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Determination of Some Epithelial-to-Mesenchymal Transition (EMT) Activation Traits and Mesenchymal Features of an Acquired 5-Fluorouracil (5-FU) Resistant MCF-7 Breast Cancer Cell line

Erebi Patricia Tawari

Department of Chemical Pathology, Faculty of Basic Clinical Sciences, College of Health Science, Niger Delta University, Bayelsa State, Nigeria

A B S T R A C T

Mesenchymal flexibility in breast cancer includes the acquisition of mesenchymal traits and reepithelialization to form an organized adjuvant mass at a metastatic location by epithelial carcinoma cells inside a primary tumour. This plasticity has effects on the development of breast cancer causes metastasis and can potentially impact the response to treatment. The objective of this study was to determine the invasive features and migration traits of acquired resistant MCF-75FU10µM breast cancer cell lines. Data attained from this study showed that the resistant cell lines MCF-75FU10µM (1st Generation and 5th Generation) exhibited greater migratory and invasive activities than the wild-type cell line MCF-7 (control). This implies that invasive and migrating traits could be induced via acquired resistance.

Keywords: Epithelial-to-Mesenchymal Transition, Acquired resistance, MCF-7 breast cancer, 5-fluorouracil.

1.0 Introduction

One of the principal cause of mortality in patients with breast cancer is developing metastases (Van Zijil et al., 2011). Breast carcinoma is the most commonly detected cancer in women and ranks second in terms of cancer-related fatality in females (DeSantis et al., 2019; Siegel et al., 2019). The epithelial–mesenchymal transition (EMT) phenomenon is a reversible procedure in which epithelial cells mislay their appearances and develop mesenchymal cell traits, with altered expression of cell adhesion molecules and cytoskeleton. According to Lambert et al., (2017) and Moustakas and de Herreros (2017), EMT is a biological process that is significant and can be categorized into three types: the first kind typically happens in embryonic growth, the next kind is related with adult tissue regeneration, and the last kind occurs during the evolution of cancer.

The evolution of EMT includes alterations in cell-matrix linkage, cytoskeleton remodeling, and disruption of cell-cell adhesion and cellular polarity. It is associated with improved invasive and migrating characteristics (Micalizzi et al., 2010). EMT inducers in cancer include stroma crosstalk, metabolic alterations, innate and adaptive immunological responses, hypoxia, cytokines, and growth factors released by the cancer microenvironment, as well as antitumor medication treatment. Because the EMT process is typically not finished in cancer cells, the tumor cells have diverse epithelial and mesenchymal genes and are in numerous transitional phases. Equated to cells with a whole EMT phenotype, these crossbreed cells in partial EMT have the ability to wander in groups and exhibit more aggressiveness (Jolly et al., 2015). The cells thus acquire motility-invasive characteristics that enable them to change between mesenchymal and epithelial states in a highly flexible and dynamic way. EMT causes epithelial cells to become migratory and invasive, losing their epithelial cell traits in favor of a mesenchymal phenotype.

In the course of developing carcinoma, epithelial cells dissociate from the main cancer, attach to and infiltrate the adjacent stroma, enter blood arteries, and travel to other tissues and organs where they can spread and develop into secondary tumors. The mechanisms necessary for epithelial cells from primary tumors to metastasize are very similar to those of cells going through EMT at the site of arrest, followed by a change from mesenchymal to epithelial (Chaffer et al., 2007; Thiery, 2003).

In EMT, adherent junctions are lost, cytokeratins and E-cadherin, which are epithelial-specific markers, are downregulated, and mesenchymal markers like fibronectin, N-cadherin, and vimentin are upregulated. This results in a fibroblastoid invasive phenotype and resistance to anoikisis and apoptosis (Garside et al., 2012; Micalizzi et al., 2010; Wells et al., 2008). One characteristic of carcinoma EMT is the forfeiture of E-cadherin, which is required for cells to acquire invasive characteristics. This is associated with an upsurge in the expression of the mesenchymal marker N-cadherin. Numerous malignancies' enhanced invasiveness and metastasis are correlated with vimentin overexpression (Wang Alan et al., 2015, Vergara et al., 2016).

1.1 Justification and Study Objectives

This study identified some Epithelial-to-Mesenchymal Transition (EMT) activation features of the acquired resistant cell line MCF-$7_{5FU10\mu M}$. A transwell invasion assay (Boyden chamber assay) was utilized to study the invasive and migratory properties of the cells and an *in vitro* wound-healing assay (scratch assay) for migration. The EMT biomarkers, vimentin, E-Cadherin, and N-cadherin of the resistant cells were examined Western blot analysis.

2.0 Methodology

2.1 Cell lines and reagents:

The MCF-7 breast cancer cell line was used to produce the resistant cell line MCF-$75_{FU10\mu M}$ was bought from ATCC, Middlesex, UK. Using a stepwise concentration-increasing technique, the MCF-7 breast cancer wild type cell line was continuously and repeatedly grown in media containing 5-fluorouracil (5-FU) (Sigma, Dorset, UK).

2.2 Migration assay

A popular and inexpensive technique for studying cell migration is the scratch or wound healing assay. $0.5x10^6$ cells of MCF-$7_{5FU10\mu M}$ test cells and wild-type MCF-7 cells control cells were sowed in a 6-well plate with 500µL of 10% FBS-containing RPMI media for 24 hours at 37°C and 5% CO_2. The scratch or wound healing assay is a popular and inexpensive way to study cell migration. $0.5x10^6$ cells of MCF-$7_{5FU10\mu M}$ test cells and wild-type MCF-7 cells control cells were planted in a 6-well plate with 500µL of 10% FBS-containing RPMI medium for 24 hours at 37°C and 5% CO_2. To ensure that a cell monolayer had grown properly, cells were visually inspected one day later (Liang et al., 2007). Using a 200ul plastic pipette tip, a small, linear area of cells is scraped off to form a "wounded." After discarding the FBS-containing media, 500µL of 10% FBS-containing RPMI medium and 5% glutamine were added to each well to ensure that any floating dead cells were removed. Cells were then subjected to fresh media containing 5% glutamine and 1% PenStrep.

A snapshot of the scratch area was taken at 0 hours, 24 hours, and 48 hours. Under a microscope, the cells may be seen moving from the uninjured portions into the scratched areas. The percentage migration was analysed using the image J software to follow the exposed area's drop over time until the "wound" is closed. Single cells, loosely connected populations (mesenchymal cells), or sheets of cells (epithelial cells) can all be seen as a result of cell migration.

2.3 Invasion assay

The cell culture inserts were nurtured for one hour at room temperature after 300 µl of warm, serum-free media was introduced to rehydrate the basement membrane layer. Following the conclusion of the incubation period, the media was cautiously discarded. 300 µl of MCF-$7_{5FU10\mu M}$ test cells and a suspension of wild-type MCF-7 cells control cells with $0.5–1x10^6$ cells/ml in serum-free media were placed into each implant. The plate was filled with 500 µl of media that contained 10% fetal bovine serum in the lowest well. Following a 48-hour incubation period, the medium was cautiously extracted from the implant. Moist cotton-tipped swabs were used to get rid of cells from the interior of the inserts.

Following that, 400 µl of cell stain solution was added to a well containing the insert, and it was left to remain at room temperature for ten minutes. After a couple mild washes in a beaker of water, the inserts were air dried. The inserts were placed in a well with 200 µl of extraction solution and nurtured for 10 minutes in an orbital shaker. After adding 100 µl of each sample to a 96-well plate, the optical density of each sample was measured at 560 nm.

2.4 Western blotting analysis:

Staining using primary antibodies and appropriate HRP conjugated secondary antibodies allowed for the determination of the protein expression levels. Santa Cruz, Dallas, Texas, USA, provides the primary antibodies for the EMT markers vimentin, E-Cadherin, and N-cadherin.

2.5 Determination of EMT characteristics in Acquired resistant cells MCF-$7_{5FU10\mu M}$

EMT indicators vimentin, E-Cadherin, and N-cadherin were identified by western blot in order to investigate the epithelial to mesenchymal transition (EMT) in MCF-$7_{5FU10\mu M}$ cells. The invasive and migratory characteristics of the cells were also assessed using the transwell invasion test (Boyden chamber assay) and the *in vitro* wound-healing assay (scratch assay) for migration.

3.0 Results

3.1 EMT Markers in Acquired resistant cells MCF-7$_{5FU10\mu M}$

When compared to the parent cell MCF-7, which served as the control, the acquired resistant cells MCF-7$_{5FU10\mu M}$ (1st Generation and 5th Generation) were found to exhibit increased expression of the EMT markers vimentin and N-cadherin, along with a corresponding decrease in E-cadherin expressions. This cadherin switch suggests the cells acquired a more mesenchymal feature (Figure 1). A loading control was applied using tubulin.

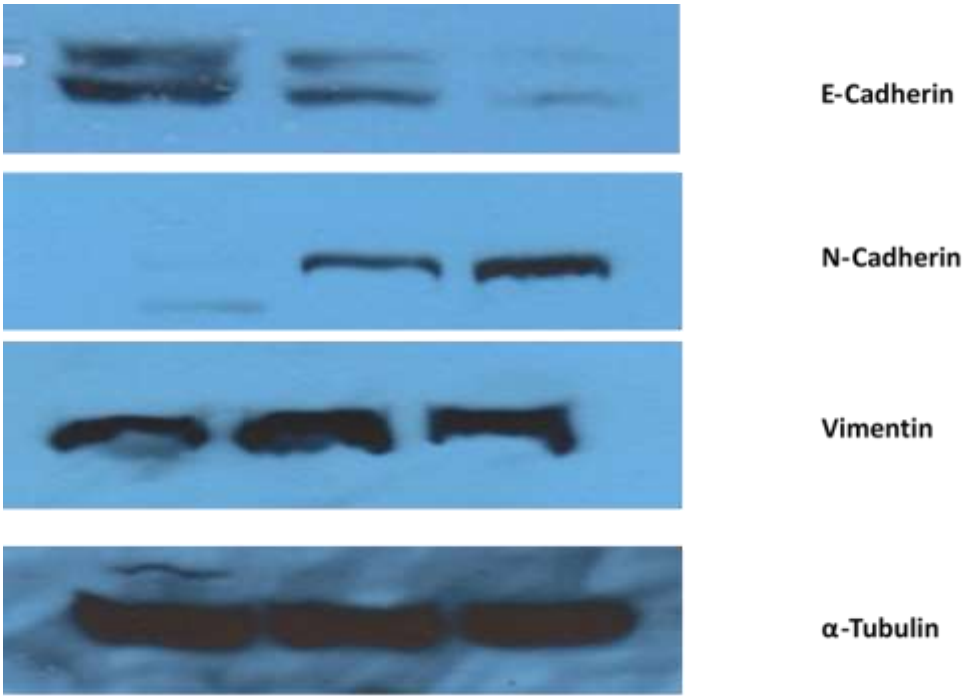

Figure1: Western Blots of EMT markers (vimentin, E-Cadherin, and N-cadherin) in MCF-7 wild-type (WT) control cells and acquired resistant MCF-7$_{5FU10\mu M}$ (1st Generation and 5th Generation) cells (α-tubulin was the loading control).

3.2 Transwell Invasion Assay of the Resistant Cell lines

Images from the matrigel invasion assay (Figure 2) showed that the acquired resistance cells MCF-7$_{5FU10\mu M}$ (1st Generation and 5th Generation) were more invasive than the wild-type MCF-7 cells.

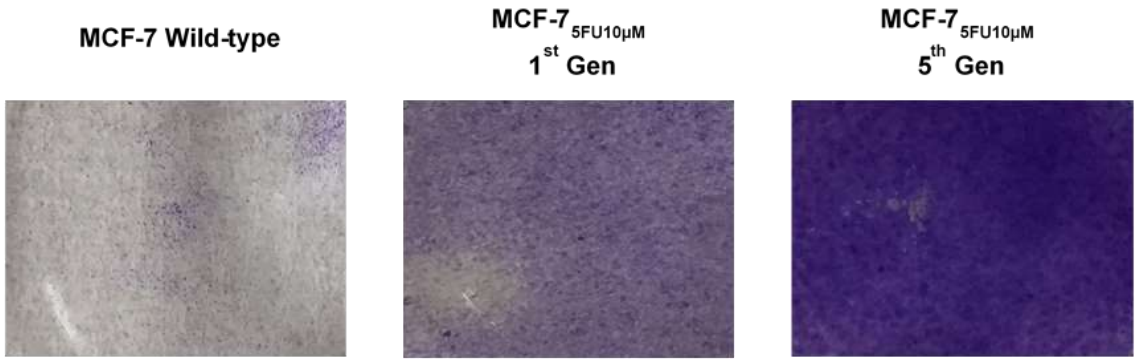

Figure 2. Representative Matrigel Invasion assay Images of MCF-7 wild-type (WT) control cells and acquired resistant MCF-7$_{5FU10\mu M}$ (1st Generation and 5th Generation) cells.

3.3 Migration assay of the Resistant Cell lines

Pictures (20X magnification) captured under a light microscope from the wound healing scratch experiment (Figures 3). The resistant cells MCF-7$_{5FU10\mu M}$ displayed increased migratory properties after 48 hours, as the scratch test revealed that the resistant cells' wound healing happened more quickly when compared to the wild-type MCF-7 control cells. According to this, the resistant cells developed a more mesenchymal appearance.

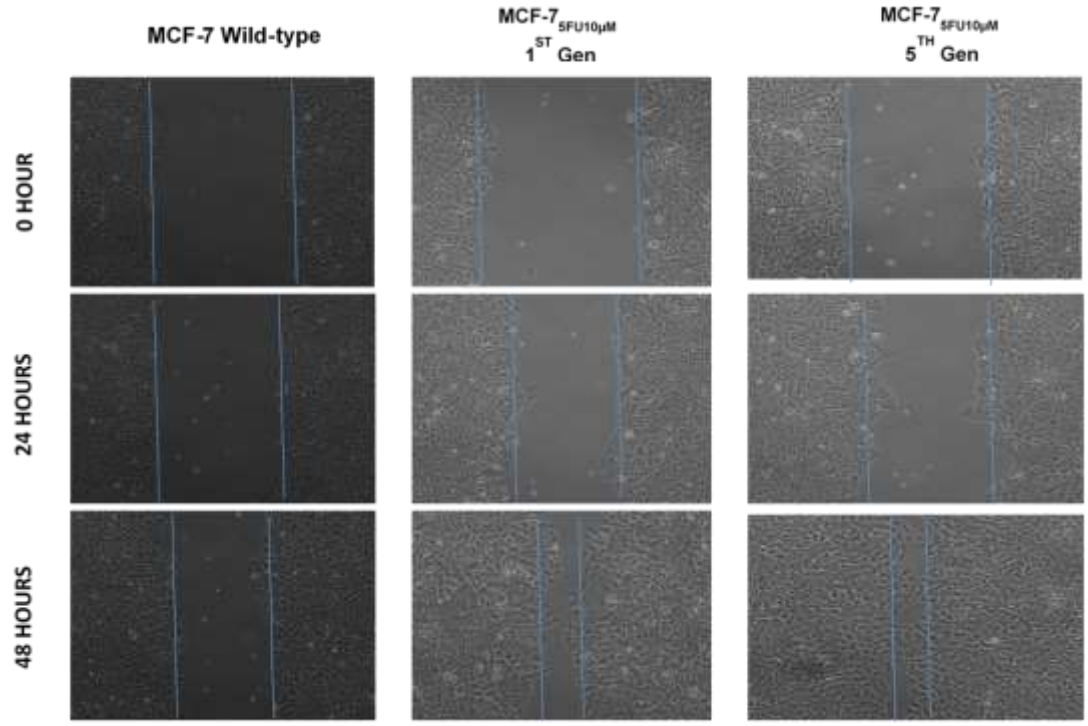

Figure 3. Representative Migration (wound healing) assay Images of MCF-7 wild-type (WT) control cells and acquired resistant MCF-$7_{5FU10\mu M}$ (1st Generation and 5th Generation) cells.

4.1 Discussion

In cancer cell metastasis, the epithelial-mesenchymal transition (EMT) is a risky progression that causes epithelial cells to enhance their mesenchymal physiognomies, such as increased movement and motility. The hallmarks of EMT include the overexpression of mesenchymal cell markers such vimentin, fibronectin, and N-cadherin (Wang Alan et al., 2015) and the loss of epithelial cell biomarkers comprising of cytokeratins and E-cadherin (Alan et al., 2008). These modifications in the expression of markers for epithelial and mesenchymal cells cause the adhesion between the transitional cell and neighboring epithelial cells to diminish, and the production of enzymes that break down the extracellular matrix to increase. This leads to the loss of apical-basal cell polarity, cytoskeleton reorganization, and gene expression reprogramming in epithelial cells, all of which accelerate the development of an invasive phenotype in cancer metastases (Vergara et al., 2016). In divergence to the control MCF-7 cells, which showed the opposite pattern, the resistant MCF-$7_{5FU10\mu M}$ cells showed the loss of E-cadherin along with overexpression of the mesenchymal markers N-cadherin and vimentin, which are hallmarks of carcinoma EMT and required for cells to acquire invasive properties, as shown in figure 1. Numerous malignancies exhibit enhanced invasiveness and metastasis when N-cadherin and vimentin are overexpressed and E-cadherin is lost (Wang Alan et al., 2015, Vergara et al., 2016).

The main causes of people with breast cancer having a bad prognosis are high invasion and metastasis. Metastatic spread of primary tumors accounts for over 90% of cancer-related fatility (Christofori, 2006). The cell invasiveness of the resistant cell lines MCF-$7_{5FU10\mu M}$ (1st Generation and 5th Generation) was examined using the Matrigel assay. In comparison to wild-type MCF-7 cells, the acquired resistant cells showed improved invasive characteristics and increased activity after three days, demonstrating mesenchymal phenotype and EMT activation (Figure 2). These results were consistent with previous research by Lee et al. (2017) and Li et al. (2014). The acquired resistant cells showed an upsurge in the invasiveness of the cancer cells, with the 5th generations of cells exhibiting higher invasion compared to the MCF-$7_{5FU10\mu M}$ 1st Generation and control cells MCF-7.

Numerous methods have been used to comprehend cell migration, including the *in vitro* wound healing (WH) assay (Martinotti and Ranzato, 2020), also referred to as the *in vitro* scratch assay, live-cell imaging for real-time surveillance (Kijanka et al., 2015), and the Boyden chamber assays (Woo et al., 2007). One popular and inexpensive technique for analyzing cell migration is the scratch or wound healing assay (Jonkman et al., 2014). The migratory characteristics of the resistant cells MCF-$7_{5FU10\mu M}$ (1st Generation and 5th Generation) were examined in this study using the wound healing assay.After 48 hours, the resistant cells MCF-$7_{5FU10\mu M}$ showed increased migratory properties, according to our results from images (Magnification 20X) taken by a light microscope from the wound healing scratch assay (Figures 3). The scratch test also showed that the resistant cells' wound healing happened more quickly than that of the wild-type MCF-7 control cells. According to this, the resistant cells developed a more mesenchymal appearance. The findings of this study are consistent with those of previous investigations by Huber et al. (2005).

4.2 Conclusion

More than 90% of cancer related mortality are triggered by metastasis, or the spread of principal tumors to an ancillary organs and sites. EMT characteristics cause mesenchymal cell markers (vimentin and N-cadherin) to increase while epithelial markers (E-cadherin) decrease, which results in the development of migratory and invasive characteristics.

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