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Identifying Senescence as a Mode of Chemo Resistance in Ovarian Cancer

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Abstract

Current treatments of ovarian and breast cancer result in chemo resistance all too often. It has been hypothesized that senescence-a dormant condition associated with increased age and apoptosis- may play a role in the development of chemo resistance. We performed an in-vitro study with HOSE (carboplatin-sensitive), SKOV3 (chemo resistant ovarian cancer), and CAMA1 (chemo resistant breast cancer) cell lines, which were exposed to a variety of platinum-based treatments meant to model current cover clinically relevant scenarios in terms of tumor hypoxia. They were then stained for senescence in-vitro using B-gal, and analyzed for proliferation using the Cell Counting Kit 8, trypan blue dye exclusion, and survival plating, among other methods. Real-time quantitative PCR was used to determine relative levels of gene expression for classical apoptotic and senescent markers. Our results indicate that proliferation was temporarily halted in SKOV3 and CAMA1 after treatment. Cell proliferation later resumed in these cell lines while HOSE cell underwent apoptosis. Analysis of genetic tests (such as qPCR) also revealed that SKOV3 and CAMA1 had decreased gene expression of key genes that regulate apoptosis and senescence (such as p53 and CDK2). It can be concluded from this data that senescence was in fact a mode of chemoresistance and that future treatments may want to focus on disabling cancerous cells' senescent stage.

Keywords: Ovarian cancer, Genomics, Chemo resistance, Senescence

Introduction

Rationale

Ovarian cancer affects over 240,000 women annually. More than 85% of ovarian cancers have "epithelial origin" (Bitleret., al 2011). Therefore there is an urgent need for effective treatments for ovarian cancer. Currently, platinum-based chemotherapy, with a taxane based drug and surgery are the primary treatments for ovarian cancer. Approximately 25% of patients introduced to platinum based chemotherapy experience tumor recurrence and develop resistance to treatment. Chemotherapy kills tumor cells by damaging cellular integrity. It mainly targets DNA. Chemotherapy takes away a major part of the tumor and leaves behind a few resistant cells that eventually regenerate into a new chemo resistant tumor. The overall 5-year survival rate for advanced ovarian cancer patients is still low (20–30%) and is due to chemo resistance in the primary or recurrent tumors, thus resulting in treatment failure. Although ovarian cancer patients show high response rates to initial chemotherapy after cytoreductive surgery, most patients also develop resistance to chemotherapy during treatment (Katsaros et al., 1999). Our study aimed to investigate a possible mode of chemo resistance, senescence. The development of chemo resistance through hypoxia was also another target of our study, as well as the pathways and genes involved in the avoidance of apoptosis.

Background on Epithelial Ovarian Cancer

Epithelial ovarian cancer is the fourth leading cause of death from female cancers in the world (Ayesha et al. 2009). A significant portion of women with a positive response to the first treatment will develop recurrence within 18 months. During 2003, over 24,400 new cases and 14,300 deaths were expected from epithelial ovarian cancer.

But a 5-year survival improvement occurred in the last three decades with more aggressive surgical management and development of more effective chemotherapy (Copeland et., al 2017). Currently, platinum-based chemotherapy is the main treatment for epithelial ovarian cancer.

Senescence

Somatic cells include all cells of a living organism other than the reproductive cells.

Somatic cells show a spontaneous decline in growth rate in continuous culture, which is unrelated to elapsed time. Cells enter into a quiescent but viable state termed replicative senescence. It is characterized by cells that are multinucleated and do not respond to mitogens or apoptotic stimuli. Cellular senescence is a state of cell cycle arrest started by many stimuli like telomere shortening, stress factors, or chemical moieties (Weiner-Gorzelet., al 2015). Multiple hypotheses state that cellular senescence is a natural antitumor program. Senescent cells demonstrate many characteristic including changes in "morphology and molecular signaling" (Weiner-Gorzelet., al 2015). Chemotherapy kills tumor cells by damaging cellular integrity, most commonly that of the DNA. To limit cell death from this damage and the cellular stress that it causes, tumor cells may enter a senescent state, sometimes long-term. In addition, "recent findings suggest that cellular senescence is a natural mechanism to prevent undesired oncogenic stress in somatic cells that has been lost in malignant tumors" (Mar et al).

HOSE, SKOV3, and CAMA1 ovarian cancer cell lines

Cell lines have similar genetic makeup because they are taken from one cell. It is a tool used to analyze molecular cell markers and cellular behavior in a controlled experimental condition. In this experiment, carboplatin-sensitive, or human ovarian surface epithelial (HOSE) and platinum-resistant (SKOV3) ovarian cancer cell lines, in addition to CAMA1 platinum resistant breast cancer cells, were exposed to various combinations of chemotherapeutic drugs designed to cover clinically relevant scenarios in terms of tumor hypoxia. Studies show that harmful and normal human ovarian surface epithelial (HOSE) cells are different in their growth factors stimuli response (Tsao et., al 1995). CAMA1 is a breast cancer cell line is used in this study because it is closely related to ovarian cancer cell lines and is related to chemo resistance. Unlike ovarian cells, CAMA1 is a luminal-type human breast cancer cell line that displays rounded morphology in adherent tissue culture. Genetically, CAMA1 is similar to ovarian cancer because the cells have oncogenic mutations in PTEN and p53 and amplification of the cyclin D1 gene (Ji et. al, 1994). If ovarian cancer cell lines show improvement of current anticancer treatments and prevent the recurrence of a cancerous tumor, then it can correlate to breast cancer, as well.

Objective

"Given that the ultimate goal of cancer research is to find the definitive cure for as many tumor types as possible, exploration of cellular senescence to drive towards antitumor therapies" may influence the outcome of new drugs (Mar et al 2011). In the present paper, we will review the potential of cellular senescence to be used as a target for anticancer therapy

Materials and Methods

Materials

HOSE 6-3, a normal human ovarian surface epithelial cell line that has been immortalized by HPV E6/7 ORF (obtained from an affiliated research lab) cell line was used to study ovarian cancer proliferation. All cell lines were maintained in DMEM 10% fetal bovine serum. SYBR Green I fluorescent dye, reverse transcriptase and RNase inhibitor was purchased from Applied Biosystems, Foster City, CA. Transwell assays and reagents were obtained from BD Bioscience. RNEasy, DNEasy were obtained from Qiagen. Cisplatin, Carboplatin, and Taxane were purchased from Sigma and reconstituted with PBS. Primers were supplied by Sigma Genosys. Cell counting kit-8 was purchased from Dojindo Molecular Technologies, Inc. ROS-Glo assay was purchased from Promega. Cell invasion and scratch assay kits were supplied by Millipore. Cellular senescence kits were purchased from Dojindo Molecular Technologies and Abcam.

Methods

Pathway Analysis

DAVID, Ingenuity, and gPROFILER are bioinformatics databases used to analyze large gene/protein lists and gain biological meaning. Analysis was useful in gaining a better understanding of candidate genes involved in chemoresistance in ovarian cancer. Once an uploaded gene list is obtained, analysis can be performed in order to classify the genes based on different mechanisms and pathways that coincide with our study. PubMed meta analysis can be done with smaller gene lists of interest to gain information on chemo resistant mechanisms.

Chemotherapy Sensitivity and Survival

The breast and ovarian cell lines HOSE, SKOV3, and CAMA1 were tested for IC20, IC50 and IC80 carboplatin sensitivity levels. Cells were seeded at 10^3 cells/well in a 96 well plate and treated with platinum and/or taxol treatments at a concentration range of 1–100 μg/ml in 100 μl of fully supplemented medium. After a period of 72 hours, cytotoxicity was assessed by trypan blue dye exclusion viability assay using 1:100 dilutions in cell culture suspensions.

Additionally, the CCK-8 assay was used to measure cell survival after treatment with drugs following the manufacturer's protocol and absorbance measured at 450 nm using the Biotek EL800 spectrophotometer. For survival plating, cells were harvested the 36 hours after chemotherapy treatment using trypsinization, counted, and a specific number of cells (500 and 250 cells) was plated in petri dishes in triplicate for clonogenic assay. "Dishes were placed in an incubator and left there for 9 days until large clones (> 1 mm) were formed (50 cells or more). Staining of colonies was done by removing medium and washing cells twice with PBS. Fixation and staining of clones was done with a mixture of 0.5% crystal violet in 50/50 methanol/water for 30 min" (Karl et al 2012). Dishes were rinsed with water and left for drying at room temperature. Counting of clones was done on the following day.

Cell Proliferation Assay

Cells were seeded into 6-well plates at 10,000 cells/mL in DMEM supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin from Invitrogen. Cells were washed in PBS, trypsinized, and counted using a 1:1 0.4% trypan blue solution every three days for growth and viability analysis. Viable cells with no blue stain and dead cells with blue stain were counted using a hemocytometer. Proliferation and metabolic activity was determined using alamar blue dye assay and spectrophotometry using the Biotek EL800 at 600nm.

Scratch Assay

This method is used to measure the migration of individual cells in vitro and overall to mimic migration of cells in vivo. The scratch assay can be performed on either native cells or transfected cells to study the effect of specific protein overexpression on cell migration. The lab used scratch assay on transfected cells. A "wound" or scratch is made through the monolayer of cells. The ability for the cells to migrate towards the center of the gap between the cells is tested. The Cell Comb Kit assay and manual from Millipore was used.

Invasion Assay

An invasion assay was performed to count the number of cells with the ability to invade through an extracellular matrix. These characteristics reflect those found in tumor cells. The assay used was the CHEMICON Cell Invasion Assay kit. Cells were plated on a 24-well tissue culture plate and the EC-MatrixTM layered cell culture inserts were used in the invasion chamber. Cells were serum starved for a period of 16 hours before the assay was performed. The serum-starved cells were placed on the 24-well tissue culture plate. 750 μl of DMEM with 15% FBS were added into the lower wells as a chemo attractant for the starved cells. Cells were incubated for a period of 24 hours. The invading cells that passed through the membrane fixed with 4% methanol in PBS were stained accordingly with crystal violet. The results of the invading cells were counted with a bright field microscope.

ROS-Glo H2O2 Assay

"Reactive oxygen species (ROS) are chemically reactive molecules that contain oxygen. ROS are beneficial to the cell, having roles in cell signaling and as natural byproducts of normal metabolism" (Alfassa and Sallam, 2012). ROS can also lead to cellular damage, or oxidative stress, as a result of environmental factors (e.g., chemotherapy). The ROS-Glo assay was performed according to the manufacturer's instructions. Luminescence was measured on a Molecular Device's SpectraMax i3x plate reader (excitation 485nm, emission 520nm).

Colorimetric Caspase-3 Apoptosis Assay

The caspase 3 Assay Kit (Colorimetric) (ab39401) measures the activity of caspases using spectrophotometric detection. It was used according to the manufacturer's instructions.

Real-time qPCR

Total cellular RNA was isolated using RNeasy kit and 1ug of RNA was reverse transcribed to cDNA using the SuperScript One Step RT-PCR system by Invitrogen, according to the manufacturer's instructions. cDNA at a 1:10 dilution was used for all PCR reactions and primers were designed by Sigma. Primer sequences are as follows: Caspase 3,5′-TGACTGGAAAGCCGAAACTC 3′ and 5′-AGCCTCCACCGGTATCTTCT-3′; Caspase 8,

5′- CCGAGCTGGACT TGTGACC -3′ and 5′- CTGCCCAGTTCTTCAGCAAT-3′ and HPRT,

5′ CTTCCTCCTCAGACCGCTTT -3′ and 5′- TTTCCAAATCCTCGGCATAA- 3′. Amplified

Fragment sizes were 122, 118 and 145 bp for Caspase 3, Caspase 8 and HPRT, respectively. All PCR reactions were performed on CFX-96 Bio-Rad RT System in triplicate and validated by the presence of a single peak in the melt curve analysis. Changes in gene expression were calculated relative to the HPRT control. PCR products were electrophoresed through 1.0% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination. Band intensity calculated using Image-J software (Bio- Levels of mRNA were expressed as the ratio of band intensity relative to that for control).

Senescence

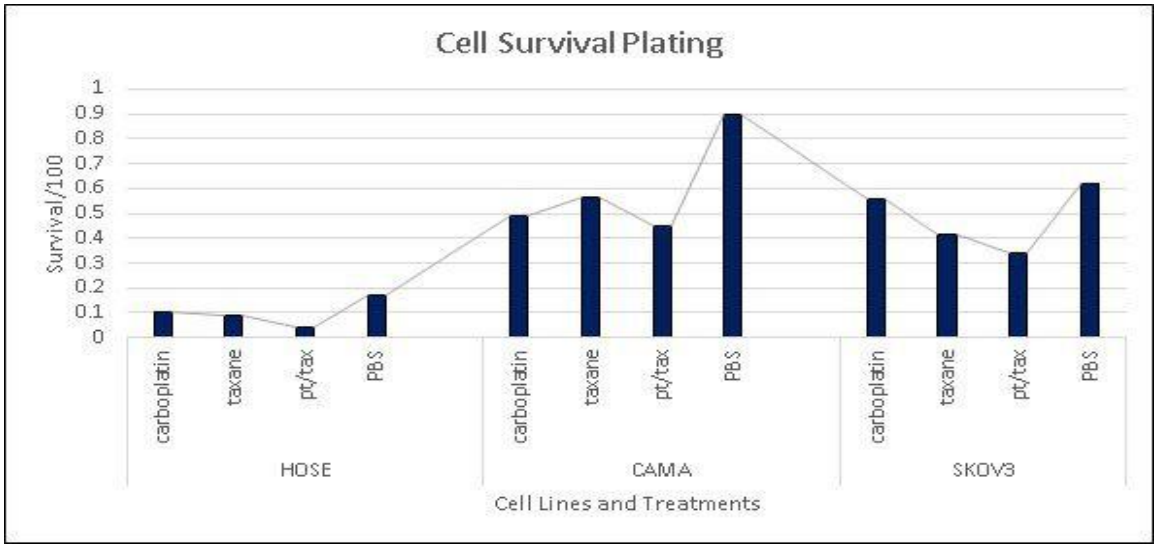
Senescence induced by chemotherapy regimens were stained using Millipore's senescence-associated β-galactosidase (SA-β-gal) assay or Abcam Senescence Detection Kit (ab6535) according to the manufacturer's protocol. After a 72 hour treatment with carboplatin, cells were washed 1x with phosphate buffered saline, fixed in a 1x fixative (provided) and stained using a freshly prepared and protected from light SA-βgal solution. Next, the plates were wrapped in aluminum foil to protect them from light and were placed in an incubator at 37°C and 5% CO2 for 48 hours. The cells were removed from the incubator and the stain was aspirated off the plates. Cells that were stained for senescence-associated β-galactosidase activity were stained blue under standard light microscopy. The percentage of positively blue stained cells was determined by scoring six random high power fields per well in a 6-well plate.

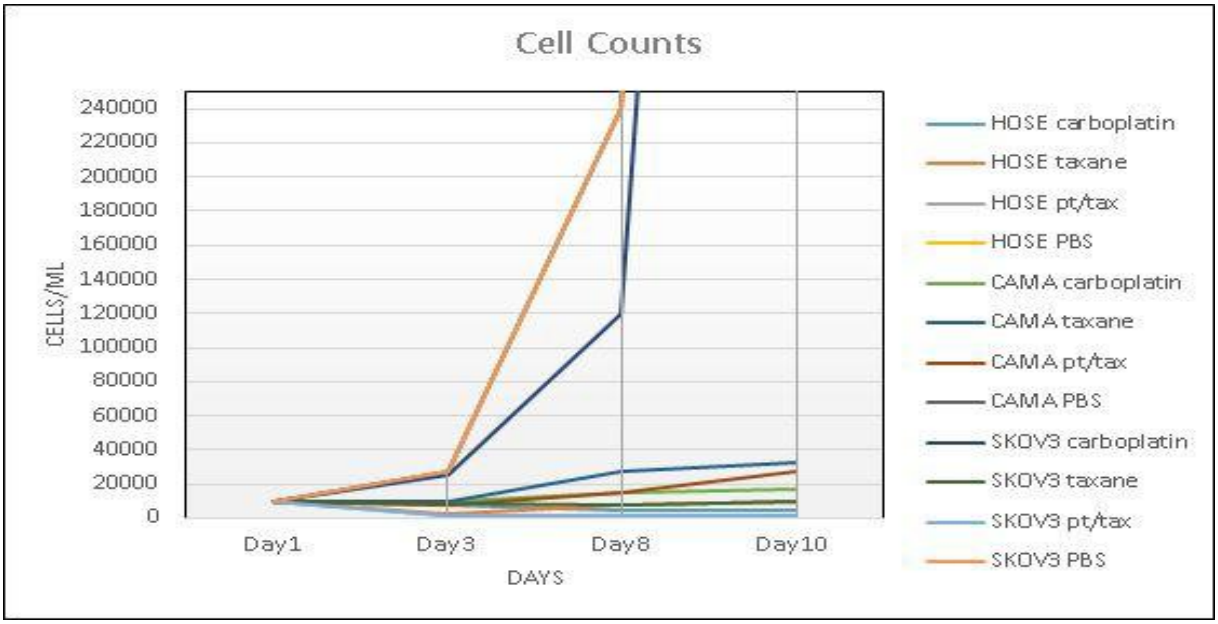
Results

David Pathway Analysis

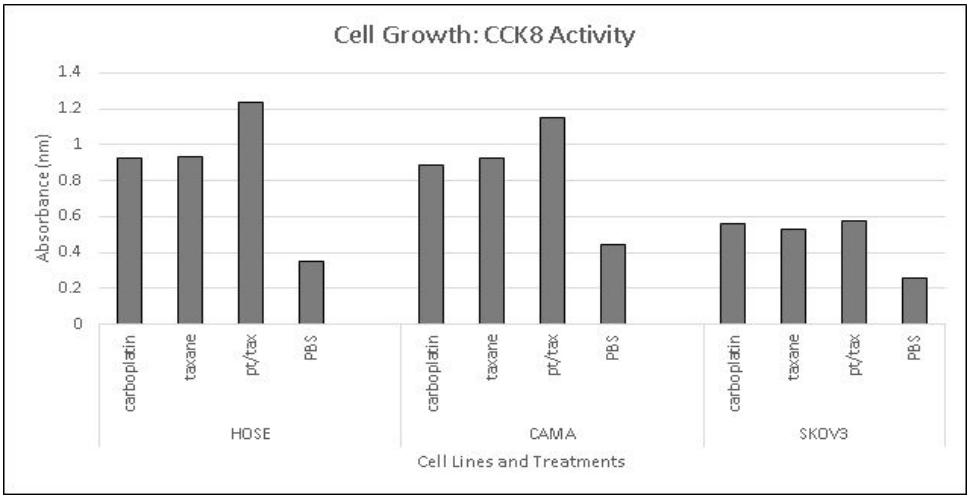
Signal transduction pathways highlighted include: transcription regulation genes, intracellular transport, glucose metabolic process, EMT, cellular response to stress, negative regulation of gene expression, and apoptosis and senescence. Common genes of interest highlighted in our bioinformatics screen included TP53, PTEN, CASP3, CDKN1A, CDK2, and ID1. We validated these genes by running gene expression analysis on our cell lines. RNA is reverse transcribed and the resulting cDNA is used as a template in the qPCR reaction to detect and quantitate gene expression products.

Survival Assay



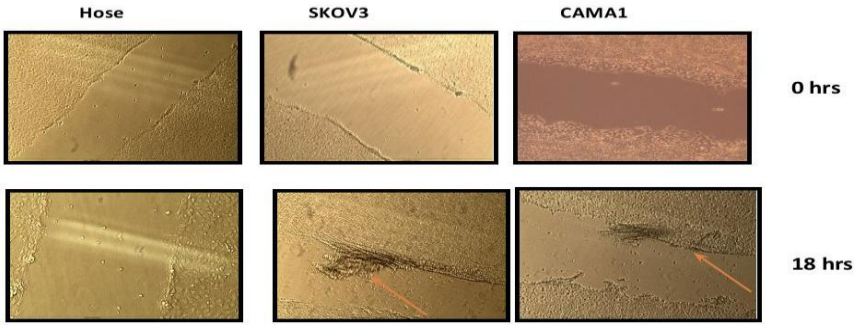


CCK8



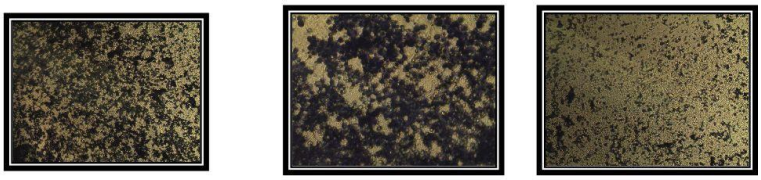
As shown by the graphs and tables above, SKOV3 and CAMA1 showed increased proliferation compared to HOSE.

Scratch Assay



The above picture shows that SKOV3 displayed significant cell migration. CAMA1 also shows cell migration after 18 hours. Hose shows very little migration. All cell lines were treated with carboplatin before the assay was performed.

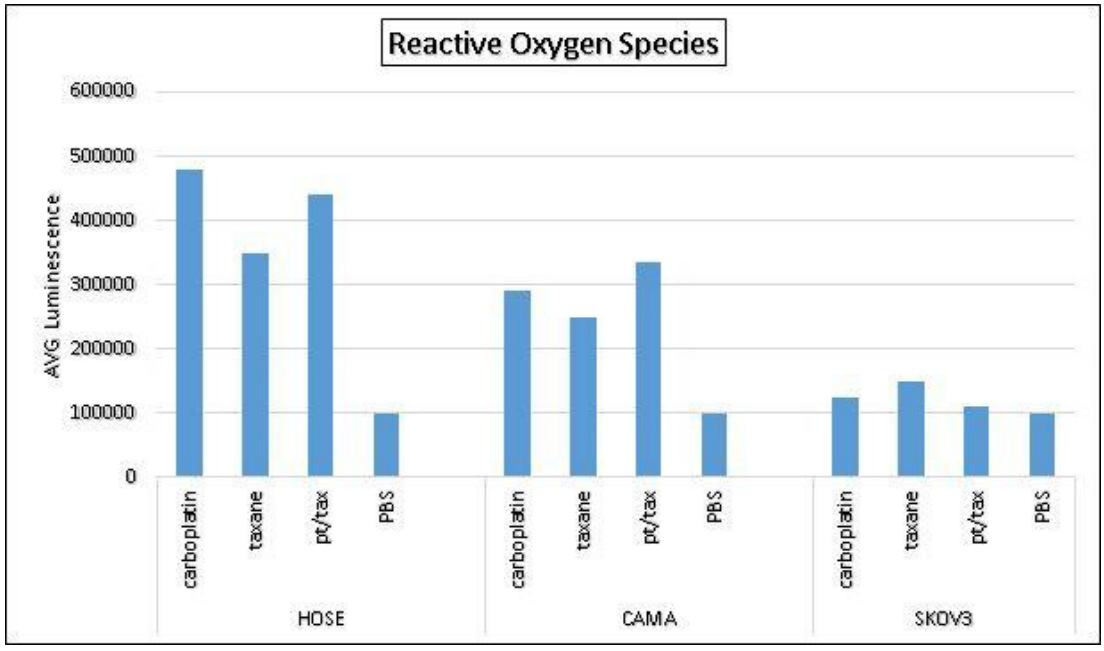
Invasion Assay



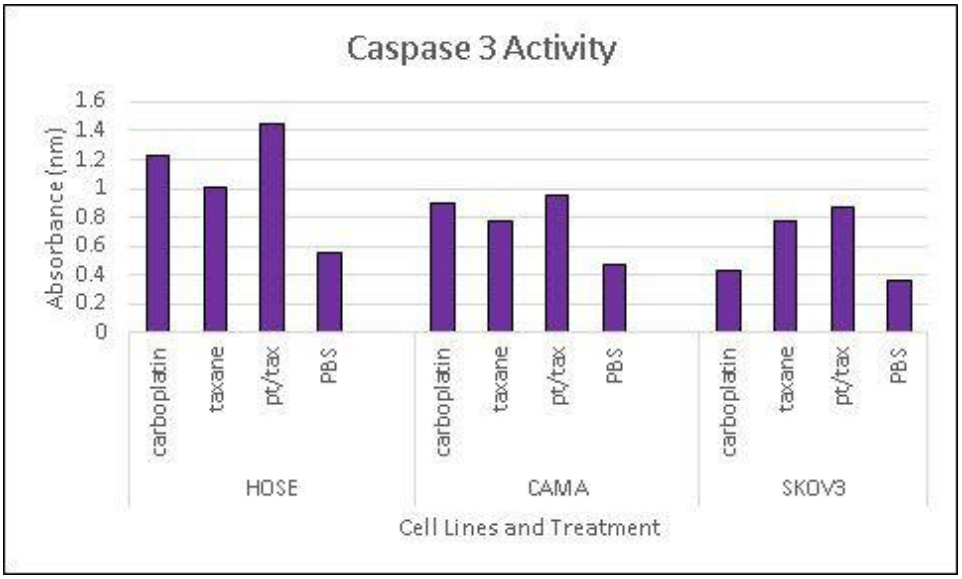
SKOV3 + Carbo CAMA1 +Carbo HOSE 6-3 +Carbo

The invasion assay above shows the aggressiveness of each cell line after treatment with carboplatin. SKOV3 and CAMA1 are recorded as exhibiting aggressive behavior. Hose does not exhibit this aggressive growth.

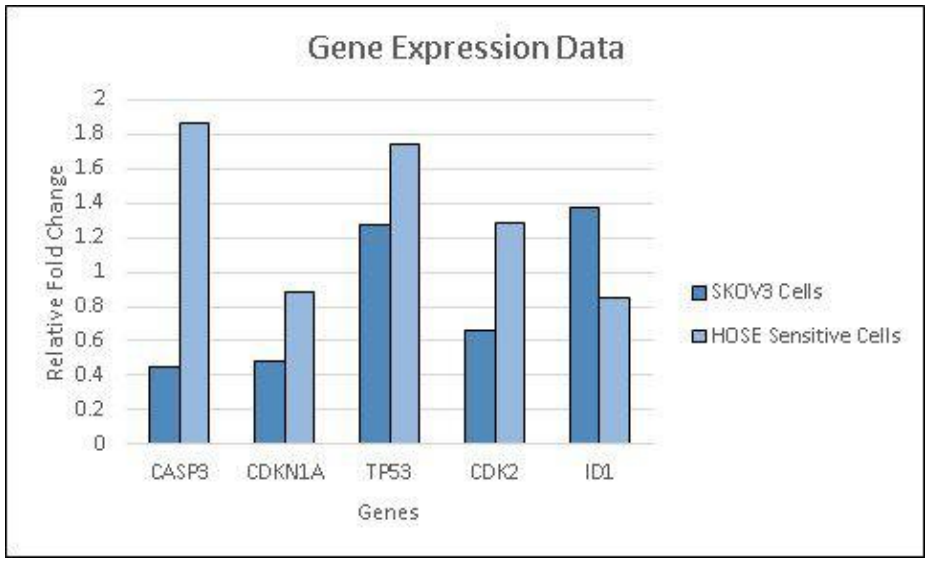
ROS-Glo H2O2 Assay

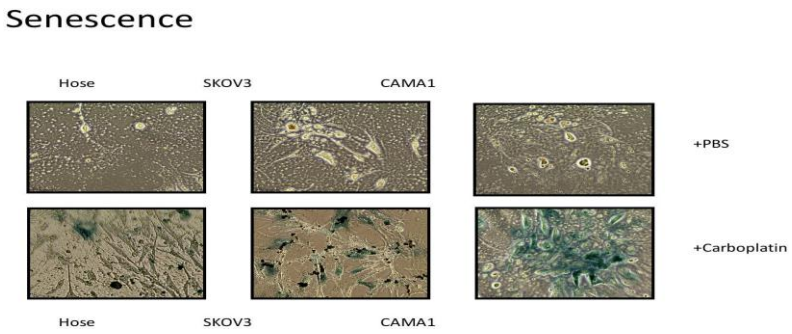


Colorimetric Caspase-3 Apoptosis Assay

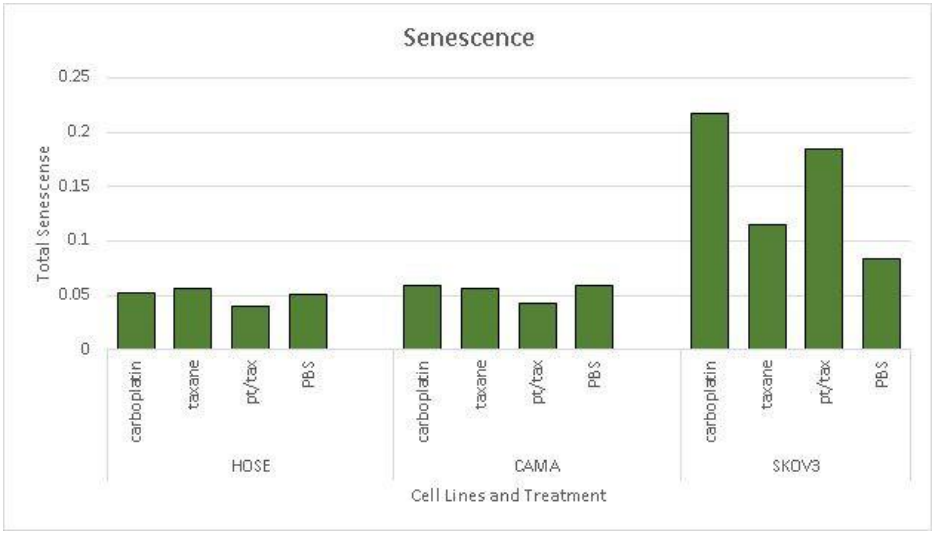


Real-time qPCR





The blue stains in the above picture are cells in a senescent state. SKOV3 cells have a notable number of blue stains.



Discussion

The survival assay, CCK8 assay, scratch assay, invasion assay, ROS-Glo H2O2 Assay, and Colorimetric Caspase-3 Apoptosis Assay all draw similar expected conclusions. SKOV3 (ovarian cancer) and CAMA1 (breast cancer) cell lines showed much more aggressive behavior after an extended time following platinum based treatment compared to the HOSE (normal human ovarian surface epithelial cells) cell line.

The survival assay showed that on average, after treatment with carboplatin, HOSE cells had a 0.102% survival rate compared to 0.492% for CAMA1 and 0.557% for SKOV3. This significant difference between the two cancerous cell lines compared to the HOSE cells lines points to the conclusion that CAMA1 and SKOV3 cell lines are able to avoid apoptosis after treatment. The CCK assay results point to suspicious dormant behavior in the CAMA1 and SKOV3 cell lines immediately after treatment. Cell proliferation is decreased directly after treatment, which provides evidence that the two chemoresistant cell lines enter a senescent stage that temporarily halts all activity. The HOSE cells continues to proliferate after treatment before apoptosis is ultimately triggered, and do not enter a senescent stage.

The scratch assay visually showed that SKOV3 and CAMA1 had similar cell migration after 18 hours. They both had much more migration and therefore increased vitality compared to the HOSE cell lines during an extended period of time following treatment with apoptosis inducing drugs. The invasion assay also visibly showed the increased aggressiveness of CAMA1 and SKOV3 compared to HOSE. These two cell cells lines had more invasive cells in their colonies that were able to pass through a membrane. The membrane models the epithelial membrane that theoretically prevents the cells from reaching the circulatory system when present in vivo.

"The ROS-Glo H202 Assay measured the level of hydrogen peroxide, a reactive oxygen species (ROS), found directly in the cell culture or in defined enzyme reactions" (Promega 2016). Increased levels of ROS result in more cell death after treatment with chemotherapy.

Increased levels of ROS are revealed through increased luminescence. The HOSE cells that were treated with carboplatin had an average luminescence (in relative light units) of 480,000, compared to 290,000 RLU for CAMA1, and 125,000 RLU for SKOV3. The noticeable difference between the two cancerous cell lines (CAMA1 and SKOV3) and the normal epithelial cell line (HOSE) further points out the ineffectiveness of apoptosis inducing drugs on chemoresistant cancers.

Caspase-3 is an enzyme that is involved in the execution of apoptosis. Increased levels of caspase-3 indicate increased apoptotic bodies (ABs). The Caspase-3 assay uses cell staining to identify caspase-3 activity. Our data indicates that HOSE had the most activity, and therefore the

most apoptotic bodies. The average percent of apoptotic bodies in the carboplatin treated HOSE colony was 78.35%. CAMA1 and SKOV3 populations had 69.33% and 24.15% of the population experience apoptosis (respectively). It can be concluded that chemotherapy was more effective in HOSE than SKOV3 and CAMA1.
Real-time qPCR (Quantitative polymerase chain reaction) reveals certain DNA sequences and helps quantify their presence. Further research can then be done to determine the role of those identified sequences. After running qPCR tests on HOSE and SKOV3, it was revealed that CASP3, CDKNIA, TP53, and CDK2, along with other genes had increased expression in HOSE. These genes are mainly involved in regulating the cell cycle and triggering apoptosis. In addition, TP53 in particular has been associated with both apoptosis and senescence. It can be concluded that decreased gene expression of CASP3, TP53, and other genes that regulate the cell cycle and apoptosis, go hand in hand with senescence. A decrease in gene expression may even trigger or lead to a cancerous cell entering the senescent stage and becoming chemoresistant after treatment. Further research is need to support this.
The test for senescence using Millipore's senescence-associated β-galactosidase

(SA-β-gal) assay or Abcam Senescence Detection Kit shows significant blue-stained cells in the SKOV3 colonies. The CAMA1 cells closely compare with the HOSE colony, though. The blue stain indicates cells in the senescent stage. The senescent stage can almost be classified as a hibernation state for cancerous cells. In normal cells, it plays a role in natural apoptosis and is a common aspect of aging in cells. Once a cell enters the senescent stage in response to various stressors, proliferation is arrested. Past research has shown that senescence plays a role in age related diseases, and it is currently hypothesized that the senescent stage is manipulated by chemoresistant cancerous cell lines in order to avoiding induced apoptosis after treatment with chemotherapy.

As the results from our tests show, one chemoresistant cell line entered the senescent stage after being introduced to a variety of platinum based treatments meant to replicate current treatments in hospitals. This partially confirms past hypothesis, but explores aspects of chemoresistance that have not been extensively explored yet, such as the link between senescence and decreased gene regulation. Statistical evaluation further shows that both HOSE and CAMA1 had p-values from a t-test of approximately 0.9. Therefore, these two cell lines are not significantly different. SKOV3 had a p-value of approximately 0.08, which still does not qualify as significantly different, but it is close to being significantly different with a p-value of 0.05. While this is not the results that were hoped for, an interesting trend is apparent. While both CAMA1 and SKOV3 are chemoresistant and exhibit deregulation of genes such as p53, only SKOV3 exhibited somewhat of a senescent state in the actual senescence assay, even though both SKOV3 and CAMA1 had senescent-like behavior in the proliferation and vitality assays.

This may be explained by possible incorrect procedures when staining CAMA1, unknown factors (such as contamination), or the possibility that more factors are involved in the connection between similar genetic expression and senescence as a mode of chemoresistance.

Senescence "has emerged as a potentially important contributor to aging and age-related disease, and it is an attractive target for therapeutic exploitation," not just in age-related diseases, but also in cancer (Childs, et al. 2016). Senescence may be a significant tool in the recurrence of tumors. Our in vitro observation that chemotherapy agents not only promote apoptosis but can also induce cellular senescence in SKOV3 indicates that genes that control senescence might also determine treatment outcome. It is also observed that chemotherapeutic drugs induced senescence in p53- and p16-defective tumor cell lines (like breast and ovarian) and therefore may be simultaneously playing a dual role in apoptosis and senescence.

Conclusions and Future Work

The goal of this research was to investigate senescence as a mode of chemoresistance in ovarian and breast cancer cell lines and to pinpoint genes whose expression was associated with hypoxia-induced chemoresistance and the incitement of senescence. We achieved these goals by using SKOV3 (ovarian cancer), HOSE (normal epithelial cells), and CAMA1(breast cancer cells), which were treated with various forms of platinum-based chemotherapy and experienced induced hypoxia. These treatments resulted in SKOV3 and CAMA1 cells entering a senescent stage, which led us to the conclusion that senescence serves as a form of "hibernation" for cancerous cells. This dormant period allows them to avoid cell death during treatment, then continue to proliferate afterwards. During this experiment, we also learned that p53 was a gene that played a role in chemoresistance, as proved by past literature and previous studies, including our study. Since ovarian and breast cancer are p53- and p16-defective tumor cell lines, this gene may be simultaneously playing a dual role in apoptosis and senescence.

Our conclusions coincide with past studies on the nature of cancer and its unique molecular events that govern the emergence of aggressive therapy-resistant cells with unique cell properties after chemotherapy. However, our results may have been influenced by a few factors that were out of our control. For example, during the course of the experiment, a few cell wells became infected with mold or bacteria and had to be discarded. Since we did an in vitro study, some deviation from typical conditions and behaviors can be expected. The best results would be those from actual tumor samples. In the future, our conclusions can be refined by duplicating this experiment with actual tumor samples instead of in vitro grown cell colonies. Different cell lines can also be examined to see if senescence is a common pathway for chemoresistance in other forms of cancer.

Due to time limitations, we were unable to run a qPCR test on CAMA1. If we were to redo our experiment, we would include this data to further support our conclusions. The use of a qPCR in another similar experiment could be used to explore gene expression in hypoxia by revealing certain DNA sequences and quantifying their presence. Finally we would use a Western Blot test to identify specific protein expressions of the genes involved in apoptosis and the cell cycle (such as p53).

After the completion of this investigation, some questions still remain. While we investigated the role that senescence plays in the development of chemoresistance, we are not entirely sure just how senescence functions in the deregulation of the cell cycle. Certain genes and pathways that influence this deregulation may still lie undiscovered, as well. Ultimately, the goal of this research is to help develop an anticancer treatment that does not lead to chemoresistance. This is still a work in progress in the research field. Our research helps brings modern medicine a step closer to a better cure. Overarchingly, improved understanding of the molecular mechanisms underlying drug resistance in cancer cells could be useful for devising targeted therapeutic approaches. Senescence is just one of the many pathways cells take to outcompete chemotherapy. It is our aim to increase understanding of molecular cues in cancer cells, which can result in better outcomes than those currently achieved using conventional approaches alone.

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