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Membrane Metalloendopeptidase (MME) positively regulates Phosphoinositide 3-Kinase (PI3K) signaling in triple negative breast cancer

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Introduction: Apart from skin cancers, breast cancer is the most common cancer diagnosis for women living in the United States. Breast cancer is commonly categorized into four subtypes based on shared molecular features: Luminal A, luminal B, HER2-enriched and triple negative. Of these, triple-negative breast cancers are the most aggressive and most difficult to treat. The lack of receptors commonly targeted in the other subtypes restricts targeted therapy for triple-negative breast cancers. Thus, better understanding the biology driving triple-negative breast cancers is an important ongoing question in cancer research.

Membrane metalloendopeptidase (MME), also known as neprilysin, is a cell-surface protease with broad substrate specificity toward multiple peptides including endothelin-1. While MME has been primarily studied in the context of its activity in the brain and cardiovascular system, it has also been implicated in several cancers. Our previously published research found that in some breast cancers epigenetic suppression of MME led to increased invasion and was associated with poor prognosis. In our current study, we asked whether MME's peptidase activity was associated with changes in primary cancer-related signaling pathways.

Pathogenic variants of the phosphoinositide 3-kinase (PI3K) pathway are common in breast cancer. PI3K is typically activated by receptors and leads to the phosphorylation and activation of the key pathway mediator, Akt. This pathway is negatively regulated by phosphatase and tensin homolog (PTEN). Phosphorylation by Akt of its numerous substrates has pleiotropic downstream effects on cell survival, protein synthesis, metabolism, proliferation and migration. However, results of clinical trials investigating the utility of drugs targeting this pathway in triple-negative breast cancer patients with PI3K gene variants have been mixed.

Here, we present evidence that MME positively regulates PI3K signaling in triple-negative breast cancer cells.

Methods: *Bioinformatics Analysis*- We merged breast cancer cell-line datasets from The Cancer Cell Encyclopedia (CCLE, Broad Institute) and breast tumor sample datasets from The Cancer Genome Atlas (TCGA, The National Cancer Institute) to consolidate genomic, proteomic and phosphoproteomic and phenotypic data. We first filtered triple negative breast cancers, then dichotomized tumor samples and cell lines by mean MME mRNA expression values from RNAseq data. We applied 2-tailed t-tests to reverse phase protein array (RPPA) values to determine which proteins were significantly differentially expressed between high- and low- MME breast cancer cell lines or tumor samples. Statistically significant proteins ($p<0.05$) identified from this screen were entered into the STRING database (string-db.org) to identify which pathways were most associated with MME mRNA expression differences in triple-negative breast cancers. The HMS LINCS database (lincs.hms.harvard.edu) was also used to analyze breast cancer cell line viability to pharmaceutical compounds.

Cell lines- MDA-MB-231 cells, BT-20 cells, HCC-38 cells, HCC-70 cells, MDA-MB-468 cells, HCC-1143 cells and HCC-1599 cells were all purchased from ATCC and grown according to ATCC recommendations. MDA-MB-468 cells were grown at room air in a humidified 37C incubator. All other cell lines were grown in a 5% carbon dioxide incubator at 37C.

Plasmids, siRNA, antibodies and inhibitors-MME (Myc-DDK-tagged) expression plasmid was ordered from Origene (RC223013). siRNA against MME was purchased from Thermo Scientific. DL-thiorphan was purchased from Cayman Chemicals. Endothelin-1 was purchased from Sigma Aldrich, reconstituted and diluted as instructed by the manufacturer and used at a concentration of 10 micromolar. Antibodies were purchased from Abcam (PTEN antibody, ab267787) or Cell Signaling (CD10/MME, 57451; pan Akt, 4691; phospho-Akt(Thr308), 13038). All chemicals and antibodies were reconstituted and diluted as instructed by the manufacturers.

RT-PCR- 500 ng RNA was used for reverse transcription and RT-PCR (Bio-Rad iScript, SYBR Supermix). The delta-delta CT method was used for analysis, and both inverse CT values (2^{-}DCt) and fold-change values were calculated and analyzed.

Western blotting- Cells were lysed using RIPA buffer and protease and phosphatase inhibitor cocktail according to manufacturer protocol (ThermoFisher). Western blotting, detection and amplification kits (Opti-4CN, Bio-Rad) were used throughout the blotting, according to manufacturer's protocol. Semi-quantitative analysis was conducted using ImageJ software and posted protocols for imaging blots.

Results: Bioinformatics analysis of TCGA breast cancer samples and CCLE breast cancer cell lines sorted by MME expression revealed that MME mRNA expression was significantly associated with protein expression changes in the phosphoinositol-3-kinase (PI3K) pathway in both triple negative breast cancer samples and cell lines. This finding prompted us to further investigate whether MME directly regulates PI3K signaling.

We first transfected the triple-negative breast cancer cell line MDA-MB-231 with MME expression plasmid and found a significant decrease in PTEN mRNA. Western blots of PTEN confirmed this change at a protein level as well. Similarly, inhibiting endogenous MME in HCC-38 cells with thiorphan, a small molecule MME inhibitor resulted in increased PTEN mRNA and protein levels. Immunohistochemistry analysis of breast cancer samples for MME and PTEN protein demonstrated that these proteins were largely expressed in a mutually exclusive manner at the cellular level.

To determine whether MME-induced PTEN alterations affected downstream PI3K signaling, we analyzed MME-transfected MDA-MB-231 cells for alterations in Akt phosphorylation using Western blots. We found that MME-transfected cells had increased active, phosphorylated Akt compared to control cells. These results were repeated with additional triple-negative breast cancer cells and inhibiting MME in HCC-38 cells with thiorphan resulted in decreased phospho-Akt levels.

Finally, we assessed the effect of MME activity on cell viability in the presence of a PI3K pathway inhibitor, GDC-0068. While we found no significant effect of GDC-0068 on PI3K-wildtype triple negative breast cancer cell lines regardless of MME expression, we observed that in the PI3K-variantcell line HCC-38, GDC-0068 inhibited viability as expected. However, inhibiting endogenous MME activity in these cells reduced the impact of GDC-0068 on cell viability. Analyzing cell line viability data from the LINCS HMS breast cancer dataset confirmed our laboratory result that MME expression associates with an improved response to GDC-0068 in triple-negative breast cancer cells with PI3K pathogenic variants.

Conclusion: Together, our data suggests that MME negatively regulates PTEN, and therefore positively regulates PI3K signaling, in triple negative breast cancer. Initial data predicts that MME may alter responsiveness to PI3K inhibitors in some breast cancers. These findings could ultimately improve the ability to predict TNBC response to therapies targeting the PI3K pathway.

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