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Triple Negative Breast Cancer Cell Lines with TP53 Mutations are Able to Undergo Cell Death

Veronica L. Stallings[1], Kashenya M. Gurley[2], Checo J. Rorie[3]

[1, 2, 3]North Carolina Agricultural and Technical State University, Department of Biology,
1601 East Market Street, Greensboro, North Carolina 27411, United States of America

Abstract: ***Triple-negative breast cancer (TNBC) is a complex disease that lacks the expression of the estrogen, progesterone, and HER2 receptors. Over 80% of TNBC cells have a mutated p53 tumor suppressor gene. The p53 pathway is responsible for a cells arrest or death response to stress. Here we show that the TNBC cell lines HCC1806 and HCC70 expresses the p53 protein and its downstream targets p21 and Puma. This study also revealed that the native forms of the p53 protein were not detected by immunofluorescence assays. Here we analyzed the cell death responses in triple-negative breast cancer cell lines HCC70 and HCC1806. We exposed the TNBC cell lines HCC70 and HCC1806, and a normal breast cell line AG11132 to the chemotherapeutics staurosporine, Actinomycin D, and doxorubicin for 24 hrss. We observed the physical characteristics of apoptosis in both HCC70 and HCC1806. We also performed Western blot analysis to reveal that Parp was cleaved in the TNBC cell lines. In conclusion, cell death responses and apoptosis occurred in the TNBC cell lines HCC70 and HCC1806 even though they have mutated p53 genes. Therefore, more experiments need to be conducted to determine whether apoptosis occurs in a p53-dependent or independent manner.***

Keywords: triple negative breast cancer, TNBC, p53, apoptosis

1. Introduction

Breast cancer is the most common cancer in women and the second leading cause of cancer death after lung cancer and heart disease, in the United States [1, 2]. Breast cancer affects both men and women, although the cancer is more common in women. In 2015, approximately 230,000 women are expected to be diagnosed with invasive breast cancer and approximately 40,500 are expected to die from breast cancer [1, 2]. There are several risk factors for developing breast cancer in women such as heredity, the early onset of menarche, body mass index, and the number of births [3]. Breast cancer consists of several different subtypes including triple-negative breast cancer which is characterized by the absence of the estrogen, progesterone, and HER2/Neu receptors and therefore do not respond to hormone therapies [4, 5]. Triple-negative breast cancer (TNBC) accounts for about 15% of all breast cancers, has high mitotic indexes and is highly invasive [5].

The prognosis for TNBC patients is poor due to the lack of effective therapy since there are no known biomarkers, and the tumor suppressor p53 gene is known to be mutated in approximately 84% of triple-negative breast cancer cases [6]. The tumor suppressor protein p53 is a master regulator of the cell acting as a transcription factor. Wild-type p53 functions to inhibit the proliferation of abnormal cells [7] by regulating the expression of other key proteins including p21, a cyclin dependent kinase (CDK) inhibitor. Inhibition of cyclin B1-CDK1 by p21 in G1 of interphase leads to cell cycle arrest to allow for double strand break repair [8]. If the double strand breaks are left unrepaired, p53 activates pro-apoptotic proteins p53-Upregulated Modulator of Apoptosis (PUMA). Mutations in p53 can change the function resulting in p53 no longer able to respond to stress signals, however, some mutant p53 has been reported to still be able to elicit some transcriptional activity [9]. Mutant p53 has been associated with high grade breast cancer including triple-negative breast cancer [5, 10, 11], however, it has not been fully determined at which stage of carcinogenesis p53 mutations occur or the consequences of those mutations on the aggressive behavior of TNBC. This study seeks to characterize the cell death and apoptotic responses of triple-negative breast cancer cell lines with reported p53 mutations to chemotherapeutics Actinomycin D, doxorubicin and staurosporine.

2. Materials and Methods

2.1. Cell Lines and Culture

Triple-negative breast cancer cell lines HCC1806 and HCC70 were purchased from American Type Culture Collection (Manassas, VA). HCC70 was derived from the mammary gland of a 49 year old African American female who suffered from a primary ductal carcinoma. The HCC70 p53 gene has a point mutation at codon 248 resulting in a CAG to CCG transition mutation of the protein (Wistuba 1998). HCC1806 was derived from a 60 year old African American female who suffered from a primary squamous cell carcinoma. The HCC1806 gene has an insertion mutation of two bases at codon 256 resulting in a frameshift mutation (Wistuba 1998). Normal breast cell line, AG11132, was purchased from Coriell Institute for Medical Research (Camden, NJ). AG11132 is a normal breast cell line that was derived from a 19 year old African American female undergoing a mastectomy. AG11132 expresses the ER, PR, and HER2 and is p53 wild-type. Cell lines HCC1806 and HCC70 were both grown in RPMI-1640 (ThermoScientific; Rockford, IL) containing 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin (P/S) (Hyclone Laboratory; Logan Utah). AG11132 was grown in MEGM (Lonza; Walkersville, MD). All cell lines were maintained in 5% CO2 at 37°C. All of the cells were grown to 95% confluence, counted, pelleted, and then stored at -80°C.

2.2. Chemotherapeutic Drug Treatments

The cell lines were grown to 95% confluence and then exposed to chemical treatments. Following exposure, cells were photographed at 20x magnification using an inverted light microscope to assess physical apoptosis. Each drug was diluted in dimethyl sulfoxide (DMSO) to working concentrations of 1 µM of Actinomycin D, 15 µM of Doxorubicin, and 1 µM of Staurosporine. Actinomycin D belongs to the Actinomycin family of antibiotics, which is produced by *Streptomyces*. Actinomycin D functions through the inhibition of gene transcription thereby minimizing translation and protein production leading to widespread genotoxic stress. Doxorubicin is one of the more widely used anthracycline chemotherapy drugs that inhibits transcription leading to genotoxic stress. Staurosporine is an alkaloid isolated from *Streptomyces staurosporeus.* Staurosporine is used regularly in the lab to study protein kinases and apoptosis. Staurosporine inhibits protein kinases by binding to ATP sites leading to genotoxic stress and ultimately apoptosis. A vehicle control of 4 µL of DMSO was also added to 2 mL of media and used as a solvent control. The cell lines were exposed to the chemical treatments for 24 hrs in a 37°C/5% CO_2 incubator. All of the cells, including floating or dead cells, were collected into Eppendorf tubes and stored as cell pellets at -80°C until further analysis.

2.3. Western Blot Analysis

Total proteins were extracted from the cell pellets using Radio-Immunoprecipitation Assay (RIPA) buffer mixed with a protease inhibitor cocktail (PIC), and then separated using SDS-PAGE electrophoresis and transferred to PVDF (Millipore) membrane paper. Membranes were then blocked in 5% non-fat dry milk for 30 min and washed twice with 1X PBS/0.1% Tween (PBST). Membranes were probed with primary antibodies with a final 1:1000 dilution in PBST. Membranes were rocked overnight at 4°C. The following day, the membranes were washed 3X in PBST for 10 minutes each. The membranes were then probed with secondary antibodies diluted 1:1000 in PBST rocked for 1 hrs at room temperature. The secondary antibody was removed and membranes were washed with PBST 3X for 10 minutes each. The membranes were then exposed to Pierce Enhanced Chemiluminescence Reagent Substrates (ECL) (Thermo Scientific; Rockford, IL) for 1 min on each side. The membranes were wrapped in Saran wrap and placed in film cassettes. X-ray film was placed on top of the covered membrane in the cassette, exposed, and then developed.

2.4 Antibodies

p53 (D0-1 and PAB-240) mouse monoclonal antibodies (Santa Cruz Biotechnology; Santa Cruz, CA), Anti-Tubulin (DMA1) mouse monoclonal antibody (Sigma-Aldrich; St. Louis, MI), and p21 (F-5) mouse monoclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA) were generated in mice. Anti-PUMA rabbit monoclonal antibody (Upstate Signaling Solution; Lake Placid, NY), Anti-Actin (20-33) rabbit monoclonal antibody (Sigma Aldrich; St. Louis, MI), and PARP ½ (H-250) rabbit polyclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA) antibodies were generated in rabbits. The p53, Anti-Tubulin, and p21 antibodies were detected using Donkey Anti-Mouse secondary antibody purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PUMA, Anti-Actin, and PARP were detected using donkey anti-rabbit secondary antibody purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.5. Immunofluorescence Analysis

Cells were grown in 6-well tissue culture plates and then fixed in 4% paraformaldehyde for 5 min and then washed twice with 1 mL of cold 1X PBS 5 min each. The cell membranes were permeabilized with 1 mL of Triton X-100 for 5 min, and then washed twice with 1 mL of cold 1X PBS for 5 min each. The cells were blocked in 5% non-fat dry milk for 30 min followed by three 1X PBS washes for 3 min each. The cells were then probed with the following primary antibodies diluted 1:100 in 1X PBST for 2 hrs: p53 (D0-1), p53 (PAB-240), Tubulin (DMA1) and Actin (20-33). Anti-Mouse and Anti-Rabbit secondary antibodies were used as negative controls. The primary antibodies were removed, and the cells were then washed 3X with 1 mL of cold 1X PBS for 5 min each. The cells were then probed for 1 hr with the appropriate secondary antibodies (1:100 dilution in 1X PBST) conjugated to Texas Red (Vector Laboratories, Inc.; Burlingame, CA). The cells were then washed 3X with 1 mL of cold 1X PBS for 5 min each. The nuclei of the cells were stained with DAPI (Vector Laboratories, Inc.; Burlingame, CA) for 5 min. Coverslips were added to each well. Plates were then covered with aluminum foil to block direct light from interacting with fluorescence until exposure using Olympus 1X71 Inverted Microscope.

3. Results

3.1 Western Blot Analysis of p53 Pathway Proteins in Breast Cell Lines

Figure 1 displays the Western blot protein expression analysis of p53 and its pathway proteins PUMA and p21. The cells were grown to 95% confluency and then protein was extracted using RIPA buffer for Western blot analysis. The p53 protein is expressed in all cell lines. The p53 downstream target PUMA was expressed in both of the TNBC cell lines, HCC70 and HCC1806 cells, but not expressed in the AG11132 normal breast cells. Interestingly, the p53 downstream target p21 was expressed in all three breast cell lines at equal levels.

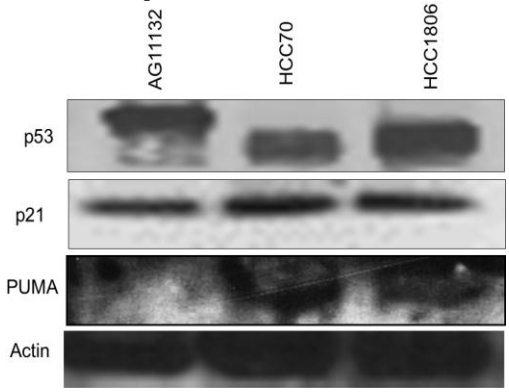

Figure 1: Western Blot of p53 Pathway Proteins in Normal (AG11132) and TNBC (HCC70, HCC1806) Cell Lines.

Actin was used as a loading control. PUMA and p21 are downstream targets of p53.

3.2 Immunofluorescence Analysis of Native p53 Pathway Proteins in TNBC Cell Lines

Figure 2 displays the immunofluorescence of native or un-denatured p53 protein expression in HCC70 cell lines. Native or un-denatured p53 protein was detected in HCC70 cells with the p53 (DO-1) antibody, however native p53 protein was not detected with the p53 (PAB-240) antibody. Tubulin was used as a positive control and detected in the cells, while anti-mouse secondary was used as a negative control. DAPI staining was used to indicate the location of the nucleus.

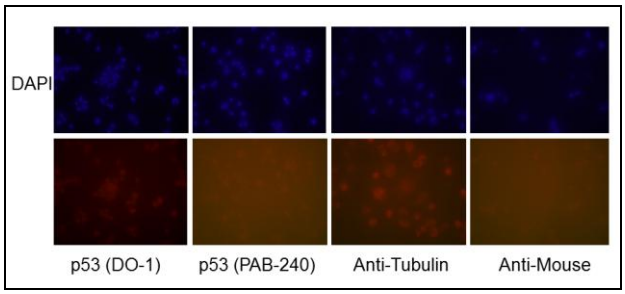

Figure 2: Immunofluorescence Images of HCC70 Cells.

Figure 3 displays the immunofluorescence of native p53 protein expression in HCC1806 cell lines. Native or un-denatured p53 protein was not detected in HCC1806 cells with either the p53 (DO-1) antibody, or with the p53 (PAB-240) antibody. Tubulin was used as a positive control and detected in the cells, while anti-mouse secondary was used as a negative control. DAPI staining was used to indicate the location of the nucleus.

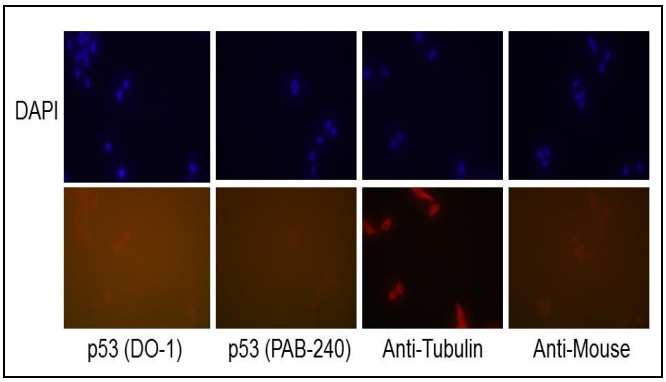

Figure 3: Immunofluorescence Images of HCC1806 Cells

3.3 Cell Death Response of TNBC Cell Lines to Chemotherapeutics

Figure 4 reveals that the TNBC cell line HCC70 is able to elicit a cell death response to chemotherapeutics despite having mutant p53 caused by a point mutation [12]. Figure 4 show light micrographs of HCC70 cells physical response to treatment exposure. Apoptotic or dead cells are indicated with red arrows. The cells are rounded, blebbing, and floating in the growth media.

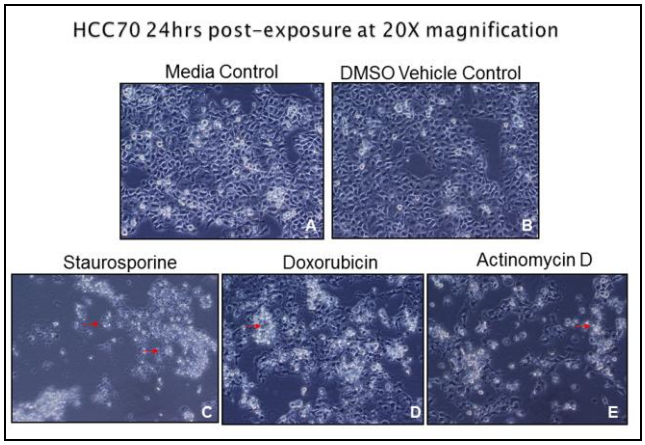

Figure 4: Light Microscopy of HCC70 Cell Line Death Response to Chemotherapeutics 24hr Post-Exposure. A is the media only control, B is the DMSO solvent control, C is staurosporine treatment, D is doxorubicin treatment, and E is Actinomycin-D treatment.

Figure 5 reveals that the TNBC cell line HCC1806 is able to elicit a cell death response to chemotherapeutics despite having mutant p53 caused by a frameshift mutation [12]. Figure 5 show light micrographs of HCC1806 cells physical response to treatment exposure. Apoptotic or dead cells are indicated with red arrows. The cells are rounded, blebbing, and floating in the growth media.

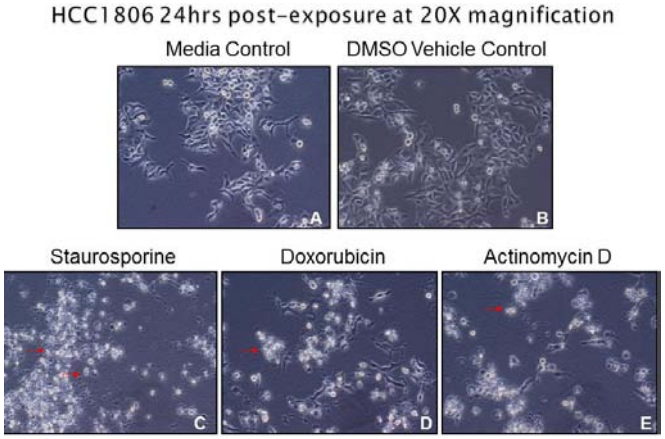

Figure 5: Light Microscopy of HCC1806 Cell Line Death Response to Chemotherapeutics 24hr Post-Exposure. A is the media only control, B is the DMSO solvent control, C is staurosporine treatment, D is doxorubicin treatment, and E is Actinomycin-D treatment.

Figure 6 reveals that both the TNBC cell lines HCC70 (Fig. 6A) and HCC1806 (Fig. 6B) are able to elicit a cell death response to chemotherapeutics as indicated by PARP cleavage. PARP is cleaved from a 116 kDa protein into 89 kDa and 27 kDa protein fragments.

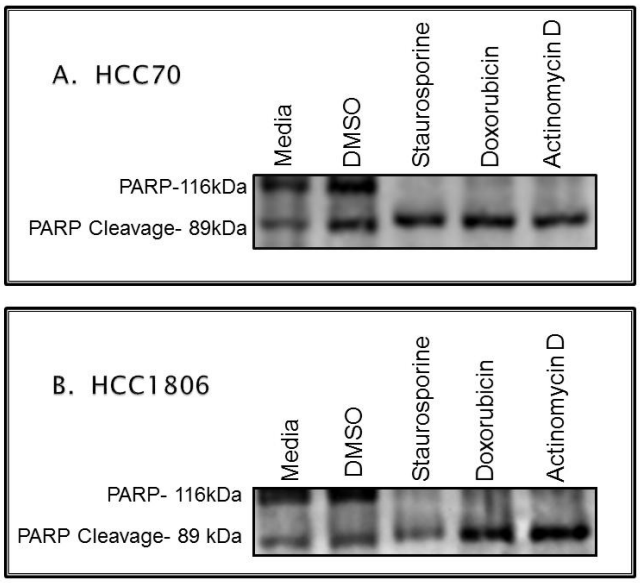

Figure 6: Western Blot of PARP Cleavage in Normal (AG11132) and TNBC (HCC70, HCC1806) Cell Lines 24hrs Post-Exposure to Chemotherapeutics. Cleaved PARP 89 kDa protein indicate apoptosis. (A) HCC70 Cell Lines. (B) HCC1806 Cell Lines.

4. Discussion

Gazdar et al, used immunostaining to characterize cell lines from breast cancer patients. According to Gazdar et al, HCC70 expresses positive mutant-p53 while HCC1806 is negative for p53 protein expression [13]. However, according to Lacroix et al, HCC1806 expresses mutant-p53 protein [14]. Here we confirm the findings previously reported and established that the TNBC cells express p53 protein, even though the p53 gene is mutated. Under normal cell conditions, wild-type p53 protein levels are kept low which occurs through interaction with MDM2. MDM2 targets p53 protein for ubiquitination and degradation [15]. When MDM2 levels are high, MDM2 inhibits p53 from function as a transcription factor. However, when cells are stressed, the p53-MDM2 complex separates and p53 protein levels increase due to its activation and then p53 relocates to the nucleus where it functions as a transcription factor and activates downstream targets including p21 and PUMA. Here we show that p53 is concentrated in the nucleus of the HCC70 cells, while the antibodies were not able to detect p53 in the HCC1806 cells.

The p53 (DO-1) antibody recognizes wild-type and mutant p53 through the epitope located between acid residues 11-25 within N-terminal region of the protein. However, the p53 (PAB-240) antibody reportedly only recognizes mutant p53 under non-denaturing conditions through the epitope located between amino acid residues 156-214 [16, 17]. Here we revealed in HCC70 cells that p53 was detected with the p53 (DO-1) antibody, but not the p53 (PAB-240) antibody. Detection of p53 in the HCC70 cells with only the p53 (DO-1) antibody suggests that the missense mutation may not have a dramatic effect on the conformation of p53 in these cells. The p53 (PAB-240) antibody recognizes an epitope that is located in a region within the protein's tertiary structure and cannot be recognized unless a dramatic conformational change occurs [16, 17]. Here we show that in HCC1806 cells that the p53 protein was not detected with either the p53 (DO-1) or (PAB-240) antibodies, which suggests that the insertion mutation has altered the reading frame and that the epitope may not be recognizable or existent. These findings provide some insight into whether p53 could still be activated to trigger the cellular response to stressors including chemotherapeutics.

Apoptosis and cell death occurred in each triple-negative breast cancer cell lines. Evidence of apoptosis could be observed in HCC70 and HCC1806 after 24 hrs exposure to the chemotherapeutics. Cell shrinkage, cell rounding, and blebbing are all physical signs of apoptosis and were observed in both the HCC70 and HCC1806 cell lines. Treatment with staurosporine and doxorubicin induced the most damage in HCC70 after 24 h of exposure compared to Actinomycin D. However, staurosporine induced slightly more apoptosis in HCC1806 compared to doxorubicin and Actinomycin D. We also confirmed that apoptosis was induced in the TNBC cell lines indicated by the distinctive cleavage of PARP protein from a 116 kDa sized protein into an 89 kDa and 27 kDa protein fragments revealed in Western blot analysis. These response differences may be due to the overall effect of the drug in HCC70 and HCC1806 cell lines which may also be related to p53 gene mutational status.

More experiments need to be conducted to determine whether the apoptotic and cell death responses and p53 downstream target activation occurs in a p53-dependent or p53-independent manner. Future studies surrounding p53 mutations may help determine whether p53 mutational status is necessary for apoptosis or its transactivational activity. Ectopic expression of wild-type p53 in the TNBC cell lines which have mutant p53 may shed some light on the intrinsic and extrinsic pathways as a means of inducing apoptosis in triple-negative breast cancer cell lines. Time-points would also be an important tool to analyze p53 expression and the p53 pathway. Tracking p53 expression levels may indicate when activity occurs optimally with correlated downstream target activation may indicate when apoptosis is triggered. However, to establish whether p21, PUMA, or any other downstream p53 targets are activated in a p53-dependent manner, Chromatin Immunoprecipitation (ChIP) Assays could provide great insight. Immunoprecipitation will be beneficial in studying proteins that associate with mutant p53 including ATM or MDM2 to characterize the consequence of the mutational status of p53 in the TNBC cell lines. We would also like to do chemical titration studies to determine the thresholds that may play a role in triggering cell death responses in the context of p53 gene status. Currently there is limited experimental and p53 functional research on triple-negative breast cancer, particularly with HCC70 and HCC1806 breast cancer cell lines. The findings in this research in conjunction with future work are important in establishing the consequence of p53 mutational status on triple-negative breast cancer.

5. Acknowledgements

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Author Profile

Veronica L. Stallings is currently a biology teacher. She received her B.S. in Biology degree from the University of North Carolina at Greensboro in Greensboro, North Carolina, USA; and her Master's of Science degree in Biology in 2011 at North Carolina Agricultural and Technical State University in Greensboro, North Carolina, USA.

Kashenya M. Gurley is currently a research assistant in the Chemistry Department at North Carolina Agricultural and Technical State University in Greensboro, North Carolina, USA. She received her Bachelor of Science degree in Chemical Engineering in 2001 from North Carolina Agricultural and Technical State University in Greensboro, North Carolina, USA; and she received her Master's of Science degree in Biology in 2009 from North Carolina Agricultural and Technical State University.

Checo J. Rorie is an Assistant Professor in the Department of Biology at North Carolina Agricultural and Technical State University in Greensboro, North Carolina, USA.