Novel Role of Hypoxia in Ovarian Cancer Chemo Resistance through Epigenetic Regulation of HIF1α

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Abstract

Ovarian cancer is the fifth deadliest cancer in woman, and epithelial malignancies account for 90% of cases. Tumor recurrence after chemotherapy or radiation remains a major obstacle to successful ovarian cancer treatment. Despite the large number of studies, molecular events that govern the emergence of aggressive therapy-resistant cells after chemotherapy are poorly defined. Genetic modifications, such as copy number variation (CNV), play an important role in controlling the expression of genes that are involved in chemo resistance. We analyzed CNV data that is publically available through the Cancer Genome Atlas and others. Of particular interest was the transcription factor HIF1α which plays an integral role in oxidative stress response such as those induced by chemotherapy reagents? The present study provides evidence for the rare escape of tumor cells from drug-induced cell death by entering a non-cycling senescent state. We report the adaptive response of human ovarian surface epithelium cells to CoCl$_2$, a chemical hypoxia-mimicking agent resulting in a senescent-like state of chemo resistant cells. The effect of the treatment was evaluated on CNV of HIF-1α gene expression, cell proliferation, survival, and tumor invasiveness.

We show here that CNV duplication events of HIF1α results in an oxidative stress response in cells leading to chemo resistance through the induction of cellular senescence. Understanding the molecular events associated with chemo resistance will ultimately lead to better patient treatment and outcomes.

1. Introduction

Epithelial ovarian cancer is the fifth leading cause of cancer death among women in the United States, accounting for 5% of cancer deaths (Jemal et al., 2011). Though rare, most patients are diagnosed with advanced disease due to the non-specific nature of early symptoms and lack of effective screening strategies. For the approximate 75% of women diagnosed with stage III or IV disease, the likelihood of long term disease-free survival ranges from 15 to 20% (Hoskins et al., 1991; McGuire et al., 2003; Barnholtz-Sloan et al., 2003). Early screening is difficult because obvious signs and symptoms such as pelvic pain, as cites or problems urinating, do not present until after the tumors have progressed to advanced stages.

Despite the recent advances in chemotherapy drugs, the survival rate for ovarian cancer is still very low due to the high rate of relapse with cancer cells that are resistant to chemotherapeutic drugs. Current treatments of chemotherapy utilize a combination of platinum drug and a taxane drug. Platinum induces apoptosis, while the taxane interferes with cell division (Barnholtz-Sloan et al., 2003). Although initial treatment is effective, all patients that experience relapse display chemo resistant tumors (Jemal et al., 2011). This requires more aggressive therapies to be used, which take a larger toll on the patient, and are less effective at treating the tumors (McGuire et al., 2003).

Researchers have been trying to discover the mechanisms, which cause these initially sensitive tumors to develop resistance. DNA copy number variation (CNV) is one such mechanism that shows promise[3]. CNV is a frequent, dynamic and complex form of genetic diversity, where in which copy number differences are observed between two or more genomes (Feuk L, 2006). CNVs are larger than 1 kilobase (kb) in size and involve gains or losses of genomic DNA. Recently, these genomic rearrangements have been linked to a variety of diseases with the resulting deletion or duplication events that take place in the cell (Sebat J, 2004). CNVs occur commonly in the genome and have been implicated in risk of complex diseases including schizophrenia (Need et al., 2009), neuroblastoma (Diskin et al., 2009), and prostate cancer (Lawrence et al., 2008; Liu et al., 2009). With the advent of large-scale computer information processing, it is now possible to analyze CNV on a genomic level. The analysis performed by the Cancer Genome Atlas (TCGA) and other groups has revealed great insights in ovarian cancer genomic alterations allowing us to compare the genomes of sensitive tumors with those that are resistant. Genomic duplication and micro deletion events were frequent throughout the genome. Of specific interest were genes involved in transcription regulation, including MYC and HIF1α (Bell D, 2011).

HIF-1 is the major transcription factor involved in the adaptive response to hypoxia and consists of HIF-1 alpha and HIF-1 beta subunits. HIF1α plays a crucial role in the transcription of many genes, including those involved in oxygen homeostasis, glucose uptake within cells, angiogenesis, and both pro and anti-apoptotic actions (Donghoon Y et al., 2006; Carmeliet P, 1998; Yu EZ et al., 2004). Studies have shown that hypoxia, oxygen concentration is less than the physiological minimum required for the cell, can induce mitotic arrest. The minimum oxygen concentration necessary for a cell varies in different areas of the body. HIF-1α is a gene that regulates the homeostatic response to hypoxia. Additionally, hypoxia has the ability to induce cellular senescence. Cellular senescence is a programmed response to remove cells with damaged DNA from the proliferative population as they age. Senescence can be induced by telomere shortening, DNA damage, or oncogene activity. At the ends of every strand of DNA are telomeres, non coding regions of DNA that protect the coding regions from being lost due to replication. After each replication the telomeres become shorter. When the telomeres become too short, cells undergo senescence in order to prevent the proliferation of cells with damaged DNA (Yu EZ et al., 2004). One way the human body tries to prevent the proliferation of cancerous cells is a pathway known as cellular senescence. Cellular senescence is one of the most fundamental aspects of cell behavior, and is thought to play a crucial role in regulating cellular lifespan both *in vitro* and *in vivo*.

Moreover, tumor hypoxia induces cellular senescence and increases the resistance of cancer cells to chemotherapy (Yu EZ et al., 2004). Hypoxia-inducible factor-1α (HIF-1α) is the main transcriptional factor activated by hypoxia and it plays a key role in reprogramming tumor growth.

In this paper, we aimed to determine how CNV through duplication of the gene HIF1α plays role in chemo resistance of ovarian cancer. Our functional analysis screen has revealed the activation of the cellular senescence pathway in human ovarian surface epithelial (HOSE). In order to study the effects of hypoxia *in vitro* we used medium infused with 50 μM $CoCl_{2\,and}$ studied the effects of it on HIF-1α gene expression at protein levels. In this paper we determined that CNV duplication of HIF1α induced by hypoxic events induces senescence in HOSE cells. Our study indicates that oxidative stress caused by chemotherapeutic agents generates senescent-like cells which play an important role in tumor chemo resistance. We suggest that premature senescence is a mechanism of tumor suppression.

2. Methods

2.1 Materials

HOSE 6-3, a normal human ovarian surface epithelial cell line that has been immortalized by HPV E6/7 ORF (obtained from Dr. S. W. Tsao, Cold Spring Harbor Laboratory, and NY) 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. Antibodies were obtained from Cell Signaling. The Western Lightning ECL-plus detection system was obtained from Perkin Elmer. Trans well assays and reagents were obtained from BD Bioscience. RNEasy, DNEasy obtained from Qiagen. Cisplatin and Carboplatin were purchased from Sigma. Primers were supplied by Sigma Genosys. Cell counting kit-8 was purchased from Dojindo Molecular Technologies, Inc. Neutral red dye uptake was purchased from Sigma. MTT viability assay kit was supplied by Roche

2.2 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 weashed in PBS, trypsinized in 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 spectrophotmetry using the Biotek EL800 at 600nm.

2.3 Chemotherapy Sensitivity and Survival

The ovarian cell line HOSE was tested for IC20, IC50 and IC80 carboplatin sensitivity levels. Cells were seeded at 10^3cells/well in a 96 well plate and treated with carboplatin at a concentration range of 1–100 µg/ml in 100 µl of fully supplemented DMEM. After a period of 72 h 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.

2.4 Colorimetric Caspase - 3 Apoptosis Assay

The caspase 3 Assay Kit (Colorimetric) (ab39401) was used according to the manufacturer's instructions. It is an assay to measure the activity of caspases using spectrophotometric detection. Briefly, cell lysates were prepared after treatment with carboplatin in Hose +HIF1α and Hose control. Samples were treated with 2X reaction buffer/DTT and DEVD-pNA. After 2 hours incubation at 37°C, results were read at 405-nm using the Biotek EL800 spectrophotometer. Comparison of triplicate plates using the absorbance of p-NA from our +HIF1α treated samples and our control was used to determine the fold increase in caspase 3 activity.

2.4 Cell treatment

Exponential-phase ovarian cancer cells were plated into 6-well plates and allowed to adhere for at least 24 h. When the cell density reached approximately 75%, the cells were treated with 10 μg previously cloned human HIF-1α cDNA fragment encoding amino acids 432–528 in a pGEX2T vector using Lipofectamine following the manufacturer's instructions (Invitrogen). Non-targeting cDNA was obtained from Invitrogen and used as our control.

2.5 MTT Assay

MTT assays (Mosmann, 1983; Hansen *et al*., 1989) were used to quantify cell number/density of viable cells by, introduction of 1mg/ml of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide to $1x10^3$ cells and incubation for 4h at 37°C. One volume of 20% SDS, 50% *N*, *N*-dimethylformamide was added and the plates were incubated overnight at 37°C. The level of Formazan, the metabolite of MTT was measured at 600 nm using the Biotek EL800 spectrophotometer and means of the triplicates were calculated. Following non-cellular background subtraction, all data were normalized to the MTT conversion activity of solvent-treated control cells.

2.6 Chemotherapy Sensitivity and Survival

The ovarian cell lines HOSE 6-3 was tested for Carboplatin and Doxorubicin sensitivity to determine the IC20, IC50 and IC80 values. Briefly, the cell line was treated with either chemotherapy drug at a concentration range of 1-100ug/ml in 100ul of fully supplemented DMEM. After a period of 72 hrs the drug was aspirated and proliferation was assessed by MTT assay. The Hose 6-3 $CoCl_2$ or PBS control treated cells were challenged with the IC50 dose of chemotherapy reagent. The cell line was seeded at 10^3 cells per well in a 96 well plate and subjected to a 72 hour treatment followed by a 72 hour recovery period. After treatment the cell proliferation was assessed by MTT assay, Netral Rerd Dye Uptake, and CKK-8 following the manufacturer's instructions.

2.7 Real-time qPCR

Total cellular RNA was isolated using RNeasy kit and 1ug of RNA was reverse transcribed to cDNA using the Super Script 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: HIF1α (5'

ACTB (5'-TTGCCGACAGGATGCAGAAGGA-3' and 5'-AGGTGGACAGCGAGGCCAGGAT-3'). 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 actin control.

2.7 Immunoblotting.

Samples were harvested, total protein extracted and protein concentration determined using the Bradford Assay. Western blotting was performed using 5–10 µg of protein lysates and electrophoresis with the Invitrogen E-Page® system. After transfer of lysates from gel to PVDF membrane, blots were blocked and hybridized for immunodetection using anti-rabbit monoclonal antibody against HIF1α, diluted 1:1,000 overnight, followed by incubation with anti-rabbit secondary antibody (horseradish peroxidase HRP-conjugated), diluted 1:10,000. Membranes were washed in TBS-Tween at room temperature. Anti-rabbit monoclonal β tubulin was used as a loading control. Membranes were washed and protected from light. The Western Lightning ECL-plus (Perkin Elmer) was used for signal detection. NIH Image J software was used to quantitate intensity values for protein levels.

2.8 Senescence

Senescence induced by HIF1α were stained using Millipore's senescence-associated β-galactosidase (SA-β-gal) assay following the manufacturers protocol. After 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% CO_2 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.

2.9 Wound Healing Assay

Cell migration was assessed by the ability of the cells to migrate into a cell-free area. Briefly, cells were plated 1×10^5 cells in full growth medium on 6-well plates and grown for 24 h to reach confluence. The monolayers were then wounded by scratching with a plastic yellow pipette tip. After washing, the cells were incubated in growth medium for 12-16hrs and observed under a microscope. The wound closure was estimated as the ratio of the remaining wound area relative to the initial wounded area using Image J software. Experiments were repeated at least three times.

2.10 Cellular Invasion

Invasive cells are able to move through an extracellular matrix into neighboring tissues. Cellular invasion can be measured in vitro using a Boyden chamber. Briefly, 2.5 x 10^4 serum starved cells are seeded in the top of the insert in serum-free media, while serum acts as chemo attractant and is placed in the well below. Migratory cells move through the pores toward the chemo attractant below. Cells that had invaded to lower surface of the Matrigel-coated membrane were fixed with 4% methanol in PBS, stained with 1:1 dilution of crystal violet, and counted in five random fields using a bright field microscope. The number of invading cells was quantified and normalized to controls.

2.11 Transformation Assay

The Millipore Cellular transformation Kit was used to determine transformation of our low passage HOSE cells. Cells were seeded at 1.25×10^5 cells per well in 6-well dishes with or without transfection of HIF1α according to the manufacturer's protocols. Two days later, the cells were transferred to 10 cm plates. After reaching confluence, they were kept for 21 days in DMEM containing 7% calf serum, after which they were stained with 0.5% crystal violet. Quantitation plotted is the means and standard errors of duplicate assays and is representative of at least six experiments.

2.12 Statistical analysis

The statistical significance of the differences in data between each treatment groups was determined by applying Student's *t*-test. A *P*-value 0.05 was considered significant. Unless otherwise noted, experiments were repeated three times with up to 10 replicates per trial.

3. Results

3.1 Gene Candidate Selections

Hypoxia, frequently found in the center of solid tumors, is a major obstacle in the development of effective cancer chemotherapy. We hypothesized that tumors have taken advantage of molecular mechanisms to increase transcription of genes resulting in resistance to platinum treatment. We have used the variable, platinum resistance, clinically defined as tumor growth 6 months or less after treatment ceases. To identify DNA CNV changes we utilized publically available data from TCGA using representational oligonucleiotide microarray analysis (ROMA). We performed statistical tests to identify genes that when expression has changed can segregate sensitive from resistant patients. The CNV data was then associated with available expression data to identify which genes are significantly up regulated and oncogenic or down regulated and suppressive (**Table 1**).

Common amplifications encoded *CCNE1, MYC*, and *HIF1*, while common deletions included BRCA1 and TP53 (Bolton et al., 2012). We identified HIF1α among the top 10 genes associating with resistance when expression levels are up regulated. HIF1α is a basic helix-loop-helix subunit of the heterodimeric transcription factor hypoxia-inducible factor 1. HIF1α has been observed to play a role in chemo resistance of several tumors, including gastric (Liu L, 2007). Genes predicted to have high expression correlations will allow us to better determine which functions are potentially altered in tumor samples. Furthermore, uncommon focal amplifications were seen in the HIF1 transcriptional regulator, therefore making it an unusal candidate to select for further analysis (**Fig 1B**).
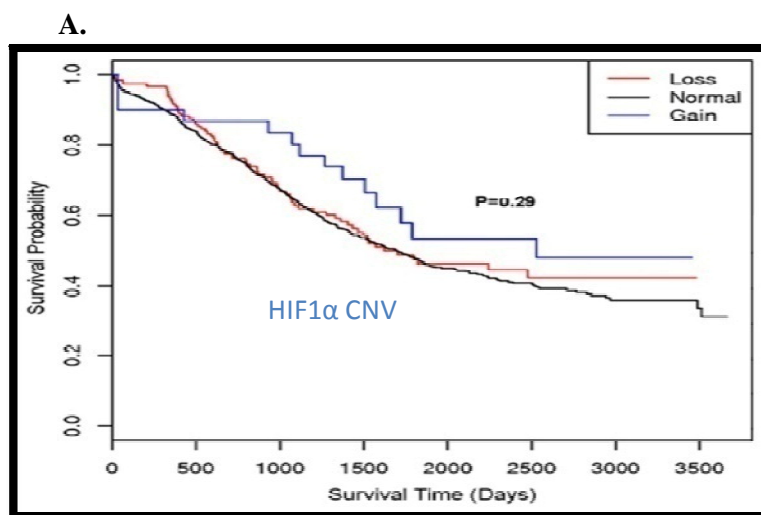
Table 1: Significantly mutated genes in Ovarian Cancer

Gene	Chromosome	Gene Name	Function Summary
BRCA1	17	breast cancer 1, early onset	BRCA1 is a tumor suppressor involved in pathways such as DNA damage repair, ubiquitination and transcriptional regulation to maintain genomic stability. Required for appropriate cell cycle arrests in both the S-phase and the G2 phase of the cell cycle.
BRCA2	13	breast cancer 2, early onset	Involved in double-strand break repair and/or homologous recombination. May participate in S phase checkpoint activation.
CCNE1	19	Cyclin E1	Regulates G1 to S transition of the cell cycle; over expressed in many tumors leading to a deregulated levels of protein and kinase activity relative to the cell cycle and inducing chromosome instability
CDK12	17	Cdc2-related kinase, arginine/serine-rich	Involved in RNA splicing.
CSMD3	8	CUB And Sushi Multiple Domains 3	Protein Coding gene. Diseases associated with CSMD3 include certain forms of epilepsy. An important paralog of this gene is CR1L.
FAT3	11	FAT Atypical Cadherin 3	May play a role in the interactions between neurites derived from specific subsets of neurons during development
GABRA6	5	gamma-aminobutyric acid (GABA) A receptor, alpha 6	GABA, the major inhibitory neurotransmitter in the vertebrate brain, mediates neuronal inhibition by binding to the GABA/benzodiazepine receptor and opening an integral chloride channel.
HIF1α	14	Hypoxia inducible factor 1, alpha	Functions as a master transcriptional regulator of the adaptive response to hypoxia.
MYC	8	Avian Myelocytomatosis Viral Oncogene Homolog	Transcription factor and oncogene, erythropoietin early response gene, interacting with various cellular factors including MAX, YY1, AMPHL. Important for stimulating genes required for proliferation; induces apoptosis in sensitive cells
NF1	17	Neuro fibromin 1	Stimulates the GTPase activity of Ras. NF1 shows greater affinity for Ras GAP, but lower specific activity. May be a regulator of Ras activity.
RB1	13	retinoblastoma 1	Key regulator of entry into cell division that acts as a tumor suppressor. Acts as a transcription repressor of E2F1 target genes.
TP53	17	tumor protein p53	Acts as a tumor suppressor in many tumor types; induces growth arrest or apoptosis depending on the physiological circumstances and cell type. Involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for this process.

Table 1: Selected ovarian cancer genes selected based off statistical analysis of TCGA data and expression analysis. Genes highlighted in red are up regulated and genes highlighted in green are down regulated.

3.2 In Vitro Platinum Screen

It was unclear if our gene candidate identified functionally from our bioinformatics analysis altered platinum resistance in the cell. Thus we performed an *in vitro* carboplatin resistance assay. HOSE 6–3 (onward, will be referred to as HOSE) was selected to perform functional studies. HOSE is a normal human ovarian surface epithelial cell line that has been immortalized by introduction of the HPV E6/7 ORF. This cell line has been previously used in the study of chemoresistance (Siu et al., 2012) and has been found to be sensitive to carboplatin treatment.

We were most interested in genes that were had altered CNV and were transcriptionally amplified. **Table 1** lists our top candidates for cDNA transfection selection. The induction of transcription caused by CNV was modeled by the introduction of cDNA clones. Since we several candidates and only could test for one at a time, we chose to further validate *HIF1α* for this study. To be more confident of our candidate, each cDNA clone was individually verified for carboplatin resistance using three concentrations of carboplatin, the IC20, the IC50, and the IC80. **Fig 1A** illustrates the basic experimental design.

A.

B.

Fig 1: **Schematic representation of experimental plan and tumor analysis. (A)** Part one consisted of a bioinformatic analysis of publically available data on The Cancer Genome Atlas and Genome Wide Association Studies databases. Next, we performed an in-vitro carboplatin screen. Finally, various functional tests were done to understand chemoresistance mechanisms. **(B) Kaplan–Meier plot** showing the segregation between carboplatin therapy resistant and sensitive patient groups on the genomic segment surrounding HIF1α. The lines represent loss, gain, and normal copy number for HIF1α.

3.3 Increased Expression of HIF1α Induces Platinum Resistance

We went on to study the effect of *HIF1α* amplification on carboplatin resistance using our in vitro cell culture model to further validate our screening methodology. The homeostatic response to hypoxia is mediated by this gene and it has already been correlated with poor survival in several studies (Braicu et al., 2014, and Lu et al., 2010). Its expression is amplified in a number of other solid tumors (Zhong et al., 1999).

Cells infected with *HIF1α* cDNA were assayed by qPCR and Western blot to ensure efficient amplification of *HIF1α* expression levels (**Fig 2A, Fig 2B**). Our results indicate that after IC50 dose treatment of carboplatin, induction of *HIF1α* expression resulted in a survival advantage (**Fig 2C**). As it is a transcription factor, amplification of *HIF1α* expression may increase carboplatin resistance by up regulating several genes important in survival, thus increasing cell growth and increasing the number of cells surviving treatment. To address this concern, growth assays were done over the course of 9 days without the addition of carboplatin, demonstrating that amplification of *HIF1α* expression in HOSE cell lines had a moderate effect on the number of cells growing (**Fig 2C**). A 3-day Bromodeoxyuridine (BrdU) assay was also used to measure the level of actively dividing cells. The BrdU assay exhibited a selective growth advantage for *HIF1α* amplified cells when compared to their control counterparts in the HOSE cell lines. This result was statistically significant, p<0.05.
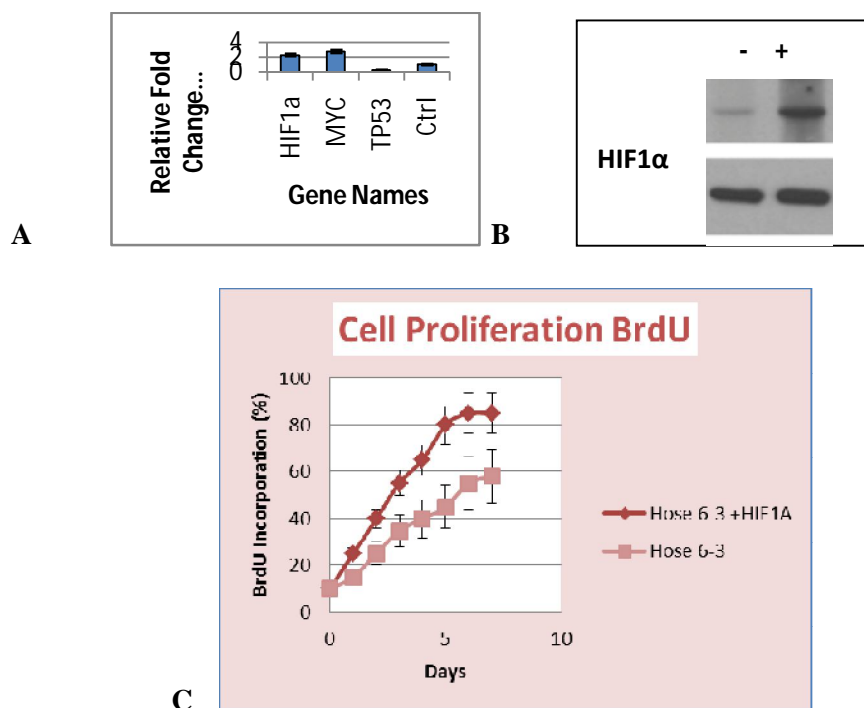
Fig 2: Expression and Growth Summary Analysis for HIF1α.

(A) RNA was prepared separately from each sample cell line with either cDNA over expression of HIF1α and MYC, or knockdown using siRNA for TP53. qPCR analysis was performed for each sample and the data combined. Data was normalized to housekeeping gene, *Actin (Ctrl)*. Error bars indicate the standard error of the mean after three independent trials (s.e.m.). Statistical analysis by *t*-test. **(B)** Western blot was used to determine translational activity of HIF1α, with Actin as the control antibody. Lane indicated with + includes the cDNA for HIF1α whereas the – indicates lysates with no transfection. **(C)** Effects of +HIF1α on cell proliferation according to BrdU incorporation assays. HOSE cells were incubated with IC50 carboplatin for 72 h. The data are the mean ± SD (n=3. Data represents three independent experiments, with three replicates per trial.

3.4 Amplification of *HIF1α* expression Modulates Apoptosis through Caspase-3 Activity

An alternative role for *HIF1α* in carboplatin resistance could be an effect on apoptosis. The deregulation of the apoptotic pathway is often shown to be involved in resistance (Fojo et al., 2001 and Morrison et al., 2011), and this process can also be epigenetically deregulated (Balch et al., 2010). To determine if *HIF1α* uses apoptosis to induce carboplatin resistance, we plated HOSE control and HOSE +*HIF1α* cell lines with and without carboplatin. After 72 hours, the percent viable cells to dead cells were determined using trypan blue stain exclusion from live cells as well as CCK-8 (cell counting kit-8), which determines cell viability in cell proliferation and cytotoxicity assays. The results showed fewer dead cells in the +HIF1α cell line compared to the control HOSE cell line in the presence of carboplatin (**Fig 3A**).

To determine if any markers of apoptosis are affected by the loss +*HIF1α,* levels of activated caspase 3 were measured using western blot (data not shown), qPCR, and colorimetric enzymatic analysis. The caspase family of cysteine proteases plays a key role in apoptosis. Caspase 3 is synthesized as an inactive pro-enzyme that is processed in cells undergoing apoptosis and can be cleaved and activated by upstream caspase 8/9 targets. This cleavage activates the enzyme and can be readily detected in vitro. Our results indicate suppression of gene expression, protein expression, and enzymatic activity of Caspase 3 (**Fig 3B**) in +HIF1α cells when compared to control cell line. These results suggest that aberrant apoptosis signals are a factor in how +HIF1α CNV confers resistance to carboplatin.
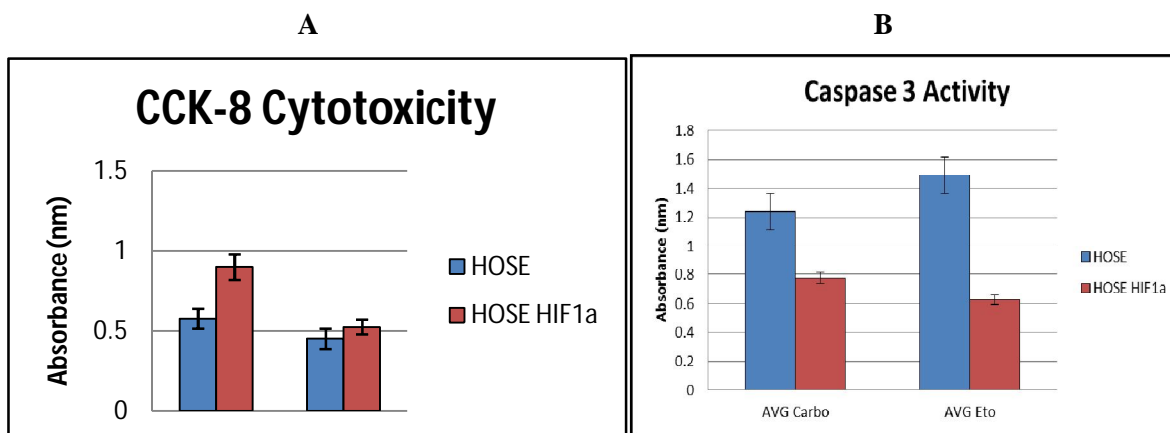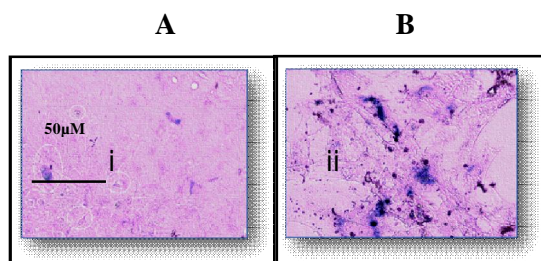
A   **B**

Figure 3: Cytotxicity, cellular proliferation, and Caspase 3 activity measurement. (A) After treatment with carboplatin and etoposide (control), cells containing HIF1α convey increased survival to chemotherapy treatment. (B) Caspase 3 apoptotic activity was measured and HOSE cells treated with either carboplatin or etoposide indicated increased caspase 3 activity compared to the HOSE +HIF1α. (C) These results were confirmed with qPCR for Caspase 3 expression.

3.5 +HIF1α Induced Cells Results in Increased Senescence

Tumor cells have the ability to maintain viability following chemotherapeutic exposure by undergoing alternative cellular fates such as cellular senescence. Tumor environmental stresses such as starvation, hypoxia and DNA damage induced by chemotherapy drugs have been shown to induce such fates as senescence (Saab, 2010) and it has been shown to be significantly associated with cancer cell survival and chemoresistance (Watson et al., 2009). Furthermore, senescent cells adopt a flat enlarged morphology and cease proliferation at subconfluent densities despite the presence of serum (reviewed by Votja, 1995). An increasingly important function of p53 is to monitor the genome to determine whether or not a cell is viable to enter the proliferative population. In tumor cells, oncogenic activation has the ability to suppress its function or can delete the gene altogether. Low levels of p53 become more prominent following DNA damage, certain oncogenic insults, hypoxia, and a variety of other cellular stresses (reviewed by Ko and Prives, 1996). Activation of p53 prevents cell proliferation by inducing cell-cycle arrest or apoptosis. To further explore whether the observed decreased cell death in the *+HIF1α* knockdown was due to protection from senescence or changes in p53 status, we performed the Millipore Cellular Senescence Assay and an ELISA for p53 quantification. According to our results of the SA- βgal assay, compared to control HOSE cells, +HIF1α Hose cells stained moderately more positive for SA-βgal after 72 hours carboplatin treatment (**Fig 4A**). Additionally, the quantitative analysis of p53 status demonstrates a no change in p53 amounts in the HOSE +HIF1α cells after treatment with carboplatin.
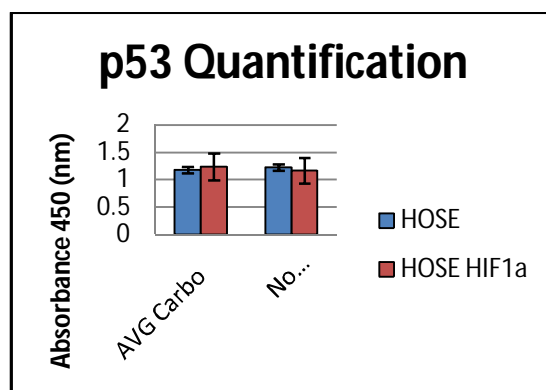
A   **B**

Figure 4: Senescent features of HOSE cells. (A) Representative image of HOSE (i) and HOSE +HIF1α (ii) cells after treatment for 72 hours carboplatin. (B) Changes in p53 quantification were measured using ELISA. As you can see from the graph, there was no significant difference in p53 amounts between HOSE and HOSE +HIF1α cell lines treated with or without carboplatin.

3.6 *HIF1α* Induction Confers an Invasive and Tumorigenic Phenotype *in Vitro*

In addition to its role in affecting apoptosis and senescence to confer carboplatin resistance, *HIF1α* is also a known transcription factor that is a major regulator of cell adaptation to hypoxic stress and plays a critical role in on cogenesis and angiogenesis (Birner et al., 2001). Therefore, we decided to explore whether *HIF1α* displayed these oncogenic properties in ovarian cancer in culture. To measure tumorigenicity, we first assayed whether cell lines with the +*HIF1α* required a basement membrane, and performed experiments for growth in soft agar. After 3 weeks of growth, +*HIF1α* cells when compared to the control cell lines indicated no change in colony growth in both size and number (results not shown). In addition, a Matrigel assay was used to quantify invasive potential of the cells. After 36 h there was a marked increase in the number of cells invading when +*HIF1α* was induced compared to the control cells (**Fig 6B**). In addition, a scratch assay was also performed to evaluate migratory potential. In comparison to the control cell lines, we observed that the Hose cells harboring the HIF1α cDNA had an increased migration (**Fig 6A**). Collectively, these results suggest that in ovarian cancer cells, a increase in the level of *HIF1α* is associated with oncogenic features, such as increased invasiveness and tumorigenicity.
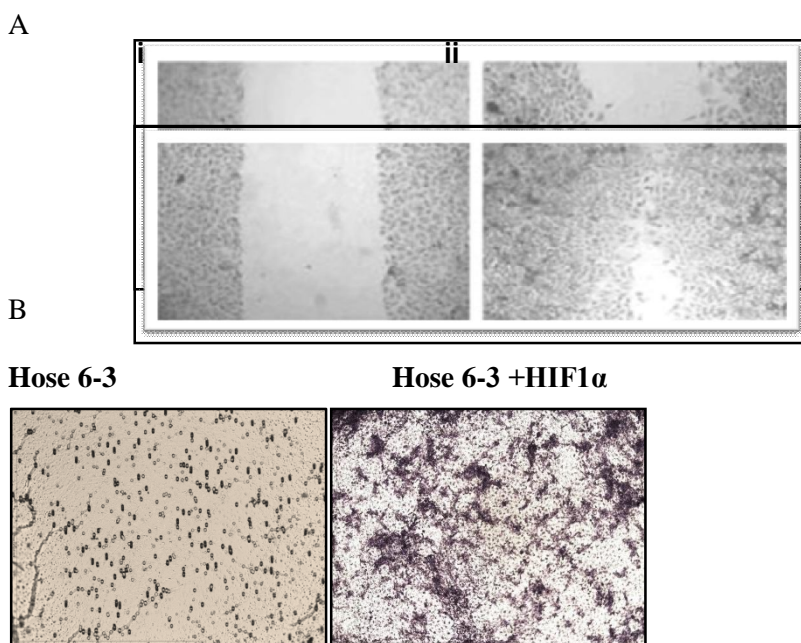

Figure 6: Cell migration/wound healing assay (A) Transwell cell invasion assay (B) were done using Hose 6-3 cancer cells at 0 and 24 h, in the presence of HIF1α over expression results in a more tumorgenic phenotype. **A/Bi** and **A/Biii** represent HOSE control and **A/Bii** and **A/Biv** represent HOSE +HIF1α.

4. Discussion

The effect of hypoxia on proliferation and tumor genesis has been well documented in the context of immortal cell lines (Votja, 1995). However, the analysis of the role hypoxia inducible factor 1-alpha plays in this pathway to confer resistance to drug treatment has yet to be established. As drug resistance is one of the major barriers to the successful treatment of malignancies, investigation of the mechanisms of drug resistance and approaches to overcoming it has been widely performed in the past decades. Ovarian cancer is a common cancer in women and the most lethal gynecologic malignancy. The high mortality rate is attributable to the asymptomatic nature of the early stage of the disease, the lack of reliable screening tests, and the development of drug resistance. While over expression of genes is crucial for control of normal cell growth and is a hallmark of cancer, it also has the potential to affect how tumors respond to chemotherapy (Hanahan, 2011). We have studied the genome of ovarian cancer to identify genes activated by DNA CNV and demonstrate that activation of the hypoxia induced factor, HIF1α functionally increases resistance to platinum drugs. To our knowledge, this is the first time this molecular signature of the gene has been implicated in therapy response.

Single nucleotide polymorphisms GWASs have yielded great insights into the etiology several complex diseases including ovarian cancer (Song et al., 2009; Bolton et al., 2010). With much unexplained variation in ovarian cancer outcome, investigation of additional inherited factors remains warranted. Here, in the first germ line ovarian cancer CNV analysis, we have harnessed CNV data gleaned from dense genome-wide SNP genotyping in order to address the hypothesis that copy number variation is associated with survival.

Our results indicate an increased presence of SA- βgal activity in the +*HIF1a* cells. SA- βgal is a protein that is activated in senescent cells. This indicates that the over expression of *HIF1a* leads to a higher rate of cells induced into senescence. We also found a higher survival percentage in the cells that exhibited an over expression of *HIF1a*. To determine whether the increased survivability was due to +*HIF1a* and not a change in p53 activity we performed a p53 quantification assay along with the senescence assay. The results showed there was no difference in p53 activity between both cell lines. From these results we can infer +*HIF1α* may play a role in the induction of senescence as a means for chemo resistance. The non-replicative state of senescence may be a pathway that cancer cells try to exploit in order to evade chemotherapeutic treatments. Chemotherapy works to target rapidly dividing cells, thus many patients lose their hair during treatment. The harsh chemicals in chemotherapeutic treatment often create a toxic environment around the tumor in order to kill it. The cancer cell may use various innate mechanisms in order to adapt and survive in the toxic environment. One of these innate mechanisms is the oxidative stress pathway. If the oxidative stress pathway is activated, the cell can enter into senescence and be removed from the replicative population. If a cancer cell that was once rapidly replicating suddenly ceases replication, the chemotherapeutic drugs may no longer target it. The development of chemo resistant tumors leads to a worse prognosis for the patient. From our results, we can infer that +*HIF1α* may play a role in the induction of senescence as a means for chemo resistance.

In order to efficiently and reliably apply the current observations of genetic-phenotype association and the value of prediction models based on common low-penetrance alleles to the clinic, further validation and confirmation using larger sample collections are indispensable. More research on this topic must be done in order to fully understand the role that over expression of *HIF1a* has on the induction of senescence and how it relates to chemo resistance. Cancer continues to evolve and we must adapt and evolve along with it in order to one day find a viable treatment option.

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