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THE ROLE OF OXIDATIVE STRESS IN THE ESTABLISHMENT OF RESISTANCE TO CISPLATIN IN EPITHELIAL OVARIAN CANCER CELLS

by

JIMMY BELOTTE

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2016

MAJOR: PHYSIOLOGY

Approved By:

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Advisor   Date

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DEDICATION

This dissertation is dedicated to my beautiful family: my loving wife Mitchell Alexis and our beautiful daughters, Sophie and Sidney. Without their love and support it would have been impossible to stay the course and bring this work to completion. I would also like to dedicate this work to my parents who understood and taught their children that education was the only opportunity for a brighter and honorable future to grow beyond our very modest surroundings; to my brothers and sisters for their love and unequivocal support throughout. Last but not least, my in-laws who are overwhelmingly supportive and loving.

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Counselors introduce/explain the study to the eligible patients, consent them, and arrange for the blood draw. We would also like to thank Dr. Michael A. Tainsky and Nancy Levin, Karmanos Cancer Institute, Detroit, MI, for their support and for providing the DNA samples for this study. The Microscopy, Imaging, and Cytometry Resources (MICR) Core is supported, in part, by NIH Center Grant P30CA22453 to The Karmanos Cancer Institute, Wayne State University, and the Perinatology Research Branch of the National Institutes of Child Health and Development.

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LIST OF ABBREVIATIONS

AGTC: Applied Genomics Technology Center

AGTC: Applied genomics technology center

AIF: Apoptosis-inducing factor

AKT: Ak mouse strain -thymoma (viral proto-oncogene serine-threonine kinase

ANOVA: Analysis of variance

ATCC: American Type Culture Collection

Atg: Autophagy-related genes

ATP7β: ATPase7 beta

BAD: Bcl-2-associated death promoter

BAK: Bcl-2 homologous antagonist killer

BAX: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma 2

Bcl-Xl: B-cell lymphoma-extra large

BER: Base excision repair

BID: BH3 interacting-domain death agonist

BLT2: Leukotriene B4 receptor-2

CA-125: Cancer antigen 125

Cas9: CRISPR associated protein 9

CAT: Catalase

cDNA: Complementary deoxyribonucleic acid

CGD: Chronic granulomatous disease

CI: Confidence interval

COL6A3: Collagen alpha-3 (VI)

CRISPR: Clustered regularly interspersed short palindromic repeats

CRS: Cytoreductive surgery

CTR1: Copper transporter 1

CYBA: Cytochrome b-245, alpha polypeptide

CYBA: Cytochrome b, alpha chain

CYBB: Cytochrome b, beta chain

DISC: Death-inducing signaling complex

DNA: Deoxyribonucleic acid

ds oligos: Double stranded oligonucleotides

ECM: Extracellular matrix

ELISA: Enzyme-linked immunosorbent assay

EOC: Epithelial ovarian cancer

ER: Endoplasmic reticulum

ERK1/2: Extracellular signal-regulated kinases 1/2

FACS: Fluorescence-activated cell sorting

FADD: Fas-Associated protein with Death Domain

FAP: Fibroblast activation protein alpha

FasL: Fas-Fas ligand

FBS: Fetal bovine serum

FIGO: International Federation of Gynecology and Obstetrics

FLIP: FLICE-like inhibitory protein

GSH: Glutathione

GSR: Glutathione reductase

GSSG: Glutathione disulfide

GST: Glutathione s-transferase

GWAS: Genome-wide association studies

H_2O_2: Hydrogen peroxide

HIF-1α: Hypoxia inducible factor

HNPCC: Hereditary nonpolyposis colon cancer syndrome

$HO^{\bullet}$: Hydroxyl radical

HOCl: Hypochlorous acid

HR: Hazard ratio

IAP: Inhibitors of apoptosis proteins

IC_{50}: Half maximal inhibitory concentration

IL-6R: Interleukin-6 receptor

iNOS: Inducible nitric oxide synthase

IRB: Internal review board

K-M: Kaplan-Meier

KCIGR: Karmanos Cancer Institute's Genetic Registry

KSR1: Kinase suppressor of Ras 1

LIF: Leukemia inhibiting factor

LR: Likelihood ratio

MAF: Minor allele frequency

MAPK: Mitogen-activated protein kinases

MCL-1 Induced myeloid leukemia cell differentiation protein Mcl-1

MDA: Malondialdehyde

MDR: Multidrug drug resistance protein

MDR: Multi-drug resistant markers

MDSC: Myeloid-derived suppressor cells

MLH: MutL homolog

MLH1: MutL1 homolog

MLH2: MutL2 homolog

MMR: DNA mismatch repair

MPO: Myeloperoxidase

mRNA: Messenger RNA

MSH: MutS homolog

MSH2: MutS2 homolog

MSH6: MutS6 homolog

mTOR: Mammalian target of rapamycin

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N_2O_3: Dinitrogen trioxide

NACT: Neoadjuvant chemotherapy

NAD(P)H: Nicotinamide adenine dinucleotide phosphate

NER: Nucleotide excision repair

NO: Nitric oxide

NO_2^-: Nitrite

NO_3^-: Nitrate

NOS: Nitric oxide synthase

NOX: NAD(P)H oxidase

Nrf2: Nuclear factor-erythroid 2 p45-related factor 2

$O_2^{\bullet -}$: Superoxide

OCIC: Ovarian Cancer-Initiating cells

OFP: Orange fluorescent protein

$ONOO^{-}$: Peroxynitrite

OS: Overall survival

PCR: Polymerase chain reaction

PFI: Platinum-free interval

PGE (2): Prostaglandin E2

PI3K: Phosphoinositide 3-kinase

PK: Pharmacokinetics

POSTN: Periostin

RNA: Ribonucleic acid

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

Shh: Notch, Sonic hedgehog (Shh)

SMAC: Small mitochondria-derived activator of caspases

SNO: S-nitrosothiol

SNP: Single nucleotide polymorphism

SOD: Superoxide dismutase

STAT-3: Signal transducer and activator of transcription-3

TAM: Tumor associated macrophages

TME: Tumor microenvironment

TNFR: Tumor necrosis factor receptor

TRAIL: TNF-related apoptosis-inducing ligand

US: United States

VDAC: Voltage-dependent anion channels

XO: Xanthine oxidase

γ-GCS: γ-glutamylcysteine synthase

CHAPTER 1: INTRODUCTION AND BACKGROUND

Ovarian cancer

Ovarian cancer represents the malignant transformation of the female gonad. There are three main types of ovarian cancer: epithelial, which comprises the majority, germ cell, and sex cord-stromal, representing less than 2% of the cases. The histopathologic classification of epithelial ovarian cancer (EOC) includes serous, endometrioid, clear-cell, and mucinous. Genomic studies of high-grade serous cancer have identified molecular subtypes that are associated with distinct biology and clinical outcome. Recent evidence suggests two different entities based on molecular signatures: type-1 and type-2 EOC. Type-1 is slow-growing and has a "cure" rate of about 90% as compared to the more aggressive type-2, which encompasses the majority of cases [1]. Type-2 EOC typically expresses mutations in tumor-suppressor and/or DNA repair genes [1]. Regardless of molecular subtype, mortality from EOC is high in patients with advanced-stage and high-grade serous EOCs. Although the exact etiology of EOC in the general population remains elusive, BRCA1/2-mutation carriers and those affected with Lynch II syndrome are considered to be high risk [2, 3]. Indeed, a strong family history of EOC is the single most important risk factor [2, 4]. Nulliparity is another risk factor; however, oral contraceptive use, tubal ligation, pregnancy, and lactation confer protection against the disease [4-6]. Evidence suggests that factors involved in cell pluripotency and self-renewal, key characteristics of stem cells, are implicated in cancer biology [7-19]. Specifically, the existence of human ovarian cancer-initiating cells (OCICs) with stemness properties and enhanced resistance to cisplatin and paclitaxel have been reported [13]. Among different proposed theories for the pathogenesis of EOC, the "incessant ovulation" model is the most widely accepted [20]. Accordingly, cyclical ovulations without intervening pregnancy will, overtime, increase the

likelihood of chronic inflammation and generation of reactive oxygen and nitrogen species (ROS and RNS) that may induce various mutations, and concomitant with impairment of repair mechanisms will initiate tumorigenesis [21-23]. Typically, the treatment of ovarian cancer requires both cytoreductive surgery (CRS) and platinum/taxane combination chemotherapy [24-27]. Recently, targeted agents such as anti-angiogenic drugs have been introduced in the management of ovarian cancer patients.

Epithelial ovarian cancer is the deadliest of all gynecologic cancers with an estimated 22,280 new cases and 14,240 deaths expected in 2016 in the US alone [28]. The 5-year survival rate for localized ovarian cancer is approximately 90%, but drops dramatically to 60% for regional disease and 20% for metastatic disease. Because early-stage ovarian cancer presents with nonspecific symptoms, frequently the diagnosis is not made until after the malignancy has spread beyond the ovaries [29]. The high mortality rates for this type of malignancy can be largely attributed to a lack of screening methods for the general population and the acquisition of resistance to the current chemotherapeutics [29].

Cisplatin resistance

Resistance to chemotherapy can be either intrinsic, occurring at the onset of treatment, or acquired, when the disease recurs despite an initially successful response [30-32]. Generally, the approach to relapsed disease is stratified based upon the amount of time that has elapsed between the completion of platinum-based treatment and the detection of relapse, established as the platinum-free interval (PFI) [33]. Patients with a PFI of less than six months are considered platinum-resistant as opposed to those with a PFI greater than the six-month cutoff.

Approximately 75% of patients with advanced disease will respond initially to platinum-containing chemotherapy. However the majority will relapse within two years of completing

treatment, while 25% of patients are intrinsically resistant [30, 34, 35]. Chemoresistance greatly limits the range of possibilities for subsequent treatments, because some tumors become resistant not only to the initial drug but also to new therapeutic agents with different mechanisms of action [31]. Attempts to overcome drug resistance are central to both clinical and basic molecular research in cancer chemotherapy [30].

Cisplatin,the prototype of the platinum drugs, is well characterized with a long track record in both basic science and clinical research. Carboplatin, a platinum compound, has replaced cisplatin in the treatment of EOC because of its superior toxicity profile. The mechanisms of action of cisplatin involve both cytoplasmic and nuclear reactions [23, 36-39]. After being actively transported to the intracellular compartment by the copper transporters (CTR1), the cisplatin molecule becomes activated by a series of aquation reactions that consist of the substitution of one or both cis-chloro groups with water molecules (Figure 1) [40-42]. Cisplatin avidly binds DNA, with a predilection for nucleophilic N7-sites on purine bases. This leads to the generation of protein–DNA complexes as well as of DNA–DNA inter- and intra-strand adducts, with the latter being more lethal. Generally p53 will be activated following DNA damage. This will lead to cell cycle arrest for DNA repair, or activation of the apoptosis pathway with cytochome c release. Of note, it is possible that mutations occur during the DNA repair process. Additionally, cisplatin is known to generate ROS by the activation of nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase in the cell membrane and the mitochondria[43-45]. The glutathione/glutathione disulfide (GSH/GSSG) pathway is very important to scavenge ROS and activate drug efflux pumps, which can expel cisplatin from the

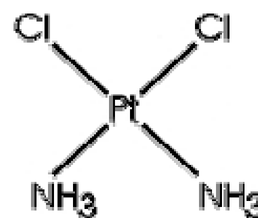


Figure 1. Molecular structure of cisplatin.

cell. The imbalance between the production and elimination of free radicals and reactive metabolites leads to a state of oxidative stress and subsequent damage of important biomolecules and cells, with potential impact on the whole organism [46].

Several possible mechanisms of cisplatin resistance have been previously described; including decreased intracellular aquation, decreased uptake and accelerated efflux due to increased activity of ATP-binding cassette and soluble carrier transporter families[47, 48]. Here, we have classified those mechanisms as follow: 1) pharmacokinetic (PK) variations, 2) activation of DNA repair, 3) decrease cell death such as apoptosis and autophagy, and alterations of cell signaling pathways, 4) cancer stem cells, 5) tumor microenvironment and, 6) oxidative stress.

1) Pharmacokinetic variations

Pharmacokinetics is a branch or pharmacology that describes how the body affects a specific drug after administration through the mechanisms of absorption, distribution, metabolism, and the elimination of the drug [49]. The PK properties of drugs may be affected by elements such as the site of administration, the absorptive surface, the volume of distribution, and more importantly the genetic makeup and morphometric characteristics of the individual receiving the specified drug [50, 51]. Genetic variations in expression and polymorphisms of enzymes that are involved in some of these processes have been identified, and are in part responsible for some of the inter-patient variations of drug effects. Similarly, the morphometric parameters such as height, weight and overall body composition; as well as disease status and environmental factors may play important roles.

Several studies have demonstrated the role of copper transporters to decreased tumor response to platinum compounds [52-56]. Clinically relevant concentrations of cisplatin were

shown to downregulate the CTR1, by internalization followed by proteasome-mediated degradation [57-59]. Additionally, the Wilson disease protein, also known as ATP7B, is a P-type cation transport ATPase that functions as a monomer, exporting copper out of the cells was reported to correlate with cisplatin resistance in preclinical studies[60-62]. However when studied in patients, its expression level was not significantly correlated with the known multi-drug resistant markers (MDRs), but found to be an independent prognosticator [63, 64]. Additional studies in both ovarian and endometrial cancers further validate ATP7B as predictive marker of chemoresistance for cisplatin [63-65].

Once inside of the cell, cisplatin becomes activated through an aquation reaction. This reaction occurs spontaneously in the cytoplasm, owing to the relatively low concentration of chloride ions (2–10 mM) when compared with the extracellular milieu (100 mM), and leads to the generation of highly reactive mono-and biaquated cisplatin forms. Cisplatin avidly binds to cytoplasmic nucleophilic species, including GSH, methionine, metallothioneins and other cysteine-rich proteins, and is then sequestered. As a result, the effective intracellular concentration of cisplatin is diminished leading to the development of chemoresistance.

Voltage-dependent anion channels (VDACs) are a class of porin ion channel located in the outer mitochondrial membrane [66]. They regulate the flux of proteins, small molecules and ions between the cytosol and the mitochondria [67]. Additionally, they have been involved in several key biologic processes such as energy metabolism, calcium transport and apoptosis [67-71]. At near zero mitochondrial membrane potential, the VDAC pores open, and allow the liberation of cytochrome c in the cytosol and trigger apoptosis[72-76]. Cisplatin has been shown to bind VDACs and trigger cell death as a preliminary step in the apoptosis process prior to cytochrome c release. Remarkably, VDAC-depleted cancer cells are highly resistant to cisplatin

treatment [67, 69, 70, 77-79]. Several PK mechanisms appear to contribute to cisplatin-resistant ovarian cancer, ranging from the downregulation of key membrane transporters such as CTR1, to altered localization of ATP7A, to sequestration of activated cisplatin compounds [58, 59, 80-87]. Depletion of the VDACs have been reported in cervical and head and neck cisplatin-resistant cancers, however, there is no direct evidence that this specific mechanism is linked to ovarian cancer [76, 77]. Based on the available evidence, interplay of PK factors seems to contribute to the suboptimal intracellular concentration of activated cisplatin, therefore establishing the cisplatin-resistant EOC phenotype.

2) Activation of DNA repair

Three excision repair pathways exist to repair single stranded DNA damage: nucleotide excision repair (NER), base excision repair (BER), and DNA mismatch repair (MMR). The bulk of cisplatin lesions are removed from DNA by the NER system [88-90]. Damaged nucleotides are excised from DNA upon incision on both sides of the lesion, followed by DNA synthesis to reconstitute genetic integrity. Amplification of this process has been correlated with cisplatin resistance in multiple preclinical models and validated clinically [91-93]. While the BER pathway can recognize specific non-bulky lesions in DNA, it can correct only damaged bases that are removed by specific enzymes. The MMR pathway only targets mismatched base pairs that are seldom seen in cisplatin-induced DNA lesions, thus not proven to be a significant mechanism of cisplatin resistance in that fashion [65, 94, 95]. The MMR-related proteins, MutL (MLH) and MutS (MSH) which participate in the recognition of GpG inter-strand adducts, will attempt to repair cisplatin adducts but fail, leading to apoptosis. Defects in MLH1 and MSH6 are associated with increased level of translesion synthesis, whereby DNA synthesis is not blocked but proceeds beyond the cisplatin effects [96]. Indeed, methylation-dependent silencing

of MLH1 has been shown to predict poor survival in ovarian cancer patients [97]. MLH1, MSH2, and MSH6 are responsible for the hereditary nonpolyposis colon cancer syndrome (HNPCC) including 2% of ovarian cancers patients. Interestingly, previous clinical report of HNPCC-ovarian cancer patients suggests an association between the syndrome with decreased response to cisplatin and aggressive tumor [98].

Cisplatin-induced inter-strand adducts can lead to the so-called double-strand DNA break lesions that are normally repaired by homologous recombination machinery. Two critical components of the homologous recombination machinery system are encoded by BRCA1 and BRCA2, two genes that are frequently mutated in familial breast and ovarian cancers. Although BRCA1/2 tumors respond better to chemotherapy including cisplatin, additional mutations that restore the function of the enzymes appear to confer resistance to platinum compound [99].

Altogether, these observations highlight the contributions of several of the DNA repair mechanisms in cisplatin-resistant ovarian cancer. The above-mentioned studies, if validated in clinical trials by other investigators, will pave the way for targeted interventions in order to reverse the chemoresistance.

3) Decreased cell death

The process of apoptosis can be initiated by a diverse range of extracellular and intracellular signals, that may positively or negatively affect the process itself [100-104]. Upon stimulation, the cell undergoes organized degradation of cellular organelles by activated proteolytic caspases. Caspases are proteins that are highly conserved, cysteine-dependent aspartate-specific proteases. There are two types of caspases: initiator caspases (caspase 2, 8, 9, 10) and effector caspases (caspase 3, 6, 7) [104]. The activation of initiator caspases requires

binding to a specific oligomeric adaptor protein. Effector caspases are then activated by these active initiator caspases through proteolytic cleavage.

Several proteins are involved in the apoptosis process, but two main methods of regulation have been identified. The first (*intrinsic*) pathway targets mitochondria functionality by directly transducing the signal via adaptor proteins to the apoptotic mechanisms [68, 102]. The second (*extrinsic*) death-receptor pathway, can be explained by two models, the tumor necrosis factor (TNF)-induced and the Fas-Fas ligand (FasL)-mediated, both involving receptors of the TNF receptor (TNFR) family coupled to extrinsic signals. The interaction between Fas and FasL results in the formation of the death-inducing signaling complex (DISC), which contains the FADD, caspase-8 and caspase-10. Upon activation, a balance between pro-apoptotic: Bcl-2-associated X protein (BAX), BH3 interacting-domain death agonist (BID), Bcl-2 homologous antagonist killer (BAK), or Bcl-2-associated death promoter (BAD) and anti-apoptotic: B-cell lymphoma-extra-large (Bcl-Xl) and B-cell lymphoma 2 (Bcl-2) members of the Bcl-2 family is established. The pro-apoptotic homodimers are required to make the mitochondrial membrane permeable for the release of caspase activators such as cytochrome c and small mitochondria-derived activator of caspases (SMAC). There also exist others like: caspase-independent apoptotic pathway that is mediated by AIF, the endoplasmic reticulum (ER) stress pathway, and the p53-dependant pathway. Alternatively, several anti-apoptotic pathways have been described such as the receptor-associated mechanisms: the Decoy receptors, the FLICE-like inhibitory protein (FLIP), the inhibitors of apoptosis proteins (IAP) family members, nuclear factor-kappa beta (NF-kB) and its relative anti-apoptotic members, the phosphoinositide 3-kinase (PI3K)/serine-threonine kinase (Akt)/mammalian target of rapamycin (mTOR) pathway, and the mitogen-activated protein kinases (MAPK) Pathways.

Lethal cisplatin-induced DNA damage activates signaling cascades leading to apoptosis. Genetic and epigenetic alterations in the components of this complex signaling network have been associated with variable levels of resistance to cisplatin. Reportedly, ovarian cancer patients carrying wild-type TP53 have a higher probability to benefit from cisplatin-based chemotherapy than patients with TP53 mutations [105, 106].

Autophagy involves cell degradation of unnecessary or dysfunctional cellular components through the actions of lysosomes [107]. At the molecular level, several autophagy-related genes (Atg) regulate this process that is known to be initiated from mTOR complex1-derived stress signals [108]. Also, intact nuclear p53 positively influences autophagy through a variety of ways[109]. Alternatively, autophagy can be more complex and specific involving a chaperone-mediated pathway [110]. Autophagy has been linked to cisplatin resistance in both ovarian and lung cancer cells by increasing the expression of key autophagy markers [111, 112]. Surprisingly, mTOR inhibitors paradoxically enhanced response to cisplatin in head and neck carcinomas [111]. Other pathways implicating ER stress, chaperone mediated autophagy, among others may play a role in response and resistance to cisplatin however; the precise mechanisms are not yet clarified [113].

Modifications in cell signaling pathways or factors that regulate and execute apoptosis, have the potential to influence cisplatin sensitivity. Several proteins (including death receptors, cytoplasmic adaptors, pro- and anti-apoptotic members of the Bcl-2 protein family, caspases, calpains, mitochondrial intermembrane proteins, and many others) have been shown to modulate the response to cisplatin and other drugs [38, 79, 114-116]. However, only some predict cisplatin responsiveness in the clinical setting and are undergoing clinical trials. For example, conclusive clinical data on the association between the pro-apoptotic Bcl-2 family members

BAX and BAK and cisplatin sensitivity are missing, but elevated levels of their anti-apoptotic counterparts including Bcl-2, Bcl-Xl and myeloid cell leukemia sequence 1 (MCL-1) reportedly correlate with cisplatin resistance and tumor recurrence in multiple clinical scenarios [117-120].

Altogether, these observations suggest that multiple factors are likely to affect the molecular mechanisms that underlie cisplatin resistance.

4) Cancer stem cells

A growing body of evidence suggests that factors involved in cell pluripotency and self-renewal, keys characteristics of stem cells, are implicated in cancer biology [7-19]. Indeed, since the isolation of human embryonic stem cells (ESCs), significant interests have been generated in science and medicine because of their therapeutic potential [121]. The genes and signaling pathways that regulate the stem cell state are referred to as the "stemness" signature [122]. Self-renewal and pluripotency is a complex process that demands the cooperation of both intrinsic and extrinsic pathways to maintain the state of stemness and undergo clonal expansion [123-127]. The transcription factors Sox2, Nanog and Oct4 form the core regulatory positive feedback-loop that sustains the stem cells capacity for self-renewal and pluripotency. Numerous pathways such as Leukemia inhibiting factor (LIF), Notch, Sonic hedgehog (Shh) and Wnt signaling have also been linked to tumorigenesis and stemness [128-136]. Cancer stem cells (CSCs) are thought to be associated with poor outcomes such as high relapse rate and resistance to standard chemotherapy [9-11, 137-139]. Both in human and animal models, Sox2 overexpression has been associated with malignant tumor formation and poor outcomes in several cancers [8, 10, 138, 140-144]. When aberrations of these stemness regulatory pathways ensue as a consequence of p53 mutations, massive clonal expansion of a malignant phenotype with self-renewal potential becomes possible.

Evidence suggests the existence of human OCICs with stemness properties and enhanced resistance to cisplatin and paclitaxel [13]. The emergence of CSC subpopulations in a tumor poses a real challenge since these cells can be slow growing and not affected by standard chemotherapy. Reportedly, after initial response to chemotherapy, the repopulation of the tumor bulk can occur, resulting progression and recurrence. The lack of improvement of overall survival in EOC raises the possibility that other mechanistic pathways, not currently targeted, are involved in the biology of the tumor.

5) Tumor microenvironment

The tumor microenvironment (TME) represents the intimate stroma that surrounds the tumor and includes the surrounding blood vessels, and other connective tissue components such as: the extracellular matrix (ECM), inflammatory cells, fibroblasts, bone marrow-derived inflammatory cells; as well as immune cells, and signaling molecules [145, 146]. There is a dynamic relationship between the TME and the tumor. The cells in the TME can affect the growth and evolution of malignant cells; while the tumor cells, by releasing extracellular signals, promoting tumor angiogenesis and induce peripheral immune tolerance. Several TME players have been linked to chemoresistance as well as to the platinums. Here, we present a non-exhaustive summary of original work studying this specific field.

Periostin (POSTN) is a human protein that functions as a ligand for alpha-V/beta-3 and alpha-V/beta-5 integrins to support adhesion and migration of epithelial cells [147]. Its overexpression in the cancer microenvironment, mediated by the AKT pathway, was found to be correlated with poor prognosis in EOC patients and associated with platinum resistance [148]. Also, low-affinity leukotriene B4 receptor-2 (BLT2) and its ligand, leukotriene B4, were highly upregulated in cisplatin-resistant SKOV-3 ovarian cancer cells and played critical roles in

mediating the chemoresistance through the activation of signal transducer and activator of transcription-3 (STAT-3) and the subsequent up-regulation of interleukin-6 (IL-6) [149]. Fibroblast activation protein alpha (FAP) is overexpressed by fibroblasts present in the microenvironment of many tumors. FAP expression in EOC cells was found to be associated with poorer clinical outcomes. It was suggested that anti-FAP therapy could be a highly effective novel treatment for EOC, especially in cisplatin-resistant cases [150].

Tumors can produce exosomes that stimulate inflammation via myeloid-derived suppressor cells (MDSCs) [151]. This group of cells include tumor associated macrophages (TAMs), mainly of the M2 phenotype, which is known to actively promote tumor growth [152]. Evidence suggests that resistance to chemotherapy is associated with increased levels of IL-6 and prostaglandin E2 (PGE (2)), two inflammatory mediators known to skew differentiation of monocytes to M2 macrophages. Treatment with cyclooxygenase-inhibitor and/or monoclonal antibody against interleukin-6 receptor (IL-6R) prevented M2-differentiation [153].

The COL6A3 gene encodes the alpha-3 chain of collagen type VI. It is responsible for microfibril formation and extracellular matrix communication [154, 155]. Differential gene expression profiling identified COL6A3 as one of the most highly upregulated genes in cisplatin-resistant ovarian cancer cells compared to their cisplatin-sensitive counterparts [156]. The authors suggest that tumor cells may directly remodel their microenvironment to increase their survival in the presence of chemotherapeutic drugs [156].

Ultimately, we have recently demonstrated that angiogenesis was impaired in chemoresistant EOC cells, and suggested that the lack of drug delivery to the tumor site as a potential mechanism for the emergence of the chemoresistant phenotype [157]. Although the contribution of the TME in the establishment of chemoresistance in several tumors has been

suggested, however, conclusive evidence supporting the associations between cisplatin-resistant ovarian cancer and the various components of the TME is still lacking.

6) Oxidative stress

Clinical, epidemiological and basic science investigations have provided evidence supporting the role of ROS and RNS in the etiology of cancer [58, 158-164]. Oxidative stress is defined as an imbalance between the production of oxidants and the biological system's inability to eliminate these oxidants by antioxidants [165].

Oxidative stress and ovarian cancer

Oxidative stress has been implicated in the pathogenesis of several malignancies including ovarian cancer [166, 167]. Moreover, there is evidence that ovarian cancer patients also have decreased levels of circulating antioxidants [167]. Reactive species (both ROS and RNS) including superoxide ($O_2^{\bullet-}$), hydroxyl radical ($HO^{\bullet}$), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), nitric oxide (NO) and, hypohalous acids are free radical molecules that are highly destructive to cellular functions [168, 169]. Various enzyme systems produce ROS including the mitochondrial electron transport chain, cytochrome P450, lipoxygenase, cyclooxygenase, the NAD(P)H oxidase complex, xanthine oxidase (XO), and peroxisomes [170, 171]. Mitochondrial O_2 metabolism is the dominant source of $O_2^{\bullet-}$ that results from incomplete coupling of electrons and H^+ with O_2 in the electron transport chain. Under normal conditions, ROS and RNS are maintained within narrow parameters by scavenging systems, as would be expected where those enzymes are involved in cell signaling, while ROS/RNS formed after exposure to oxidative stress can activate signaling molecules [159, 170-177]. The redox balance is maintained by various enzyme systems that neutralize toxic reactive oxygen and nitrogen species. Superoxide dismutases (SOD) catalyze the conversion of $O_2^{\bullet-}$ to H_2O_2, which then can be converted to water

by catalase (CAT) or glutathione peroxidase (GPx) coupled with glutathione reductase (GSR) [178]. Other important scavengers include thioredoxin coupled with thioredoxin reductase, and glutaredoxin, which utilizes GSH as a substrate. Additionally, glutathione S transferase (GSTs) are involved in detoxification of varieties of environmental carcinogens and xenobiotics by catalyzing their conjugation to GSH, and subsequent removal from the cell [179]. Glutathione plays a central role in maintaining redox homeostasis, and the GSH-to-oxidized-GSH ratio provides an estimate of cellular redox buffering capacity [170, 180]. Moreover, evidence suggests that increase in oxidative stress mediated by the GSH/GSSG complex results in enhanced activity of GS-X-MRP1 efflux pump. This pump is known to decrease the intracellular effective drug concentration, therefore is considered one of the mechanisms of multiple drug resistance including platinum compounds [181-184].

Cancer cells are known to be under intrinsic oxidative stress resulting in increased DNA mutations or damage, genome instability, and cellular proliferation [46, 178, 185-187]. The persistent generation of cellular ROS is a consequence of many factors including exposure to carcinogens, infection, inflammation, environmental toxicants, nutrients, and mitochondrial respiration [188-191]. Nitration of tyrosine residues to 3-nitrotyrosine, a hallmark of inflammation as well as a footprint of many types of oxidative injury, may play a role in the pathogenesis of human ovarian cancers [192]. Specifically, an increased expression of pro-oxidant enzymes such as inducible nitric oxide synthase (iNOS), myeloperoxidase (MPO), NAD(P)H oxidase, as well as an increase in NO as indicated by increased nitrate/nitrite (NO_3^-/NO_2^-) has been shown in EOC tissues and cells [193-195]. Also, EOC cells manifested lower apoptosis, which was markedly induced by inhibiting iNOS by L-NAME, indicating a strong link between apoptosis and NO/iNOS pathways in these cells [194]. Myeloperoxidase

utilizes NO, produced by iNOS, as a one-electron substrate generating NO^+, a labile nitrosating species that is rapidly hydrolyzed forming NO_2^- as an end product [196-199]. The ability of MPO to generate NO^+, from NO, led us to believe that not only does MPO play a role in S-nitrosylation of caspase-3, thus inhibiting caspases-3 activity, which is indicative of lower apoptosis in EOC cells, but also highlights a possible cross-talk between iNOS and MPO [193]. Myeloperoxidase, an abundant hemoprotein previously believed to be present solely in neutrophils and monocytes, plays an essential role in immune surveillance and host defense mechanisms, and can contribute to 3-nitrotyrosine formations *in vivo* and directly modulate inflammatory responses via regulation of NO bioavailability during inflammation [192, 200]. Silencing MPO gene expression utilizing MPO specific siRNA induced apoptosis in EOC cells through a mechanism that involved S-nitrosylation of caspase-3 by MPO [193]. Additionally, we have compelling evidence that leads us to believe that MPO may serve as a source of free iron under oxidative stress, where both NO and $O_2^{\bullet-}$ are elevated [201]. Iron reacts with H_2O_2 and catalyzes the generation of highly reactive $HO^\bullet$, thereby increasing oxidative stress, which in turn increases free iron concentrations by the Fenton and Haber–Weiss reaction [202].

Collectively, we now have substantial evidence to believe that altered oxidative stress may play a role in maintaining the oncogenic phenotype of EOC cells. In addition, epidemiologic studies have demonstrated the role of family history as an important risk factor for ovarian cancer [203]. Mutations in BRCA genes are currently utilized to evaluate risk for breast and ovarian cancer, however, are not ideal because the mutations are so rare (1 out of 500 individuals), thus, the overall impact on mortality is small [204]. Additionally, the burden of cancer is being correlated with common, weakly penetrant alleles that are often classified as sporadic (i.e. without a heritable basis) [204]. Non-synonymous single nucleotide

polymorphisms (SNPs) substitute encoded amino acids in proteins, and are more likely to alter the structure, function, and interaction of the protein [205]. Therefore SNPs are good candidates as disease-modifiers and have been associated with an altered cancer risk [205].

We have recently characterized EOC tissues and cells to manifest a persistent pro-oxidant state with the upregulation of several key oxidant enzymes including: myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS), and nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase with concurrent decrease in apoptosis compared to normal human ovarian tissues and cells. More importantly, shutting down the expression of one or more of these key oxidant enzymes reduced the pro-oxidant state, and significantly induced apoptosis. Single-nucleotide polymorphisms (SNPs) are point mutations that are selectively maintained in populations and are distributed throughout the human genome at an estimated overall frequency of at least one in every 1000 base pairs. Several SNPs in key oxidants and antioxidants enzymes have been associated with various cancers including ovarian. Therefore, we are proposing the following hypothesis: *cisplatin treatment induces mutations in key oxidant and antioxidant enzymes resulting in further enhancement of oxidative stress and the acquisition of resistance in EOC cells.* To test this hypothesis, we are proposing then following specific aims:

Specific Aim 1: To determine the association of specific single nucleotide polymorphisms (SNPs) in key oxidant and antioxidant enzymes with EOC risk and survival in patients.

The rationale of this aim is based on the fact that oxidative stress is strongly associated with several cancers, including ovarian. Known specific SNPs in oxidant and antioxidant genes may alter their expression profile and enzymatic functions. These SNPs have been reported to be associated not only with cancer risks, but also patient response to treatment and survival. The hypothesis of this aim is that *specific SNPs in key oxidant and antioxidant enzymes are associated with overall patient survival.* To achieve this aim, we will perform a case-control

study using stored blood samples of research participants from the Karmanos Cancer Center. Individuals (n=143) recruited were divided into controls (n=94),) and ovarian cancer cases (n=49). Samples will undergo DNA extraction followed by TaqMan® SNP genotype analysis for rs4880 manganese superoxide dismutase (*MnSOD*), rs4673 (*NAD(P)H* oxidase (*CYBA*), rs3448 glutathione peroxidase (*GPX1*), rs2297518 inducible nitric oxide synthase (*iNOS*), rs1002149 glutathione reductase (*GSR*), and rs1001179 catalase (*CAT*). We will perform a multivariate analysis for identification of confounding variables and potential predictors of risk. Additionally, to study the impact of the SNPs on overall survival, Cox regression and Kaplan-Meier survival analyses will be used.

Specific Aim 2: To determine the association of key oxidant and antioxidant enzymes as well as specific SNPs in these enzymes with the development of cisplatin resistance in EOC cells.

The rationale of this aim is based on previous findings showing an association between the altered redox enzymes and EOC, in both patients and human cell lines. The hypothesis of this aim is that *the acquisition of resistance to cisplatin in EOC cells is associated with enhanced pro-oxidant profile, as well as specific SNPs in key oxidant and antioxidant enzymes.* To achieve this aim, we will utilize two human EOC cell lines, MDAH-2774 and SKOV-3 and their cisplatin resistant counterparts. We will perform TaqMan PCR genotyping, real-time RT-PCR, ELISA, and Griess assay to study the expression profile of the following genes: *CYBA/NOX4*, *iNOS*, *CAT*, *SOD3*, GSR and *GPX1*. To analyze the difference in the expression profiles of these genes for sensitive compared to resistant cells, we will use a Student's t-test.

Specific Aim 3: To determine whether specific SNP(s) in key oxidant and antioxidant enzymes cause the acquisition of cisplatin resistance in EOC cells.

The rationale of this aim is based on the established fact that cisplatin treatment causes DNA damage, and the observation that specific SNPs in the redox enzymes were found to be

associated with poor survival in patients. The hypothesis of this aim is: *specific SNPs in key oxidant and antioxidant enzymes cause cisplatin resistance*. To achieve this aim, we will utilize the CRISPR/Cas9 system to generate point mutations in sensitive EOC cells corresponding to the SNP genotype of the chemoresistant MDAH-2774 and SKOV-3 EOC cells. The cells will then be tested for cisplatin resistance using the MTT viability assay using the IC_{50} method. Results will be analyzed with regression analysis and student t-tests.

CHAPTER 2: GENERAL METHODS

Cell Culture

This is a well-established technique in our laboratory [193]. Human EOC cell lines, MDAH-2774 (CRL-10303) and SKOV-3 (HTB-77), were obtained from American Type Culture Collection (ATCC, Manassas, VA). The MDAH-2774 cell line was developed from ascitic fluid cells from a patient with adenocarcinoma in 1972, and forms tumors in nude mice [206]. SKOV-3 cells were developed from ascitic fluid of a 64-year-old Caucasian female. Cell lines are cultured in McCoy's 5A medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin and 10% heat-inactivated fetal bovine serum (FBS) at 37°C in 5% CO_2.

Two parent human EOC cell lines (SKOV-3 and MDAH-2774) were utilized in developing cells resistant to cisplatin. Cells were exposed to a stepwise increase in cisplatin (Sigma Aldrich, St Louis, MO) over the course of a year, with final cisplatin IC_{50} concentrations of 1.87 µM and 3.31 µM for SKOV-3 and 1.70 µM and 6.34 µM for MADH-2774, sensitive and resistant respectively. The Trypan Blue Dye Exclusion Method was used to confirm resistance as follows: once the desired level of resistance was achieved, a 2-week incubation period without cisplatin and subsequent reintroduction of cisplatin was performed. Resistance was confirmed with the Vybrant® MTT Cell Proliferation Assay Kit (Life Technologies, Carlsbad, CA), according to the manufacturers protocol. The IC_{50}, concentration of the drug at which 50% of the cells are alive, was determined using regression analysis of the MTT dose-response cell proliferation assay.

Protein Extraction

Cell lysates were prepared utilizing cell lysis buffer (Cell Signaling Technology, Danvers,

MA) supplemented with Protease Arrest (G-Biosciences, St. Louis, MO). Total protein concentration of cell lysates was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) per the manufacturer's protocol.

Purification of DNA and the TaqMan SNP Genotyping Assay for SNPs

DNA, from blood samples or cells, was isolated by the Applied Genomics Technology Center (AGTC, Detroit, MI). DNA was extracted with QIAamp DNA mini kit per the manufacturer's protocol (Qiagen, Valencia, CA) [207]. The TaqMan® SNP Genotyping Assay Sets (Applied Biosystems, Carlsbad, CA) (NCBI dbSNP genome build 37, MAF source 1000 genomes) were used to genotype selected SNPs described in Table 1. The AGTC performed this assay and analysis was done utilizing the QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems).

Table 1: Characteristics of single nucleotide polymorphisms examined for genotyping.

Gene (RS)	SNP	NCBI MAF	Chromosomal location	Known AA Switch	Effect on activity
CAT (rs1001179)	C-262T	0.123	11p13	Unknown	Decrease
CYBA (rs4673)	C242T	0.303	16q24.3	Tyr to His	Decrease
GPX1 (rs3448)	C-1040T	0.176	3p21.31	Unknown	Unknown
GSR (rs1002149)	G201T	0.191	8p12	Unknown	Unknown
SOD2 (rs4880)	T47C	0.371	6q25.3	Val to Al	Decrease
MPO (rs2243828)	T-764C	0.23	17q22	Unknown	Decrease
NOS2 (rs2297518)	C2087T	0.173	17q11.2	Ser to Leu	Increase

AA; amino acid, Ala; alanine, *CAT*; catalase, *CYBA*; NAD(P)H oxidase subunit (NOX4), *GSR*; glutathione reductase, *GPX*; glutathione peroxidase, His; histidine, Leu; leucine, MAF; minor allele frequency, *SOD2*; manganese superoxide dismutase, *MPO*; myeloperoxidase, NCBI; National Center for Biotechnology Information, *NOS2*; nitric oxide synthase, Ser; serine, SNP; single nucleotide polymorphism, Tyr; tyrosine, Val; valine.

ELISAs

Unless otherwise stated, all assays were performed utilizing cell lysate according to the manufacturer's protocols.

The Superoxide Dismutase Assay Kit (Cayman Chemical, Ann Arbor, MI) detects SOD

activity by measuring the dismutation of $O_2^{\bullet-}$ generated by XO and hypoxanthine. The standard curve generated using this enzyme provides a means to accurately quantify the activity of all three types of SOD (Cu/Zn-, Mn-, and Fe-SOD). One unit of SOD, detected at 460 nm, is defined as the amount of enzyme needed to exhibit 50% dismutation of $O_2^{\bullet-}$.

CHAPTER 3: RISK AND SURVIVAL ASSOCIATIONS BETWEEN SPECIFIC SNPS IN OXIDANT AND ANTIOXIDANT ENZYMES AND PATIENTS WITH EPITHELIAL OVARIAN CANCER

(This chapter contains previously published material [208]. See Appendix B)

Abstract

We sought to evaluate the association of SNPs in key oxidant and antioxidant enzymes with increased risk and survival in patients with EOC. The rationale for this part of the study is based on the fact that oxidative stress is strongly associated with several cancers, including ovarian. Known specific SNPs in oxidant and antioxidant genes may alter their expression profile and enzymatic functions. These SNPs have been reported to be associated not only with cancer risks, but also patient response to treatment and survival. The hypothesis is that *specific SNPs in key oxidant and antioxidant enzymes are associated with a pro-oxidant profile, ovarian cancer risk and overall patient survival.* We performed a case-control study using stored blood samples of research participants from the Karmanos Cancer Center. Individuals (n=143) recruited were divided into controls (n=94) and ovarian cancer cases (n=49). Samples were subjected to DNA extraction followed by TaqMan® SNP genotype analysis for rs4880 *SOD2*, rs4673 *CYBA*, rs2333227 *MPO*, rs3448 *GPX1*, rs2297518 *iNOS*, rs1002149 *GSR*, and rs1001179 *CAT*. We have performed a multivariate analysis for identification of confounding variables and potential predictors of risk. Additionally, to study the impact of the SNPs on overall survival, Cox regression and Kaplan-Meier survival analyses were done.

Introduction

Epidemiologic studies have clearly established the role of family history as an important risk factor for both breast and ovarian cancers [203]. Mutations in *BRCA* are currently utilized to evaluate risk for breast and ovarian cancer. They are not ideal because the mutations are so rare (1 out of 500 individuals), thus, the overall impact on mortality rate is small [204]. Genomic

variations between individuals have been increasingly used in the practice of medicine [209-213]. A SNP occurs because of point mutations that are selectively maintained in populations and are distributed throughout the human genome at an estimated overall frequency of at least one in every 1000 base pairs [214]. Non-synonymous SNPs substitute encoded amino acids in proteins, and are more likely to alter the structure, function, and interaction of the protein [215]. Recent evidence demonstrates an association between enzymatic activity altering SNPs in oxidative DNA repair genes and antioxidant genes with human cancer susceptibility [216]. Additionally, a pro-oxidant state has been implicated in the pathogenesis of several malignancies, including ovarian cancer [166, 195]. The current study is based on the fact that certain SNPs present in key oxidants and antioxidants enzymes result in altered enzymatic activity as well as our previously published work delineating the role of oxidative stress in ovarian cancer. Recently, a SNP in *MPO* (rs2333227), which confers a decreased MPO activity, has been associated with early stage ovarian cancer such that those with the G allele are at an increased risk for ovarian cancer [217-219]. Additionally, a SNP in *SOD2* has also been associated with increased risk for ovarian cancer [219]. Increased enzyme activity is also associated with a SNP in *iNOS* (*NOS2*, rs2297518) [220]. An increase in NAD(P)H oxidase activity has been associated with a specific SNP in the *CYBA* gene (rs4673) and is also associated with an increased risk for other diseases where oxidative stress plays a critical role in their pathophysiology, including cardiovascular disease, asthma, and diabetic nephropathy [221-223]. A SNP in *CAT* (rs1001179) and *MnSOD* (*SOD2*, rs4880), also result in a decreased enzyme level of CAT and activity of SOD [224-226]. We have selected several SNPs in key oxidant and antioxidant enzymes based on their effect on function or association with cancer.

This chapter summarizes our efforts to determine whether specific SNPs in key oxidant

and antioxidant enzymes are associated with the increased risk as well as overall survival of ovarian cancer patients.

Methods

Human Subjects

Specimens were collected according to the Wayne State University Human Subject Committee (IRB) protocol number 1102009344, IRB# 024199MP2F(5R), expiration date 01/11/13.

Women and Minorities

This study includes samples from women as it is assessing a gender specific disorder of which many are minorities commensurate with the clinical population which is 82% African-American's in the city of Detroit, where Wayne State University and the Karmanos Cancer Center are located.

Study design

We performed a case-control study comparing female subjects with and without EOC and determined whether there is an association with several selected SNPs in established redox genes. Eligible women were 19 to 80 years of age and were previously recruited through the Karmanos Cancer Institute's Genetic Registry (KCIGR), Detroit, MI. Research activities and method of consent were conducted with the approval of Wayne State University Institutional Review Board (IRB#024199MP2F(5R)). Informed written consent forms were utilized and permission was granted for the collection of blood samples and for access to medical records for all subjects.

Patient Population

Recruited individuals (n=143) were divided into controls (94), which include: healthy volunteers (n=18), high-risk *BRCA1/2* negative (n=53), high-risk *BRCA1/2* positive (n=23) and

ovarian cancer cases (n=49). Controls were selected primarily from research subjects, considered high-risk for breast and ovarian cancers, without ovarian cancer that underwent genetic screening for *BRCA1/2* carrier status. Of note, the criteria used for screening included personal history of breast and ovarian cancers, family histories of breast and ovarian cancers, and *BRCA1/2* mutations. Additionally, healthy volunteers were also recruited as controls from the metropolitan Detroit area with no such histories. Cases were selected based on histopathology-confirmed primary diagnosis of EOC. All participants, except healthy volunteers, had previously undergone *BRCA1/2* testing and the results were made available to us.

Samples used for this study were collected from participants recruited between 1999 and 2012. DNA samples were utilized to determine the presence of polymorphisms in the genes described in Table 1. The SNPs were chosen based on previously reported associations with several cancers [227-235]. Of the 143 subjects, 49 (34.3%) had a primary diagnosis of ovarian cancer while 94 (65.7%) without cancer served as controls. For the ovarian cancer cohort: 13 (26.5%) were *BRCA1/2* positive as compared to 34 (69.4%) *BRCA1/2* negative; 2 (4.1%) cases were missing. The data is normally distributed with the age of enrollment ranged from 18 to 90 with a mean of 52 ± 15 and a median age of 52. The age at diagnosis ranged from 23 to 77, with a mean of 52 ± 11 and a median age of 52. The racial distribution was 88.8% (Caucasian), 8.4% (African-American) and 2.8% (Other). Personal and family histories of breast cancer, ovarian cancer, other cancers, and *BRCA1/2* were quantified. The frequencies of the presence of the SNP (heterozygous plus homozygous mutant) compared to homozygous wild type were determined for each gene studied.

Purification of DNA and the TaqMan SNP Genotyping Assay for SNPs

DNA, from blood samples, was isolated by the Applied Genomics Technology Center

(AGTC, Detroit, MI) and TaqMan was performed as described in *General Methods*.

Statistical analysis

Data were analyzed using SPSS (IBM, Armonk, New York) for Mac V.22. The following tests were conducted: descriptive statistics, multivariate analysis, Cox regression and Kaplan-Meier survival analyses. The variables selected for the analyses include genotypes, age at diagnosis, and age at enrolment, personal and family histories of breast, ovarian, and *BRCA1/2* mutations, in addition to other malignancies. Using the median age at diagnosis/enrolment as a cut point, we dichotomized the “age at diagnosis” variable. The “race” variable was categorized as: Caucasian, African-American or Other. We consolidated the following tumor and clinical variables into binary categorical schemes: International Federation of Gynecology and Obstetrics (FIGO) stages into early (IIA-IIIB) and advanced (IIIC-IV); FIGO grades (G1/2) and (G3); histology (serous and other). For all the genes studied, the “genotype” variable was dichotomized using the following scheme: homozygous wild type versus homozygous mutant plus heterozygous mutant. To compare cases to controls on the selected demographic, clinical, and genotypic characteristics, we performed Pearson Chi-square analysis. The recurrence rate was determined as the percentage of patients that have gone into remission, but the disease has returned months or years later, based on physical examination, radiological studies and serum CA-125 levels.

Cox regression and Kaplan-Meier analyses of variables as a predictor of overall survival

To study the impact of the SNPs on overall survival, Cox regression analyses were performed using the above-listed variables and classification schemes, using the likelihood ratio forward stepwise method. Several method simulations were performed such as: forced entry (ENTER), forward LR (likelihood ratio), etc. The forward LR was chosen for the final analysis.

This method is a stringent model that selects the strongest predictors of the outcome to be included in the final model. All patients received the standard of care after tumor board discussion. Details on treatment characteristics were not available. Additionally, Kaplan-Meier survival curves were generated for the variables selected by the model. Statistical significance was established at P-value less than 0.05 for all analyses.

Results

We performed side-by-side comparison between ovarian cancer cases and controls using Pearson chi-square analysis. Racial distribution was statistically similar between the groups (P>0.05). As expected, "personal or family history of ovarian cancer", "personal or family history of other cancers" and "advanced age" were significantly different between the groups and

Table 2. **Comparison of cases and controls based on demographic, personal or family history of cancer, and genotypic characteristics.**

	Controls %	Ovarian Cancer %	P-value (Pearson Chi-square, 2-tailed)
Age at enrolment (n=125)	n (76)	n (49)	<0.001*
< median	51 (67.1)	8 (16.3)	
> median	25 (32.9)	41 (83.7)	
Race (n=143)	n (94)	n (49)	0.365
Caucasian	81 (86.2)	46 (93.9)	
African-American	10 (10.6)	2 (4.1)	
Other	3 (3.2)	1(2.0)	
Personal / Family History of Cancer (Yes)	n (94)	n (49)	
Breast (n=105)	67 (71.3)	38 (77.6)	0.480
Ovarian (n=81)	33 (35.1)	48 (98.0)	<0.001*
BRCA1/2 (n=23)	18 (19.1)	5 (10.2)	.168
Other Cancer (n=69)	39 (44.3)	30 (62.5)	.043*
SNP (Yes)			
NOS2 (n=49)	34 (37.4)	15 (30.6)	0.424
CYBA (n=92)	57 (60.6)	35 (71.4)	0.201
MPO (n=56)	36 (39.1)	20 (41.7)	0.771
GSR (n=36)	27 (30.3)	9 (19.6)	0.180
GPX1 (n=61)	40 (43.5)	21 (42.9)	0.943
CAT (n=49)	30 (31.9)	19 (38.8)	0.412
SOD2 (n=103)	66 (72.5)	37 (75.5)	0.703

*P < 0.05, 0.05, CAT; catalase, CYBA; NAD(P)H oxidase subunit (NOX4), GPX; glutathione peroxidase, GSR; glutathione reductase, MnSOD; manganese superoxide dismutase, MPO; myeloperoxidase, NOS2; inducible nitric oxide synthase.

are known ovarian cancer risk factors ($p < 0.05$, Table 2). Comparative analyses for *MnSOD*,

rs4880, *CYBA*, rs4673, *GPX1*, rs3448, *NOS2,* rs2297518, *MPO*, rs2243828, *GSR*, rs1002149, and *CAT*, rs1001179 did not find a significant difference between the cases and controls (Table 2). Out of the 49 ovarian cancer cases, 38 (77.5%) were further analyzed by the Cox regression method, and 11 (22.5%) were dropped due to missing data. The majority of the cases were serous histology, advanced stage, and high-grade tumors (Table 3). The recurrence rate was found to be 60.5%.

Table 3. Stage, grade and pathologic characteristics of the cancer cases.

Tumor characteristics	**Number (%)**
Stage (n=38)	
IA-IIIB	10 (26.3)
IIIC-IV	28 (73.7)
Total	38 (100)
Grade (n=38)	
G1/2	6 (15.8)
G3	32 (84.2)
Total	38 (100)
Histology (n=38)	
Serous	34 (89.5)
Clear cell	1 (2.6)
Endometrioid	1 (2.6)
Total	38 (100)

The CAT SNP is a predictor of shorter survival

At the time of these analyses, there were 26 deaths (18.2%) and 117 (81.8%) subjects alive. Among the SNPs examined, only *CAT* (rs1001179) was identified as a predictor of shorter survival by the Cox regression model with a hazard ratio (HR) of 3.68 (95% CI: 1.149-11.836, p=0.028) (Table 4). As expected, "age at diagnosis" greater than the median (52) was found to be a significant predictor of death with an HR of 2.78 (95% CI: 1.022-7.578, p=0.045) (Table 4A). The variables selected for the analyses, but rejected by the model are listed in Table 4B.

Table 4A. Cox regression forward likelihood ratio (LR).

Variables in the Equation				
			95% CI for HR	
	Significance	**HR**	Lower	Upper
Age at Diagnosis > Median	.045*	2.782*	1.022	7.578
CAT (CT+TT)	.028*	3.688*	1.149	11.836

Table 4B. Variables analyzed by cox regression but rejected by the model.

Variables not in the Equation		
	Score	Significance
Race (Caucasian)	.580	.446
Stage (III-IV)	.708	.400
Grade (high)	.708	.400
GSR (CT+TT)	.411	.522
GPX1 (CT+TT)	.000	.988
MnSOD (CT+TT)	1.020	.312
NOS2 (CT+TT)	2.084	.149
CYBA (CT+TT)	1.229	.268
MPO (CT+TT)	.178	.673
Histology (serous)	1.016	.314

Kaplan-Meier (K-M) survival analysis factored by *CAT* genotype, which used 84.6% of the deaths, demonstrated a statistically significant median overall survival difference (108 [95% CI: 79-137] versus 60 [95% CI: 40-80] months, P<0.05) and a mean overall survival difference (182 [95% CI: 75.5-288] versus 47 months [95% CI: 31-60], P<0.05) for subjects with the normal genotype as compared to the *CAT* SNP genotype (Figure 2).

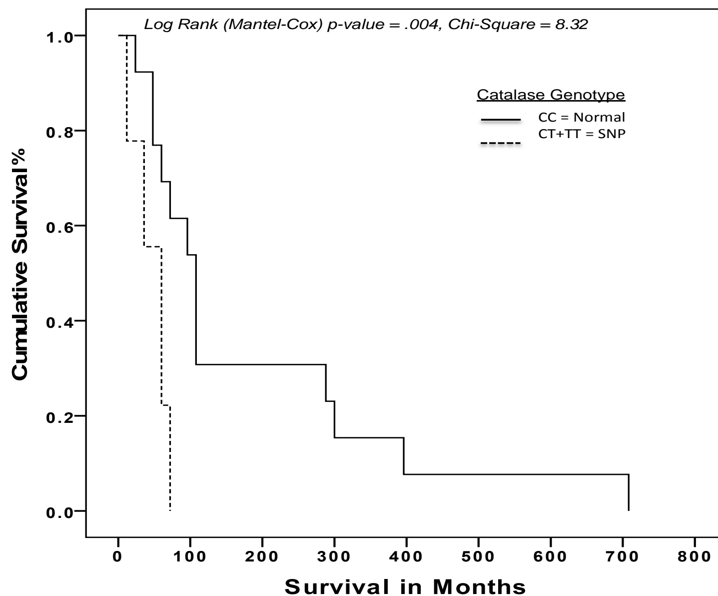


Figure 2. Kaplan-Meier overall survival curves for ovarian cancer patients utilizing a specific catalase SNP. The solid curve represents cases with (CC) homozygous wild-type genotype as compared to the dashed curve, which represents cases with homozygous mutant plus heterozygous mutant (CT+TT) genotypes. The X-axis represents patient survival in months; the Y-axis represents cumulative survival percentage. Chi-square P-value < 0.05 is considered statistically significant.

Discussion

A large body of evidence suggests that ovarian cancer patients have decreased levels of circulating antioxidants and higher levels of oxidative stress [23, 166, 167, 178, 193, 195, 236]. We have reported the existence of a persistent pro-oxidant state in EOC that included increased

expression of key pro-oxidant enzymes such as iNOS, NAD(P)H oxidase, and MPO [193-195]. Interestingly, the expression of MPO in EOC cells and tissues came as a surprise as it is an oxidant-generating enzyme typically found in cells of myeloid origin [217]. We have also determined that MPO can produce the nitrosonium cation (NO^+) utilizing NO produced by iNOS. This is important because NO^+ causes s-nitrosylation of caspase-3, and inhibition of its activity, resulting in a decrease in apoptosis [193]. This mechanism further explains the observation that EOC cells manifest significantly decreased apoptosis and increased survival [193, 194, 197, 198]. Interestingly, the evaluation of mutations in the various redox enzymes in the form of SNPs is an active area of scientific research [237-245]. Genetic polymorphisms are known to be associated with cancer susceptibility and can be determined by studying functional polymorphisms in genes that control the levels of cellular reactive oxygen species and oxidative damage, including SNPs for genes involved in carcinogen metabolism (detoxification and/or activation), antioxidants, and DNA repair pathways [246]. For example, germline mutations in *BRCA1* or *BRCA2* are associated with ovarian cancer at a rate of only 20-40%, suggesting the presence of other unidentified mutations in other genes as an etiology [215, 247, 248]. Additional genetic variations, many of which have been identified in recent genome-wide association studies (GWAS), have been hypothesized to act as low to moderate penetrant alleles, which contribute to ovarian cancer risk, as well as other diseases [215, 249]. In support of this, recent studies have also associated genetic polymorphisms in genes involved in suppression of tumorigenicity as well as those involved in cell cycle with ovarian cancer [250, 251].

For this study, we sought to evaluate the association of specific SNPs in key oxidant and antioxidant enzymes with increased risk and overall survival of ovarian cancer. The analysis of the patient population revealed that the average age at diagnosis and racial distribution of those

diagnosed with ovarian cancer were consistent with known risk factors for ovarian cancer, specifically, women of North American decent and those over 50 years old. Currently we demonstrated that there is no association between the selected SNPs and risk of developing ovarian cancer (Table 2). It is important to emphasize the fact that although the selected SNPs for this study were not found to be associated with ovarian cancer risk, additional change of function SNPs for these enzymes exist and should be explored further. Of the SNPs studied when examining survival, we found the *CAT* SNP (rs1001179) to be a significant predictor of death when present in ovarian cancer patients as illustrated by the Cox regression and K-M survival analyses (Table 4A and Figure 2). Specifically, ovarian cancer patients with the *CAT* SNP died significantly sooner than those without it (Figure 2). The *CAT* SNP (rs1001179) is found in the promoter region of the *CAT* gene, substituting allele C with T at position -262 in the 5' region of chromosome 11 and is correlated with decreased enzyme activity level [224]. Catalase is a very important and ubiquitous enzyme involved in the degradation of two molecules of H_2O_2 to water and oxygen. The current findings are consistent with several other studies, which linked this specific SNP with risk, response to adjuvant treatment and survival of cancer patients [227, 228, 233, 252]. Specifically, low serum CAT levels were associated with adverse prognosis for ovarian cancer [230]. Our data provides a possible explanation for low serum CAT levels, which may be a result of a *CAT* SNP that lowers enzymatic activity. Moreover, mechanistic studies have identified H_2O_2, a result of oxidative stress, enhance angiogenesis and tumor invasiveness through several pathways including: hypoxia inducible factor 1-alpha, p38 MAPK and snail [253, 254]. It appears that the final common pathway culminates to epidermal growth factor (EGF)-induced down-regulation of epithelial cadherin expression that can be inhibited by exogenous CAT [253]. Epithelial-cadherin is a cell-cell adhesion glycoprotein encoded by the *CDH1* gene

in humans, which has been characterized as a tumor suppressor [255, 256]. Its loss of function is correlated with several solid tumors including ovarian and thought to contribute to tumor progression and metastasis [257].

It is important to emphasize that the lack of association between the selected SNPs in this study with ovarian cancer risk does not definitively answer this important question because additional change-of-function SNPs for these enzymes exist and should be explored further. Recent genetic studies have linked MPO to lung and ovarian cancers by demonstrating a striking correlation between the relative risk for development of the disease and the incidence of functionally distinct *MPO* polymorphisms [258]. In breast cancer, the presence of the *CAT* SNP (rs1001179), was shown to confer increased risk [233]. We have selected numerous additional SNPs based on their effect on enzyme activities or association with cancer. Several SNPs in *NOS2* have been associated with gastric, esophageal, skin and urogenital cancers [229, 231]. Also, SNPs in *MnSOD*, *GPX1*, *GPX4*, *CAT* were found to be associated with prostate cancer [233].

Other studies have found a SNP in *SOD2* (rs4880) and a SNP in *MPO* (rs2333227) to be associated with increased risk for ovarian cancer [217]. The *MPO* SNP we have analyzed in this study is in 100% concordance with SNP rs2333227 [259]. Thus, in addition to examining other changes of functional SNPs, increasing the size of our cohort may be sufficient to reach statistical significance in several of the SNPs chosen for this study. The strength of our study includes the comprehensive nature of redox genes studied and the translational aspect of our approach by assessing simultaneously clinical and genotypic characteristics of the population. We believe the fact that our control cohort is heterogeneous represents strength, because it includes patients considered at high risk for *BRCA1/2* mutation and those without any

established risk factors for ovarian cancer, reflecting the baseline risk group (general population). Interestingly, patients who tested negative for *BRCA1/2* mutations, as well as, those with family history of *BRCA1/2* mutations but also tested negative for *BRCA1/2* should be considered at a higher risk profile than the general population. More importantly, to our knowledge, we are the first to report an association between the presence of this specific *CAT* SNP and ovarian cancer survival. The study has several limitations such as small sample size, the retrospective nature inherent to case-control studies, and the geographic restriction of the population. In our patient population, the recurrence rate was found to be 60.5%; however, the exact date of recurrence was not established making the computation of progression-free survival (PFS) impossible. We acknowledge that the determination of PFS would have strengthened our findings as PFS has often been used as a primary endpoint or a surrogate to overall survival in clinical trials [260-265].

It is now evident that oxidative stress plays a major role in the pathogenesis of cancer including ovarian. The exact mechanisms remain to be clarified. In this preliminary study we were able to show that a specific *CAT* SNP is associated with poor survival in ovarian cancer patients. Further studies examining other SNPs in key oxidants and antioxidant enzymes with higher number of patients will be needed to establish this link. SNPs in these enzymes may serve as potential markers for ovarian cancer, which are urgently needed. Our study indicates a strong association with the *CAT* SNP and survival of ovarian cancer patients, and thus may serve as a prognosticator.

CHAPTER 4: ASSOCIATION BETWEEN KEY OXIDANT AND ANTIOXIDANT ENZYMES AS WELL AS SPECIFIC SNPS IN THESE ENZYMES WITH THE DEVELOPMENT OF CISPLATIN RESISTANCE IN EPITHELIAL OVARIAN CANCER CELLS

(This chapter contains previously published material [266]. See Appendix C)

Abstract

In this chapter, we will describe the association of key oxidant and antioxidant enzymes as well as specific SNPs in these enzymes with the development of cisplatin resistance in EOC cells. The foundation of this study is based on previous findings showing an association between the redox enzymes and EOC in both patients and human cell lines; also the conclusion of the previous chapter showing an association between key SNPs in the redox enzymes with decreased overall survival. We hypothesize that *the acquisition of resistance to cisplatin in EOC cells is associated with a pro-oxidant profile, as well as specific SNPs in key oxidant and antioxidant enzymes.* To achieve this, we have utilized two human ovarian cancer cell lines, MDAH-2774 and SKOV-3. For both cell lines, we utilized cisplatin sensitive and cisplatin resistant cells. DNA was extracted from cells for TaqMan PCR genotyping, RNA was extracted for gene expression using real-time RT-PCR, protein was utilized for enzymatic activity using ELISA, and media for NO_2^-/NO_3^- levels as a surrogate of iNOS activity using the Griess assay. We have studied the expression profile of the following genes: *CYBA, NOS2, CAT, SOD2, GSR* and *GPX1*. To analyze the difference in the expression profiles of these genes for sensitive compared to resistant cells, we have used a Student's t-test for gene expression, protein activity, and nitrite/nitrate levels.

Introduction

Oxidative stress is critical for tumorigenesis of EOC and may be linked to cisplatin resistance. Specifically, an increased expression of pro-oxidant enzymes such as iNOS, MPO,

NAD(P)H oxidase, as well as an increase in NO as indicated by increased nitrate/nitrite has been shown in EOC cells [193-195]. Also, EOC cells manifested lower apoptosis, which was markedly reduced by inhibiting iNOS and MPO, indicating a strong link between apoptosis and NO/iNOS and MPO pathways in these cells [194]. Reduced caspase-3 activity is indicative of decreased apoptosis. The platinum-based drugs represent the chemotherapy backbone in the medical management of EOC. Platinum-resistant ovarian cancer is very common and have been touted as a major contributor to poor survival in ovarian cancer patients. Although the exact mechanism of cisplatin resistance in EOC remains elusive, available evidence suggests a role for oxidants and antioxidants in the development of resistance to chemotherapy. Establishing the redox profile of EOC cells is a critical step to understand the underpinnings of the potential relationships between oxidant and antioxidants with diverse key metabolic processes.

In this chapter, we will present our analysis determining whether key oxidant and antioxidant enzymes are associated with chemoresistance in EOC cells.

Methods

Experimental design

Cell Culture and establishment of cisplatin resistant cell lines. Culture of EOC cell lines MDAH-2774 and SKOV-3 and their chemoresistant counterparts are described in *General Methods*. For measurement of messenger RNA (mRNA), activity and NO_2^-/NO_3^- levels, cells (1.2×10^6) were seeded in 100-mm dishes (Corning, Corning, NY) and allowed to rest for 24 hours followed by media replacement and cell collection 24 hours later.

RNA Isolation and Reverse Transcription of cDNA, for Real-time RT-PCR

Total RNA was extracted from EOC cells using an RNeasy Mini Kit (Qiagen) according to the protocol provided by the manufacturer. A 20 µL cDNA reaction volume utilizing 1 µg

RNA was prepared using the Superscript VILO Master Mix (Life Technologies, Carlsbad, CA), as described by the manufacturer's protocol. Quantitative real-time RT-PCR was performed using a QuantiTect SYBR Green RT-PCR kit (Qiagen) and the Cepheid 1.2f Detection System (Cepheid, Sunnyvale, CA). Human oligonucleotide primers were designed with the aid of the software program, Beacon Designer (Premier Biosoft, Palo Alto, CA). Human oligonucleotide primers that amplify variable portions of the protein coding regions are established and verified by our laboratory, as previously described, and are listed in Table 5 [267-270]. The RT-PCR was performed in a 25 µl total reaction volume including 12.5 µl of 2x QuantiTect SYBR Green RT-PCR master mix, 5 µl of 10x diluted cDNA template for *GSR* and 2 µl of undiluted cDNA template for *iNOS*, 3 µl of undiluted cDNA template for, *p22*^{phox}, and *CAT*; and 1 µL of undiluted cDNA template for *GPX1, p22*^{phox}*, β-actin*; and 0.2 µM/L each of target specific primers designed to amplify each gene. Standards with known concentrations and lengths (base pairs [bp]) were designed specifically for genes interested using the Beacon Designer software (Premier Biosoft), allowing for construction of a standard curve using a 10-fold dilution series. A specific standard for each gene allows for absolute quantification of the gene in a number of copies, which can then be expressed per µg of RNA. The RT-PCR reaction conditions were programmed as follows: An initial cycle was performed at 95°C for 900 secs for *GSR* and 1000 sec for *iNOS,* and 95 °C for 60 seconds for *GPX1*, *p22*^{phox}, and 1800 sec for *CAT*. This was followed by 35 cycles of denaturation at 95 °C for 15 secs, annealing for 30 secs 55°C for *CAT*, 59 °C for *GSR*, 30 seconds at 59°C for *GPX1*, 54 °C for *p22*^{phox}, 72 °C for *β-actin* and, 58°C for *iNOS*. A control, containing all the reaction components except for the template, was included in all experiments. All experiments were performed in triplicate.

Table 5. Human Oligonucleotide primers.

Accession Number	Gene	Sense (5'-3')	Antisense (3'-5')	Amplicon (bp)
NM_001752	CAT	GGTTGAACAGATAGCCTTC	CGGTGAGTGTCAGGATAG	105
NM_000637	GSR	TCACCAAGTCCCATATAGAAATC	TGTGGCGATCAGGATGTG	116
NM_000581	GPX1	GGACTACACCCAGATGAAC	TTCTCCTGATGCCCAAAC	96
NM_003102	SOD3	GCCTCCATTTGTACCGAAAC	AGGGTCTGGGTGGAAAGG	78
AY288918	NOX4	AACCTTCTTGCTGTATAAC	TGCTTAGACACAATCCTA	77
NM_000101	$p22^{phox}$	GTACTTTGGTGCCTACTC	GGAGCCCTTCTTCCTCTT	82
NM_001101	β-actin	ATGACTTAGTTGCGTTACAC	AATAAAGCCATGCCAATCTC	79
NM_000625	NOS2	GGCACAGAACTTAAGGATGG	TTGTTAGGAGGTCAAGTAAAGG	145

Purification of DNA and the TaqMan SNP Genotyping Assay for SNPs

DNA, from cells, was isolated by the Applied Genomics Technology Center (AGTC, Detroit, MI) and TaqMan was performed as described in *General Methods*.

ELISAs

Protein was extracted and concentration determined as described in *General Methods.* Unless otherwise stated, all assays were performed utilizing cell lysate according to the manufacturer's protocols.

The Catalase Assay Kit (Cayman Chemical, Ann Arbor, MI) utilizes the peroxidatic function of CAT for determination of enzyme activity and is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. Formaldehyde produced is measured spectrophotometrically with Purpald as the chromogen, which is detected at 540 nm.

The Glutathione Reductase Assay Kit (Abcam, Cambridge, MA) measures GSR activity by its ability to reduce GSSG to GSH, which reacts with 5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) to generate TNB, which can be detected at 405 nm. Absorbance was read once a

minute for 5 minutes and slope was utilized to calculate GSR activity.

The Glutathione Peroxidase Assay Kit (Abcam, Cambridge) measures GPx activity by GPx's ability to reduce Cumene Hydroperoxide while oxidizing GSH to GSSG. GSR reduces the generated GSSG to GSH with consumption of NADPH. The decrease of NADPH (easily measured at 340 nm) is proportional to GPx activity. Absorbance was read once a minute for 5 minutes and slope was utilized to calculate GPx activity. The rate of decrease in the absorbance 340 is directly proportional to the GPx activity in the sample.

The nitrate/nitrite colorimetric assay (Cayman Chemical, Ann Arbor, MI) was used to measure the levels of stable NO by-products, NO_2^- and NO_3^-, as an indication of NO production. Due to the fact that the proportion of NO_2^- and NO_3^- is variable and cannot be predicted with certainty, the sum of both NO by-products is a more accurate indicator of NO production. The assay was performed utilizing cell culture media. Absorbance was detected at 540 nm and a standard curve for nitrite was utilized to determine total NO_2^- and NO_3^-.

Statistical Analysis

Unpaired t tests were used to compare controls and the resistant cell lines. Statistical significance was established at P-value less than 0.05 for all analyses.

Results

Cisplatin-resistant SKOV-3 EOC cells have lower $p22^{phox}$ mRNA levels compared to their chemosensitive counterparts

The $p22^{phox}$ mRNA levels were significantly decreased (from 272.78 ± 18.44 to 104.65 ± 3.61 fg/µg RNA) in cisplatin-resistant MDAH-2774 EOC cells ($P<0.05$, Figure 3), and in SKOV-3 EOC cells (from 380.37 ± 38.61 to 70.12 ± 4.25) ($P<0.05$, Figure 3), as compared to chemosensitive controls.

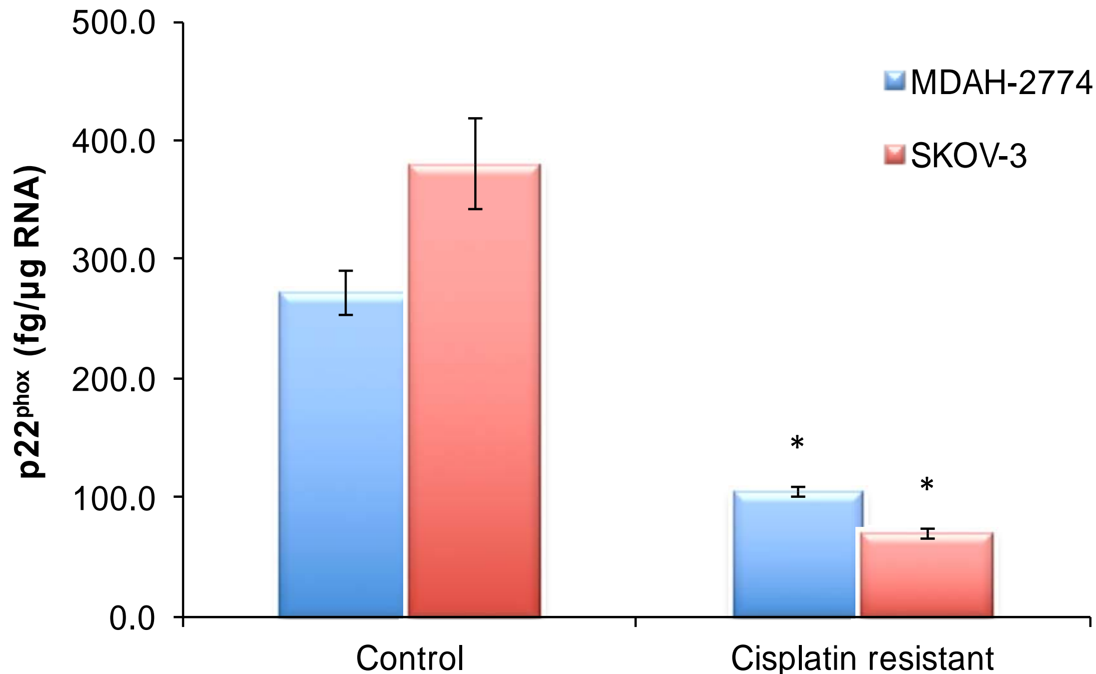


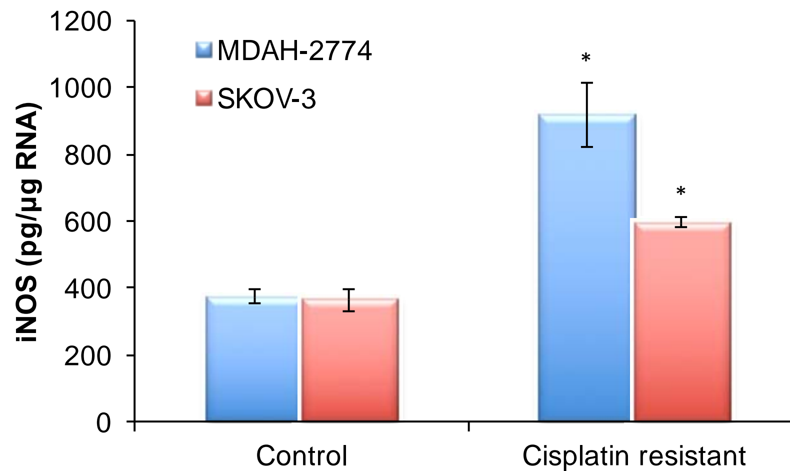
Figure 3. ***p22**^{phox}* **mRNA levels in sensitive EOC as compared to their resistant counterparts.** RNA was isolated from both sensitive and resistant EOC cell lines (MDAH-2774 and SKOV-3). Real-time RT-PCR was utilized to measure mRNA. *P<0.05 denotes statistical significance. Error bars represent standard deviation.

Cisplatin-resistant EOC cells have higher iNOS mRNA levels and NO_2^-/NO_3^- levels

The *iNOS* mRNA levels were significantly increased from 374.18 ± 20.54 to 916.26 ± 96.01 pg/µg RNA in MDAH-2774, and from 366.31 ± 32.67 to 597.34 ± 13.27 pg/µg RNA in SKOV-3 cisplatin-resistant EOC cells when compared to sensitive counterparts ($P < 0.05$, Figure 4A) [266].

Correspondingly, NO_2^-/NO_3^- levels were significantly increased in MDAH-2774 cisplatin-resistant cells from 12.26 ± 0.04 µM/L to 16.51 ± 0.06 µM/L, and from 8.2 ± 0.0 µM/L to 13.7 ± 0.2 µM/L in SKOV-3 cisplatin-resistant EOC cells when compared to their sensitive counterparts ($P < 0.05$, Figure 4B).

A



B

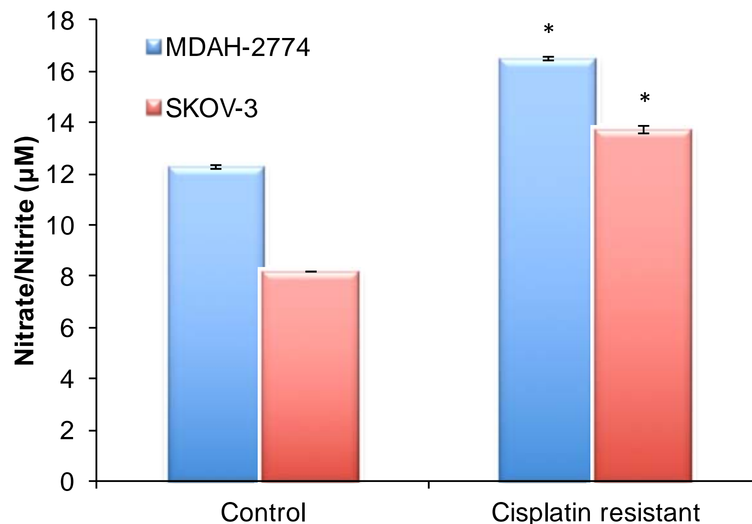


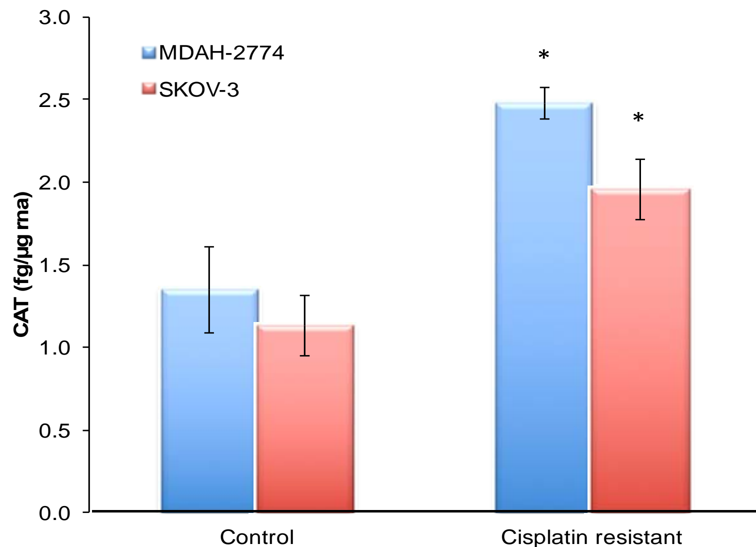
Figure 4. *iNOS* mRNA and nitrate/nitrite levels in sensitive EOC as compared to their resistant counterparts. RNA and protein were isolated from both sensitive and resistant EOC cell lines (MDAH-2774 and SKOV-3). Real-time RT-PCR was utilized to measure mRNA and Griess assay measuring nitrate/nitrite levels in protein were utilized. *P<0.05 denotes statistical significance. Error bars represent standard deviation of the mean.

Chemoresistant EOC cells have an increase in CAT mRNA levels and activity as compared to their chemosensitive counterparts

Catalase mRNA levels were increased from 1.13 ± 0.18 to 1.96 ± 0.18 fg/µg RNA in SKOV-3, and from 1.35 ± 0.26 to 2.48 ± 0.09 fg/µg RNA in MDAH-2774 (P<0.05, Figure 5A). Correspondingly, CAT activity also increased in cisplatin-resistant SKOV-3 (from 6034.66 ± 487.48 to 7884.37 ± 138.66 nmol/min/mL) and MDAH-2774 (from 6156.28 ± 213.32 to 8823.37

± 257.32 nmol/min/mL) when compared to their sensitive controls (P < 0.05, Figure 5B).

A



B

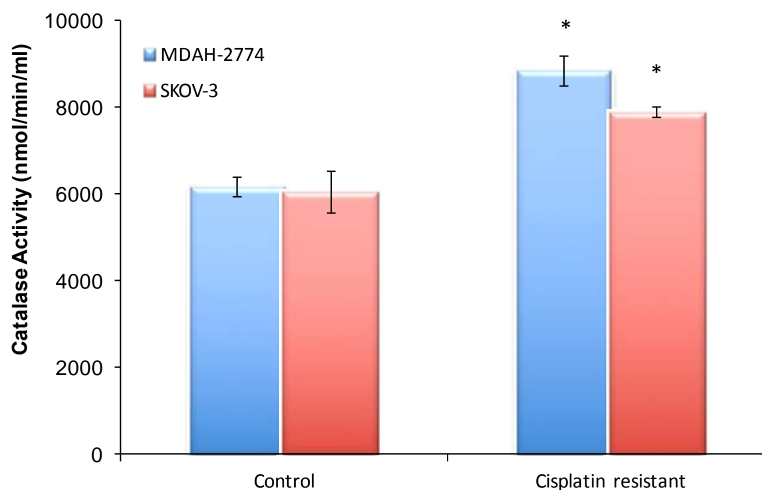


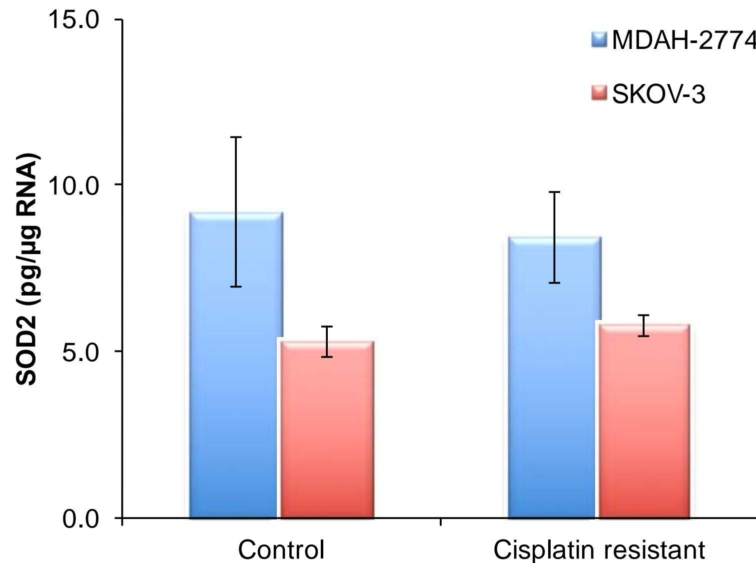
Figure 5. Catalase *mRNA* and activity levels in sensitive EOC as compared to their resistant counterparts. RNA and protein were isolated from both sensitive and resistant EOC cell lines (MDAH-2774 and SKOV-3). Real-time RT-PCR to measure mRNA and ELISA to measure protein levels were utilized. *P<0.05 denotes statistical significance. Error bars represent standard deviation of the mean.

Chemoresistant EOC cells have decreased SOD activity as compared to their chemosensitive counterparts

The *SOD2* mRNA levels decreased (from 9.18 ± 2.25 and 5.27 ± 0.46 to 8.42 ± 1.34 and 5.79 ± 0.30 pg/µg RNA) in MDAH-2774 (P>0.05, Figure 6A) and SKOV-3 respectively (P>0.05,

Figure 6A), as compared to their chemosensitive controls. The activity of SOD decreased in cisplatin-resistant MDAH-2774 (from 30,111.06 ± 388.11 to 26,959.88 ± 1011.41 U/ml/mg protein) and SKOV-3 (from 40,373.40 ± 809.48 to 27,955.97 ± 1600 U/ml/mg protein) when compared to respective sensitive controls ($P < 0.05$, Figure 6B).

A



B

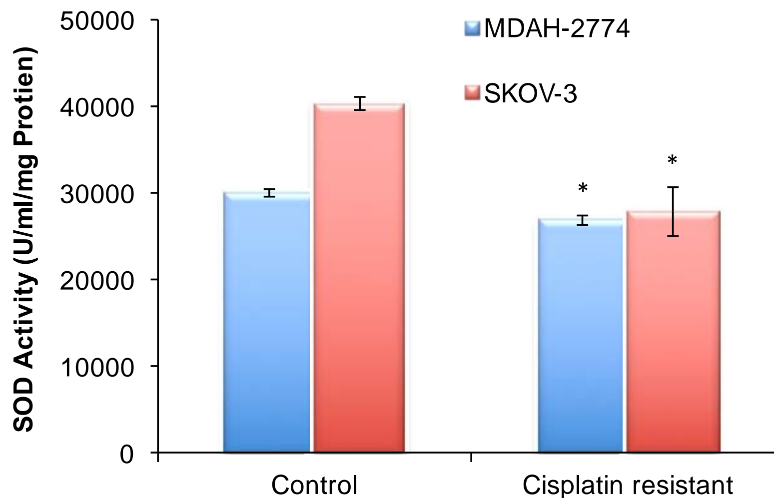
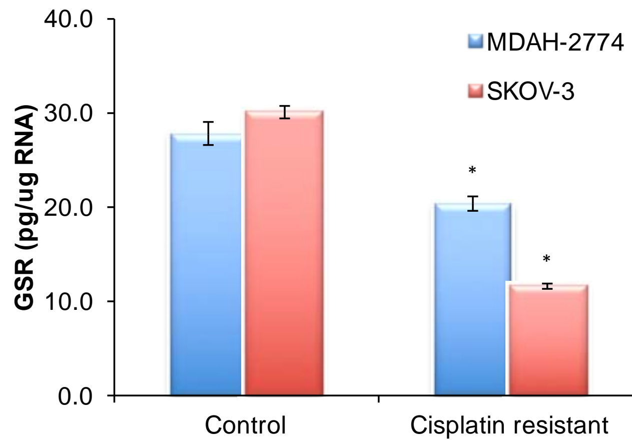


Figure 6. *SOD2* mRNA and activity levels in sensitive EOC as compared to their resistant counterparts. RNA and protein were isolated from both sensitive and resistant EOC cell lines (MDAH-2774 and SKOV-3). Real-time RT-PCR to measure mRNA and ELISA to measure protein levels were utilized. *$P<0.05$ denotes statistical significance. Error bars represent standard deviation of the mean.

Chemoresistant EOC cells have a decrease in GSR mRNA levels and activity as compared to their chemosensitive counterparts

There was a significant decrease in the mRNA expression and activity of GSR in both cisplatin-resistant EOC cell lines when compared to their sensitive counterparts (Figure 7) [266]. The GSR mRNA levels decreased in cisplatin-resistant MDAH-2774 (from 27.7 ± 1.2 to 20.3 ± 0.7 pg/µg RNA) and SKOV-3 (from 30.1 ± 0.6 to 11.6 ± 0.25 pg/µg RNA) when compared to their chemosensitive controls. (P < 0.05 Figure 7A).

A



B

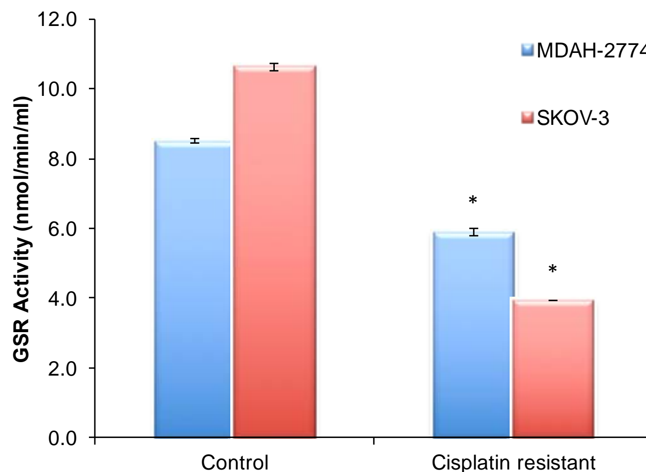


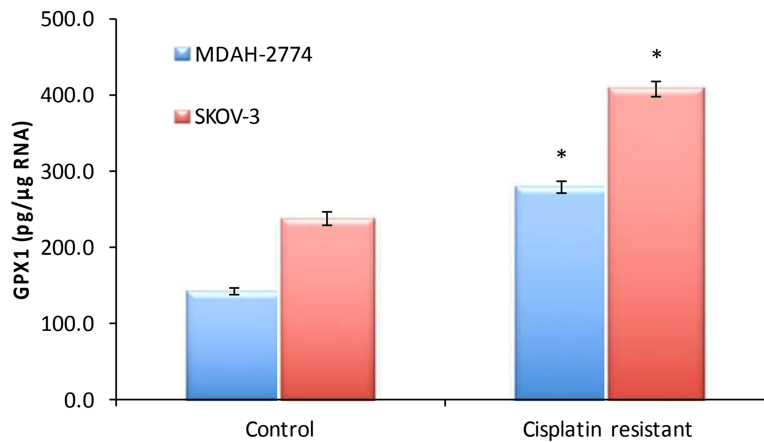
Figure 7. *GSR* mRNA and protein activity levels in sensitive EOC as compared to their resistant counterparts. RNA and protein were isolated from both sensitive and resistant EOC cell lines (MDAH-2774 and SKOV-3). Real-time RT-PCR to measure mRNA and ELISA to measure protein levels were utilized. *P<0.05 denotes statistical significance. Error bars represent standard deviation of the mean.

Correspondingly, GSR activity also decreased in cisplatin-resistant MDAH-2774 (from 8.5 ± 0.07 to 5.9 ± 0.1 nmol/min/mL) and SKOV-3 (from 10.6 ± 0.1 to 3.9 ± 0.0 nmol/min/mL) when compared to respective sensitive controls ($P < 0.05$, Figure 7B).

Chemoresistant EOC cells have an increase in GPX1 mRNA levels and activity as compared to their chemosensitive counterparts

There was a significant increase in the mRNA expression of GPX1 and activity of GPx in both cisplatin-resistant EOC cell lines when compared to their sensitive counterparts (Figure 8).

A



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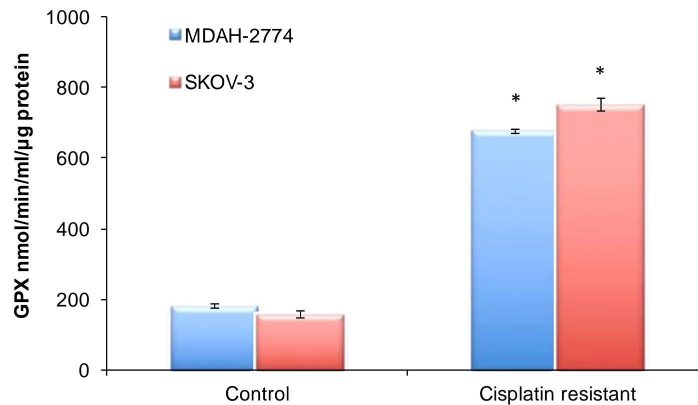

Figure 8. *GPX1* mRNA and activity levels in sensitive EOC as compared to their resistant counterparts. RNA and protein were isolated from both sensitive and resistant EOC cell lines (MDAH-2774 and SKOV-3). Real-time RT-PCR to measure mRNA and ELISA to measure protein levels were utilized. *$P<0.05$ denotes statistical significance. Error bars represent standard deviation of the mean.

There was a significant increase in GPX1 mRNA levels (from 141.95 ± 4.13 and 236.95 ± 40.17 pg/µg RNA to 279.22 ± 17.39 and 407.75 ± 20.32 pg/µg RNA) in MDAH-2774 and SKOV-3

EOC cells, respectively (P<0.05, Figure 8A), as compared to their chemosensitive controls.

Similarly, GPx protein activity increased in cisplatin-resistant MDAH-2774 (from 180.83 ± 4.5 to 675.57 ± 5.25 nmol/min/ml/µg protein) and SKOV-3 (from 155.47 ± 9.91 to 750.81 ± 17.71 nmol/min/ml/µg protein) when compared to respective sensitive controls ($P < 0.05$, Figure 8B).

Chemoresistant EOC cells are associated with mutations in GPX1, SOD2, and CYBA

For both cell lines, our genotyping data did show the presence of the selected SNPs, except for *GPX1,* in the cisplatin-sensitive EOC cells. The cisplatin-resistant cells revealed the presence of point mutations in *GPX1*, *CYBA,* and *SOD2* genes. The mutations in the *SOD2* and *CYBA* genes correspond to the selected SNPs in *SOD2* and *CYBA*; and the reversal of the *GPX1* SNP. There was a single nucleotide change from T to C in *GPX1* and *SOD2*, and from C to T in *CYBA* in the cisplatin-resistant cells compared to their sensitive counterparts. No change has been observed for *CAT*, *NOS2*, or *GSR*. The data suggest an association between the mutations and possibly the SNPs, and cisplatin resistance (Table 6).

Table 6. Cisplatin resistance is associated with nucleotide change in *CYBA, GPX1* and *SOD2.*

	Genotype *(indicates change to SNP)*					
Cell Line and Treatment	***CAT* (C→T)**	***GPX1* (C→T)**	***GSR* (G→T)**	***SOD2* (T→C)**	***CYBA* (C→T)**	***NOS2* (C→T)**
MDAH-2774 Sensitive	**C/C**	**C/T**	**G/G**	**T/T**	**T/C**	**C/C**
MDAH-2774 Cisplatin resistant	**C/C**	**C/C**	**G/G**	**T/C**	**TT**	**C/C**
SKOV-3 Sensitive	**C/C**	**C/T**	**G/G**	**T/T**	**T/C**	**C/C**
SKOV-3 Cisplatin resistant	**C/C**	**C/C**	**G/G**	**T/C**	**TT**	**C/C**

DNA was isolated from both sensitive and resistant EOC cell lines (MDAH-2774 and SKOV-3). Genotyping was done utilizing TaqMan probes. For both cell lines, there is a single nucleotide change from T to C in the *SOD2*, and *GPX1* genes; C to T for *CYBA* in cisplatin resistant cells compared to their sensitive counterparts. No change has been observed for *CAT*, *GSR*, and *iNOS*.

Discussion

In this study, we have shown that both MDAH-2774 and SKOV-3 cells displayed decreased expression of *CAT* and *GSR* with the development of cisplatin resistance (Figure 5A and B). Many lines of evidence have linked *CAT* expression and activity to cancer and resistance to chemotherapy [178, 271-274]. For example, genetic polymorphisms involving C262T SNP was associated with increased CAT expression and concomitant reduction of breast cancer risk [271, 272]. It has also been reported that MCF-7 breast cancer cells overexpressing CAT not only had impaired proliferation and migration capacities but also, were more sensitive to anticancer treatments when compared to parent cells [273]. Additionally, others have demonstrated that both CAT and GPx can inhibit the cisplatin-induced down regulation of Bcl-2, a powerful anti-apoptosis protein [274].

Glutathione is a powerful antioxidant that plays an important role in preventing ROS-induced damages to vital cellular functions [275-277]. However, it was shown that once cancer is established, high levels of GSH can be deleterious by preventing the cytotoxic effect of various chemotherapeutic agents through detoxification and increased activity of efflux pump [278-280]. Conversely, it has also been reported that low levels of GSH are linked to impaired immune response and tumor progression [281-283]. The latter is more aligned with our findings, because low expression of GSR is indicative of low GSH and oxidative stress since GSH will not be adequately regenerated from its oxidized form (GSSG). Together, the antioxidant mechanisms involving both CAT and GSR, when impaired may adversely impact the response to cisplatin by failing to restore the oxidative balance.

For both cell lines, *CYBA* was overexpressed in cisplatin-resistant cells supporting the theory that tumor cells are under intrinsic oxidative stress that may play a role in chemotherapy

resistance and tumor progression. Indeed, previous report suggests impairment of apoptosis-induced *CYBA* upregulation conferred resistance to chemotherapy by over-activation of MEK/ERK pathway in liver tumor cells [284].

Our results suggest that increased expression of iNOS correlates with the development of resistance to cisplatin. The NO synthase family includes the calcium/calmodulin mediated isoenzymes eNOS (endothelial) and nNOS (neuronal), in addition to the non-calcium-dependent iNOS [285]. Earlier studies have shown that NO donors can induce apoptosis at high concentrations, whereas preventing it at low physiologic levels [286, 287]. Moreover, as compared to normal cells, cancer cells have significantly increased level of iNOS [288].

Previously, we have reported that increased iNOS activity can lead to S-nitrosylation of caspase-3 and decreased apoptosis in EOC cells [193]. Also, we have demonstrated that high levels of iNOS in EOC cells are associated with high levels of vascular endothelial growth factor (VEGF) production and angiogenesis induction [194]. Additionally, other groups have reported an inverse relationship between the expression of eNOS/nNOS and iNOS that suggests elevated expression of iNOS coupled with decreased eNOS/nNOS expression may be associated with p53-mediated cisplatin resistance in EOC [289]. Conversely, low expression of iNOS in head and neck squamous cell carcinoma was shown to be associated with resistance to cisplatin/taxol-induced apoptosis mediated through survivin [290]. The physiology of the NO synthase family is fairly complex and not fully elucidated; however, they appeared to be involved in cisplatin resistance in many cancers.

Interestingly, the genotyping data revealed point mutations in the *SOD2, GPX1*, and *CYBA* genes for cisplatin resistant cell lines (both MDAH-2774 and SKOV-3) as compared to their sensitive counterparts. Wild-type *SOD2* and *GPX1* genes encode for antioxidant proteins,

which functions are to mitigate oxidative stress; while the *CYBA* gene encode for oxidants.

The nucleotide change (T to C) observed in the *SOD2* gene is consistent with SNP (rs4880), which results in a decrease in the SOD enzymatic activity due to an amino-acid change (valine to alanine). As mentioned above, the cisplatin-sensitive cells carry the *GPX1* SNP (rs3448) which was reversed in the cisplatin-resistant cells. No specific amino-acid change or enzymatic function alterations have been consistently reported; however, in the next chapter we will provide new insights on the impact of *GPX1* genetic editing and cisplatin resistance.

The *CYBA* gene encodes for the cytochrome b-245 alpha light chain protein ($p22^{phox}$), which in humans is involved in $O_2^{\bullet-}$ production. It is a component of the NOX complex. The nucleotide change observed in the *CYBA* gene is consistent with SNP (rs4673), which has been reported to a decrease in NOX4 enzymatic activity due to an amino-acid change (tyrosine to histidine).

The present study suggests a potential relationship between the oxidative state and the development of cisplatin resistance in ovarian cancer. Additionally, our data suggest a link between previously reported SNPs in *SOD2*, *NOS2*, *GPX1* and *CYBA* genes with cisplatin-resistant EOC cells. These findings represent a new direction in our quest to unravel the exact role of oxidative stress in the establishment of cisplatin resistance in EOC cells and will be the foundation of the next chapter.

CHAPTER 5: CAUSAL RELATIONSHIP BETWEEN *SOD, CYBA*, AND *GPX1* SPECIFIC SNPS AND ACQUISITION OF CISPLATIN RESISTANCE IN EPITHELIAL OVARIAN CANCER CELLS

Abstract

In this chapter, we will discuss whether there is a causal relationship between *SOD2, CYBA*, and *GPX1* specific SNPs and acquisition of cisplatin resistance in EOC cells. The rationale of this study is based on the established fact that cisplatin treatment causes DNA damage, and the observation that specific SNPs in the redox enzymes were found to be associated with poor survival in ovarian cancer patients. Additionally, our findings from Chapter 4 demonstrated an association between cisplatin resistant cells and point mutations corresponding to specific SNPs in several redox enzymes. The hypothesis of this study is: *specific SNPs in key oxidant and antioxidant enzymes cause cisplatin resistance.* To achieve this, we utilized the CRISPR/Cas9 system to generate point mutations corresponding to specific SNPs in selected genes in sensitive MDAH-2774 and SKOV-3 cell lines. Cells were enriched for the specific point mutation by flow cytometry. The enriched cells were then cultured with increasing doses (0.5 to 5 μM) of cisplatin and tested for resistance using the MTT cell viability assay. IC_{50} values were derived from equations generated by regression analyses of the MTT cisplatin dose-response curves. Cisplatin IC_{50} values from SKOV-3 cells without the point mutation and those with the point mutation were compared to determine cisplatin resistance.

Introduction

Oxidative stress can result in increased DNA mutations or damage, genome instability, and cellular proliferation; which have been implicated in the pathogenesis of several malignancies including ovarian cancer [46, 166, 167, 178, 185-187]. Moreover, there is evidence of decreased circulating antioxidants in ovarian cancer patients [167]. We have previously reported that cisplatin resistant EOC cells were associated with an enhanced oxidative

state, indicating that oxidative stress may play a role in maintaining the oncogenic phenotype of EOC cells [291]. In an attempt to determine the mechanisms underlying this observation, we selected and tested for the presence of specific functional SNPs known to be associated with cancer. In the parent cell lines, only *GPX1* revealed the presence of the SNP. Surprisingly, we found point mutations in *CYBA, SOD2* and *GPX1* (reversal of the SNP) that were identical to the selected SNPs, indicating that exposure to cisplatin or other factors may have been responsible for the occurrence of *de novo* mutations. Indeed, pharmacokinetically, cisplatin avidly binds and damages DNA, hence creating the possibility of translesion synthesis, whereby DNA synthesis is not blocked but proceeds beyond cisplatin due to specific classes of polymerases [88, 96].

Here, we sought to test whether there is a causal relationship between the point mutations corresponding to known SNPs in key redox genes and cisplatin resistance in SKOV-3 and MDAH-2774 EOC human cell lines.

Methods

Experimental design

Culture of EOC cell lines MDAH-2774 and SKOV-3 and their chemoresistant counterparts are described in *General Methods*.

Following design of a genomic DNA target sequence for *SOD, CYBA* and *GPX1*, the GeneArt CRISPR kit (Life Technologies, Carlsbad, CA) was used to generate double stranded oligonucleotides (ds oligo), per the manufacturer's protocol. The ds oligo was cloned via a ligation reaction into a CRISPR vector. Cloning of the CRISPR vector was performed in chemically competent *E. coli* included in the kit. These cells are ideal for high-efficiency cloning and plasmid propagation, and allow stable replication of high-copy number plasmids. Transformed *E. coli* were transferred onto Luria broth (LB) agar plates containing 100 μg/ml

ampicillin and grown overnight resulting in ampicillin-resistant colony formation. A single colony was grown in LB medium containing 100 μg/ml ampicillin overnight and subsequently purified using the HI Pure Plasmid Mini Prep Kit (Life Technologies), per the manufacturer's protocol. For transfection of the plasmid into our EOC cells, cells were plated (400,000) in a 6-well dish and allowed to grow overnight. Cells were transfected once they reached ~80% confluency. 250 μl of Opti-MeM (Life Technologies) was used for transfection and included the specific concentration of the CRISPR vector in a 1:3 ratio of the TransIT-X2 transfection reagent (Mirus Biologicals, Madison, WI). The cells were incubated for 24 hours and the presence of fluorescence served as confirmation of transfection of the cells as the CRISPR system contains an orange fluorescence protein reporter.

The CRISPR transfected cells were enriched using Fluorescence-activated cell sorting (FACS) (Figure 9).

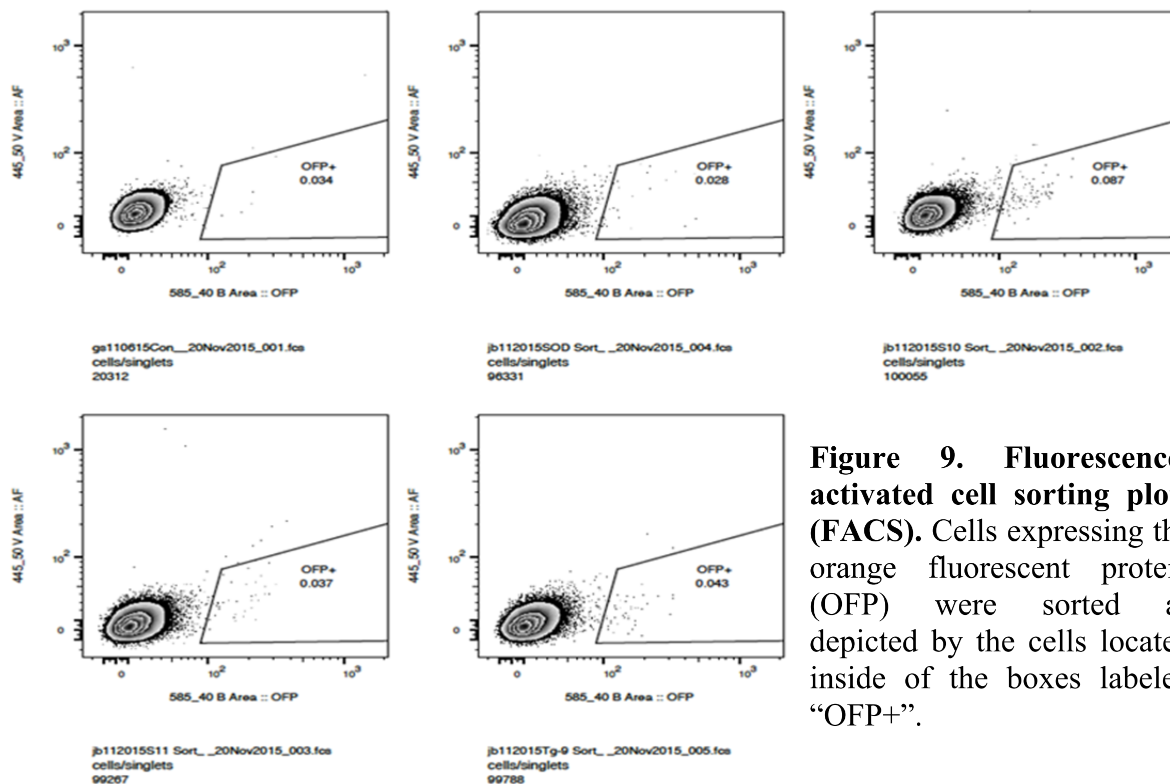

Figure 9. Fluorescence-activated cell sorting plots (FACS). Cells expressing the orange fluorescent protein (OFP) were sorted as depicted by the cells located inside of the boxes labeled "OFP+".

Cell sorting was performed at the Wayne State University Microscopy, Imaging, and Cytometry (MICR) Resources Core Facility. Cells expressing the orange fluorescent protein (OFP) were sorted on a Sony SY3200 cell sorter equipped with 355 nm, 405 nm, 488 nm, 561 nm, and 642 nm lasers (Sony Biotechnology, San Jose, CA). The SKOV-3 EOC cell lines with the SOD point mutation were further analyzed for activity using an ELISA, as described in General Methods. Additionally, cell viability will be determined utilizing the Vybrant MTT Cytotoxicity Assay (Invitrogen) following treatment with increasing doses of cisplatin (0.5 to 5 μM) for 72 hours.

Statistical analysis

For each MTT viability assay, a cisplatin dose-response curve was generated in the form of a scatter plot with a linear regression line. Then, the predicted cisplatin IC50 value was determined using the regression equation. Comparisons between cell lines were done using the predicted cisplatin IC50 values, with lower values indicate cisplatin sensitivity and higher values cisplatin resistance. Baseline cisplatin IC50 values were previously established for both MDAH-2774 and SKOV-3 cell lines and served as controls, as described in Chapter 2. P-values were based on the predicted minimum and maximum 95% CIs. Again, to analyze the difference in protein activity of SOD between controls, cisplatin resistant and point mutation-induced EOC cell lines, unpaired t-tests were used. For all analyses, P-value less than 0.05 is considered statistically significant.

Results

Induction of the point mutation corresponding to a known function SNP in SOD2 resulted in lower SOD activity in both MDAH-2774 and SKOV-3 EOC cell lines.

We have successfully transfected both MDAH-2274 and SKOV-3 chemo-naïve cells with the CRISPR/Cas9 system and demonstrated a significant decrease in activity of SOD in both the

MDAH-2774 and SKOV-3 EOC cell lines following the induction of the point mutation corresponding to a specific SNP (Figure 10).

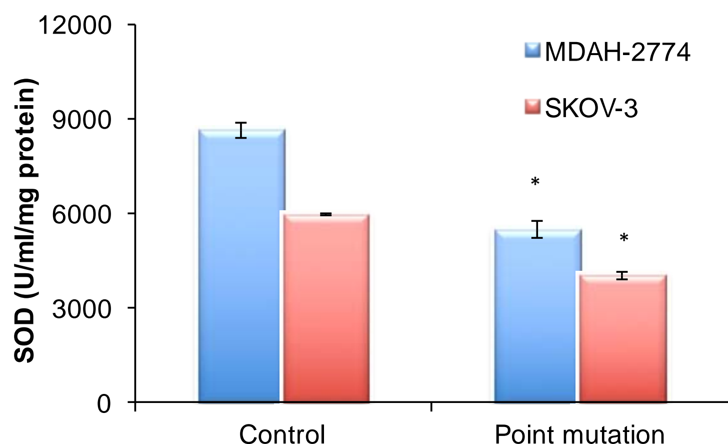

Figure 10. SOD activity levels for the point mutation-induced EOC cells as compared to untreated controls. ELISA results show a significant reduction of the SOD activity in the point mutation-induced EOC cells as compared to untreated counterparts. *P<0.05 denotes statistical significance. Error bars represent standard deviation.

Both MDAH-2774 and SKOV-3 cisplatin resistant EOC cell lines demonstrated a higher cisplatin IC_{50} compared to their sensitive counterparts

The cisplatin IC_{50} for MDAH-2774 cisplatin resistant cells was 6.34 (95% CI [6.19-6.49]) compared to 1.70 (95% CI [1.66-1.74]) for their cisplatin sensitive counterparts (P<0.05), a 2.73-fold change (Figure 11, Table 7). Similarly, the cisplatin IC_{50} for SKOV-3 cisplatin resistant cells was 3.31 (95% CI [3.27-3.35]) compared to 1.87 (95% CI [1.83-1.91]) for their cisplatin sensitive counterparts (P<0.05), a 0.77-fold change (Figure 12, Table 7).

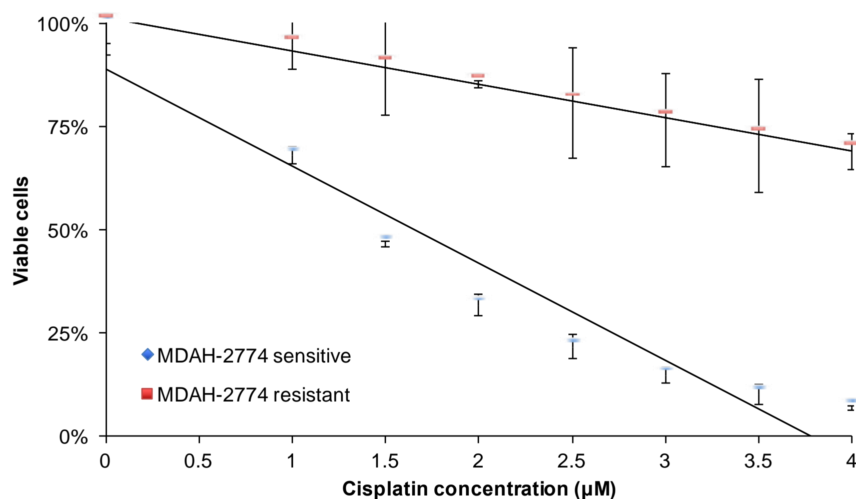

Figure 11. Cell viability assay for MDAH-2774 human EOC cells. The red curve depicts resistant cells as compared to the blue curve, which depicts sensitive cells. IC_{50}: the dose of cisplatin that kills 50% of cells.

Table 7. Predicted cisplatin IC_{50} for SKOV-3 EOC cells.

Cell Line	Predicted IC_{50}	95% CI min	95% CI max	Fold-change	P-value
SKOV-3 sensitive	1.87	1.83	1.91	0.00	<0.05
SKOV-3 resistant	3.31	3.27	3.35	0.77	<0.05
Point Mutation-Induced Sensitive SKOV-3 EOC Cells					
MnSOD	2.79	2.77	2.80	0.49	<0.05
GPX1	3.60	3.58	3.62	0.92	<0.05
CYBA	1.73	1.70	1.77	-0.08	<0.05

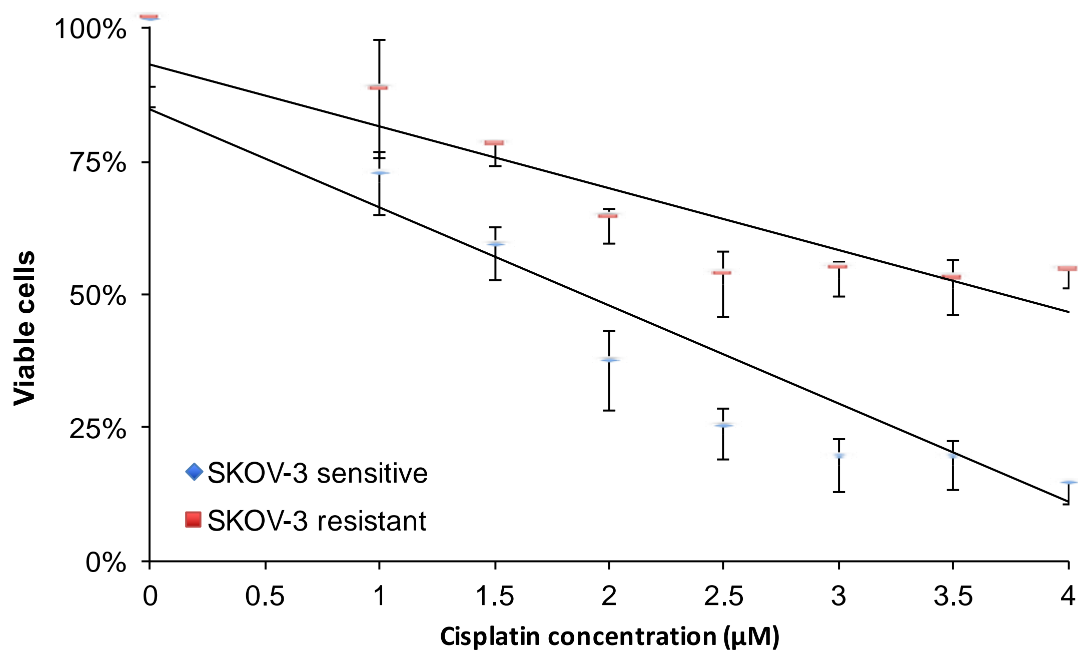

Figure 12. Cell viability assay for SKOV-3 human EOC cells. The blue curve depicts resistant cells as compared to the red curve, which depicts sensitive cells. IC_{50}: the dose of cisplatin that kills 50% of cells.

SKOV-3 cisplatin sensitive cells demonstrated a shift in the cisplatin IC_{50} after introduction of point mutations

The SKOV-3 EOC cells with the *SOD2* point mutation (Table 6) revealed a cisplatin IC_{50} of 2.79 (95% CI [2.77-2.80]) compared to 1.87 (95% CI [1.83-1.91]) for their cisplatin sensitive counterparts (P<0.05), indicating that the introduction of the point mutation corresponding to the *SOD2* SNP (rs4880) rendered the cells resistant to cisplatin by a 0.49-fold change (Figure 13,

Table 7). Similarly, the SKOV-3 EOC cells with the *GPX1* point mutation revealed a cisplatin IC_{50} of 3.60 (95% CI [3.58-3.62]) compared to 1.87 (95% CI [1.83-1.91]) for their cisplatin sensitive counterparts ($P<0.05$), indicating that the introduction of the mutation corresponding to the reversal of *GPX1* SNP (rs3448) rendered the cells resistant to cisplatin by a 0.92-fold change (Figure 14, Table 7). Interestingly, the SKOV-3 EOC cells with the *CYBA* point mutation revealed a cisplatin IC_{50} of 1.73 (95% CI [1.70-1.77]) compared to 1.87 (95% CI [1.83-1.91]) for their cisplatin sensitive counterparts ($P<0.05$), indicating that the introduction of the point mutation that corresponds to *CYBA* SNP (rs4673), sensitized the cells to cisplatin, by a -0.08-fold change (Figure 15, Table 7).

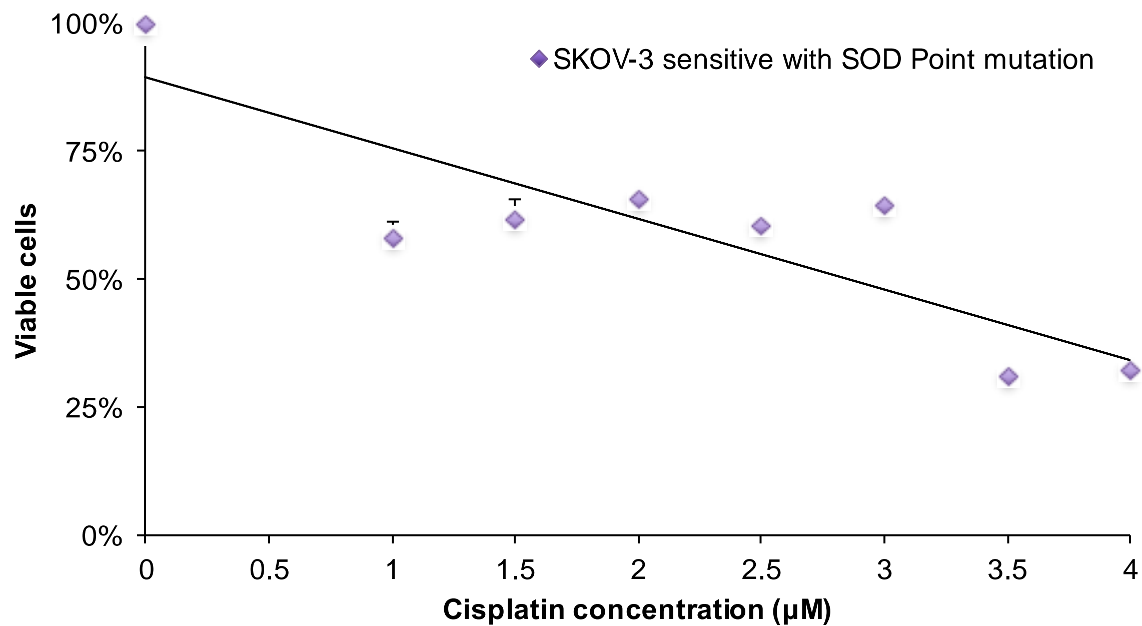

Figure 13. Cell viability assay for SKOV-3 human EOC cells harboring the *SOD* point mutations corresponding to SNP (rs4880). IC_{50}: the dose of cisplatin that kills 50% of cells.

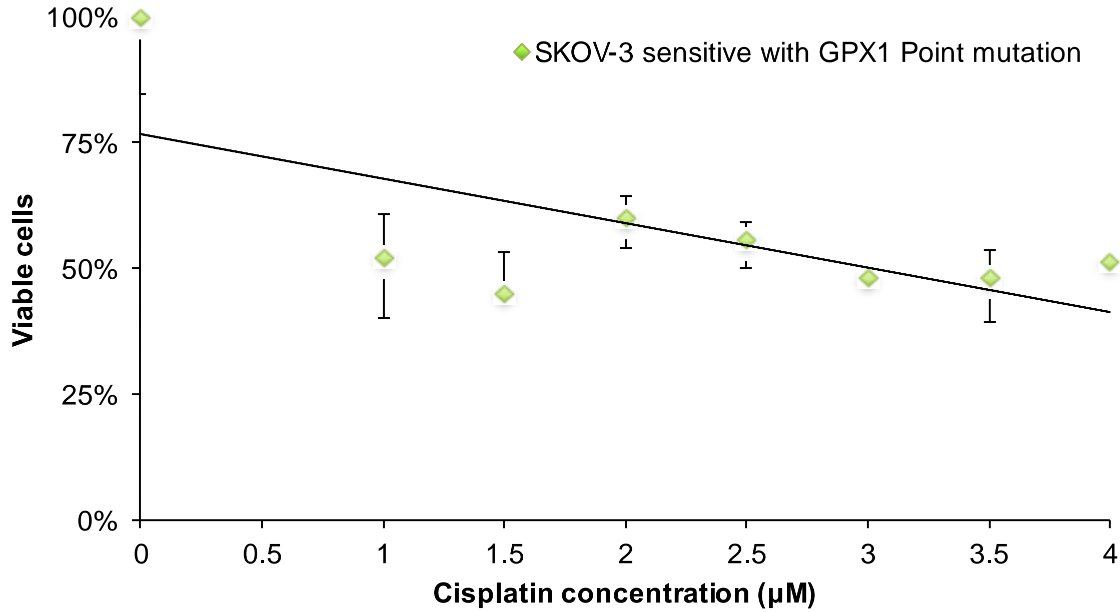

Figure 14. Cell viability assay for SKOV-3 human EOC cells harboring the *GPX1* point mutation corresponding to the reversal of SNP (rs3448). IC_{50}: the dose of cisplatin that kills 50% of cells.

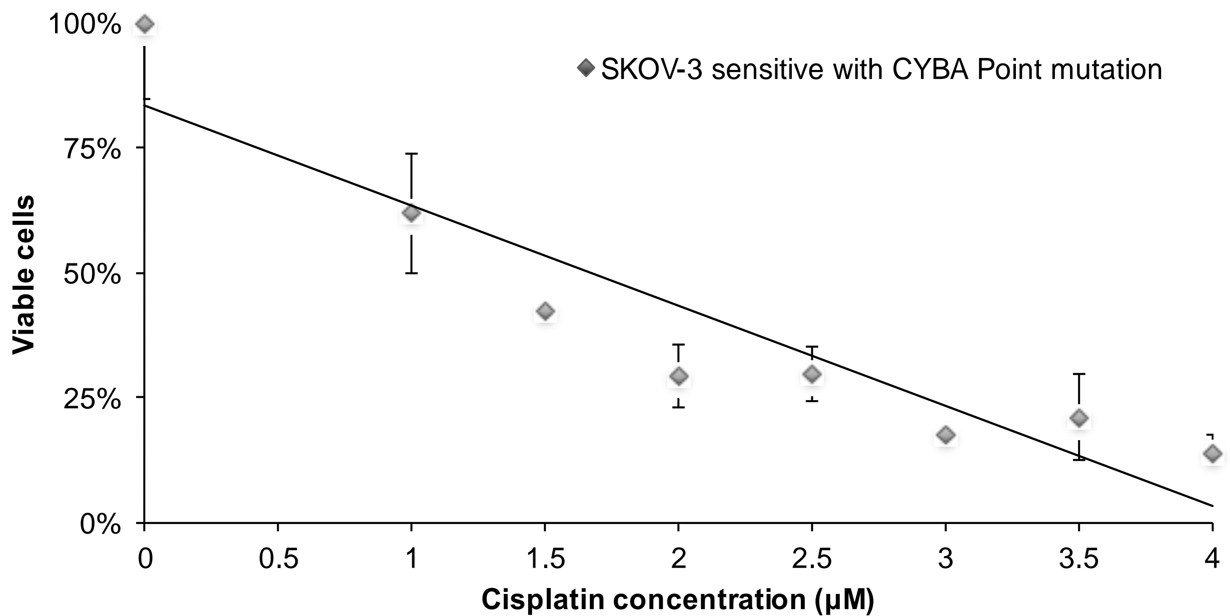

Figure 15. MTT cell viability assay for SKOV-3 human EOC cells harboring the *CYBA* point mutation corresponding to SNP (rs4673). IC_{50}: the dose of cisplatin that kills 50% of cells.

Discussion

In this study, we have used the CRISPR/Cas9 system to successfully generate point mutations in SKOV-3 chemosensitive EOC cells. Subsequently, we have established cisplatin IC_{50} values for SKOV-3 sensitive, resistant and mutation-induced cell lines. By comparing the IC_{50} values, we determined that the cells with point mutations for *GPX1* and *SOD* have become resistant to cisplatin. Interestingly, we found that the creation of the point mutation in the *CYBA* gene increases their sensitivity to cisplatin. As outlined in the previous chapter, the point mutations which correspond to specific SNPs in*, SOD2* and *CYBA* were originally observed in both SKOV-3 and MDAH-2774 cisplatin-resistant cell lines. For both cell lines, the *GPX1* SNP was observed only in the cisplatin-sensitive cells.

The SKOV-3 EOC cells were found to be highly resistant to cisplatin when the specific *GPX1* mutation is present. Moreover, the magnitude the cisplatin resistance, as indicated by the IC_{50} value, was greater in the SKOV-3 cells with the CRISPR-induced *GPX1* point mutation, than the resistant controls. This indicates that *GPX1* is a major contributor of cisplatin resistance and is consistent with the literature. The *GPX1* gene located at 3p21.31 encodes for GPx, which is one of the most important antioxidants in humans and functions in the detoxification of hydrogen peroxide [292-294]. Variations in *GPX1* (rs3448) were associated with overall prostate cancer risk with an odds ratio of 0.62 for the mutant (TT) versus the wild-type (CC) (95% confidence interval, 0.44-0.88); as well as breast cancer risk [295, 296]. Decreased levels or activity of GPx is associated with colorectal cancer in humans; however in esophageal cancer, its expression is thought to drive aggressive growth and metastasis, partly through cisplatin resistance [292, 297]. *GPX1* variants heavily influenced cellular biology, suggesting that different *GPX1* variants affect cancer risk differently [298].

Associations between high levels of GSH, glutathione transferase and chemoresistance in patients with ovarian cancer have been widely reported [297, 299-302]. These observations are consistent with the known role of the MDR membranous proteins pumps, as they could be modulated by the GSH/GSSG system. These proteins, when activated, export several chemotherapeutic drugs, including cisplatin, outside of the cells thereby decreasing their effective concentrations. In one study, it was suggested that the GSH system may be involved in the mechanism of chemoresistance in ovarian cancer, as explained by the upregulation of GSH and MRP expression modulated by gamma-GCS [303].

The *SOD2* point mutation was also found to be associated with cisplatin resistance. Intriguingly, the IC_{50} exhibited by the sensitive SKOV-3 EOC cells with the *SOD2* mutation was higher than the SKOV-3 control EOC cells but lower than the SKOV-3 cisplatin resistant EOC cells, where the mutation was originally observed. This discrepancy suggests that, additional factors may contribute to the cisplatin resistance in these cells. We demonstrated that the *SOD2* mutated cells manifest a decrease in activity consistent with what has been reported for the SNP (rs4880).

Evidence in several cancers suggests that antioxidants such as SOD may decrease cell killing, hence promoting cisplatin resistance [304-307]. Indeed, salinomycin, an antibacterial drug with anticancer activity, induces cell death in cisplatin-resistant colon adenocarcinoma (SW620) cells via down-regulations of SOD and GPx activities. These observations were associated with a decrease of the Bcl-2/Bax ratio, indicating a tilt towards apoptosis [305]. In cisplatin-resistant urothelial carcinoma (NTUB1) cells, cisplatin-induced overexpression of the CCAAT/enhancer binding protein delta (CEBPD, C/EBPdelta, NF-IL6beta) was found to be associated with decrease of ROS through the upregulation of *SOD1* via direct promoter

transactivation. This has led to decrease ROS-triggered apoptosis, hence cisplatin resistance [306]. Similar observation was found in other systems and implicated other antioxidants such as CAT and GPx [306, 307].

Conversely, in gastric cancer, antioxidants were found to be beneficial. Ginkgo biloba extract (EGb) 761 was reported to possess antitumor activity that may be related to the KSR1-mediated ERK signaling pathway. Specifically, EGb 761 reduced the expression of various cisplatin and etoposide-induced mitogenic genes; the malondialdehyde (MDA) content; and elevated the activities of SOD and GPx. EGb 761 enhanced the chemotherapy sensitivity and reversed the chemoresistance through suppression of the KSR1-mediated ERK1/2 pathway in gastric cancer cells, and the underlying mechanism may be related to its antioxidant activity [304].

The mutant TT genotype of the *CYBA* gene translates into a decrease in activity of the protein, thereby decrease the generation of $O_2^{\bullet -}$ and limiting oxidative stress. *CYBA* or *CYBB*, encodes for $p22^{phox}$ or NOX2 respectively and mutations in both lead to chromic granulomatous disease (CGD) [308]. Contrary to *CYBB*, *CYBA* is susceptible to a relatively high number of SNPs that could influence the level of ROS generation. These SNPs are associated with hypertension, coronary artery disease, coronary heart disease, and, cerebral ischemic diseases [309-311]. Stable overexpression of $p22^{phox}$ increases cisplatin resistance in oral squamous cell carcinoma, which was reversed by the knockdown of the gene. The mechanism involves preventing nuclear localization of cisplatin [312]. This observation in consistent with our findings with the presence of the mutant TT genotype, indicating that lower expression or activity of p22phox confers sensitivity to cisplatin. Conversely, *CYBA* was among five genes that were found to be lost through methylation, and were associated with tumor progression and

metastasis in malignant melanoma [313]. Although one of the genes was strongly associated with cisplatin resistance, *CYBA* was not [313].

We strongly believe that, thus far, our data does not fully explain the link between low *CYBA* expression and cisplatin resistance. However, if validated, it would be worthwhile to investigate the underlying mechanisms; the resolution of which may lead to the discovery of new cisplatin sensitizing drugs with significant impact on improved survival outcome. We attempted to establish causality between specific SNPs in *CYBA*, *SOD2*, and *GPX1* and the acquisition of cisplatin resistance in SKOV-3 EOC cells. It would be important to confirm these findings in MDAH-2774 cell line. Moreover, reversing the mutation in both SKOV-3 and MDAH-2774 EOC cell lines, and demonstrating cisplatin sensitivity will further test the hypothesis that specific SNPs in key oxidant and antioxidant enzymes cause cisplatin resistance.

CHAPTER 6: SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

The main objective of the present work is to determine whether oxidative stress is responsible for the acquisition of cisplatin resistance in EOC. As stated above, previous evidence from both our laboratory and others suggests that oxidative stress not only plays an important role in tumorigenesis, but also cancer progression and aggressiveness. We have demonstrated that: 1) the overall survival rate is shorter in ovarian cancer patients patients that carry a specific SNP in the *CAT* gene, 2) cisplatin resistant human EOC cell lines manifest a pro-oxidant state, 3) point mutations corresponding to known SNPs in *SOD*, and *CYBA* genes are present in cisplatin resistant cells, while absent in their sensitive counterparts, suggesting a strong association between cisplatin resistance and those SNPs, 4) for both cell lines, the cisplatin-sensitive parent cells carry a specific *GPX1* SNP, reversal of which, makes the cells resistant to cisplatin, and 5) we have successfully generated point mutations in the sensitive EOC cells that correspond to the SNP genotype in our chemoresistant EOC cells using CRISPR/Cas9 system. Additionally, we have established cisplatin IC_{50} values for sensitive, resistant and mutation-induced cell lines, as well as demonstrated the acquisition of cisplatin resistance with *GPX1* and *SOD* mutations. Lastly, we found that the presence of the *CYBA* mutation in the sensitive SKOV-3 cells renders them more sensitive to cisplatin as indicated by a lower IC_{50}.

Epithelial ovarian cancer remains a deadly disease. In fact, it is the deadliest of all gynecologic cancers with an estimated 22,280 new cases and 14,240 deaths expected in 2016 in the US alone [28]. Although a relatively rare disease, the mortality rate of EOC remains relatively unchanged for the last four decades. Among the keys factors driving these statistics are: the lack of universal screening stools for the general population making prevention and early detection a real challenge; and the limited opportunities for cure due the intrinsic limitations of

surgery and the development of resistance to standard chemotherapy. Therefore, it is of public health interest to continue to support both basic science and translational research in this field.

Future studies testing the impact of scavenging oxidants and/or adding antioxidants to chemotherapy-resistant EOC cell lines are needed. Indeed, we are designing experiments using patient-derived cisplatin-resistant EOC cells treated with CAT-SKL, a novel drug previously reported to increase mitochondrial CAT activity [314, 315]. Similarly, we are testing whether treating the EOC cells with SOD, GPx and GSR purified proteins as well as their inhibitors influences their response to cisplatin exposure. Preliminary results suggest that antioxidants may behave differently in sensitive and resistant EOC cells. Understanding the precise mechanism by which the EOC redox profile relates to the development of cisplatin resistance will have the potential to significantly impact survival of ovarian cancer patients.

APPENDIX A

HIC Protocol Approval Letter

(All samples were collected and stored while Protocol was active)

IRB Administration Office
87 East Canfield, Second Floor
Detroit, Michigan 48201
Phone: (313) 577-1628
FAX: (313) 993-7122
http://irb.wayne.edu

NOTICE OF FULL BOARD CONTINUATION APPROVAL

To: Michael Tainsky
Oncology
Karmanos Cancer Institute

From: James Chinarian, M.D. or designee J. Chinarian /BD
Chairperson, Medical/Pediatric Institutional Review Board (MP2)

Date: November 14, 2013

RE: IRB #: 024199MP2F(5R)
Protocol Title: 1972: Cancer Genetics Research Program
Funding Source: Unit: Molecular Biology & Genetics
Protocol #: 1102009344

Expiration Date: November 13, 2014

Risk Level / Category: Pediatric: 45 CFR 46.404 - Research not involving greater than minimal risk
Adult: Research not involving greater than minimal risk

Continuation for the above-referenced protocol and items listed below (if applicable) were **APPROVED** following Full Board review by the Wayne State University Institutional Review Board (MP2) for the period of 11/14/2013 through 11/13/2014. This approval does not replace any departmental or other approvals that may be required.

- Actively accruing participants
- Medical Research Informed Consent with HIPAA authorization (Revision Date: 12/19/2011)
- Medical Research Informed Consent – Skin Punch Biopsy Consent Form with HIPAA authorization (Revision Date: 12/19/2011)
- Medical Research Informed Consent Form – Healthy Control (Revision Date: 12/05/2011)
- Medical Research Informed Consent –Proxy Consent Form with HIPAA authorization (Revision Date: 12/19/2011)
- Parental Permission/Research Informed Consent with HIPAA authorization (Revision Date: 05/10/2013)
- Request to Obtain Medical Records
- The Karmanos Cancer Institute Genetics Registry Contact Form
- Personal & Family Cancer History Screener Form
- Study Brochure
- Cancer Genetics Research Program Letter to Patient Doctors & Participant Recruitment Letter

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- Federal regulations require that all research be reviewed at least annually. You *may* receive a "*Continuation Renewal Reminder*" approximately two months prior to the expiration date; however, it is the Principal Investigator's responsibility to obtain review and continued approval ***before*** the expiration date. Data collected during a period of lapsed approval is unapproved research and can *never* be reported or published as research data.
- All changes or amendments to the above-referenced protocol require review and approval by the IRB **BEFORE** implementation.
- Adverse Reactions/Unexpected Events (AR/UE) must be submitted on the appropriate form within the timeframe specified in the IRB Administration Office Policy (http://www.irb.wayne.edu/policies-human-research.php).

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1. Upon notification of an impending regulatory site visit, hold notification, and/or external audit the IRB Administration Office must be contacted immediately.
2. Forms should be downloaded from the IRB website at **each** use.

APPENDIX B

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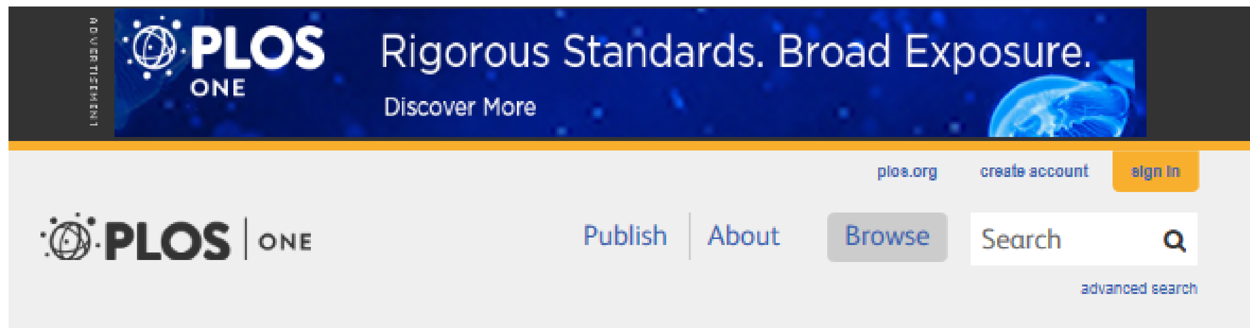

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ABSTRACT

THE ROLE OF OXIDATIVE STRESS IN THE ESTABLISHMENT OF RESISTANCE TO CISPLATIN IN EPITHELIAL OVARIAN CANCER CELLS

by

JIMMY BELOTTE

December 2016

Advisor: Dr. Ghassan Saed

Major: Physiology (Reproductive Sciences Concentration)

Degree: Doctor of Philosophy

Epithelial ovarian cancer is the deadliest of all gynecologic cancers with an estimated 22,280 new cases and 14,240 deaths expected in 2016 in the US alone. This high mortality rate can be partially attributed to a lack of universal screening and the development of resistance to the recommended chemotherapeutics. Typically, the treatment of ovarian cancer requires both cytoreductive surgery (CRS) and platinum/taxane combination chemotherapy. Initially, 50–80% of patients with advanced disease will achieve complete clinical response. Unfortunately, most will relapse within 18 months with chemoresistant disease. Thus, understanding the mechanisms of platinum resistance is critical in order to improve the care of ovarian cancer patients. Several theories to explain cisplatin resistance have been proposed but failed to translate into clinical practice. Typically drug resistance mechanisms are multifactorial but can be broadly categorized as follows: 1) pharmacokinetic, resulting in inadequate intratumor cisplatin concentration, 2) tumor micro-environment, involving membrane transporters by reducing cisplatin uptake or increasing efflux, 3) increased inactivation and sequestration of cisplatin, 4) activation of DNA repair and antiapoptotic mechanisms, 5) decreased autophagy and, 6) cancer-cell specific mechanisms such as: acquired somatic mutations and epigenetic changes and persistence of slow

growing cancer stem cells that maintain the cancer phenotype.

We have recently characterized EOC tissues and cells to manifest a persistent pro-oxidant state with the upregulation of several key oxidant enzymes including: myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS), and nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase with concurrent decrease in apoptosis compared to normal human ovarian tissues and cells. More importantly, shutting down the expression of one or more of these key oxidant enzymes reduced the pro-oxidant state, and significantly induced apoptosis. Single-nucleotide polymorphisms (SNPs) are point mutations that are selectively maintained in populations and are distributed throughout the human genome at an estimated overall frequency of at least one in every 1000 base pairs. Several SNPs in key oxidants and antioxidants enzymes have been associated with various cancers including ovarian. Therefore, we are proposing the following hypothesis: *cisplatin treatment induces mutations in key oxidant and antioxidant enzymes resulting in further enhancement of oxidative stress and the acquisition of resistance in EOC cells.* To test this hypothesis, we are proposing then following specific aims:

Specific Aim 1: To determine the association of specific single nucleotide polymorphisms (SNPs) in key oxidant and antioxidant enzymes with EOC risk and survival in patients.

The rationale of this aim is based on the fact that oxidative stress is strongly associated with several cancers, including ovarian. Known specific SNPs in oxidant and antioxidant genes may alter their expression profile and enzymatic functions. These SNPs have been reported to be associated not only with cancer risks, but also patient response to treatment and survival. The hypothesis of this aim is that *specific SNPs in key oxidant and antioxidant enzymes are associated with overall patient survival.* To achieve this aim, we will perform a case-control study using stored blood samples of research participants from the Karmanos Cancer Center. Individuals (n=143) recruited were divided into controls (n=94),) and ovarian cancer cases

(n=49). Samples will undergo DNA extraction followed by TaqMan® SNP genotype analysis for rs4880 manganese superoxide dismutase (*MnSOD*), rs4673 (*NAD(P)H* oxidase (*CYBA*), rs3448 glutathione peroxidase (*GPX1*), rs2297518 inducible nitric oxide synthase (*iNOS*), rs1002149 glutathione reductase (*GSR*), and rs1001179 catalase (*CAT*). We will perform a multivariate analysis for identification of confounding variables and potential predictors of risk. Additionally, to study the impact of the SNPs on overall survival, Cox regression and Kaplan-Meier survival analyses will be used.

Specific Aim 2: To determine the association of key oxidant and antioxidant enzymes as well as specific SNPs in these enzymes with the development of cisplatin resistance in EOC cells.

The rationale of this aim is based on previous findings showing an association between the altered redox enzymes and EOC, in both patients and human cell lines. The hypothesis of this aim is that *the acquisition of resistance to cisplatin in EOC cells is associated with enhanced pro-oxidant profile, as well as specific SNPs in key oxidant and antioxidant enzymes.* To achieve this aim, we will utilize two human EOC cell lines, MDAH-2774 and SKOV-3 and their cisplatin resistant counterparts. We will perform TaqMan PCR genotyping, real-time RT-PCR, ELISA, and Griess assay to study the expression profile of the following genes: *CYBA/NOX4*, *iNOS*, *CAT*, *SOD3*, GSR and *GPX1*. To analyze the difference in the expression profiles of these genes for sensitive compared to resistant cells, we will use a Student's t-test.

Specific Aim 3: To determine whether specific SNP(s) in key oxidant and antioxidant enzymes cause the acquisition of cisplatin resistance in EOC cells.

The rationale of this aim is based on the established fact that cisplatin treatment causes DNA damage, and the observation that specific SNPs in the redox enzymes were found to be associated with poor survival in patients. The hypothesis of this aim is: *specific SNPs in key oxidant and antioxidant enzymes cause cisplatin resistance.* To achieve this aim, we will utilize

the CRISPR/Cas9 system to generate point mutations in sensitive EOC cells corresponding to the SNP genotype of the chemoresistant MDAH-2774 and SKOV-3 EOC cells. The cells will then be tested for cisplatin resistance using the MTT viability assay using the IC_{50} method. Results will be analyzed with regression analysis and student t-tests.

AUTOBIOGRAPHICAL STATEMENT

Jimmy Belotte, MD, F.A.C.O.G.

EDUCATION

09/2012-present	Ph.D. – Reproductive Sciences Concentration, Wayne State University, School of Medicine, Department of Physiology, Detroit, MI
10/1996-9/2003	M.D. - Universite Notre Dame d'Haiti, Faculte de Medecine et des Sciences de la Sante, Port-Au-Prince, Haiti.

POSTGRADUATE TRAINING

01/2010-062011	Wayne State University School of Medicine, Detroit, MI Ob/Gyn, PGY III/IV
07/2007-12/2009	Nassau University Medical Center, Ob/Gyn/, East Meadow, NY, / Resident, PGY I/II/III

HONORS/AWARDS

05/2014	Wayne State University, School of Medicine, Ob/Gyn., Detroit, MI, C.S. Mott Center Scientific Retreat, Best poster in Clinical Research Category.
09/2013	Wayne State University, School of Medicine, Detroit, MI, Office of Vice president for Research, Travel award
09/2011-present	Wayne State University, School of Medicine, Ob/Gyn, Detroit, MI, Women's Reproductive Health Research (WRHR) K-12 NIG grant
01/2011	Wayne State University, School of Medicine, Ob/Gyn, Detroit, MI, MFM Award, Wayne State University

SELECTED PUBLICATIONS

Original Work

1. Nusrat O, **Belotte J**, Fletcher NM, Memaj I, Saed MG, Diamond MP, Saed GM. The Role of Angiogenesis in the Persistence of Chemoresistance in Epithelial Ovarian Cancer. Reprod Sci. 2016 Apr 26. PMID: 27122375.
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Case Reports

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