Merge

Volume 8 | Issue 1 Article 3

2024

Investigating SCUBE3 Nuclear Localization in Presence of Doxorubicin Treatment in Triple Negative Breast Cancer Cells

Lillian Ergle
Mississippi University for Women, lillianfaithergle@gmail.com

Investigating SCUBE3 Nuclear Localization in Presence of Doxorubicin Treatment in Triple Negative Breast Cancer Cells

Lillian Ergle
Mississippi University for Women

I. Introduction

Signal peptide with CUB and EGF-like domain containing protein 3(SCUBE3) is a protein suspected to be involved in cancer. It is a secreted protein discovered in the early 2000s (Wu, B.-T., 2004). The gene sequence for SCUBE3 is found on the cytogenetic band 6p21.31 (Genecard, 2017). The final location of the protein with respect to the cell is the extracellular space and plasma membrane. Originally SCUBE3 was found in the umbilical vein and in human epithelial cells which have critical roles in cell differentiation, recognition, and modeling (Kumar, S., 2022). This protein has recently been looked at in cancer research with lung cancer having the most input (Wu, Y.-Y., 2011; Zhao, C., 2013; Chou, C.-H., 2013). Overexpression in SCUBE3 has been evaluated heavily in lung cancer cells (Chou, C.-H., 2013). These studies found a correlation between high expressions of SCUBE3 and cancer prognosis. Cancer cells overexpress the protein while noncancerous cells exhibit low levels. A knockdown of SCUBE3 expression has been shown to reduce tumorigenesis and cancer metastasis in vivo (Wu, Peck, SCUBE3 TGF-Beta). Since SCUBE3 is a secreted protein, it would be found mainly in the extracellular fluid or plasma membrane. When treated with Doxorubicin (DOX), the protein has been observed to translocate into the nucleus (Kumar, S., 2022). Proteins found in the nucleus bind or repress DNA (Azar, W. J., 2014); however, SCUBE3 being translocated to the nucleus does not have the ability to bind to DNA or repress it (Wu, B.-T., 2004) which means that its purpose is unknown.

Based on this observation and preliminary data, we hypothesized that nuclear SCUBE3 protein promotes the survival of cells against Doxorubicin treatment. To investigate this hypothesis, we made three constructs with mutated nuclear localization sequences (NLS). Mutated NLS will allow us to see whether the Doxorubicin treatment targets the signal sequences and sends SCUBE3 to the nucleus. Our constructs were linked to green fluorescence protein (GFP). GFP was a report protein that helped us monitor the location of SCUBE3 in the cells, and determine if putative NLS was required for SCUBE3 translocation. The reason we looked at GFP is because the cell makes its own SCUBE3 on top of the constructs we are introducing, and we will need to distinguish between these versions of the proteins within the cells. We also used immunoprecipitation to determine whether nuclear transport proteins importin-α and -β are required for SCUBE3 migration to the nucleus.

II. Materials and Methods

Cell culture and maintenance of cell lines

MDA-MB-231, MDA-MB-436, and MDA-MB-468 Human cell lines cultured from three separate individuals with TNBC were obtained from the American Type Culture collection (ATCC Manassas, VA). Three different cell lines were used to monitor the effect of SCUBE3 inhibition on the cells immunity to DOX and confirm the possible findings against one another. MDA-MB-231 and MDA-MB-436 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal Bovine serum (FBS), and neomycin analogue - G418. MDA-MB-468 was cultured in 1X Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% fetal Bovine serum (FBS), and neomycin analogue-G418. Each cell line (MDA-MB-231, MDA-MB-436, and MDA-MB-468) was stably transfected with control plasmid (pcDNA3.1(-)) or SCUBE3 constructs (wildtype SCUBE3, mutated SCUBE3 Nuclear Localization Sequence 1 (NLS1), or mutated SCUBE3 Nuclear Localization Sequence 2 (NLS2) and mutated SCUBE3 Nuclear Localization

Sequence 1&2 (NLS1&2). Transfected cells were selected with 1mg/ml G418 for MDA-MB-231 cells and 400ug/ml G418 for MDA-MB-468 cells. The selection was completed for about two weeks. Afterward the MDA-MB-231 cells were maintained in 500ug/ml G418 and MDA-MB-468 cells in 200ug/ml G418. All cells were incubated in CO_2 humidified incubator at 37°C.

Cell Viability and Proliferation Assay

Stable MDA-MB-468 cells bearing SCUBE3 wildtype or mutated constructs were seeded in 96-well plates at the amount of 1,000 cells per well with three redundant examples of each strain. The cells were incubated for five days. To test cell viability, the seeded cells were treated with 50nM concentration of Doxorubicin (DOX) for five days. After five days, the number of viable cells were determined with alamar blue dye using the BioTek LX plate reader. MDA-MB-468 was the only cell line to stabilize within the time constraints of the experiment.

RNA Isolation and qRT-PCR Analysis

Total RNA were extracted from the cancer cells, MDA-MB-468 and all five strains, using the Qiagen RNA isolation kit and following the manufacturer's recommendation. The isolated RNA were converted to cDNA using the iScript cDNA synthesis kit from BioRad. The resulting cDNA were used for Realtime PCR reaction using SYBR Green and primers for the SCUBE3 and GAPDH. The reaction mix was incubated at 95 °C for 3 minutes, 95 °C for 10 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. SCUBE3 transcript levels were determined by comparative Ct method (2−ΔΔCt). The ΔΔCt = ΔCt $SCUBE3_{target}$ − ΔCt $GAPDH_{reference}$. The results were normalized using the empty vector or vehicle control.

Statistical analysis

All experiments were performed at least once with at least three samples per strain of the MDA-MB-468 cancer cells. Results are presented as means of example and should be repeated to confirm precise value. MDA-MB-468 was the only cell line to stabilize within the time constraints of the experiment. MDA-MB-231 and MDA-MB-436 cell lines were not tested and did not result in data.

III. Preliminary Data

Doxorubicin Induces SCUBE3 Localization to the Nucleus and Expression

In our preliminary data, DOX treatment induced the nuclear localization of SCUBE3 in a dose treatment manner (Figure 1). Based on this observation, we investigated whether DOX treatment alters SCUBE3 expression. To test this, MDA-MB-468 cells were treated with different DOX doses (0nM, 25nM, 50nM, 100nM, and 200nM) for 24 hours. The RNA of

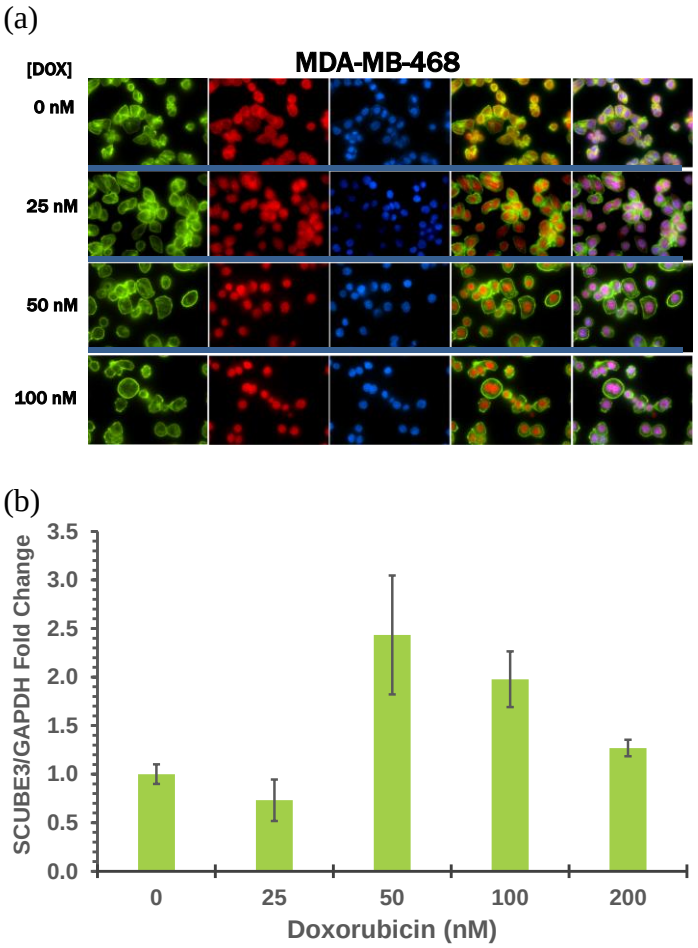


Figure 1: DOX treatment alters SCUBE3 in the cells: (a) Immunofluorescence result of MDA-MB-468 cells treated with different concentration of DOX for 24 hours. The result shows SCUBE3 protein in red, tubulin in green, and nucleus blue. (b) The realtime PCR result showing SCUBE3 level in DOX treated MDA-MB-468.

the treated and control cells, five strains of MDA-MB-468 that were created, were isolated and subjected to reverse transcription and Realtime PCR reactions. The result shows the elevation of SCUBE3 expression in the presence of DOX in a dose dependent manner (Figure 1b). The result also shows that 50nM DOX concentration induces the largest elevation of SCUBE3. It further demonstrated the impact of DOX treatment on SCUBE3.

Mutation of SCUBE3 Predicated Nuclear Localization Sequences Did Not Alter SCUBE3 Expression or SCUBE3-Driven Proliferation

Next, we wanted to address if the elevation and nuclear localization of SCUBE3 is important for the survival of the cells. First, using a bioinformatics approach, we determined two predicted-SCUBE3 nuclear localization sequences (NLS) in the SCUBE3 gene sequence with NLStradamus and Protein Subcellular Localization Prediction Tool II (PSORT II) that identified two classical NLS at $_{532}$-RKGKGRRARTPP-$_{543}$ and $_{836}$-PPPEMEILIV-$_{845}$ within the SCUBE3 linker domain.

Next, we generated SCUBE3 gene constructs with each NLS (Fig 2) by changing the lysine to methionine and arginine to glutamate amino acids. All the constructs were fused to Green Fluorescent Protein (GFP) gene. GFP is important for tracking the location of the ectopic SCUBE3 proteins in the cells. The mutations were confirmed through gene sequencing. Having generated the constructs, we assessed the effect of the mutations' SCUBE3 expression. The mRNA from cells bearing the stable SCUBE3 constructs were isolated and used to analyze SCUBE3 mRNA levels (Figure 3a) in the control, wildtype, and NLS mutant cells. The result showed elevated SCUBE3 expression in wildtype and NLS mutant cells compared to GFP cells. We observed the largest increase of SCUBE3 in cells bearing both mutations of NLS1 & NLS2. Next, we tested whether our mutations affected SCUBE3's ability to promote cell proliferation. The MDA-MB-468 cells were seeded in a 96-well plated and incubated for five days. Afterward, the cells were counted. The result showed that the wildtype and mutated SCUBE3 constructs promoted cell proliferation (Figure 3b). These results indicated that mutation of SCUBE3 NLS did not suppress SCUBE3-mediated cell proliferation.

(a)

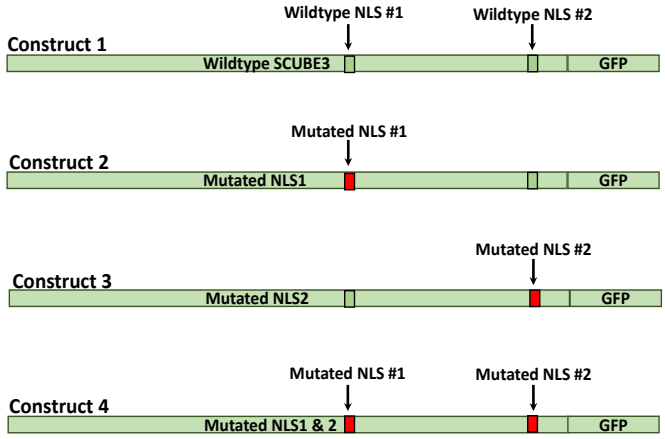
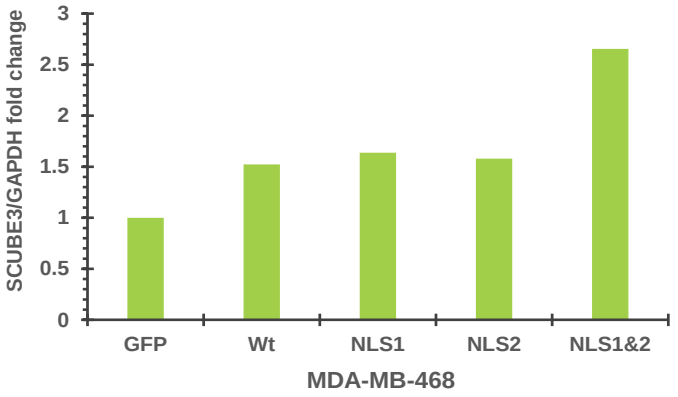
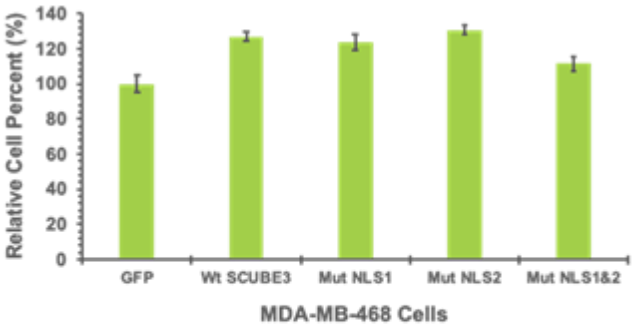


Figure 2: Two predicted-SCUBE3 nuclear localization sequences were used to generate viable constructs to determine whether these sequences were fundamental to

(a)





(b)

Figure 3: Mutated constructs did not alter SCUBE3 expression or SCUBE3-driven proliferation. (a) Realtime PCR result of SCUBE3 mRNA levels within stable MDA-MB-468 shows no effect on SCUBE3 expression. (b) Stable MDA-MB-468 cells were intubated for six days and analyzed. The result indicates no effect on SCUBE3-driven proliferation.

IV. Results

Promotes Cells' Survival

To gain insight into the functional importance of SCUBE3 in the nucleus, we treated cells bearing SCUBE3 constructs with 50nM of DOX for 24 hours and then incubated them for five days. Afterward, the cell viability analysis was performed between the different treatments. The result showed that the NLS1 and NLS2 cells have the smallest number of viable cells (Figure 4). Taken together, this result showed for the first time that SCUBE3 possesses two functional NLS sequences and that SCUBE3 actively translocates into the nucleus in the presence of DOX to promote cell survival. The cells with the double mutated NLS sequences had the highest number of viable cells.

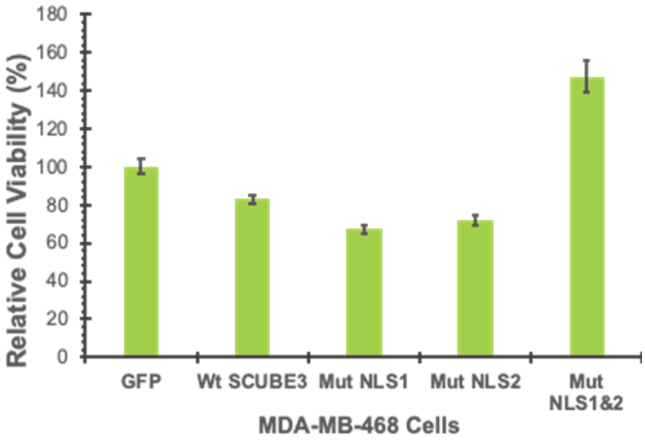


Figure 4: SCUBE3 NLS is important for cell survival. The stable MDA-MB-468 cells were treated with 50nM of DOX for 24hrs and a 6-day incubation followed before a cell viability analysis was completed. The results indicate that both NLS are functional and promotes survival.

V. Discussion

SCUBE3 is known to exert its actions through extracellular activities with cell surface proteins, such as receptors. However, our study suggests that treatment with DOX induces nuclear translocation of SCUBE3 therefore suggesting intranuclear actions. Our data indicates that the context dependent translocation of SCUBE3 may be important for cell survival during cytotoxic stress. We have identified and characterized two functional NLS sequences in the SCUBE-3 and showed that the lack of nuclear translocation of SCUBE3 has promise in preventing its pro-tumorigenic protection action against DOX.

Some proteins under 40 – 60 kDa can passively diffuse into the nucleus through the pores. However, most proteins with nuclear functions use active carrier protein transport pathways. Larger proteins such as SCUBE3, which is about 1000 kDa, theoretically cannot diffuse into the nucleus without the aid of protein transporters. The presence of two NLS suggests that SCUBE3 translocation might be aided by protein transporters.

Relative to these new findings, we investigated the functional importance of SCUBE3 in the nucleus in the presence of DOX treatment. Our results show that the mutation of the two predicted SCUBE3 NLS is important for cell survival suggesting that the loss of the wildtype sequences is important for the interaction with the classic nuclear transport proteins importin-α and -β that mediated the transportation of proteins to the nucleus. The loss of the wildtype NLS sequences suggests that the ectopic SCUBE3 failed to translocate to the nucleus. In conclusion, our data suggests that SCUBE3's translocation to the nucleus is important for cell survival in the presence of DOX-induced cytotoxicity.

Bibliography

Atif Ali Hashmi, Samreen Naz, Shumaila Kanwal Hashmi, Muhammad Irfan, Zubaida Fida Hussain, Erum Yousuf Khan, Huda Asif, & Naveen Faridi. (2019). Epidermal growth factor receptor (EGFR) overexpression in triple-negative breast cancer: Association with clinicopathologic features and prognostic parameters. *Surgical and Experimental Pathology*, *2*(1), 1–7. https://doi.org/10.1186/s42047-018-0029-0

Azar, W. J., Zivkovic, S., Werther, G. A., & Russo, V. C. (2014). IGFBP-2 nuclear translocation is mediated by a functional NLS sequence and is essential for its pro-tumorigenic actions in cancer cells. *Oncogene*, *33*(5), 578–588. https://doi.org/10.1038/onc.2012.630

Chou, C.-H., Cheng, Y.-F., Siow, T. Y., Kumar, A., Peck, K., & Chang, C. (2013). SCUBE3 regulation of early lung cancer angiogenesis and metastatic progression. *Clinical & Experimental Metastasis*, *30*(6), 741–752. https://doi.org/10.1007/s10585-013-9575-8

El-Tanani, M., Dakir, E.-H., Raynor, B., & Morgan, R. (2016). Mechanisms of Nuclear Export in Cancer and Resistance to Chemotherapy. *Cancers*, *8*(3), E35. https://doi.org/10.3390/cancers8030035

Fu, D., Pfannenstiel, L., Demelash, A., Phoon, Y. P., Mayell, C., Cabrera, C., Liu, C., Zhao, J., Dermawan, J., Patil, D., DeVecchio, J., Kalady, M., Souers, A. J., Phillips, D. C., Li, X., & Gastman, B. (2022). MCL1 nuclear translocation induces chemoresistance in colorectal carcinoma. *Cell Death & Disease*, *13*(1), 63. https://doi.org/10.1038/s41419-021-04334-y

Huo, Q., He, X., Li, Z., Yang, F., He, S., Shao, L., Hu, Y., Chen, S., & Xie, N. (2021). SCUBE3 serves as an independent poor prognostic factor in breast cancer. *Cancer Cell International*, *21*(1), 268. https://doi.org/10.1186/s12935-021-01947-3

Kim, Y., Kim, J., Lee, H.-D., Jeong, J., Lee, W., & Lee, K.-A. (2013). Spectrum of EGFR Gene Copy Number Changes and KRAS Gene Mutation Status in Korean Triple Negative Breast Cancer Patients. *PLoS ONE*, *8*(10), 1–7. https://doi.org/10.1371/journal.pone.0079014

Kumar, S., Prajapati, K. S., & Gupta, S. (2022). The Multifaceted Role of Signal Peptide-CUB-EGF Domain-Containing Protein (SCUBE) in Cancer. *International Journal of Molecular Sciences*, *23*(18), 10577.

Li, X., Wang, H., Yang, X., Wang, X., Zhao, L., Zou, L., Yang, Q., Hou, Z., Tan, J., Zhang, H., Nie, J., & Jiao, B. (2021). GABRP sustains the stemness of triple-negative breast cancer cells through EGFR signaling. *Cancer Letters*, *514*, 90–102. https://doi.org/10.1016/j.canlet.2021.04.028

Liu, X., Wang, P., Zhang, C., & Ma, Z. (2017). Epidermal growth factor receptor (EGFR): A rising star in the era of precision medicine of lung cancer. *Oncotarget*, *8*(30), 50209–50220. https://doi.org/10.18632/oncotarget.16854

Maik-Rachline, G., Hacohen-Lev-Ran, A., & Seger, R. (2019). Nuclear ERK: Mechanism of Translocation, Substrates, and Role in Cancer. *International Journal of Molecular Sciences*, *20*(5), E1194. https://doi.org/10.3390/ijms20051194

Park, H. S., Jang, M. H., Kim, E. J., Kim, H. J., Lee, H. J., Kim, Y. J., Kim, J. H., Kang, E., Kim, S. W., & Kim, I. A. (2014, January 1). High EGFR gene copy number predicts poor outcome in triple-negative breast cancer. *MODERN PATHOLOGY*, *27*(9), 1212–1222.

Pfitzer, L., Moser, C., Gegenfurtner, F., Arner, A., Foerster, F., Atzberger, C., Zisis, T., Kubisch-Dohmen, R., Busse, J., Smith, R., Timinszky, G., Kalinina, O. V., Müller, R., Wagner, E., Vollmar, A. M., & Zahler, S. (2019). Targeting actin inhibits repair of doxorubicin-induced DNA damage: A novel therapeutic approach for combination therapy. *Cell Death & Disease*, *10*(4), 302. https://doi.org/10.1038/s41419-019-1546-9

Poreba, E., & Durzynska, J. (2020). Nuclear localization and actions of the insulin-like growth factor 1 (IGF-1) system components: Transcriptional regulation and DNA damage response. *Mutation Research. Reviews in Mutation Research*, *784*, 108307. https://doi.org/10.1016/j.mrrev.2020.108307

SCUBE3 (Signal Peptide-CUB-EGF Domain-containing Protein 3) Modulates Fibroblast Growth Factor Signaling during Fast Muscle Development—Journal of Biological Chemistry*. (n.d.). Retrieved September 20, 2022, from https://www.jbc.org/article/S0021-9258(20)40481-8/fulltext

Shapira, I., Lee, A., Vora, R., & Budman, D. R. (2013). P53 mutations in triple negative breast cancer upregulate endosomal recycling of epidermal growth factor receptor (EGFR) increasing its oncogenic potency. *Critical Reviews in Oncology / Hematology*, *88*(2), 284–292. https://doi.org/10.1016/j.critrevonc.2013.05.003

Thisse, B., & Thisse, C. (2005). Functions and regulations of fibroblast growth factor signaling during embryonic development. *Developmental Biology*, *287*(2), 390–402. https://doi.org/10.1016/j.ydbio.2005.09.011

Wu, B.-T., Su, Y.-H., Tsai, M.-T., Wasserman, S. M., Topper, J. N., & Yang, R.-B. (2004). A Novel Secreted, Cell-surface Glycoprotein Containing Multiple Epidermal Growth Factor-like Repeats and One CUB Domain Is Highly Expressed in Primary Osteoblasts and Bones *. *Journal of Biological Chemistry*, *279*(36), 37485–37490. https://doi.org/10.1074/jbc.M405912200

Wu, Y.-Y., Peck, K., Chang, Y.-L., Pan, S.-H., Cheng, Y.-F., Lin, J.-C., Yang, R.-B., Hong, T.-M., & Yang, P.-C. (2011). SCUBE3 is an endogenous TGF-β receptor ligand and regulates the epithelial-mesenchymal transition in lung cancer. *Oncogene*, *30*(34), 3682–3693. https://doi.org/10.1038/onc.2011.85

Yang, M., Guo, M., Hu, Y., & Jiang, Y. (2013). Scube regulates synovial angiogenesis-related signaling. *Medical Hypotheses*, *81*(5), 948–953. https://doi.org/10.1016/j.mehy.2013.09.001

Yang, X., Hu, J., Shi, C., & Dai, J. (2020). Activation of TGF-β1 Pathway by SCUBE3 Regulates TWIST1 Expression and Promotes Breast Cancer Progression. *Cancer Biotherapy & Radiopharmaceuticals*, *35*(2), 120–128. https://doi.org/10.1089/cbr.2019.2990

Zakaria, Z., Zulkifle, M. F., Hasan, W. A. N. W., Azhari, A. K., Raub, S. H. A., Eswaran, J., Soundararajan, M., & Husain, S. N. A. S. (2019). Epidermal growth factor receptor (EGFR) gene alteration and protein overexpression in Malaysian triple-negative breast cancer (TNBC) cohort. *OncoTargets & Therapy*, *12*, 7749–7756. https://doi.org/10.2147/OTT.S214611

Zhao, C., qin, Q., Wang, Q., Zhang, J., Xu, Y., Li, W., Gu, M., Chen, S., & Deng, A. (2013). SCUBE3 overexpression predicts poor prognosis in non-small cell lung cancer. *Bioscience Trends*, *7*(6), 264–269.

Zhou, Z., Kennell, C., Jafari, M., Lee, J.-Y., Ruiz-Torres, S. J., Waltz, S. E., & Lee, J.-H. (2017). Sequential delivery of erlotinib and doxorubicin for enhanced triple negative Breast cancer treatment using polymeric nanoparticle. *International Journal of Pharmaceutics*, *530*(1–2), 300–307. https://doi.org/10.1016/j.ijpharm.2017.07.085