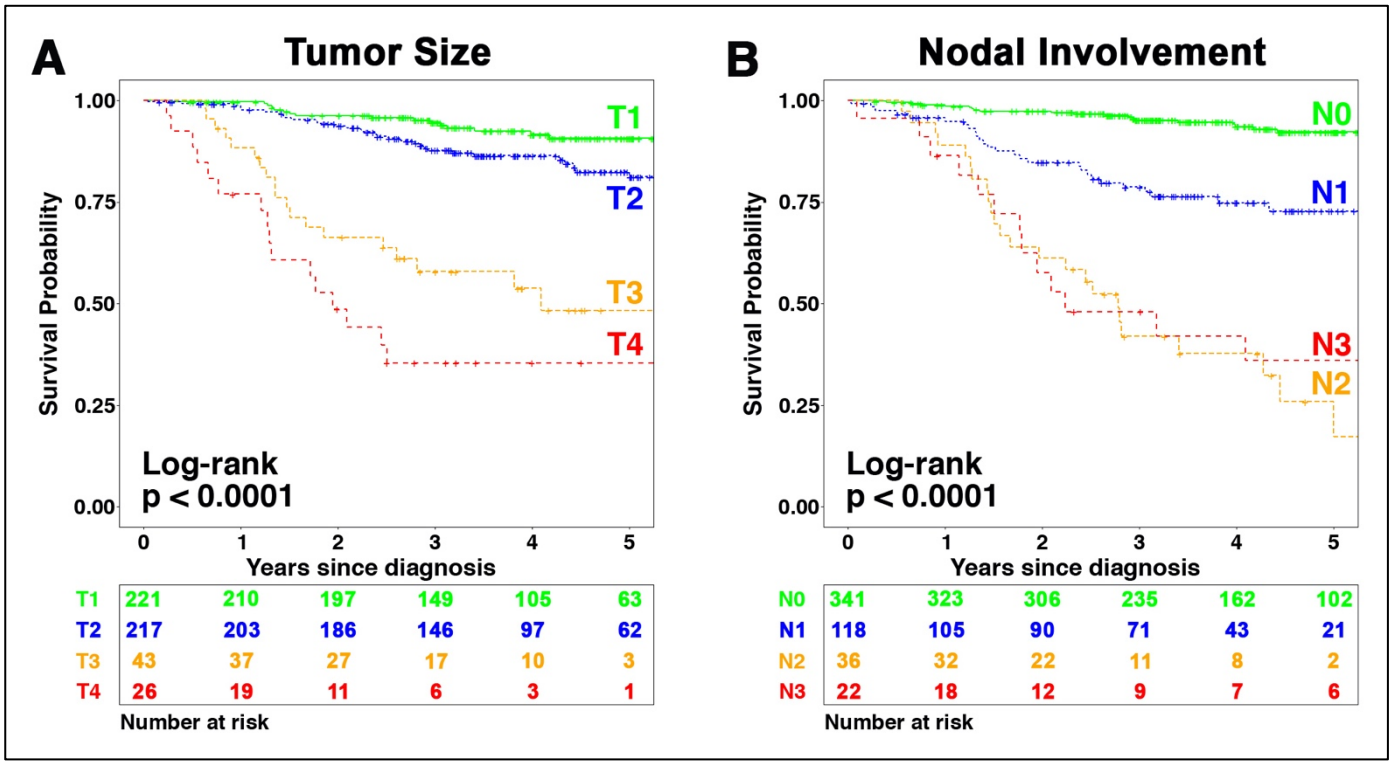


Figure 5. The 5-year survival in our local TNBC cohort was calculated based on tumor size and LN involvement. Kaplan-Meier survival curves are shown in a 5-year study. (**A**) Our local TNBC cohort was stratified based on tumor size: T1 (green dotted line), T2 (blue dotted line), T3 (orange dotted line), and T4 (red dotted line). (B) Our local TNBC cohort was then stratified based on LN involvement: N0 (green dotted line), N1 (blue dotted line), N2 (orange dotted line), and N3 (red dotted line). **Conclusion:** Our local TNBC cohort is well-separated based on the TNM staging to stratify high-risk patients, identify chemo-resistant tumor clones, detect treatment disparity and cancer disparity, forecast early tumor relapse and predict patient survival in TNBC.

3b2. We analyzed the prognostic power of SIAH in stratifying patients and predicting 10-year survival in a NACT-treated high-risk and locally advanced breast cancer cohort

We previously demonstrated the promising prognostic power of $SIAH^{ON/OFF}$ expression in a NACT-treated high-risk and locally advanced breast cancer cohort through a 5-year survival study (van Reesema *et al.*, 2016). Here, we followed up by conducting a 10-year survival study to determine the long-term prognostic value of $SIAH^{ON/OFF}$ binary code in this NACT-treated breast cancer cohort of 188 breast cancer patients of mixed molecular subtypes. The results showed that $SIAH^{ON/OFF}$ binary code is a highly prognostic biomarker in stratifying these high-risk patients, forecasting early tumor relapse, and predicting long-term survival in breast cancer. pCR post-NACT predicts the best survival in this high-risk cohort of breast cancer. Among the pIR patients, high $SIAH^{ON}$ expression in the residual tumors post-NACT predicts worst survival, whereas low $SIAH^{OFF}$ expression in residual tumors post-NACT predicts increased patient survival (**Figures 6, 7 and 8**). Moreover, among the pIR patients, low $SIAH^{OFF}$ expression in untreated and NACT-treated residual tumors predicts the best long-term survival, whereas high $SIAH^{ON}$ expression in untreated and NACT-treated residual tumors predicts the worst long-term survival. In contrast, a high $SIAH^{ON}$ expression in untreated and a low $SIAH^{OFF}$ expression in residual tumor indicates effective therapy against a highly aggressive primary tumor that resulted in intermediate survival (**2nd manuscript is in preparation**). These clinical studies on the high-risk cohorts of NACT-treated breast cancer patients provided additional molecular evidence and unambiguous results in support of this DOD-funded TNBC study as proposed.

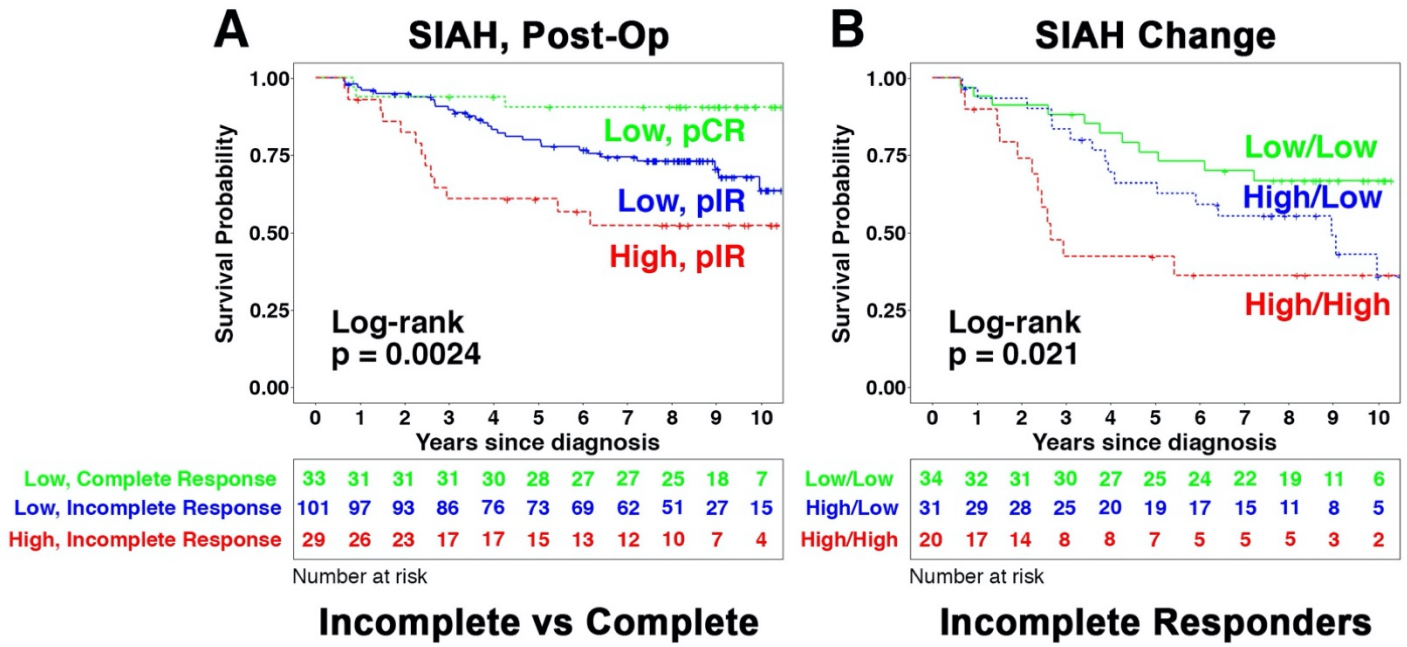


Figure 6. SIAH$^{High/Low}$ expression can be used to stratify incomplete responders into high-risk and low-risk pIR patients post-neoadjuvant systemic therapies (NST).

Kaplan-Meier survival curves are shown in a 10-year study. We found that SIAH$^{High/Low}$ expression pre-NST and post-NST, as well as NST-induced changes of SIAH expression are highly prognostic in a high-risk breast cancer cohort of mixed molecular subtypes with 10-year survival. (**A**) pIR patients whose residual tumors have high SIAH expression post-NST have the worst survival (**Red lines**), pCR patients whose residual tumors with less than 1% SIAH expression post-NST have the best survival (**Green lines**), and pIR patients whose residual tumors with low SIAH expression post-NST have intermediate survival (**Blue lines**). (**B**) The pIR patients whose untreated primary tumors and therapy-treated residual tumors have high SIAH expression pre- and post-NST have the worst survival (**Red lines**). The pIR patients whose untreated primary tumors and therapy-treated residual tumors have low SIAH expression both pre- and post-NACT have the best survival (**Green lines**). The pIR patients whose untreated primary tumors have high SIAH expression pre-NACT and therapy- treated residual tumors have low SIAH expression post-NST have intermediate survival (**Blue lines**). **Conclusion**: The tumor-specific SIAH$^{ON/OFF}$ binary classification is therapy-responsive and prognostic in stratifying pIR patients, quantifying tumor response, identifying chemo-resistant tumor clones, and predicting patient survival in incomplete responders (pIR) with residual diseases post-NST in neoadjuvant settings.

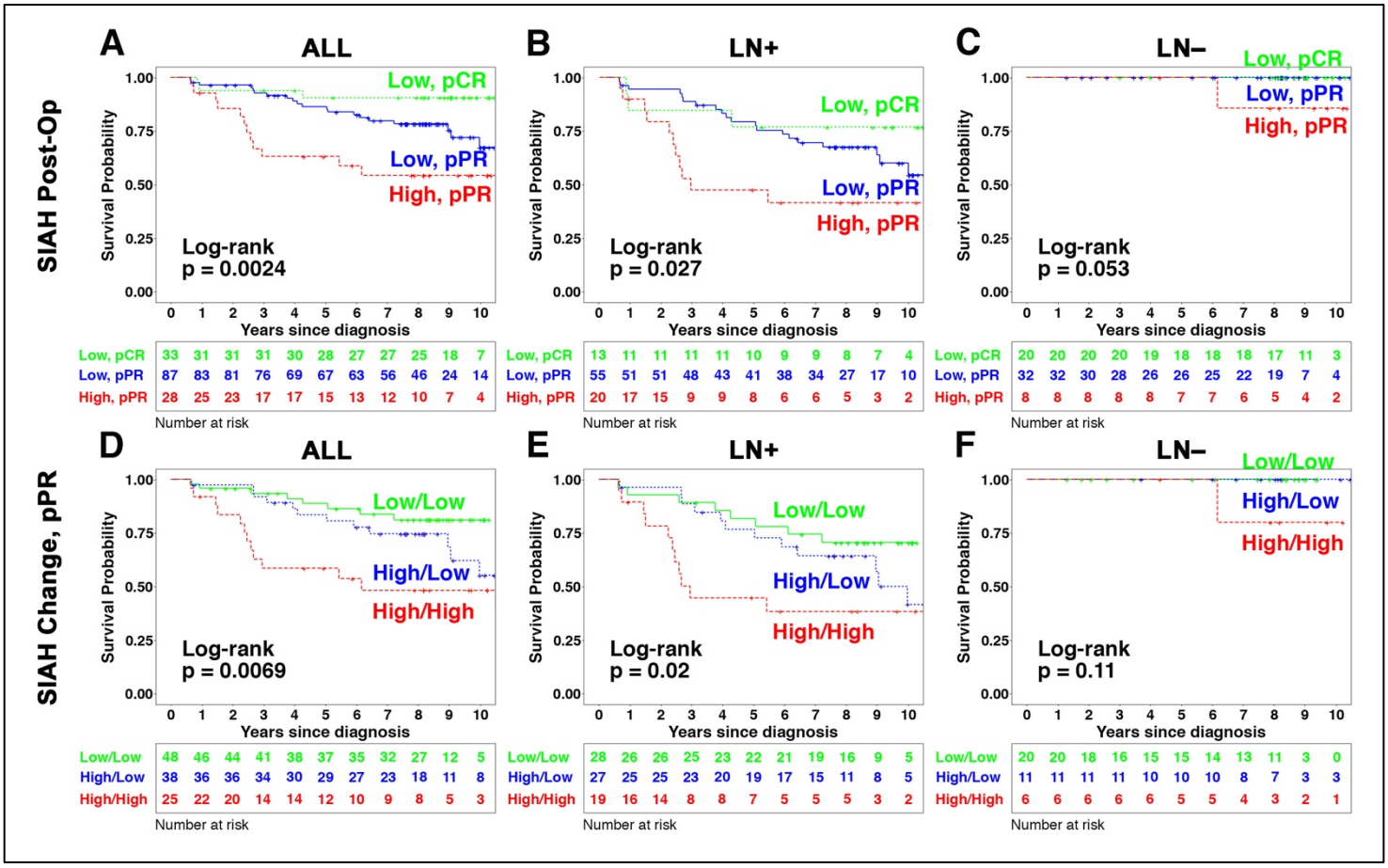


Figure 7. SIAH$^{High/Low}$ expression can be used to stratify NST-treated breast cancer patients (ALL), especially breast cancer patients with LN metastases, in neoadjuvant settings.

Kaplan-Meier survival curves are shown in a 10-year study. We separated a cohort of neoadjuvant systemic therapy (NST)-treated patient cohort into ALL patients (182 patients) (**A** and **D**), breast cancer patients with LN metastases (LN$^+$ cohort) (**B** and **E**), and breast cancer patients without LN metastases (LN$^-$ cohort) (**C** and **F**). (**A**-**C**) We found that SIAH$^{High/Low}$ expression in residual tumors post-NST is highly prognostic in a neoadjuvant-treated breast cancer cohort of mixed molecular subtypes in a 10-year survival study. pIR patients whose residual tumors have high SIAH expression post-NST has the worst survival (**Red lines**), pCR patients whose residual tumors have low SIAH expression post-NST has the best survival (**Green lines**), and pIR patients whose residual tumors have low SIAH expression post-NST has the intermediate survival (**Blue lines**) in this cohort (ALL patients and LN$^+$ cohort) with a *p* value < less than 0.05, that is statistically significant (**A** and **B**). (**D**-**F**) We also found that NST-induced changes of SIAH expression are highly prognostic in this cohort of breast cancer with mixed molecular subtypes in a 10-year survival study. Patients whose untreated primary tumors and treated residual tumors have high SIAH expression pre- and post-NST have the worst survival (**Red lines**), patients whose untreated primary tumors and treated residual tumors have low SIAH expression both pre- and post-NST have the best survival (**Green lines**), and patients whose untreated primary tumors have high SIAH expression pre-NST and treated residual tumors have low SIAH expression post-NST have intermediate survival (**Blue lines**) in this cohort (ALL patients and LN$^+$ cohort) with *P* value < less than 0.05, that is statistically significant (**D** and **E**). SIAH prognostic value in the LN$^-$ cohort is limited with *P* value > more than 0.05, that is statistically insignificant (**C** and **F**). **Conclusion**: Among breast cancer patients with LN metastases, decreased SIAH expression post-NST and therapy-induced reduction in SIAH expression, are strongly correlated with improved patient survival. Thus, SIAH$^{ON/OFF}$ binary classification is therapy-responsive and prognostic in stratifying therapy-treated breast cancer patients, quantifying tumor response, identifying chemo-resistant tumor clones, and predicting patient survival in neoadjuvant settings.

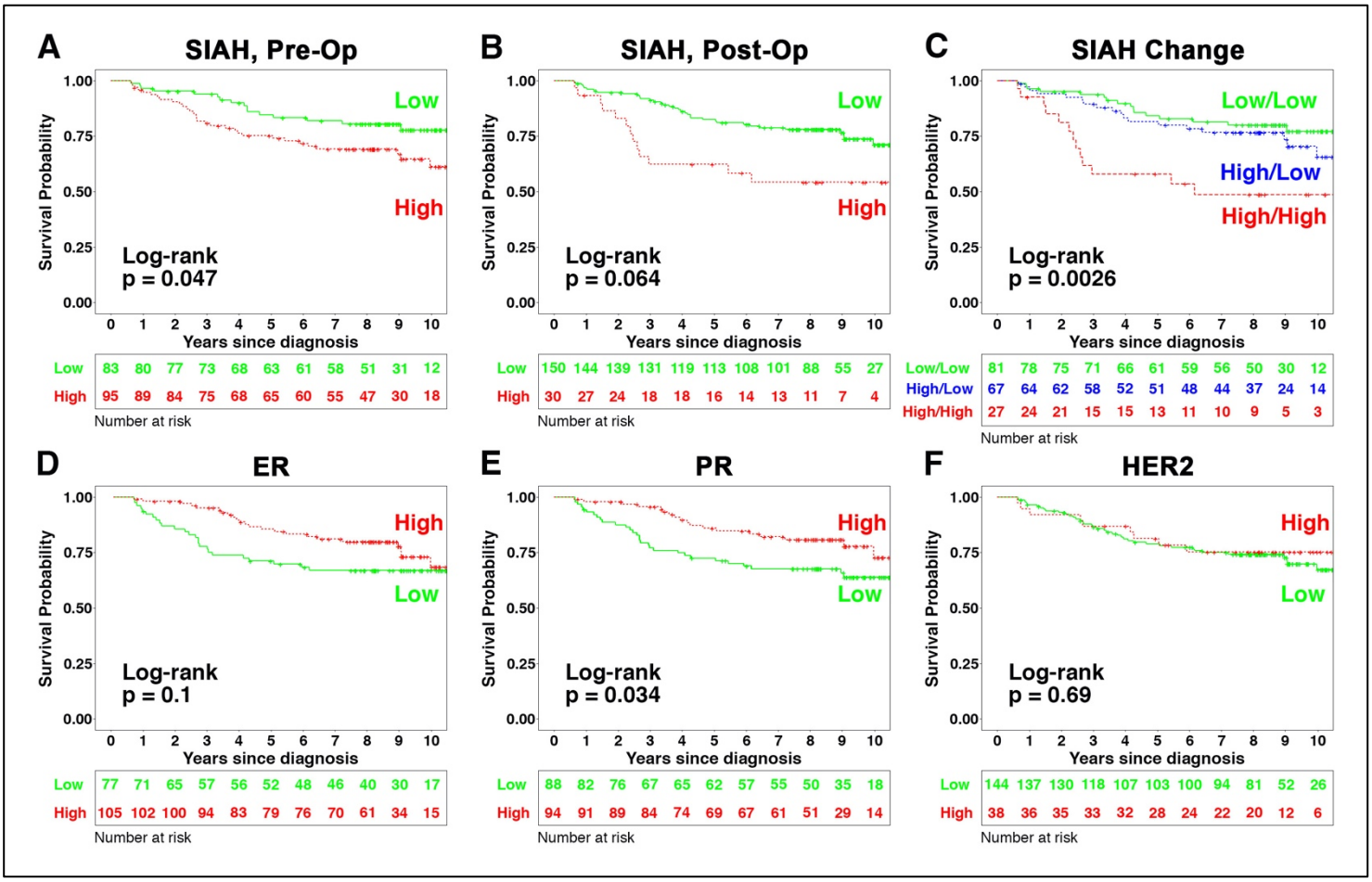


Figure 8. Therapy-induced change of SIAH$^{High/Low}$ expression is highly prognostic to stratify patients and correlate with patient survival in a 10-year survival study in a cohort of NACT-treated breast cancer.

Kaplan-Meier survival curves are shown in a 10-year study. (**A**) Tumor expression of high SIAH in untreated tumors (pre-NACT) is associated with reduced patient survival with *P* value of 0.047. (**B**) Tumor expression of high SIAH in treated tumor (post-NACT) is not associated with increased patient survival. (**C**) Therapy-induced change of SIAH$^{High/Low}$ expression is associated with patient survival with P value of 0.0026. Patients whose tumors have high SIAH expression both pre and post-NACT has the worst survival (**Red lines**), patients whose tumors have low SIAH expression both pre and post-NACT has the best survival (**Green lines**), and patients whose tumors have high SIAH expression pre-NACT and low SIAH expression post-NACT has the intermediate survival (**Blue lines**) in this cohort. (**D**) Tumor expression of ER is not associated with increased patient survival. (**E**) Tumor expression of PR is associated with patient survival with P value of 0.034. (**F**) Tumor expression of HER2 is not associated with patient survival. **Conclusion**: The tumor-specific SIAH$^{ON/OFF}$ binary code is therapy-responsive and prognostic in predict patient survival in NACT-treated breast cancer.

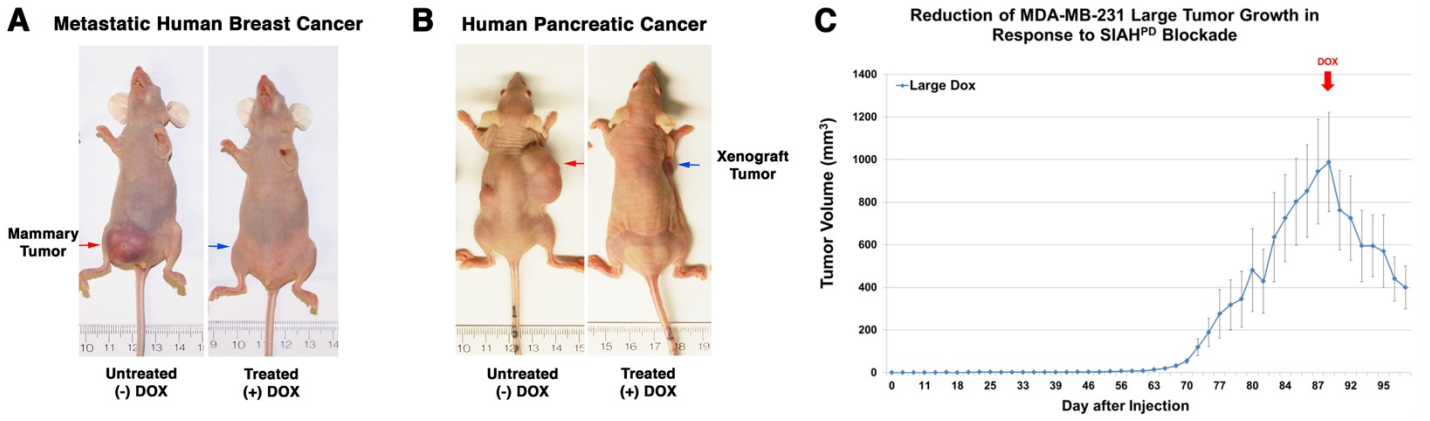


Figure 9. Anti-SIAH therapy is highly effective in inducing dramatic tumor regression at the exponential growth phases in two oncogenic K-RAS-driving TNBC and PDAC xenograft models.
DOX-induced SIAH2^{PD} targeted therapy to treat highly aggressive and malignant TNBC and PDAC at exponential tumor growth phase. **(A)** One million human TNBC cells (MDA-MB-231) and (**B**) one million PDAC (MiaPaCa) cells that carry DOX-inducible-SIAH^{PD} transgene were injected into the right and left mammary fat pads (for TNBC) and left and right flank (for PDCA) of 60 female athymic nude mice per cell line at 4-weeks of age. Growth of un-induced (–DOX) control tumors followed a stereotypical exponential growth curve. Doxycycline (DOX) was used to induce a SIAH inhibitor (SIAH^{PD} expression) when the implanted xenograft tumors reach the tumor size of small (100 mm^3), medium (200 mm^3) and/or large (1000-2000 mm^3) tumors. Inhibition of SIAH was highly effective against MDA-MB-231 tumors when they reached a large size of 1,000 mm^3. Control MDA-MB-231 xenograft mammary tumors and pancreatic MiaPaCa xenograft tumors are marked with red arrows. Tumors exposed to anti-SIAH therapy are marked with blue arrows, revealing a clear difference in tumor size and dramatic response. (**C**) Graphically represented data from experiments (A) and (B) demonstrating the effects of anti-SIAH therapy on TNBC and PDAC tumors at the exponential growth phase. **Conclusion**: The SIAH2^{PD} inhibitor is highly effective in shrinking well-established "undruggable" oncogenic K-RAS-driven malignant large TNBC and PDAC tumors at their exponential growth phases in athymic nude mice. By regressing super-large TNBC tumors so effectively in xenograft models *in vivo*, anti-SIAH2 targeted therapy has shown a good potential to become a new and potent anti-TNBC therapy for future clinical application in the clinic.

3b4. Cancer signaling pathway and kinomic analysis of anti-SIAH targeted therapy against EGFR/K-RAS-driven TNBC by using Reverse Phase Protein Array (RPPA) in collaboration with Dr. Chip Petricoin at GMU.

To understand the molecular mechanism(s) of why anti-SIAH2^{PD} targeted therapy is so effective in eradicating metastatic TNBC cell lines, MDA-MB-231 and MDA-MB-468, we collaborated with leading proteomic and kinomic expert, Dr. Chip Petricoin, and his team at GMU. About 160 proteins/phosphoproteins were quantititatively measured by the RPPA platform to identify new tumor vulnerabilities and actional targets, compensatory signaling network activation in response to anti-SIAH targeted therapies in TNBC cell lines. Together, we conducted a detailed, well-controlled, and global cancer signaling pathway analysis to identify the major changes in tumor-driving signaling pathways in response to anti-SIAH-based anti-K-RAS/anti-EGFR targeted therapy in TNBC (**Figures 10**, **11**, **12**, and **13**). In this study, we aim to identify and validate new TNBC vulnerabilities and actionable drug targets by using state-of-art Reverse Phase Protein Array (RPPA) technology and conducting phospho-protein-based kinomic cancer pathway mapping. This methods will allow us to identify major signaling pathway alterations in response to SIAH inhibition in malignant TNBC cell lines

Two commonly known and well-characterized TNBC cell lines were used in this RPPA study.

1. **MDA-MB-231** is an invasive and metastatic TNBC cell line that harbors the dual mutations in oncogenic mutated K-RAS^{G13D} and oncogenic mutated B-RAF^{G464V} (Ikediobi *et al*, 2006; Shoemaker, 2006).
2. **MDA-MB-468** is another malignant TNBC cell line that harbors an abnormally high copy number of EGFR amplification (> 350 copies), the highest EGFR expression levels, and EGFR signaling pathway and network hyperactivation (Ali & Wendt, 2017; Filmus *et al*, 1985; Kalyana-Sundaram *et al*, 2012).

3. Two oncogenic K-RAS-driven malignant pancreatic and lung cancer cell lines (MiaPaCa and A549) and one malignant cervical cancer cell line (HeLa) were used as the comparative controls in support of this RPPA study.

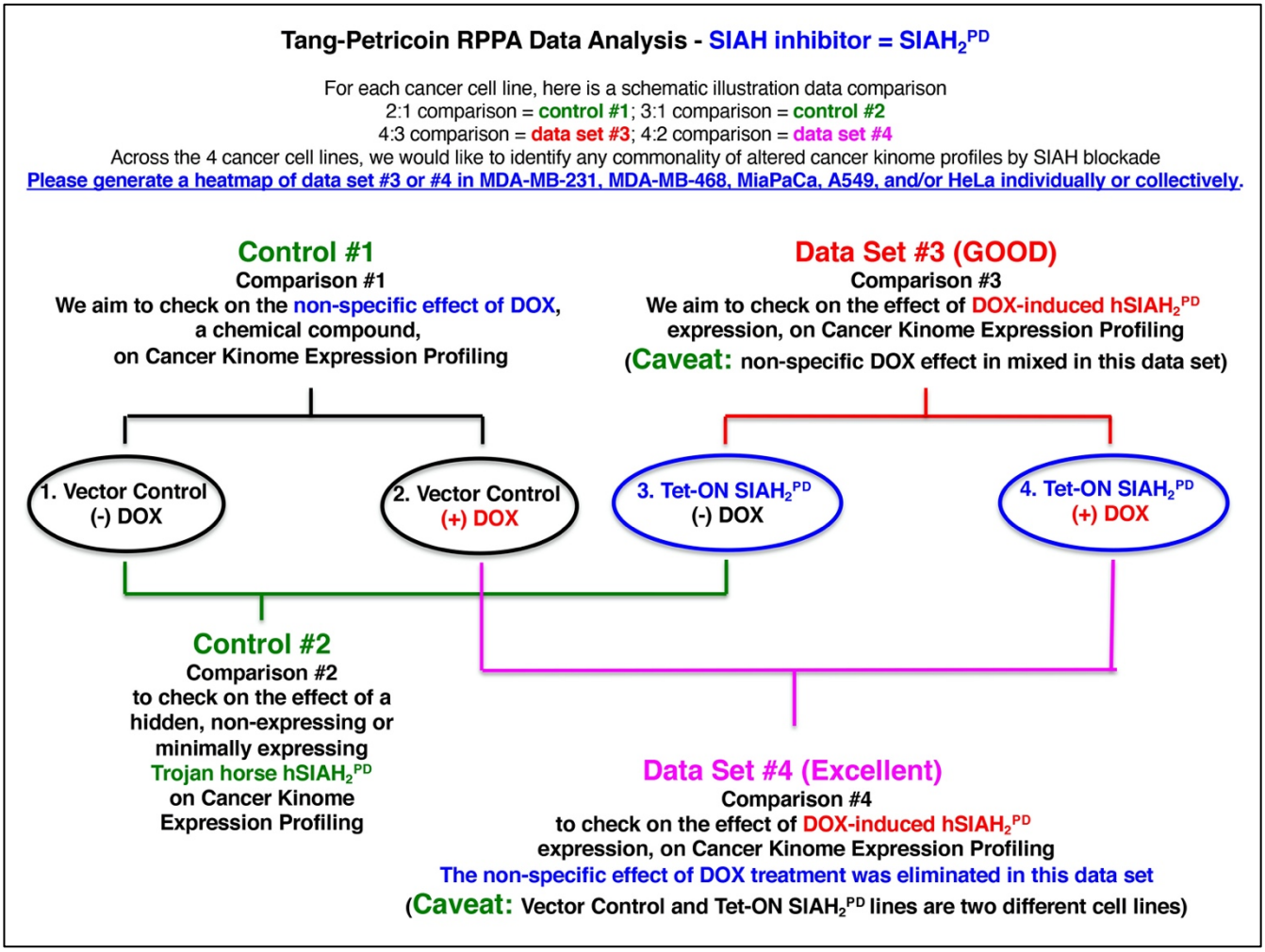
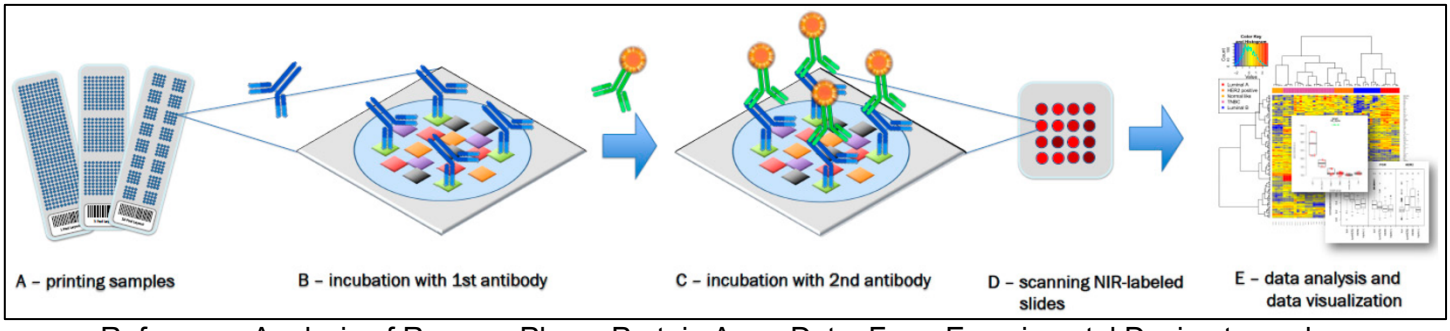


Figure 10. A schematic illustration of experimental set-up for RPPA cancer kinomic pathway studies in SIAH2-proficient and SIAH2-deficient TNBC cell lines in a pairwise fashion. We have set up these experiments in triplicates meticulously that controlled for both (+/-) DOX and SIAH2$^{ON/OFF}$ as shown.

What is RPPA?

- Reverse Phase Protein Array (RPPA) is an immunoassay that profiles small amounts of tumor lysates, enabling analysis of thousands of samples using dot-blots.
- Highly sensitive and works great for biomarker discovery.
- Tumor lysates are profiled by printing samples onto glass slides, adapted to an immunoassay platform
- Once samples are arrayed, proteins are detected with primary antibodies.
- Visualization of primary antibodies viewed by secondary antibodies



Reference: Analysis of Reverse Phase Protein Array Data: From Experimental Design towards Targeted Biomarker Discovery. *Microarrays* 2015, *4*(4), 520-539

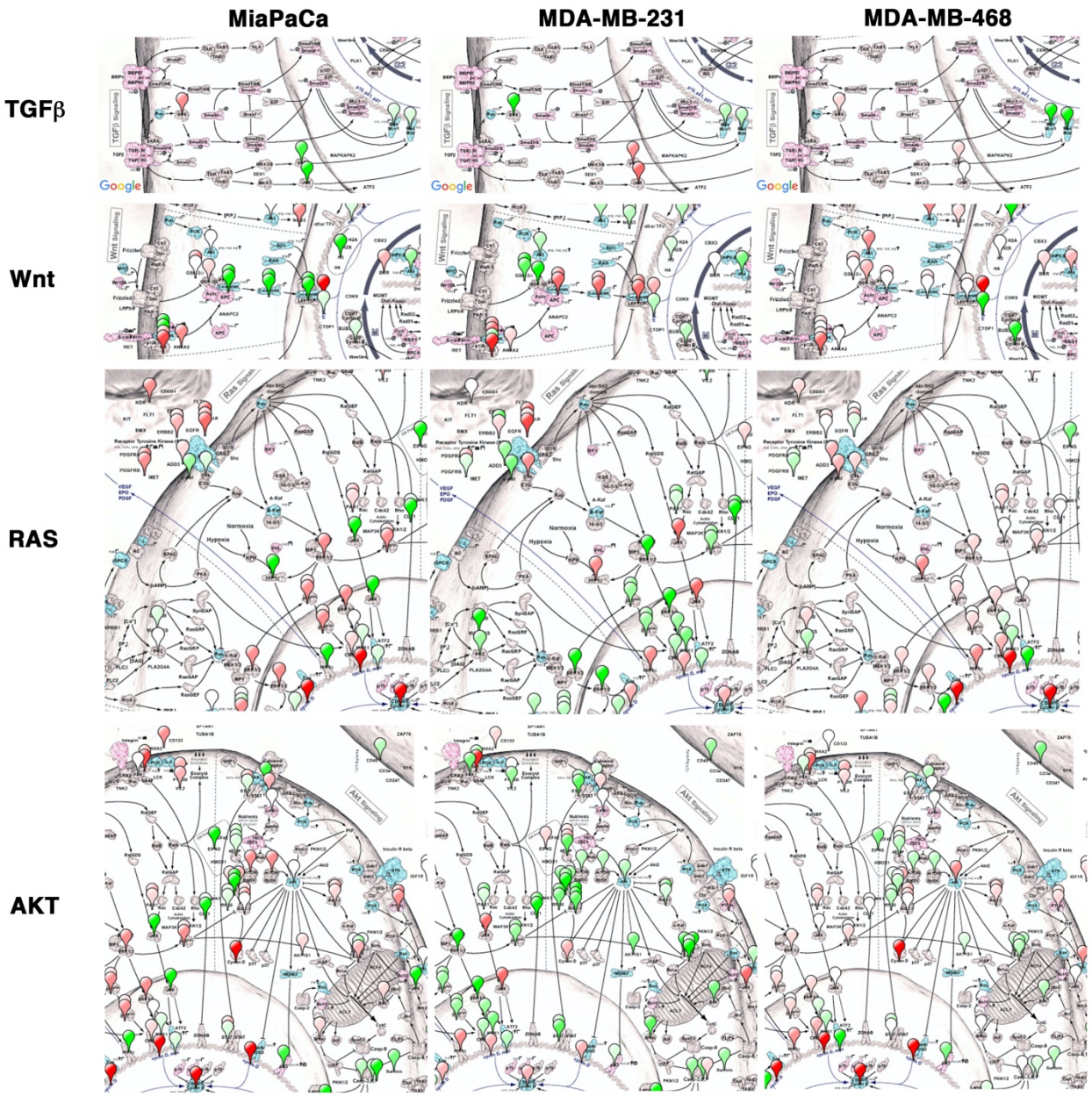


Figure 11. A cancer landscape (CScape) protein pathway activation maps of TNBC cancer signaling pathways were depicted (TGFβ, Wnt, RAS and AKT signaling pathways – Part-1).

RPPA pathway activation data were superimposed on the schematics of the corresponding signal pathway with the balloon maps. A cancer landscape (CScape) protein pathway activation map is a novel data and easy pathway visualization tool. CScape take RPPA kinomic data directly and map the expression/activation changes onto a signaling network image so that pathway activation/inactivation portraits can be easily generated and network connections clearly revealed, visualized and comprehended. The statistically significant differences in the key signaling modules in the biochemical pathways and signal transduction cascades were depicted in colored balloons, with **red** for the highest levels of phosphorylation and expression, **green** for the lowest levels of phosphorylation and expression, and **black** for no change in protein phosphorylation and expression. Higher fold differences in the SIAH2^{PD}-induced increases are shown in increasing shades of red, whereas higher fold differences in the SIAH2^{PD}-induced decrease in each unique cell line were shown in increasing shades of green. Each balloon pin is placed over the protein measured by RPPA. **Conclusion**: Major signaling alterations were detected in the TGFβ, Wnt, RAS and AKT signaling pathways in response to SIAH2^{PD} targeted therapy using the RPPA kinomic datasets extracted from the vector control (+/- DOX) and SIAH2^{PD} (+/- DOX) in in two malignant TNBC cell lines (MDA-MB-231, MDA-MB-468) in a serial pairwise comparison. A PDAC cancer cell line, MiaPaCa, was used as another control for CScape comparison since it has the oncogenic K-RAS activation.

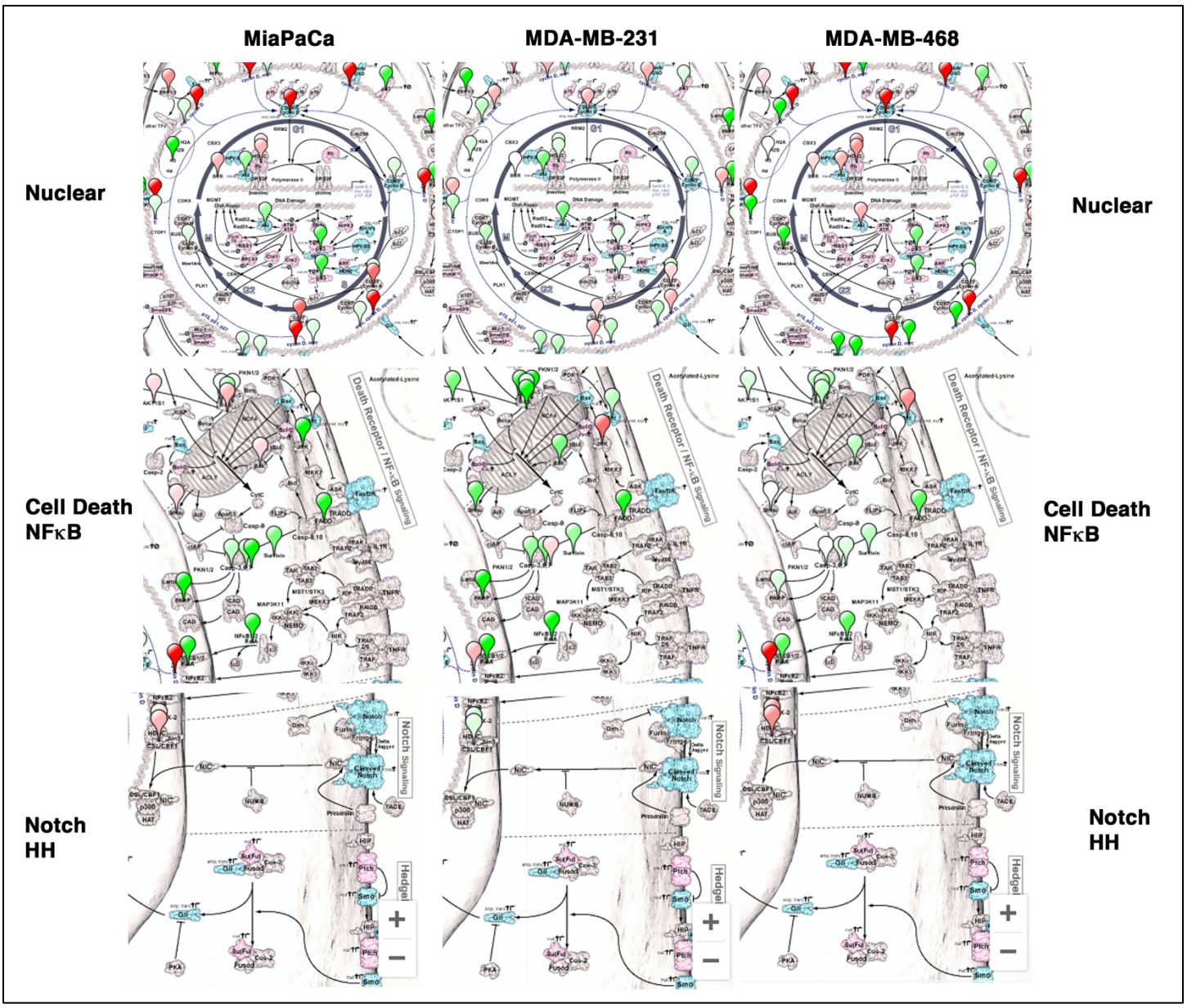


Figure 12. A cancer landscape (CScape) protein pathway activation maps of TNBC cancer signaling pathways were depicted (Nuclear, Cell Death, NFκB, Notch, and HH – Part-2).

RPPA pathway activation data were superimposed on the schematics of the corresponding signal pathway with the balloon maps. A cancer landscape (CScape) protein pathway activation map is a novel data and easy pathway visualization tool. CScape take RPPA kinomic data directly and map the expression/activation changes onto a signaling network image so that pathway activation/inactivation portraits can be easily generated and network connections clearly revealed, visualized and comprehended. The statistically significant differences in the key signaling modules in the biochemical pathways and signal transduction cascades were depicted in colored balloons, with **red** for the highest levels of phosphorylation and expression, **green** for the lowest levels of phosphorylation and expression, and **black** for no change in protein phosphorylation and expression. Higher fold differences in the SIAH2^{PD}-induced increases are shown in increasing shades of red, whereas higher fold differences in the SIAH2^{PD}-induced decrease in each unique cell line were shown in increasing shades of green. Each balloon pin is placed over the protein measured by RPPA. **Conclusion**: Major signaling alterations were detected in the Nuclear, Cell Death, NFκB, Notch, and HH signaling pathways in response to SIAH2^{PD} targeted therapy using the RPPA kinomic datasets extracted from the vector control (+/- DOX) and SIAH2^{PD} (+/- DOX) in two malignant TNBC cell lines (MDA-MB-231, MDA-MB-468) in a serial pairwise comparison. A PDAC cancer cell line, A PDAC cancer cell line, MiaPaCa, was used as another control for CScape comparison since it has the oncogenic K-RAS activation.


MDA-MB-231-SIAH2PD (+/- DOX)
4EBP1 T37/T46
ALK Y1586
ATR S428
BCL−2 S70
cABL T735
Calreticulin total
EGFR Y1148
Ephrin A3 Y799/A4 Y799/A5 Y8
ERBB2 Y877
ERG total
FYN T12
GAB1 Y627
GFAP total
GSK3aB Y279/Y216
NQO1 total
p70S6K T412
MiaPaCa-SIAH2PD (+/- DOX)
ALDH total
AMPK alpha T172
BAD S136
Caspase 9, cleaved D33
Collagen Type 1 total
COX−2 total
Cyclin D1 total
eIF2 alpha S51
phrin A3 Y799/A4 Y799/A5
PD−L1 total (Atezolizuma
A549-SIAH2PD (+/- DOX)
4EBP1 T37/T46
Adducin S662
AKT total
CD44 total
MyD88 total
p70S6K S371
S6RP S235/S236
MDA-MB-468-SIAH2PD (+/- DOX)
4EBP1 T37/T46
cABL T735
CD44 total
c−MYC total
CREB S133
EGFR Y1148
eNOS S1177
Ki67 total
MLH1 total
p70S6K T389
Ubiquitin total
VEGFR2 Y996
Hela-SIAH2PD (+/- DOX)
4EBP1 S65
4EBP1 T37/T46
BCL−2 S70
Cofilin S3
Fibronectin total
FYN T12
GAB1 Y627
LCK Y505
PARP total
PD−L1 total (SP142)
PRK1 T774/PRK2 T816
RAS total
Survivin total
Synuclein aB (Syn205) total

Figure 13. Boxed plots of the changes in individual protein levels in response to SIAH2 blockade. Statistically significant endpoints were plotted in boxed plots. There was a limited overlap in how the nine major cancer signaling pathways were dynamically altered and systemically rewired In response to $SIAH2^{PD}$ blockade based on the RPPA data extracted from these five highly aggressive and malignant cancer cell lines. Despite of the limited commonality at each individual signaling component level in these different cancer cell lines originated from distinct tumor type, there were a few concerted changes at the cancer signaling pathway levels in response to anti-SIAH2 targeted therapy: (1) K-RAS/AKT pathways are largely downregulated at multiple downstream signaling modules in MDA-MB-231 cells, a TNBC cell line; (2) Cell death and NFκB pathways are largely down-regulated in MDA-MB-231 and MDA-MB-468, two TNBC cell lines; (3) protein synthesis and DNA repair pathways are largely suppressed. In addition, there are a few compensatory signaling pathway activations to counter anti-SIAH-mediated signaling blockade in these distinct cancer cell lines with diverse tumor genetics, an assortment of different oncogenes/tumor suppressors, and dynamic interactions of the cancer network rewiring in response to $SIAH2^{PD}$ inhibition.

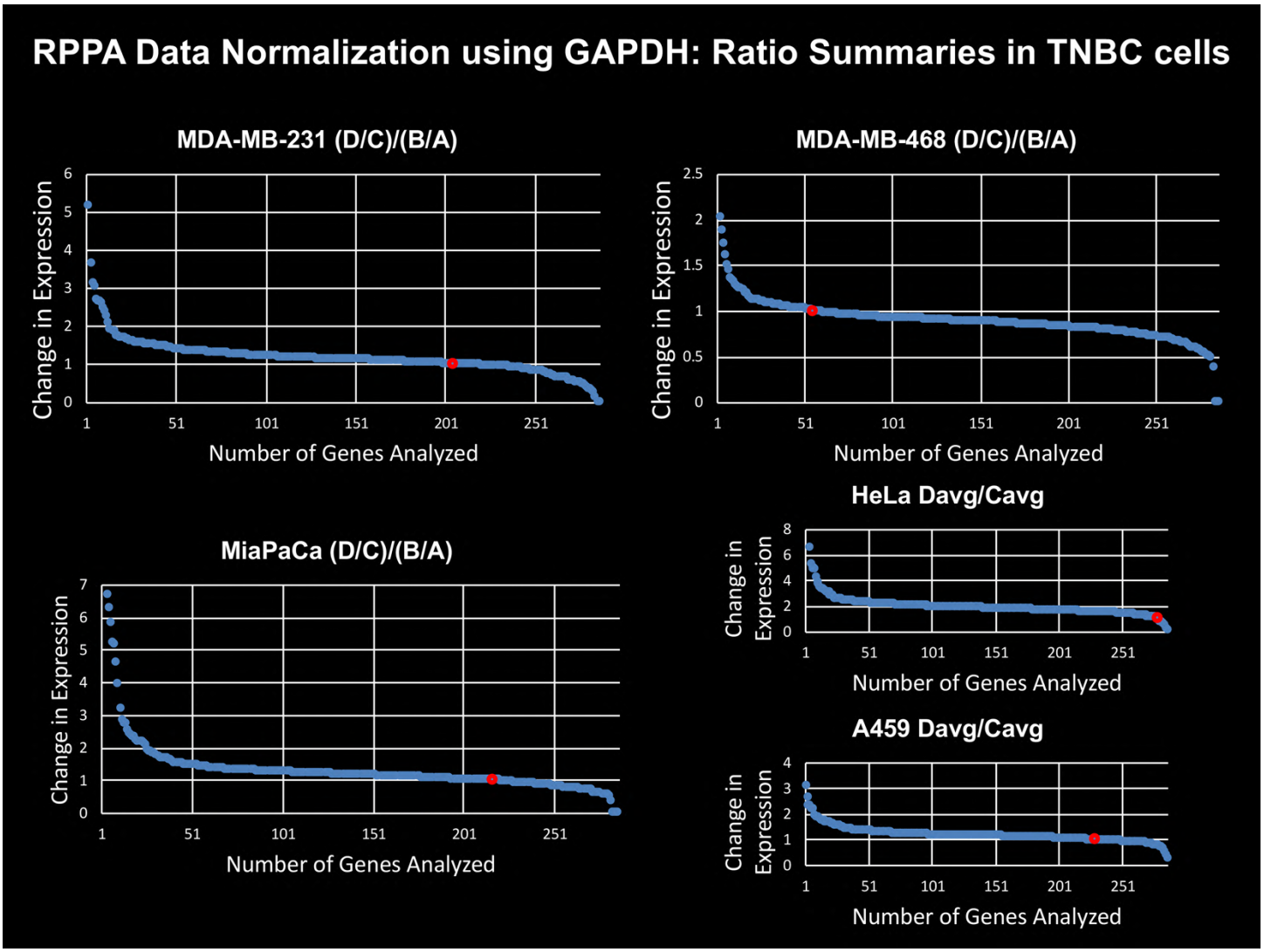
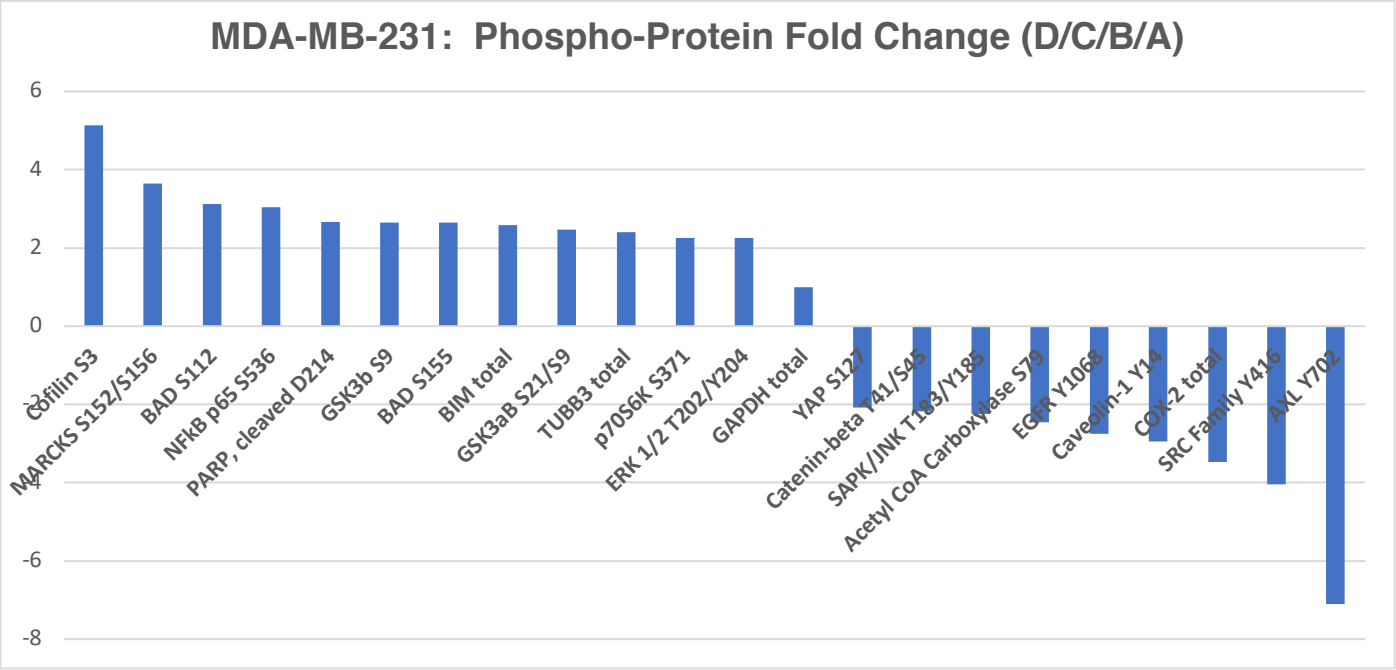


Figure 14. RPPA data normalization in EGFR/K-RAS-driven TNBC cells (MDA-MB-231 and MDA-MB-468). *Firstly*, Data clustering and group distribution were analyzed using principal component analysis (PCA) to examine the RPPA data clustering and distribution in each experimental condition: (**A group**) Tet-ON vector control (- DOX), (**B group**) Tet-ON vector control (+ DOX), (**C group**) Tet-ON $SIAH2^{PD}$ cell line (- DOX), (**D group**) Tet-ON $SIAH2^{PD}$ cell line (+ DOX). The PCA analysis showed that the RPPA data distribution in group D and group C are segregated widely in a non-overlapped fashion, whereas the RPPA data distribution in group B and group A are segregated more closely, suggesting more similarity between group B and group A. *Secondly*, principal component analysis (PCA) identified a housekeeper gene, **GAPDH**, whose expression was unaltered and unchanged under these four experimental conditions. GAPDH was found cluster around the center of the RPPA data distribution range, suggesting that GAPDH expression was very stable independent of these 4 different experimental conditions. Lastly, t **GAPDH** was used to normalize the RPPA datasets. The fold of change of the RPPA data ratio post GAPDH normalization in each cancer cell line are shown above.

- <u>Four experimental conditions:</u>
  - **Group A**: Tet-ON controlled cells without DOX induction
  - **Group B**: Tet-ON controlled cells with DOX induction
  - **Group C**: Tet-ON-SIAH2^{PD} cancer cells without DOX-induction (no SIAH2^{PD} inhibition)
  - **Group D**: Tet-ON-SIAH2^{PD} cancer cells with DOX-induction (Yes, SIAH2^{PD} inhibition)

- The ratios of D/C, B/A, and D/C/B/A were calculated after GAPDH normalization
- The up- or down-regulated phospho-proteins in the D/C/B/A datasets was shown by waterfall plots (Figure 14)



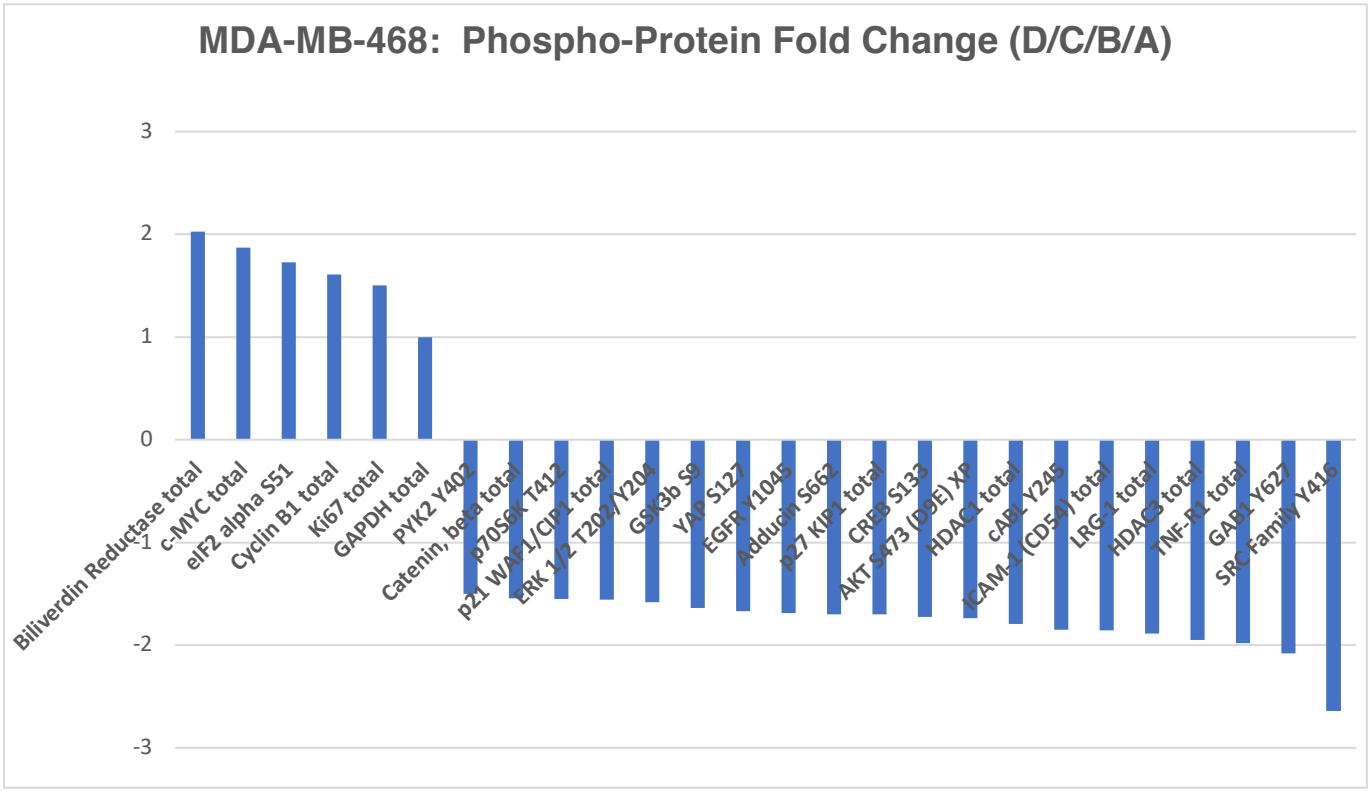


Figure 15: Phospho-proteins that are up- and down-regulated in response of SIAH2 inhibition in EGFR/K-RAS/B-RAF-driven TNBC cell lines: MDA-MB-231 and MDA-MB-468.
The four experimental conditions were used: **A group** is Tet-ON vector control (- DOX), **B group** is Tet-ON vector control (+ DOX), **C group** is Tet-ON SIAH2^{PD} cell line (- DOX), and **D group** is Tet-ON SIAH2^{PD} cell line (+ DOX). SIAH2^{PD} expression was induced in Tet-ON SIAH2^{PD} cell lines by DOX. The DOX effect was minimized from the experimental data sets D/C when deduced the DOX effect from that of the control data sets (B/A).

These three phospho-protein candidates will be subjected to further validation, interrogation and future studies.

1. **P70S6KS371**
    - Upregulated (~2.4)-fold in MDA-MB-231
    - Upregulated (~2.5)-fold in MiaPaCa

2. **PARP, cleaved D214**
    - Upregulated (~2.5)-fold in MDA-MB-231
    - Upregulated (~7)-fold in MiaPaCa

3. **Coflilin S3**
    - Upregulated (~15)-fold in MiaPaCa
    - Upregulated (~5)-fold in MDA-MB-231

Multiplex 8-color OPAL IHC staining

In collaboration of Dr. Harry Bear at Virginia Commonwealth University (VCU), Dr. Jennifer Koblinski and Dr. Michael Idowu are helping us to carry out the SIAH IHC staining using the newly established TNBC tissue microarray (TMA) at Massey Cancer Center at VCU. In addition, Dr. Jennifer Koblinski has been successful in carrying out are OPAL multicolor IHC staining using human non-small cell lung cancer (NSCLC) (**Figure 15**). Dr. Tang-Bear-Koblinski will conduct OPAL multicolor IHC staining on TNBC tumors. Dr. Koblinski and her team will provide histology services including H&E staining, IHC, ISH as well as multiplex ISH and IF (OPAL) staining. Dr. Koblinski and her team will use an Akoya Biosciences, Vectra Polaris and Infrom software, which allows for automated quantitative pathology and OPAL multicolor IHC imaging.

Immuno-oncology (I/O) is an exciting area of clinical research in TNBC. In 2020, immune checkpoint therapy by pembrolizumab or atezolizumab was approved by the FDA to treat PD-L1-positive mTNBC. We aim to determine whether SIAH can be used as a precision prognostic and predictive biomarker to guide and augment treatment decision of immune-oncology (I/O) for metastatic TNBC. Ultimately, we aim to establish SIAH as a high-resolution precision tool that can be used to guide effective immuno-oncology (I/O) therapy by demonstrating an inverse relationship between EGFR/K-RAS/SIAH pathway activation and immune suppression to treat TNBC malignancy.

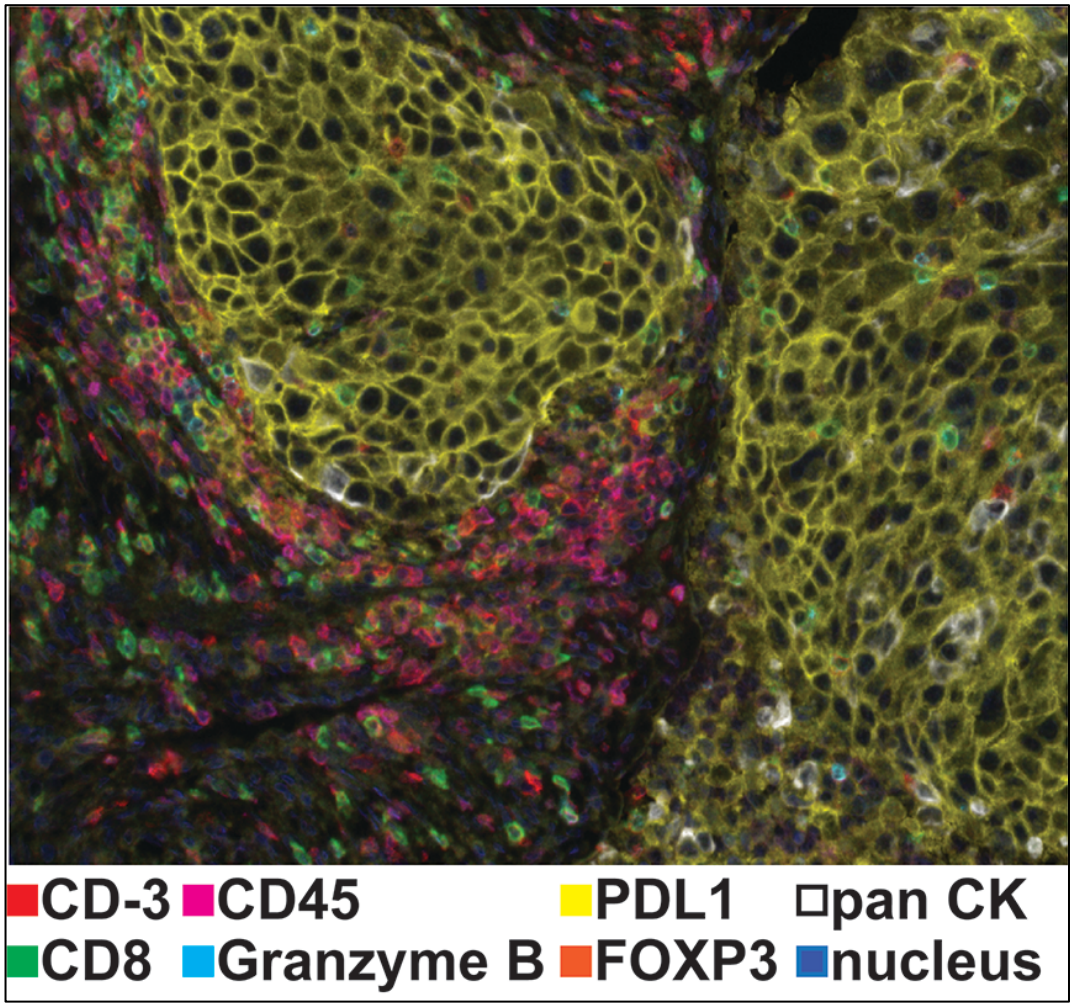


Figure 16. OPAL eight-color multiplex IHC staining of human non-small cell lung cancer (NSCLC) tumor and TME is shown. The multiplex eight-color OPAL IHC in human NSCLC tumor was successfully performed using the optimal Ab order and tumor marker following the manufacturer protocols and instructions. Tumor/tumor microenvironment (TME)/tumor immune microenvironment (TIME) are shown. The expression of PD-L1 (SP142), CD3 (T-cell lymphocytes), CD8 (cytotoxic T cells), CD45 (Memory T cells), Granzyme B (cytotoxic lymphocytes), FOXP3 (regulatory T cells) that mark the infiltrating tumor associated immune cells, pan-cytokeratin (epithelial cells) and DAPI (nucleus) are shown.
Contribution: The 8-color OPAl IHC staining in NSCLC tumors was provided by Dr. Jennifer Koblinski at VCU

3c. What opportunities for training and professional development has the project provided?

I have successfully recruited a PhD graduate student, Mr. Andrew P. Howell, who rotated in my laboratory in the Fall of 2020 and joined my lab in June 2021. Andrew has completed the principal component analysis (PCA) of the RPPA data. He is learning to use the ingenuity pathways analysis (IPA), cancer landscape (CScape) functional protein pathway mapping to categorize the feedforward, feedback, and compensatory signaling pathway activation in response to SIAH blockade in EGFR/K-RAS/B-RAF-driven TNBC cell lines.

I am honored and privileged to work with the five outstanding clinical residents (Amber L. Collier, M.D., Michael P. Lee, M.D., Caroline Dasom Lee, M.D., Lauren L. Siewertsz van Reesema, M.D., and C. Kendal Major, M.D.) as well as the five MD students (Ms, Emily L. Breeding, Ms. Jamie A. Parkerson, Ms. Angela Tang-Tan, Mr. Brandon Euker, Mr. Zakary L. Kolkey) who are assisting me with advancing this TNBC IHC project at Sentara Pathology and establishing these TNBC clinical databases at Sentara-EVMS-VOA. All ten MD students/clinical resident team members have successfully completed the required HIPAA, CITI, IRB, EPIC, iKnowMed training, and clinical database management training at

EVMS-Sentara-VOA. These five MD students are assisting me with identifying and pulling TNBC paraffin tumor blocks, organizing them, and preparing them for robotic IHC staining at Sentara Pathology. We are working on two manuscripts with our MD/PhD trainees together.

3d. How were the results disseminated to communities of interest?

Our two publications were advertised at the EVMS weekly news and the EVMS student news last year.

3e. What do you plan to do during the next reporting period to accomplish the goals?

Dr. Hoefer and Dr. Tang have disseminated our results and publications, and communicated the success pf this TNBC project to the top leadership at Sentara-EVMS-VOA who are in support of the execution and timely completion of this DOD-funded TNBC project.

4. IMPACT

4a. What was the impact on the development of the principal discipline(s) of the project?

(1) Validating SIAH as a tumor-specific, therapy-responsive, prognostic and predictive biomarker that can augment precision medicine in TNBC, and (2) developing a new anti-SIAH targeted therapy to treat and eradicate multidrug-resistant and incurable TNBC will be impactful on TNBC prognosis and treatment. $SIAH^{ON/OFF}$ binary code will allow the timely identification of high-risk TNBC patients who are likely to develop early tumor relapse and rapid systemic dissemination post-NACT. SIAH, as a new precision molecular tool, would be useful in identifying the high-risk TNBC patients who may benefit from additional adjuvant chemotherapy, immunotherapy, or other targeted agents to increase MBC patient survival. In this study, we will determine whether persistent K-RAS/SIAH/EGFR signaling pathway activation is correlated with immune suppression in chemo-resistant residual tumors post-NACT (**Aim 1**). We will determine whether a potent SIAH inhibitor, blocking K-RAS/SIAH/EGFR/HER2 signaling, can prevent metastasis of chemo-resistant TNBC tumors in a "xenopatient" mouse model (**Aim 2**). This SIAH-centered prognostic biomarker and new targeted therapy will allow us to intervene with 2^{nd} line effective therapies as soon as identify chemo-resistant TNBC tumors as early as possible post 1^{st}-line SOC therapies in the clinic.

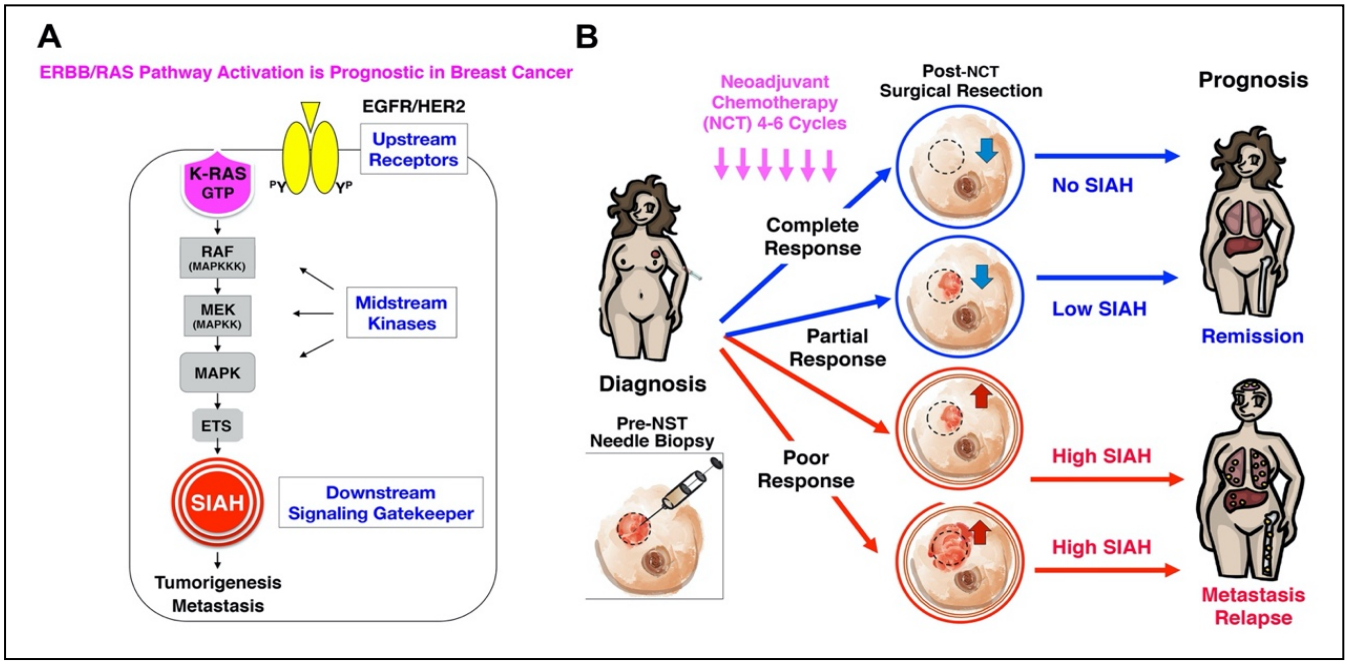


Figure 16: **Schematic illustration of the K-RAS/SIAH pathway activation in breast cancer**.
(**A**) SIAH is the most downstream "gatekeeper" signaling module identified in the canonical EGFR/RAS signal transduction pathway in human cancer cells. (**B**) Loss of SIAH expression post-NACT is correlated with EGFR/HER2/K-RAS pathway inactivation and tumor regression, whereas persistent SIAH expression is correlated with EGFR/HER2/K-RAS pathway activation, tumor progression and early relapse in high-risk partial responders.

In this study, we aim to demonstrate that *the SIAH/EGFR/K-RAS pathway activation is a major and pivotal driving force fueling the high-risk and chemo-resistant TNBC tumors that will recur and invade post-NACT, and SIAH is a key tumor vulnerability in chemo-resistant and high-grade TNBC.* $SIAH^{OFF/LOW}$ in residual tumors correlates with tumor remission and good prognosis, whereas $SIAH^{ON/HIGH}$ in residual tumors marks chemo-resistant tumor cells and predicts early tumor relapse and poor prognosis post-NACT (**Figure 15**). Hence, $SIAH^{ON/OFF}$ is a valuable prognostic binary code, $SIAH^{ON/OFF}$, that will accurately identify residual tumor clones with high molecular precision at a single tumor cell resolution post-NACT. As such, $SIAH^{ON/OFF}$ can be added to augment and enhance augment the commonly-used clinical prognostic predictors – pathologic complete response (pCR), pathologic incomplete response (pIR), and residual cancer burden (RCB) – that can aid and assist the oncologists to stratify high-risk TNBC patients, identifying chemo-resistant tumor clones, forecasting early tumor relapse, and predicting patient survival in neoadjuvant settings in the clinic.

The successful execution of this TNBC project is likely to make a major difference by providing strong logic, conceptual breakthrough in support of **a unified strategy** by focusing on EGFR/K-RAS/SIAH-signaling pathway that drives most, if not all, chemo-resistant, relapsed, and metastatic TNBC tumors, independent of changing genomic/epigenomic/transcriptomic/proteomic/kinomic/multi-Omics diversity, constant-evolving tumor/TME/TIME heterogeneity. The simplicity, efficacy, and cost-saving measure will be significant for rapid clinical translation. $SIAH^{ON/OFF}$ will provide high prognostic accuracy and molecular precision at a single tumor cell resolution to quantify therapy efficacy, measure tumor response, and test the antitumor efficacy of existing and emerging anti-TNBC therapies used in the neoadjuvant settings. This concept is simple, timely, and urgently needed to help clinicians to better monitor, measure, and manage treatment options as we treat advanced, relapsed, and metastatic TNBC in real time.

4b. What was the impact on other disciplines?

The successful execution of this TNBC project is also likely to impact several other major cancer types with high relapse rates, such as pancreatic cancer, high-grade serous ovarian cancer, non-small cell lung cancer (NSCLC), mCRC, and mTNBC that are prone to develop tumor recurrence after complete resection and intense adjuvant chemotherapy used in the clinic.

4c. What was the impact on technology transfer?

The successful execution of this TNBC project is likely to provide high-quality, unambiguous, and convincing molecular evidence to demonstrate the efficacy and validity of a SIAH-centered prognostic biomarker to forecast tumor relapse and predict patient survival, as well as the efficacy and validity of an anti-SIAH-based targeted therapy to control and eradicate undruggable and incurable stage IV metastatic TNBC resected from non-responders using the PDX models *in vivo*.

4e. What was the impact on society beyond science and technology?

Guided by our well-respected clinician/scientist leaders at DOD-Sentara-EVMS-VOA-VCU-GMU, we aim to generate high-quality data and convincing evidence in support of a future FDA-approval of adding a SIAH-centered molecular testing kit to augment the prognostic accuracy and molecular precision of the existing clinicopathological parameters by identifying the pIR patients who are the

highest risk of developing early tumor relapse and chemo-resistance post-NACT. We aim to validate and develop a new anti-SIAH-based anti-EGFR/RAS targeted strategy to control and conquer multidrug-resistant and incurable TNBC. If successful, we hope to make a difference, save more lives, and change the landscape of late-stage and metastatic TNBC in this great country at DOD-BCRP.

5. CHANGES/PROBLEMS

5a. Changes in approach and reasons for change.

None

5b. Actual or anticipated problems or delays and actions or plans to resolve them

COVID-19 impact – The TNBC IHC staining project was delayed at Sentara and IACUC animal experiments (tumor implantation experiments) was delayed at EVMS.

Due to the COVID shutdown and COVID-19 social distance restriction, we have encountered repeated delays in carrying out the IHC staining of this TNBC project at Sentara pathology.

On March 23, 2020, Virginia Governor Northam issued an order to close all public and private schools in the Commonwealth of Virginia. As a result, the COVID-19 pandemic shut down all non-essential clinical operations and all non-essential research activities at Sentara and EVMS. No new clinical research activities were allowed and no mice/experimental animals could be ordered at EVMS. As such, the TNBC IHC staining project was put on hold and repeatedly delayed, even though a purchase service agreement contract to start this TNBC IHC staining project was signed and approved to proceed at EVMS and Sentara Hospitals on April 21, 2020 (Please see the attached signed contract for details). Even though the Sentara-EVMS purchase service agreement contract was signed on April 21, 2020, right before the COVID-19 shutdown at Sentara Hospitals in Virginia, our IHC staining operation was stalled completely.

The PPE shortage and global COVID-19 pandemic has subsequently shut down all new clinical, pre-clinical, and basic science research activities at Sentara and EVMS. On March 15, 2020, Dr. Amy Tang, the PI of this project, applied and received essential worker status at EVMS to maintain the continuity of our federally-funded DOD-TNBC projects in support of basic laboratory operations. Dr. Tang has continued working to advance this DOD-BCRP-BC180907 TNBC project. EVMS research begun to reopen and ramp up in two phases: Phase I (May 15, 2020) and phase II (September 18, 2020). Nonetheless, our research progress was impeded significantly.

With the state of Virginia fully re-opening on **May 28, 2021**, we are expected to catch up on this TNBC project rapidly using the state of the art infrastructure and advanced pathology facility at Sentara Pathology Department. With the use of IHC automation capacity at Sentara pathology under the leadership of Dr. Janet Winston and Dr. Billur Samli, we are confident that we will catch up very rapidly using the seven robotic Ventana auto-stainers at Sentara pathology. We anticipate that we are scheduled to finish Aim 1 and Aim 2 in 2022 and/or 2023.

The Sentara Healthcare Hospital System is a large medical entity comprising 12 large hospitals that provides state-of-the-art healthcare in Virginia. ***Sentara Cancer Network treats ~ 25% of all breast cancer patients in Virginia***. With over 50,000 cancer patients in active surveillance at Sentara Tumor Registry, Sentara is an invaluable clinical partner for biomarker discovery, target validation, preclinical testing platform, and cancer disparity studies. Sentara's large tumor bank, multidisciplinary clinical expertise, state-of-the-art imaging facility, robotic pathology facility, modern infrastructure, and access to a large African American TNBC cohort are all clear strengths of this DOD-BC180907 Breakthrough study in the Commonwealth of Virginia, and the United States.

5c. Changes that had a significant impact on expenditures

None

5d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

None

5e. Significant changes in use or care of human subjects

No change

5f. Significant changes in use or care of vertebrate animals

No change

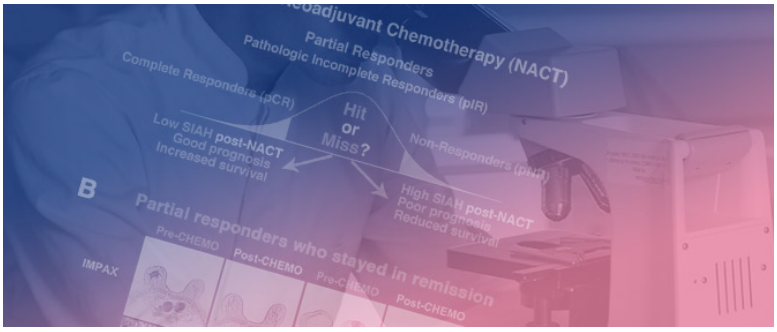
5g. Significant changes in use of biohazards and/or select agents

No change

6. PRODUCTS
Publications, conference papers, and presentations

6a. Two publications:

- *Gupta, G. K., *Collier, A. L., *Lee, D., Hoefer, R. A., Zheleva, V., Siewertsz van Reesema, L. L., Tang-Tan, A. M., Guye, M.L., Chang, D. Z., Winston, J. S., Samli, B., Jansen, R. J., Petricoin, E. F., Goetz, M. P., Bear, H. D., and A. H. Tang (2020) Perspectives on Triple-Negative Breast Cancer: Current Treatment Strategies, Unmet Needs, and Potential Targets for Future Therapies. Cancers 2020, 12(9), 2392; doi:10.3390/cancers12092392 https://www.mdpi.com/2072-6694/12/9/2392



- **This TNBC article was highlighted and featured at MDPI Cancers with a cover image as attached.**

- Gupta, G. K., Lee, D. C., Guye, M. L., Van Sciver, R. E., Lee, M.P., Lafever, A. C., Pang, A., Tang-Tan, A. M., Winston, J. S., Samli, B., Jansen, R. J., Hoefer, R. A., and **A. H. Tang** (2020) Unmet Clinical Need: Developing Prognostic Biomarkers and Precision Medicine to Forecast Early Tumor Relapse, Detect Chemo-Resistance and Improve Overall Survival in High-Risk Breast Cancer, Annals of Breast Cancer and Therapy. 2020, 4 (1) 48-57; DOI: 10.36959/739/525; PMCID: PMC7295150; PMID: 32542231
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7295150

6b. Books or other non-periodical, one-time publications

6c. Other publications, conference papers and presentations.

- Gits, H.C., **Tang, A.H.**, Harmsen, W.S., Bamlet, W.R., Graham, R.P., Petersen, G.M., Smyrk, T.C., Mahipal, A., Kowalchuk, R.O., Ashman, J.B., Rules, W.G., Owen, D., Neben Wittich, M.A., McWilliams, R.R., Halfdanarson, T., Ma, W.W., Sio, T.T., Cleary, S.P., Truty, M.J., Haddock, M.G., Hallemeier, C.L., Merrell, K.W. (2021) Intact

SMAD-4 is a Predictor of Increased Locoregional Recurrence in Upfront Resected Pancreas Cancer Receiving Adjuvant Therapy. *Journal of Gastrointestinal Oncology* (JGO-21-55). Accepted for publication

- Van Sciver, R. E., Cao, Y., and **Amy H. Tang**. A large-scale and unbiased seven-in-absentia (sina) mutagenesis screen, and *Drosophila* SINA and human SIAH1/2 inhibitor functional validation in *Drosophila* eye development. *PLOS Genetics* (manuscript in review).

- 2021.05.11 Tang invited seminar presentation at the WSU Biomedical Seminar Series at Washington State University (WSU). that was entitled “Early Detection of Tumor Relapse, Precision Medicine, and Eradication of Undruggable and Incurable Oncogenic EGFR/K-RAS-Driven Malignant Cancer”.

6d. Technologies or techniques

A prognostic SIAH/EGFR-based clinical testing kit:

We aim to develop a clinical testing kit by developing a SIAH-centered EGFR/K-RAS/SIAH pathway dependent prognostic biomarker panel to stratify high-risk TNBC partial responders, forecast early tumor relapse, increase molecular precision in TNBC prognosis of RCB II-III tumors post-NACT. We will delineate SIAH biology and SIAH pathway activation in TNBC. We will develop anti-SIAH targeted therapy as a better way of treating multidrug-resistant and incurable TNBC, aiming to minimize toxicity, translate to the clinic, and improve patient survival rates in the clinic in the future.

As enumerated in this 2nd annual progress report of DOD-BC180907, we are working on three additional manuscripts in the second year. We will continue to work hard in hopes of make a difference, saving lives, and changing the landscape of TNBC and metastatic TNBC at DOD-BCRP.

- SIAH ON = tumor-driven K-RAS/EGFR pathway is still ON in residual TNBC tumors after 1st line NACT treatment
- SIAH OFF = tumor-driven K-RAS/EGFR pathway is OFF in residual TNBC tumors after 1st line NACT treatment
- $SIAH^{ON/OFF}$ binary code is a new predictive and prognostic biomarker to quantify TNBC therapy response, forecast early tumor relapse, and predict patient survival.
- SIAH is a new TNBC target and a major TNBC tumor vulnerability, and anti-SIAH therapy can be used to synergistically reduce the mortality associated with chemo-resistant and metastatic TNBC in the future.

We are immensely grateful for the continued DOD support, and hope to continue to leverage this DOD-BC180907 grant to expedite the clinical translation and future FDA approval of SIAH for clinical application. This has the potential to improve TNBC pIR patient stratification and enhance the molecular precision of RCB post-NACT, the commonly used clinical parameter, Together, we strive to save more high-risk and locally advanced TNBC patients and address healthcare disparities for African Americans Hampton Roads Virginia with a large African American population.

6e. Inventions, patent applications, and/or licenses

SIAH monoclonal antibody was licensed for research use by UC Berkeley (30%), Howard Hughes Medical Institute (HHMI) (30%), and the Mayo Clinic (40%).

6e. Other Products

None

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7a. What individuals have worked on the project?

Amy H. Tang, Ph.D. **PI**
Professor of Cancer Biology
Leader of Pancreatic & Breast Cancer Research
Eastern Virginia Medical School

Leroy T. Canoles Jr. Cancer Center
651 Colley Avenue, Lester Hall, Room 423
Norfolk, Virginia 23501
Office: (757) 446-5664
Email: tangAH@evms.edu

Project role: PI
Nearest person month worked: 2.4-months (20% effort - cost-matched)
Contribution to Project: Dr. Tang has overseen and supervised the operation of this multi-centered clinical team in establishing, extracting, and validating the data accuracy of this local TNBC cohort. Dr. Tang has coordinated with a team of surgeons, pathologists, oncologists, cancer experts, and biostatistician to obtain the IRB approval and Sentara contract agreement, to conduct IHC staining and PDX studies, and wrote manuscripts to advance this DOD study to augment current existing prognostic panels to identify TNBC partial responders who will develop early tumor relapse as early as possible so that effective 2nd-line therapies can be added to extend patient survival post-NACT

Harry D. Bear, M.D., PhD. (Co-I)

Walter Lawrence, Jr. Distinguished Professor of Oncology;
Chairman, Division of Surgical Oncology
Professor, Departments of Surgery, Microbiology & Immunology
Director, Breast Health Center, VCU Massey Cancer Center
Medical Director, Massey Cancer Center Clinical Trials Office
Massey Cancer Center, VCU School of Medicine, VCUHS
Virginia Commonwealth University
Richmond, VA 23298
Email: harry.bear@vcuhealth.org
Office: 804-628-3242;
Fax: 804-828-4808

Project role: co-I
Nearest person month worked: 0.3-months (2.5% effort)
Contribution to Project: Dr. Bear is an internationally-renowned top surgeon leader in Virginia. Dr. Bear has served a clinical mentor for Dr. Tang on all the key aspects of this TNBC study at Sentara-EVMS-VOA. Dr. Bear has advised and guided Dr. Tang and this TNBC team so that appropriate milestones are met and statistically significant conclusions are reached at the end of this DOD study. Dr. Bear edited and revised all of our manuscripts. His leadership is pivotal to advance this DOD project in Virginia.

Emanuel F Petricoin Ph.D. (Co-I)

University Professor
Co-Director Center for Applied Proteomics and Molecular Medicine
School of Systems Biology
George Mason University
10920 George Mason Circle
Room 2006 Institute for Biomedical Innovation
Manassas, VA 20110
Email: epetrico@gmu.edu
Office: 703-993-8646; Fax: 703-993-8606

Project role: co-I
Nearest person month worked: 0.24-months (2% effort)
Contribution to Project: Dr. Petricoin is an internationally-renowned top scientist in clinical marker validation and cancer pathway mapping in the fields of proteomics and cell signaling. As an inventor of Reverse Phase Protein Microarray (RPMA) technology, Dr. Petricoin has advised and guided Dr. Tang to successfully conduct RPPA kinomic data analysis using TNBC cell lines to identify the changes in the TNBC cancer signaling pathways/intertwined signaling network in response to anti-SIAH2 blockade to shutdown oncogenic EGFR/K-RAS hyperactivation and impede tumor growth of MDA-MB-231 and MDA-MB-468 cell lines in cell line and PDX models. Dr. Petricoin edited and revised all of our manuscripts. His

leadership is pivotal to dissect the proteomic/kinomic-based cancer pathway alterations in TNBC in response to SIAH inhibition.

Richard A. Hoefer, D.D. FACS (Co-I)
Retired Surgeon
Medical Adviser, Sentara Cancer Network
Co-Director, Dorothy G. Hoefer Comprehensive Breast Center
33 King Street
Charleston, SC 29401
E-Mail: rahoefer@sentara.com

Project role:	co-I
Nearest person month worked:	0.3-months (2.5% effort – no salary support is requested)
Contribution to Project:	Dr. Hoefer has tirelessly promoted this TNBC project to Sentara top Leadership. Dr. Hoefer coordinated the institutional resources at Sentara Cancer Network to help us to identify and recruit 700 NACT-treated TNBC patients, and conduct IHC staining studies in support of this DOD project at Sentara Breast Cancers and Sentara Hospitals System. Dr. Hoefer has served as a clinical mentor for Dr. Tang. Dr. Hoefer edited and revised all of our manuscripts. His leadership is pivotal to advance this DOD project locally.

Rick Jansen, PhD, MS (Biostatistician)
Assistant Professor
Department of Public Health
Genomics and Bioinformatics Program
Center for Immunization Research and Education (CIRE)
Center for Diagnostic and Therapeutic Strategies in Pancreatic Cancer
North Dakota State University
1805 Research Park Drive/Fargo, ND 58102
Dept 2662, PO Box 6050/Fargo, ND 58108-6050
Tel: 701.231.6487; Fax 701.231.5586;
Email: rick.jansen@ndsu.edu

Project role:	Biostatistician – A paid consultant
Nearest person month worked:	12-months (10% effort)
Contribution to Project:	Dr. Jansen has performed multiple biostatistical analyses of our TNBC datasets and compared the KM survival curves of the local TNBC cohort with that of the national SEERS TNBC database. Two manuscripts are in preparation.

Janet S. Winston, M.D.
Director, Breast Pathology Services
Pathology Sciences Medical Group
Department of Pathology
Sentara Norfolk General Hospital (SNGH)
600 Gresham Drive
Norfolk, VA 23507-1904
Office Phone: 757-388-5827
Fax: 757-388-3799
Email: JSWinsto@sentara.com

Project role:	A board-certified clinical pathologist – A paid consultant
Nearest person month worked:	**0-months (0% effort)**
Contribution to Project:	As the Director of Breast Pathology Service in the Department of Pathology at Sentara Norfolk General Hospital (SNGH), Dr. Winston was scheduled to direct and conduct the IHC staining to advance this new TNBC prognostic biomarker validation study. **The IHC staining project was delayed due to COVID-19 restrictions at Sentara Hospitals.** This IHC project was approved to restart at Sentara pathology. Dr. Winston will supervise and coordinate the clinical operation of identifying, retrieving, and cutting of the paraffin tumor

blocks at Sentara Pathology Department. Dr. Winston edited and revised two manuscripts for us. Her leadership, pathology expertise, technical guidance, and active involvement are instrumental to the successful execution of this TNBC Breakthrough project.

Dr. Billur Samli
Attending Surgical and Breast Pathologist
Pathology Sciences Medical Group (PSMG)
Department of Pathology
Sentara Norfolk General Hospital (SNGH)
600 Gresham Drive
Norfolk, VA 23507-1904
Office Phone: 757-388-1158
Fax: 757-388-3799
bxsamli@sentara.com

Project role:	A board-certified clinical pathologist – A paid consultant
Nearest person month worked:	**0-months (0% effort)**
Contribution to Project:	As the Attending Surgical and Breast Pathologist at Sentara Norfolk General Hospital (SNGH), Dr. Samli was scheduled to direct and conduct the IHC staining to advance this new TNBC prognostic biomarker validation study. **The IHC staining project was delayed due to COVID-19 restrictions at Sentara Hospitals.** This IHC project was approved to restart at Sentara pathology. Dr. Samli will work independently from Dr. Winston in a double-blind fashion to review and score the IHC staining of the RAS pathway biomarkers. Dr. Samli will provide highly valuable 2nd opinions and 2nd independent scores in the IHC study. Dr. Samli edited and revised two manuscripts for us. Her leadership, pathology expertise, technical guidance, and active involvement are instrumental to the successful execution of this TNBC Breakthrough project.

7b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

No change

7c. What other organizations were involved as partners?

Organization Name: Eastern Virginia Medical School (EVMS) and Leroy T. Canoles Jr. Cancer Center (LTCCC)
Location of Organization: Norfolk, Virginia 23501
Partner's contribution to the project (identify one or more)

- Financial support: (Dr. Tang's 20% effort and salary are cost-matched at EVMS)
- Equipment: (e.g., Dr. Tang's laboratory equipment has been used to advance this DOD study).
- Facilities (e.g., EVMS core facilities, animal facility, and imaging facility will be used to advance this DOD study).
- Collaboration (e.g., Dr. Tang's MD/PhD students have made important contributions in establishing the TNBC databases, conducting clinical validation and data authentication, and performing the critical clinical work in support of this TNBC study).

Organization Name: Sentara Hospitals Systems, Sentara Cancer Network, and SNGH pathology
Location of Organization: Multiple Locations – Sentara headquarter is located at Norfolk, VA 23507
Partner's contribution to the project (identify one or more)

- Financial support; Dorothy G. Hoefer Foundation for Breast Cancer (Dr. Hoefer has provided seed money $57K to conduct pilot study and generate preliminary data in support of this DOD study).
- Equipment: (e.g., Sentara makes pathology/IHC equipment, autostainers available to Dr. Tang).
- Facilities (e.g., We are using the Sentara's state-of-art pathology facilities to advance this TNBC project).
- Collaboration (e.g., Sentara's two pathologists and their pathology staff have worked with Dr. Tang to identify and retrieve tumor paraffin blocks, cut and stain the H&E and IHC slides, conduct quality control, review and score IHC staining to advance this TNBC project).

Organization Name: Center for Applied Proteomics and Molecular Medicine, School of Systems Biology George Mason University (GMU)
Location of Organization: Manassas, VA 20110
Partner's contribution to the project (identify one or more)

- Equipment: (e.g., GMU makes RPPA equipment, data analysis software available to Dr. Tang).
- Facilities (e.g., We are using the GMU's state-of-art advanced proteomics, kinomics, cancer pathway analysis, systems biology facilities to advance this TNBC project).
- Collaboration (e.g., Dr. Petricoin and Dr. Julia Wulfkuhle will conduct the RPPA assays, global phospho-protein proteomic analysis, and clinical kinomic profiling to identify new TNBC vulnerability and actionable targets in response to SIAH blockade to control and eradicate multidrug-resistant and incurable mTNBC.

Organization Name: VCU Health System, and Massey Cancer Center
Location of Organization: Richmond, VA 23298
Partner's contribution to the project (identify one or more)

- Equipment: (e.g., VCU is making a TNBC Tissue microarray (TMA) that will be available to Dr. Tang).
- Facilities (e.g., We are exploring and expanding the capacity of using the VCU's state-of-art PerkinElmer OPAL™ multiplex automation IHC detection, BOND RX autostainer, and multi-color IHC imaging facility to advance this TNBC project).
- Collaboration (e.g., Dr. Bear and his TNBC team is constructing a new TNBC TMA, they will conduct SIAH/EGFR/Ki67/PD-1/PD-L1/CD8/CD4/CD3 multiplex OPAL IHC to validate and verify that SIAH can serve as a new prognostic biomarker to predict immune checkpoint efficacy (i.e., $SIAH^{ON}$ in residual tumors post-NACT predict immune suppressive environment, where $SIAH^{OFF}$ in residual tumors post-NACT predict immune sensitive environment as in high-risk TNBC patients with residual diseases).

Organization Name: Virginia Oncology Associates (VOA)
Location of Organization: Norfolk, VA, 23502
Partner's contribution to the project (identify one or more)

- Facilities (e.g., VOA leadership has offered staunch support and clinical guidance to Dr. Tang. VOA has offered iKnowMed clinical database training to Dr. Tang and her TNBC team members so that we can extract and confirm a multitude of chemo- and targeted therapies used to treat each and every TNBC patients in our local cohort).
- Collaboration (e.g., David Chang, M.D., Ph.D., Scott Krugger, M.D. – the IRB chair, Ms. Margot Richards and Ms. Karen Pearson – two EMR Managers at VOA, have advised, guided, taught and supported Dr. Tang and her MD/PhD students to advance this study at VOA).

8. SPECIAL REPORTING REQUIREMENTS

We have and will continue to demonstrate and adhere to the highest standards in academic honesty, data integrity, ethics in scientific rigor, high quality, and data reproducibility in science and medicine in hope to save more lives in TNBC.

9. APPENDICES

The IRB approval letters and Sentara Service Agreement

- 2020.04.21 Tang_SOW_Purchased Services Agreement_FE_4.21.20 (EVMS-Sentara)
- 2021.05.28 Tang Needle Biopsy (IRB-13-07-FB-0157) Continuing-Review (250 patients) Approval Letter
- 2020.10.02 Tang Breast Cancer IRB (Tang, 11-10-WC-0226) Continuing Review Approval Letter
- 2020.10.02 Tang Breast Cancer IRB (Tang, 11-10-WC-0226) Amendment of Data Extension Approval Letter

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