TNFα and ZEB1 Contribute to Tumorigenesis in Chemo-Resistant Breast Cancer Cells

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ABSTRACT

Cancer genes are identified by their altered expression which induces uncommon phenotypic characteristics in a variety of cancers. The modified expression of these genes may promote the stimulation or development of a neoplasm, as oncogenes do, or may impede it, as do tumor suppressor genes. Breast cancer (BC) is the most prevalent malignancy in women and has been so over the past two decades. Comprehension of the biological pathways that lead to gene expression alterations and its association with therapy response is crucial for identifying novel biomarkers or mechanisms involved in therapy response. Analysis of a selection of genes regularly mis regulated in BC and associated with epithelial-mesenchymal transition (EMT), was performed utilizing the Cancer Genome Atlas (TCGA) and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) pathway analysis. Our results indicate upregulation of tumor necrosis factor α (TNFα) in chemo-sensitive and chemo-resistant BC cells, and upregulation of zinc finger E-box binding protein 1 (ZEB1) only in chemo-resistant cells. The gene expression alterations revealed by our cells also induced cellular proliferation upon chemotherapeutic treatment. Moreover, our results indicate that interaction between TNFα and ZEB1 encourages chemoresistance in BC cells. Being that TNFα and ZEB1 promote cell displacement and invasiveness through EMT stimulation, we herein propose that these genes employ such a pathogenic mechanism to render cells resistant to chemotherapeutic treatment. EMT is an overly complex, but also reversible event; therefore, further investigation of genes involved in the inhibition of TNFα and ZEB1 are an effective strategy for cancer therapy.

Introduction

BC is the predominantly detected type of cancer among American women (Breastcancer, n.d.). Clinically, this multifaceted disease is classified into three basic therapeutic subtypes: estrogen receptor (ER) positive, human epidermal growth factor receptor 2 (HER2) (also called ERBB2) amplified, and triple-negative breast cancers (TNBCs, do not express ER, progesterone receptor (PR), nor HER2) (Centers for Disease Control and Prevention, n.d.). Some subclassifications of BC lead to a poor prognosis for patients due to their absence of distinguishable molecular targets and their display of resistance to therapy (Yin et al., 2020). Metastasis, tumor relapse, and chemotherapeutic resistance are predominant causes of death for patients with BC (Yin et al., 2020). The absence of efficacious therapies brings attention to the need for an enhanced comprehension of the molecular mechanisms leading BC advancement (May et al., 2011). It is increasingly recognized that abnormal stimulation of EMT, a dormant embryonic program, can grant cancer cells the mobile and invasive abilities related to metastasis (May et al., 2011).

EMT is a key developmental event that allows polarized epithelial cells to be reprogrammed to portray mesenchymal characteristics (May et al., 2011). During an ordinary embryonic growth process, EMT acts as a dissolver of cell-cell connections and an enhancer of natural cell movement, thereby forming the basis for the vast mobility-related characteristics needed by cells for gastrulation and organogenesis (May et al., 2011). The EMT program is normally dormant in ordinary adult tissues, but it can be reactivated as wound mending and tissue regeneration occurs (May et al., 2011). However, if EMT is not regulated, the program can induce pathologic states, including organ

fibrosis and tissue destruction (May et al., 2011). Precisely, as previously stated, epithelial cancer cells may gain characteristics needed for metastasis, namely, migration and invasiveness; these traits are induced by the EMT program (May et al., 2011). This extensive transition from epithelial cellular characteristics to mesenchymal ones is driven by the collaboration of signal transduction pathways and transcription factors (TFs) associated with EMT, which regulate the expression of genes that contribute to cell-cell communication and cell development and mobility (May et al., 2011).

Methods

Gene Analysis

The Cancer Genome Atlas (TCGA) is a publicly accessible online platform to obtain a list of related genes to BC. Moreover, DAVID was utilized for functional pathway analysis of the selected genes from TCGA. DAVID is a bioinformatics online database that is used to analyze gene lists derived from genomic experiments. It systematically extracts biological meaning from large gene and or protein lists. First, a gene list containing several common gene identifiers was uploaded. Next, an analysis using one or more text- and pathway-mining tools was performed.

Cell Culture

The chemo-resistant breast mammary epithelium (BME) is a cell line isolated from the mammary tissue of a female mouse; this cell line overexpresses oncogene Neu (American Type Culture Collection, n.d.-b). The chemo-sensitive CAMA-1 cell line was procured from a 51-year-old white female affected with breast adenocarcinoma; the cells, which were particularly obtained from the woman's pleural effusion, behave as epithelial cells (American Type Culture Collection, n.d.-a). **Figure 1** illustrates both cell lines under the microscope. The BME and CAMA-1 cryovials were obtained from ATCC; they were rapidly thawed at 37°C and placed in 100 mm Petri dishes supplemented with Dulbecco's Modified Eagle Medium (DMEM) medium with sodium bicarbonate, 10% fetal bovine serum (FBS), and 1% antibiotics. The cell cultures were maintained at 37°C in a CO_2 incubator. When both cell lines reached confluency, subculturing was performed. For splitting the cell lines, Phosphate Buffered Saline solution (PBS) was used to wash the cell; trypsin was used for detachment. Subsequently, the cells were centrifuged and plated in different well plates for further analysis and assays.

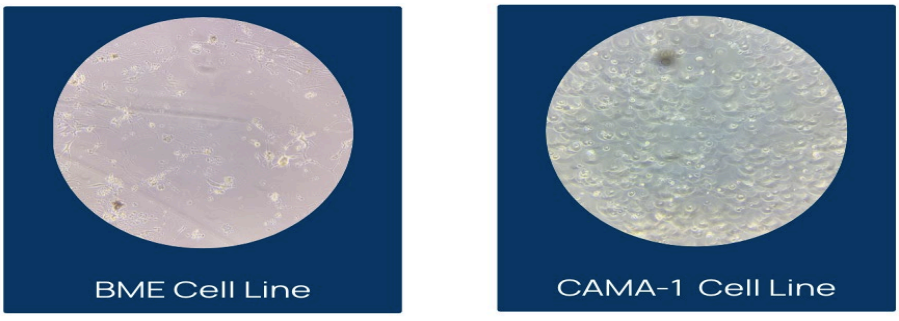

Figure 1. BME and CAMA-1 Cell Lines**.** These figures represent the two cell lines used; the pictures were taken using an inverted microscope at 10X. Both cell lines were obtained from ATCC.

Chemotherapeutic Treatment

The chemotherapy drug carboplatin was obtained from Sigma-Aldrich. Samples of BME and CAMA-1 cells were treated with 100 µg/ml of carboplatin and incubated for two days. Treated cells were designated as BME (+) and CAMA-1(+). Accordingly, untreated cell samples were referred to as BME (-) and CAMA-1(-). The control cell line was CAMA-1.

DNA Extraction and Gene Amplification

Genomic DNA Kit by IBI Scientific was used to extract the DNA from the four samples. In addition, SYBR®-Green PCR was employed to make copies of specific genes in the extracted DNA speedily. Amplification was performed using Step One Plus ™ Real-Time Polymerase Chain Reaction (RT-PCR) system (Applied Biosystems; Thermo Fisher Scientific, Inc.), RNA primers, and temperature hydrogen bond control. Samples were made with 20µl of DNA and iTaq Universal SYBR Green Supermix (which contains DNA polymerase, dNTPs, MgCl2, KCl, and other stabilizers). Primers were optimized for melting temperatures, and PCR was initiated according to the standard conditions: primary destruction of protein characteristics at 95℃ for thirty seconds, accompanied by 30 cycles of 95℃ for thirty seconds, 50℃ for 30 seconds, and 64℃ for 60 seconds. The samples were maintained at 4℃ until analyzed on a 0.7% agarose gel, stained with ethidium bromide. All PCR reactions were carried out on the CFX-96 Bio-Rad RT System in triplicate. Gene amplification was confirmed upon recognition of a single band. The specific primers are exhibited in Table 1.

Functional Analysis Using Ros-Glo, CCK-8, Caspase-3, and MTT

Ros-Glo: Oxidative stress levels were obtained from BME (+) and BME (-) cell samples utilizing the ROS-Glo H_2O_2 assay. Oxidative stress levels are indicated by the amount of hydrogen peroxide (H_2O_2) produced by a cell sample. H_2O_2 is a reactive oxygen species that arise due to the disproportion in the oxidation to antioxidant enzyme action (oxidation increases while antioxidant enzyme action decreases) in cells (Cheng et al., 2022). Furthermore, H_2O_2 induces apoptosis in cells by increasing oxidative stress levels.

CCK-8: Cell viability was evaluated using Cell Counting Kit 8 (CCK-8) by Dojindo. This assay contains WST-8, which creates a yellow dye when reduced by cell dehydrogenase activities (Dojindo, n.d.). The user manual states that the amount of dye produced is proportional to the number of viable cells. The four samples were plated in a 96-well plate. According to the protocol, each well was treated with 10 µL of CCK-8, then incubated for 1 to 2 hours (Dojindo, n.d.). Furthermore, the absorbance was measured at 450 nm.

Caspase-3: Caspase-3 Assay Kit (Colorimetric) by Abcam was employed to analyze cell apoptotic activity; this assay contains a DEVD sequence coupled with a DNA fluorophore molecule called p-nitroaniline (p-NA) (Abcam, n.d.-a). Once inside the apoptotic cell, the Caspase-3 protein recognizes and cleaves the substrate DEVD-pNA; the DNA probe enters the nucleus and binds to the DNA producing a bright fluorescent signal (Abcam, n.d.-a). According to the user's manual, 50 µL of the cell sample was placed in a 96-well plate; then, 50 µL of reaction buffer and 5 µL of DEVD-p-NA substrate were added to each well. The samples were incubated for 1 to 2 hours; the OD was measured at 400 nm.

MTT: The four cell groups were evaluated via MTT assay. MTT is a colorimetric assay to determine cell viability. The MTT molecule can be reduced to formazan and produce a purple color; this reduction only occurs in viable cells when mitochondria oxidoreductase enzymes are present (Abcam, n.d.-b). The four cell samples were plated in a 96-

well plate. Following the protocol, the medium was removed from each well and the cells were washed with the PBS solution (Abcam, n.d.-b). In addition, the cells were incubated for 95 minutes in a 50 µL of medium and 50 µL of MTT reagent. The absorbance was taken at 590 nm.

Table 1. Primers for Gene Amplification

Name	Primer Sequence
Caspase-3	5′-TGACTGGAAAGCCGAAACTC 3′ 5′-AGCCTCCACCGGTATCTTCT-3′
Caspase-8	5′-CCGAGCTGGACT TGTGACC-3′ 5′- CTGCCCAGTTCTTCAGCAAT-3′
BIM	5′-GGCTCAGCTACGCCTTCTC-3′ 5′-TCCTTCTCTGGAAACAATGACA-3′
TWIST-1	5′-GGCTCAGCTACGCCTTCTC-3′ 5′-TCCTTCTCTGGAAACAATGACA-3
HPRT	5′-CTTCCTCCTCAGACCGCTTT-3′ 5′-TTTCCAAATCCTCGGCATAA-3′
TNFα	5’-CTCTTCTGCCTGCTGCACTTTG-3’ 5’ATGGGCTACAGGCTTGTCACTC-3’
CAV1	5’-CCAAGGAGATCGACCTGGTCAA-3’ 5’-GCCGTCAAAACTGTGTGTCCCT-3’
FZD1	5'-GCTTTGTGTCGCTCTTCCGCAT-3’ 5’-TACAGCACGCTGAAGACGCCAA-3
ZEB1	5’-ATTCAGCTACTGTGAGCCCTGC-3’ 5’-CATTCTGGTCCTCCACAGTGGA-3
Actin	5’-CACCATTGGCAATGAGCGGTTC-3 5’-AGGTCTTTGCGGATGTCCACGT-3
FFBCL2	5’-ATCGCCCTGTGGATGACTGAGT-3’ 5-'GCCAGGAGAAATCAAACAGAGGC-3'
CCNE1	5’-TGTGTCCTGGATGTTGACTGCC-3’ 5’-CTCTATGTCGCACCACTGATACC-3’
KLF4	5’-CATCTCAAGGCACACCTGCGAA-3’ 5’-TCGGTCGCATTTTTGGCACTGG-3’
GSK3β	5'-CCGACTAACACCACTGGAAGCT-3' 5’-AGGATGGTAGCCAGAGGTGGAT-3'

Results

ZEB1 and TNFα are Upregulated in Chemo-resistant Breast Cancer Cell

The activity of genes responsible for breast cancer and EMT in our breast cancer cell lines were evaluated by performing RT-PCR. Agarose gel stained with ethidium bromide was used to confirm the upregulation of the gene. Gene amplification was confirmed upon recognition of a single band. Table 2 and **Figure 2** represent the RT-PCR results. The results indicate that actin (ACT), the control group, was amplified in the four cell lines. In addition, TNFα was upregulated in all the cell lines, while only the BME cell line showed an increase in ZEB1 expression.

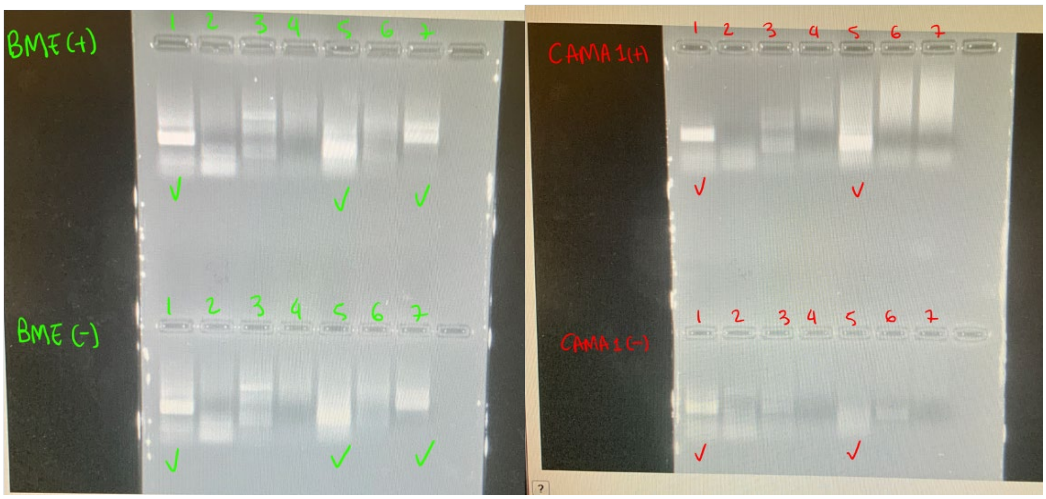

Figure 2. RT-PCR results. This figure represents the outcomes from the RT-PCR. Gene amplification was confirmed upon recognition of a single band.

Table 2. RT-PCR Results Summary.

	1 ACT	2 CAV2	3 GSK3β	4 KFL4	5 TNFα	6 TWIST-1	7 ZEB-1
CAMA-1(+)	✓				✓		
CAMA-1(-)	✓				✓		
BME (+)	✓				✓		✓
BME (-)	✓				✓		✓

The check symbol indicates an upregulation of the gene present in the cell sample.

Functional Analyses Suggest ZEB1 and TNFα Promote Chemoresistance in Breast Cancer Cell Lines

The ROS-Glo H_2O_2 assay was performed to determine the level of oxidative stress in the cell samples; this measurement is directly proportional to the amount of H_2O_2 produced by a cell sample. The average H_2O_2 luminescence readings for each of our samples are shown in Table 3 and **Figure 3**. The sample DMEM had the highest amount of H_2O_2. Both BME (+) and BME (-) cell samples had high levels of H_2O_2 activity, and there was a minute difference between the treated and untreated cell samples. This assay was not performed on the CAMA-1 cell line.

Table 3. ROS-Glo H_2O_2 assay results.

Cell Line	Average Reading	H2O2	Luminescence
BME (+)	1291.9375		
BME (-)	1384.4375		
DMEM	1783.5625		

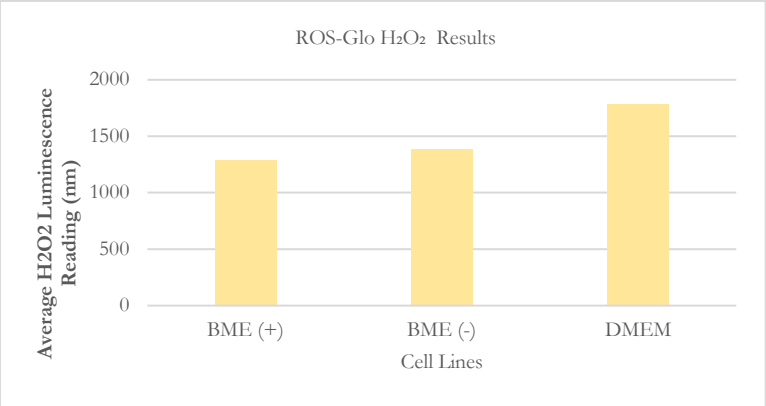

Figure 3. ROS-Glo H_2O_2 assay results. The outcome demonstrates that DMEM had the highest absorbance value. Both BME (+) and BME (-) cell samples had high levels of H_2O_2 activity.

CCK-8 and MTT assays were carried out to further investigate the survival and proliferation of the breast cancer cells. According to the CCK-8 user manual, the amount of dye produced is proportional to the number of viable cells. Table 4 and **Figure 4** illustrate the results from the CCK-8 assays. After treating the cells with the CCK-8 reagent, the results revealed that CAMA-1 (+) had a higher number of viable cells than CAMA-1 (-). Also, BME (+) had fewer viable cells than BME (-). However, the four cell lines had similar absorbance values ranging from 0.1800 to 0.2200 nm. The CCK-8 results demonstrate a non-statistically significant difference between BME and CAMA-1 cell lines treated with chemotherapy versus the non-treated. MTT is an assay used to assess metabolic activity in cells. The assay's protocol states that higher absorbance correlates with a higher number of viable cells. Table 5 and **Figure 5** exhibit the results obtained from the MTT assay. These results indicate that BME (-) and CAMA-1 (-) had the highest cellular metabolic activities (this comparison being between each individual cell line), thereby indicating a greater number of viable cells in these samples. On the contrary, BME (+) and CAMA-1 (+) showed lower metabolic activity.

Table 4. CCK-8 assay results.

	CCK-8 Absorbance Value (nm)			
Cell Line	**1**	**2**	**3**	**Mean**
CAMA-1 (-)	0.18	0.177	0.197	0.184667
CAMA-1 (+)	0.181	0.222	0.18	0.194333
BME (-)	0.214	0.215	0.218	0.215667
BME (+)	0.177	0.172	0.173	0.17400

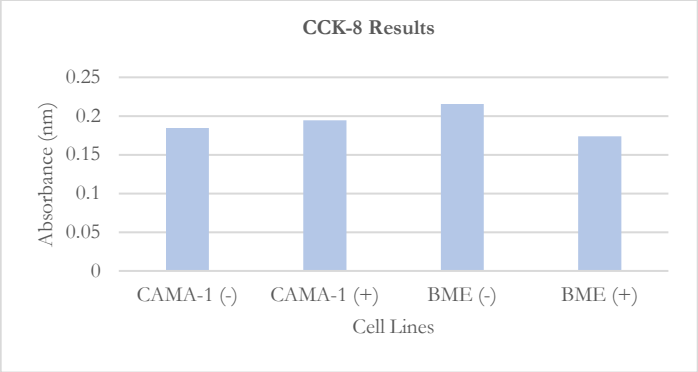

Figure 4. CCK-8 assay results. This outcome indicates that CAMA-1(+) had a higher number of viable cells than CAMA-1(-). On the other hand, the non-treated BME cells had a higher number of cells than treated BME cells.

Table 5. MTT assay results.

	Absorbance Value				
Cell Lines	**1**	**2**	**3**	**Mean**	**SD**
BME (+)	0.2133	0.00554	0.05753	0.092123	0.10811373
BME (-)	0.2217	0.06827	0.0921	0.127356	0.08256794
CAMA-1 (+)	0.16	0.05967	0.07463	0.0981	0.05412631
CAMA-1 (-)	0.2063	0.04607	0.0579	10344	0.08928994
DMEM	0.189	0.04137	0.09361	0.09361	0.08273248

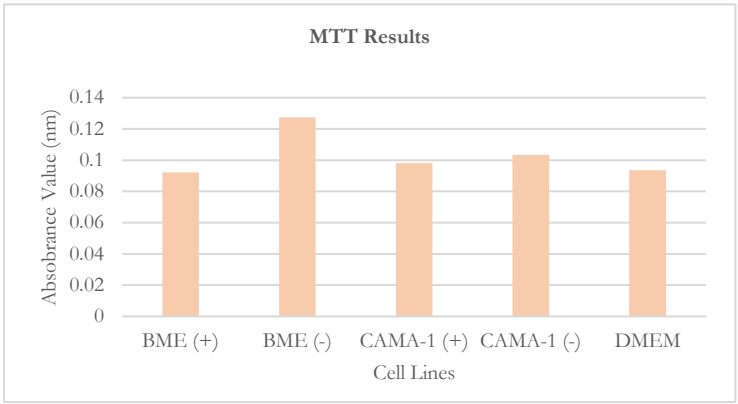

Figure 5. MTT assay results. Both figures represent the results after treating the cells with the MTT assay. BME (-) and CAMA-1(-) had the highest absorbance value, indicating a higher metabolic activity. On the other hand, BME (+) and CAMA-1(+) showed lower metabolic activity.

The Caspase-3 assay was employed to analyze cell apoptotic activity in the four cell lines. Table 6 and Figure 6 demonstrate the results from this assay. The user's manual states that higher absorbance corresponds to a higher level of apoptotic activity. The chemo-sensitive cell lines were expected to have higher Caspase-3 activity. The results indicate that CAMA-1 (+) had a higher apoptotic activity than CAMA-1 (-). On the other hand, BME (-) had a slightly

higher apoptotic activity than BME (+). CAMA-1 (+) had the greatest apoptotic activity among the four cell lines; this indicates that BME (+), despite being treated with carboplatin, did not undergo Caspase-3-initiated apoptosis.

Table 6. Caspase-3 assay results.

	Absorbance Value			
Cell Lines	**1**	**2**	**Mean**	**SD**
BME (+)	0.1237	0.1115	0.1176	0.00863
BME (-)	0.1299	0.1156	0.1227	0.01011
CAMA-1 (+)	0.1325	0.1583	0.1454	0.01824
CAMA-1 (-)	0.128	0.1164	0.1222	0.0082
DMEM	0.1107	0.1007	0.1057	0.00707

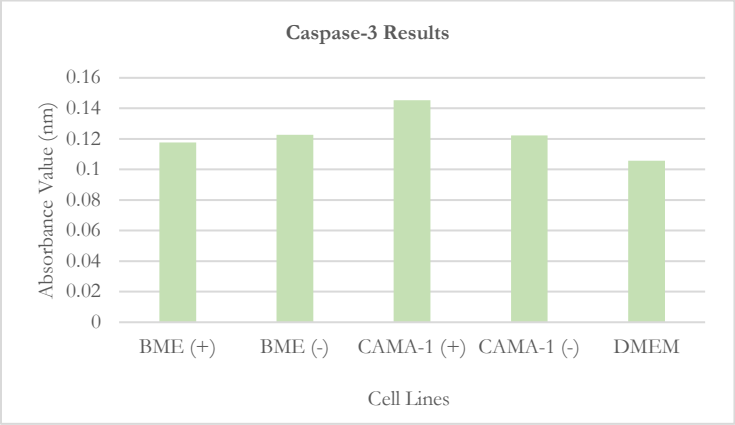

Figure 6. Caspase-3 assay results. This figure demonstrates the results from the Caspase-3 assay. CAMA-1(+) had a higher level of apoptotic activity than CAMA-1(-). BME (-) had a slightly higher apoptotic activity than BME (+). Additionally, the results indicate that CAMA-1(+) had the greatest apoptotic activity among the four cell lines.

Discussion

The most common type of cancer is breast cancer, with approximately 290,000 estimated new cases in the United States in 2022 (New Mexico Department of Health, 2022). A report by the American Cancer Society submits that BC is the second leading cause of cancer death among women in the United States (Breastcancer, n.d.). Principal causes of death for patients with breast cancer include metastasis, tumor relapse, and chemo-resistance. Our research targets chemo-resistance as a gateway to better understand breast cancer. The activation of EMT can increase chemo-resistance in cancer cells (Ashrafizadeh et al., 2020). EMT is a process in which epithelial cells undergo phenotypic changes and acquire mesenchymal characteristics (Ribatti et al., 2020). In addition, gene expression alterations contribute to the occurrence of EMT (Wang et al., 2013). Therefore, this investigation aims to improve the comprehension of the role which gene expression alteration and EMT play in BC; this ameliorated understanding will aid to develop new strategies that will reduce chemo-resistance of BC cells and promote a good prognosis for patients with BC.

The PCR results using chemo-sensitive and chemo-resistant breast cancer cells indicate that TNFα was up-regulated in all the cell samples and ZEB1 was upregulated in the chemo-resistant BME cell line only. TNFα is a cytokine that promotes inflammatory responses and is upregulated in multiple cancers (Mercogliano et al., 2020). Particularly in breast cancer, TNFα is associated with growth of neoplasm cells, elevated disease severity, augmented spreading of the disease to other sites in the body, and a poor disease outcome for the patient (Mercogliano et al., 2020). ZEB1 is a transcriptional regulator that is also recognized in cancers, and aids in the initiation of a variety of developmental pathways and neoplasm escalation to other body parts through EMT; more specifically, ZEB1 suppresses epithelial genes as EMT occurs–an event which, as previously stated, increases chemoresistance in tumoral cells (Ashrafizadeh et al., 2020; Perez-Oquendo & Gibbons, 2022).

To better comprehend the regulatory role that both TNFα and ZEB1 have in the survival and proliferation of breast cancer cells after carboplatin treatment, multiple in-vitro assays were performed. Carboplatin forms reactive platinum complexes inside the cells causing strand cross-linkage of DNA; this process affects DNA structure and inhibits DNA synthesis (Rabik & Dolan, 2007).

The ROS-Glo assay measures oxidative stress in cells, the results of which were obtained after two days treatment. Our assay results show that the BME (+) cells produced intracellular H_2O_2, thereby indicating that this cell sample was undergoing oxidative stress. ROS can be augmented by the action of TNFα in excessively activating reduced nicotine adenine dinucleotide phosphate (NADPH), thereby increasing oxidative stress (Lee & Yang, 2012).

The survival and proliferation of the breast cancer cells were further analyzed utilizing the CCK-8 and MTT assay results. The CCK-8 results reveal that the dissimilarity between BME and CAMA-1 cell lines treated with chemotherapy versus the non-treated, is non-statistically significant. Overall, there was not much change in the absorbance values between samples. Due to its classification as a chemo-resistant cell line, BME cell samples were anticipated to have a higher number of viable cells; however, this effect was not observed after 2-days treatment with carboplatin. Possibly, treatment for a longer time interval will increase BME cell survival.

The MTT assay showed that BME (-) and CAMA-1(-) had the highest cellular metabolic activities pertaining to their individual cell lines, indicating that those cell samples were the most viable. BME (+) and CAMA-1(+) showed less cellular metabolic activity, suggesting that these cell samples underwent less proliferation and were thus the least viable. Overall, there was a minute change in metabolic activity between the cells treated with and without carboplatin. Due to its chemoresistance, the BME (+) sample was expected to resist carboplatin treatment and thereby have greater cell viability than BME (-); but this effect was not observed in the results. This outcome implies that TNFα and ZEB1 interaction in these cell samples may have been hindered by human error, environmental and cultural factors such as nutrient depletion, waste product accumulation, and temperature variations, thereby impeding the cells' chemo-resistant characteristics. More trials incorporating a longer treatment time interval, or a higher carboplatin concentration may procure alternate results.

Apoptosis is highly regulated by a series of proteins that play a role in either inhibiting or promoting cell death. Caspase-3 is an initiator enzyme among these proteins, expressed by a family of proteins recognized for their proteolytic roles in the execution of apoptosis (Eskandari & Eaves, 2022). Studies suggest that cancer treatment, such as carboplatin, induces Caspase-3 activity. Therefore, Caspase-3 levels can be used as a sign of apoptosis and an indicator of efficacy in cancer treatment. Our Caspase-3 results indicate that CAMA-1(+) had the highest level of caspase-3 enzymatic activity among the cell samples. On the other hand, BME (-) had the lowest amount. These results suggest that the carboplatin treatment in the CAMA-1 cell line significantly increased caspase-3 activity. Moreover, the chemo-resistant cell line BME (+) did not undergo caspase-3-initiated apoptosis despite being treated with carboplatin.

Conclusion

Taken together, our results suggest that TNFα and ZEB1 interaction promotes chemoresistance in breast cancer cell lines; this interaction may also influence EMT activation and hijacking inflammation. Inflammation is a hallmark of

cancer as this event is a risk factor for the disease and assists cancer development (Greten & Grivennikov, 2019). TNFα and ZEB1 are markers of pro-inflammation through the induction of EMT; our results therefore indicate that these genes undertake such a course of pathogenesis to render breast cancer cells resistant to chemotherapy treatment. The upregulation of ZEB1 in the BME cell line only, which the Caspase-3 assay showed to be chemo-resistant, correlates with our idea that cancer cell chemoresistance is influenced and guided by TNFα and ZEB1 cooperation via EMT induction.

Personalized medicine is an emerging practice that uses information about an individual's genes and proteins to prevent, diagnose, and treat disease (National Human Genome Research Institute, n.d.). This approach can be used to analyze DNA from patients' tumors to identify the mutations or other genetic changes that drive their cancer (e.g., increased expression of ZEB1) (Psomagen, 2022). Our research is important because it implies carboplatin may not be suitable for patients with TNFα and ZEB1 upregulation. Physicians may therefore employ alternative platinum-based therapies, such as cisplatin or oxaliplatin, for patients with breast cancer who have an increased expression of TNFα and ZEB1. Physicians can also utilize personalized treatments that may improve prognosis of patients with TNFα and ZEB1 upregulation, including target therapy, immunotherapy, and photodynamic therapy. Further research targeting TNFα and ZEB1 would lead to an effective tactic to enhance BC prognosis, repress tumor metastasis, and reduce cancer recurrence.

Limitations

Time limitations were a hindrance on the study herein presented. This experiment was performed in four months; as such, the cell treatment time intervals may have been insufficient for the MTT and CCK8 assays to reveal significant outcomes. Moreover, time restrictions only permitted that one trial was performed employing each assay mentioned in the study. Additional trials would have contributed to the validation of our findings.

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