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New Insights into the Pathogenesis of Ovarian Cancer: Oxidative Stress

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<http://dx.doi.org/10.5772/intechopen.73860>

Abstract

Ovarian cancer is the leading cause of death from gynecologic malignancies yet the underlying pathophysiology is not clearly established. Epithelial ovarian cancer (EOC) has long been considered a heterogeneous disease with respect to histopathology, molecular biology, and clinical outcome. Treatment of ovarian cancer includes a combination of cytoreductive surgery and combination chemotherapy, with platinum and taxanes. Despite initial success, over 75% of patients with advanced disease will relapse around 18 months and the overall 5-year survival is approximately 50%. Cancer cells are known to be under intrinsic oxidative stress, which alters their metabolic activity and reduces apoptosis. Epithelial ovarian cancer has been shown to manifest a persistent pro-oxidant state as evident by the upregulation of several key oxidant enzymes in EOC tissues and cells. In the light of our scientific research and the most recent experimental and clinical observations, this chapter provides the reader with up to date most relevant findings on the role of oxidative stress in the pathogenesis and prognosis of ovarian cancer, as well as a novel mechanism of apoptosis/survival in EOC cells.

Keywords: ovarian cancer, oxidative stress, chemoresistance, apoptosis, nitrosylation, caspase-3

1. Introduction

Ovarian cancer is the fifth leading cause of cancer death; the leading cause of death from gynecologic malignancies, and the second most commonly diagnosed gynecologic malignancy; yet the underlying pathophysiology continues to be delineated [1, 2]. Epithelial ovarian cancer

has long been considered a heterogeneous disease with respect to histopathology, molecular biology, and clinical outcome. It comprises at least five distinct histological subtypes, the most common and well-studied being high-grade serous ovarian cancer (HGSOC) [3]. The majority of advanced-stage tumors are of epithelial cell origin and can arise from serous, mucinous, or endometrioid cells on the surface epithelium of the ovary or the fallopian tube [2]. The most obvious clinical implication of tumor heterogeneity is that molecular-targeted therapy, while being effective at one tumor site, may not be as effective at all of them [3].

Because early-stage ovarian cancer presents with nonspecific symptoms, most often diagnosis is not made until after the malignancy has spread beyond the ovaries [4]. Mortality rates for this type of malignancy are high because of a lack of a sensitive and specific early-stage screening method [4]. Surgical cytoreduction followed by platinum/taxane chemotherapy results in complete clinical response in 50–80% of patients with stage III and IV disease, but most will relapse within 18 months and ultimately develop chemoresistant disease [2]. Resistance to chemotherapy can either be intrinsic, occurring at the onset of treatment, or acquired, when the disease recurs despite an initially successful response [5–7]. Attempts to overcome drug resistance are central to both clinical and basic molecular research in cancer chemotherapy [5, 8]. Cancer cells are known to be under intrinsic oxidative stress, resulting in increased DNA mutations or damage, genome instability, and cellular proliferation [9–13]. The persistent generation of cellular reactive oxygen species (ROS) is a consequence of many factors including exposure to carcinogens, infection, inflammation, environmental toxicants, nutrients, and mitochondrial respiration [14–17].

The origin and causes of ovarian tumors remains under debate. Injury to surface epithelial ovarian cells due to repeated ovulation is thought to induce tumorigenesis in these cells and is known as the “incessant ovulation hypothesis.” Additionally, hormonal stimulation of the surface epithelium of the ovary has been described to initiate tumorigenesis in surface epithelial cells and is known as the “gonadotropin hypothesis.” Moreover, the fallopian tube, and not the ovary, has been suggested to be the origin for most epithelial ovarian cancer [2, 18, 19]. Nevertheless, many cases of ovarian cancer continue to be described as *de novo*.

Histopathologic, clinical and molecular genetic profiles were successfully utilized to clearly discriminate between type I and type II ovarian tumors [19]. Accordingly, type I ovarian tumors develop from benign precursor lesions that implant on the ovary and include clear cell, endometrioid, low-grade serous carcinomas, mucinous carcinomas and malignant Brenner tumors [19]. Type II ovarian tumors develop from intraepithelial carcinomas of the fallopian tube and can then spread to involve both the ovary as well as other sites, such as high-grade serous carcinomas which comprise morphologic and molecular subtypes [19]. Additionally, high-grade endometrioid, poorly differentiated ovarian cancers, and carcinosarcomas are also classified as type II tumors.

Attempts to identify specific genes in ovarian tumors to help in early detection of the disease and serve as targets for improved therapy had failed to identify reproducible prognostic indicators [2, 20–22]. Several oncogenic mutations and pathways have been identified in ovarian cancer. Specific inherited mutations in the *BRCA1* and *BRCA2* genes that produce tumor suppressor proteins, are known to be associated with a 15% increased risk of ovarian cancer overall [2]. Ovarian cancers associated with *BRCA1* and *BRCA2* mutations are much more common in

younger age patients as compared with their nonhereditary counterparts. Additionally, somatic gene mutations in RAD51C and D, HNPCC, NF1, RB1, CDK12, P53, BRAF, KRAS, PIK3CA, and PTEN have been identified in epithelial ovarian cancer. Somatic mutations in BRAF and KRAS genes are relatively common in type I tumors, while p53 mutations, RAS signaling and PIK3CA are common in type II. Additional genetic variations have been hypothesized to act as low to moderate alleles, which contribute to ovarian cancer risk, as well as other diseases [23].

Ovarian tumors are distinct from many other type of cancers as they rarely metastasize outside of the peritoneal cavity [24]. Ovarian tumors are spread into the peritoneal cavity when cells from the primary tumor detach and travel into the peritoneum where they implant into the mesothelial lining [25]. Metastases beyond the peritoneum are usually restricted to recurrent or advanced disease; however, pleural metastases were reported to be present at initial diagnosis. Moreover, the recent discovery of ovarian cancer stem cells, which manifest properties of typical cancer stem cells, in ascites is a new additional contributing factor to not only to metastasis but also to chemoresistance [25, 26].

2. Oxidative stress

Homeostasis, the balance between the production and elimination of oxidants, is maintained by mechanisms involving oxidants and antioxidant enzymes and molecules. If this balance is altered, it leads to an enhanced state of oxidative stress that alters key biomolecules and cells of living organism [13]. Oxidant molecules are divided into two main groups; oxygen-derived or nitrogen-containing molecules. Oxygen-derived molecules, also known as reactive oxygen species (ROS), includes free radicals such as hydroxyl (HO^\bullet), superoxide ($\text{O}_2^{\bullet-}$), peroxy (RO_2^\bullet), and alkoxy (RO^\bullet), as well as oxidizing agents such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3), and singlet oxygen ($^1\text{O}_2$) that can be converted to radicals [13, 27]. Nitrogen containing oxidants, also known as reactive nitrogen species (RNS), are derived from nitric oxide (NO) that is produced in the mitochondria in response to hypoxia [13]. Exposure to inflammation, infection, carcinogens, and toxicants are major sources of ROS and RNS, *in vivo* [13, 16, 27, 28]. Additionally, RNS and ROS can be produced by various enzymes including cytochrome P450, lipoxygenase, cyclooxygenase, nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase complex, xanthine oxidase (XO), and peroxisomes (**Figure 1**) [13, 28, 29].

To maintain the redox balance, ROS and RNS are neutralized by various important enzyme systems including superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione (GSH), thioredoxin coupled with thioredoxin reductase, glutaredoxin, glutathione peroxidase (GPX), and glutathione reductase (GSR) (**Figure 1**) [27]. Superoxide dismutase is known to convert $\text{O}_2^{\bullet-}$ to H_2O_2 , which is then converted to water by CAT. Glutathione S-transferase is involved in detoxification of carcinogens and xenobiotics by catalyzing their conjugation to GSH that will aid in expulsion from the cell (**Figure 1**) [27]. Indeed, the GSH-to-oxidized-GSH (GSH/GSSG) ratio is a good indicator of cellular redox buffering capacity [30, 31]. Under enhanced oxidative stress, the GSH/GSSG complex is known to stimulate the activity of the GS-X-MRP1 efflux pump, which removes toxins from cells. This mechanism has been investigated in the development of resistance to chemotherapeutic drugs [30, 31].

(Keap1), that binds Nrf2 in the cytoplasm and prevents its translocation into the nucleus, where it binds to promoters of antioxidant enzymes [13, 33]. Additionally, oxidative stress is known to activate certain signaling pathways, specifically, the MAPK/AP-1 and NF- κ B pathways, which are critical for the initiation and maintenance of the oncogenic phenotype [34].

More importantly, ROS and RNS are known to induce genetic mutations that alter gene expression as well as induce DNA damage, and thus have been implicated in the etiology of several diseases, including cancer [2, 13, 35]. Damage to DNA by ROS and RNS is now accepted as a major cause of cancer, and has been demonstrated in the initiation and progression of several cancers including breast, hepatocellular carcinoma, and prostate cancer [34]. Oxidative stress is known to modify all the four DNA bases by base pair substitutions rather than base deletions and insertions. Modification of GC base pairs usually results in mutations, whereas, modification of AT base pairs does not [36]. Modification of guanine in cellular DNA, causing G to T transversions, is commonly induced by ROS and RNS [34]. If not repaired, the transversion of G to T in the DNA of oncogenes or tumor suppressor genes can lead to initiation and progression of cancer. Oxidation of DNA bases, such as thymidine glycol, 5-hydroxymethyl-2'-deoxyuridine, and 8-OHdG are now accepted markers of cellular DNA damage by free radicals [35].

Oxidants and free radicals are known to enhance cell migration contributing to the enhancement of tumor invasion and metastasis, main causes of death in cancer patients [2, 13]. Reactive oxygen species, through the activation of NF- κ B, regulate the expression of intercellular adhesion protein-1 (ICAM-1), a cell surface protein in various cell types [13]. In response to oxidative stress, the interleukin 8 (IL-8)-induced enhanced expression of ICAM-1 on neutrophils enhances the migration of neutrophils across the endothelium, which is key in tumor metastasis [13]. Another important player that controls cell migration and consequently, tumor invasion, is the upregulation of specific matrix metalloproteinases (MMPs), essential enzymes in the degradation of most components of the basement membrane and extracellular matrix, such as type IV collagen [13, 37]. The expression of MMPs, such as MMP-2, MMP-3, MMP-9, MMP-10, and MMP-13 is enhanced by free radicals, specifically H₂O₂ and NO, through the activation of Ras, ERK1/2, p38, and JNK, or the inactivation of phosphatases [13, 37]. Indeed, the major source of cellular ROS, the NAD(P)H oxidase family of enzymes, has been linked to the promotion of survival and growth of tumor cells in pancreatic and lung cancers [2, 13].

Oxidants and free radicals are also known to enhance angiogenesis, a key process for the survival of solid tumors [13]. Angiogenesis involves the upregulation of vascular endothelial growth factor (VEGF) or the downregulation of thrombospondin-1 (TSP-1), an angiogenesis suppressor in response to oxidative stress [13]. This process is controlled by several oncogenes and tumor-suppressor genes such as Ras, c-Myc, c-Jun, mutated p53, human epidermal growth factor receptor-2, and steroid receptor coactivators [38, 39]. Additionally, oxidants and free radicals are known to stabilize HIF-1 α protein and induce the production of angiogenic factors by tumor cells.

4. Cancer cells are under intrinsic oxidative stress

Cancer cells are continuously exposed to high levels of intrinsic oxidative stress due to increased aerobic glycolysis (Warburg effect), a known process in cancer cell metabolism [10, 40].

Thus, cancer cells trigger several critical adaptations that are essential for their survival such as suppression of apoptosis, alteration of glucose metabolism, and stimulation of angiogenesis [10, 29]. Oxygen depletion, due to a hypoxic microenvironment, significantly stimulates mitochondria to produce high levels of ROS and RNS which is known to activate HIF-1 α and consequently promote cell survival in such an environment [29]. The half-life of HIF-1 α is extremely short as it is rapidly inactivated through hydroxylation reactions mediated by dioxygen, oxaloglutarate, and iron-dependent prolyl 4-hydroxylases, located in the nucleus and cytoplasm [40, 41]. Nitric oxide and other ROS, as well as H₂O₂ efflux into the cytosol due to dismutation of O₂^{•-}, can inhibit prolyl 4-hydroxylases activity, leading to the stabilization of HIF-1 α [29, 42]. More importantly, stabilization of HIF-1 α , under hypoxic conditions, can be blocked when inhibiting ROS production in mitochondria that lack cytochrome c [29, 43].

Pro-oxidant enzymes such as myeloperoxidase (MPO), inducible nitric oxide synthase (iNOS) and NAD(P)H oxidase have been associated with initiation, progression, survival, and increased risk in cancers such as breast, ovarian, lung, prostate, bladder, colorectal and malignant melanoma [21, 44]. Moreover, the expression of those key pro-oxidant enzymes was found to change based on the histological type and grade of the tumor [21, 45, 46]. Likewise, antioxidants have also been associated with initiation, progression, survival, and increased risk in cancers such as lung, head and neck, and prostate cancer [47–50]. The expression of GSR and GPX, key antioxidant enzymes, has also been reported to be altered in various types of cancer [21]. The activity and expression of SOD, a powerful antioxidant enzyme, has been reported to be decreased in colorectal carcinomas, pancreatic, lung, gastric, ovarian, and breast cancers [21, 45, 46]. Likewise, the expression and activity of CAT, a key antioxidant enzyme, was reported to be decreased in breast, bladder, and lung cancers but increased in brain cancer [21, 45, 46]. Antioxidant enzymes play a critical role in maintaining the redox balance in the presence of microenvironment stress, and thus, alteration of this balance may provide a unique and complex microenvironment for cancer cell survival.

5. Ovarian cancer cells manifest a persistent pro-oxidant state

Recent evidence suggests that oxidative stress is a critical factor in the initiation and development of several cancers, including ovarian cancer [40, 51]. Consistently, it has been reported that ovarian cancer patients manifested significantly decreased levels of antioxidants and higher levels of oxidants [10, 22, 40, 51–53]. An enhanced redox state, resulting from increased expression of key pro-oxidant enzymes and decreased expression of antioxidant enzymes, has been extensively described in epithelial ovarian cancer (EOC) [52–54]. We have previously reported that MPO, a hemoprotein present solely in myeloid cells that acts as a powerful oxidant, and iNOS, a key pro-oxidant enzyme, are highly expressed and co-localized to the same cell in EOC cells [53]. These two enzymes, MPO and iNOS, work together to inhibit apoptosis, a hallmark of ovarian cancer cells. Nitric oxide, produced by iNOS, is used by MPO as a one-electron substrate to generate nitrosonium cation (NO⁺), a labile nitrosating species, resulting in a significant increase in S-nitrosylation of caspase-3, which inhibits apoptosis [53, 55, 56]. Indeed, attenuating oxidative stress by inhibiting MPO or iNOS significantly induced

apoptosis in EOC cells [54]. Moreover, the remarkably higher levels of iNOS/NO, produced by EOC cells, resulted in the generation of high levels of nitrate and nitrite, powerful protein nitration agents that are known to stimulate the initiation and progression of tumor cells [53]. Under oxidative stress, where both NO and $O_2^{\bullet-}$ are elevated, MPO was reported to serve as a source of free iron which reacts with H_2O_2 and generated highly reactive hydroxyl radical (HO^{\bullet}), further increasing oxidative stress [22, 53]. Additionally, EOC cells are also characterized by enhanced expression of NAD(P)H oxidase, a potent oxidant enzyme that is known to be the major source of $O_2^{\bullet-}$ in the cell. Such high levels of $O_2^{\bullet-}$ combined with significantly high levels of NO generates peroxynitrite, another powerful nitrosylation and nitration agent, which modifies proteins and DNA structure and function in cells [57].

Recently we have gathered compelling evidence demonstrating that talc, through alteration of the redox balance, can generate a similar pro-oxidant state in both normal ovarian epithelial and ovarian cancer cells. Talc and asbestos are both silicate minerals, and the carcinogenic effects of asbestos have been extensively studied and documented in the medical literature [58]. Asbestos fibers in the lung initiate an inflammatory and scarring process, and it has been proposed that ground talc, as a foreign body, might initiate a similar inflammatory response [58]. Although there is strong epidemiological evidence to suggest an association between talc use and ovarian cancer, the direct link and precise mechanisms have yet to be elucidated. We investigated the effect of talc on both oxidants and antioxidants in normal ovarian epithelial and ovarian cancer cell lines. There was a marked increase in mRNA levels of the pro-oxidant enzymes, iNOS and MPO in talc treated ovarian cancer cell lines and normal ovarian epithelial cells, all as compared to their control, as early as 24 hours. Additionally, there was a marked decrease in the mRNA levels of the antioxidant enzymes CAT, GPX, SOD3, but with a marked increase in GSR, and no change in GST, in talc treated ovarian cancer cell line and in normal ovarian epithelial cells, all compared to their control, as early as 24 hours (*data not published*). Thus, there is a direct effect of talc on the molecular levels of oxidant and antioxidants, elucidating a potential mechanism for the development of ovarian cancer in response to talc.

6. Biomarkers for the early detection of ovarian cancer

The discovery of MPO expression in ovarian EOC cells and tissues was surprising, as it is only expressed by cells of myeloid origin. Intriguingly, the combination of serum MPO and free iron was reported to potentially serve as biomarkers for early detection of ovarian cancer [22]. A robust detection method based on molecular profiles for ovarian cancer has not yet been developed because the disease exhibits a wide range of morphological, clinical and genetic variations during its progression. The search for non-invasive, cost-effective ovarian cancer biomarker tests has been ongoing for many years. Immunizations of mice with ovarian cancer cells has led to hybridoma validation by ELISA, while flow cytometry analysis permitted the discovery of cancer antigen (CA)-125 and mesothelin [59]. Furthermore, the screening of an array of 21,500 unknown ovarian cDNAs hybridized with labeled first-strand cDNA from ten ovarian tumors and six normal tissues led to the discovery of human epididymis protein 4 (HE4) [60]. Most interestingly, HE4 is overexpressed in 93% of serous and 100% of endometrioid

EOCs, and in 50% of clear cell carcinomas, but not in mucinous ovarian carcinomas [61]. Thus, HE4 was identified as one of the most useful biomarkers for ovarian cancer, although it lacked tissue-specificity [60, 62–64]. Secreted HE4 high levels were also detected in the serum of ovarian cancer patients [65]. Additionally, combining CA-125 and HE4 is a more accurate predictor of malignancy than either alone [66–68].

Multi-marker panels have the potential for high positive predictive values (PPVs), but careful validation with appropriate sample cohorts is mandatory and complex algorithms may be difficult to implement for routine clinical use [59]. Panels of biomarkers have been extensively investigated to improve sensitivity and specificity and have included some of the most promising reported markers such as CA72-4, M-CSF, OVX1, LPA, prostacin, osteopontin, inhibin and kallikrein [69–71]. However, most of these tests frequently require certain equipments and complex computational algorithms that may not be available in a standard immunoassay laboratory, [32]. Among postmenopausal women in the U.S., only 1 in 2500 women are reported with ovarian cancer. Due to this low prevalence of the disease, a screening method that yield a 75% sensitivity and 99.6% specificity to achieve a PPV value of 10% to be effective for the detection of all stages of ovarian cancer [72]. To date, there is no single biomarker available that met these requirements.

The established role of MPO in oxidative stress and inflammation has been a leading factor in the study of MPO as a possible marker of plaque instability and a useful clinical tool in the evaluation of patients with coronary heart disease [73]. Recent genetic studies implicated MPO in the development of lung cancer by demonstrating a striking correlation between the relative risk for development of the disease and the incidence of functionally distinct MPO polymorphisms [74]. Myeloperoxidase levels reported for various inflammatory disorders are coincidentally lower than those levels found in all stages of ovarian cancer. A previous study reported normal serum MPO and iron levels as 62 ± 11 ng/ml and 96 ± 9 μ g/dl, respectively [75]. However, there was a significant increase in serum MPO and iron levels to 95 ± 20 ng/ml and 159 ± 20 μ g/dl, respectively, in asthmatic individuals [75]. Although there was an increase in this reported serum iron, these levels still fell within the normal range (50 to 170 μ g/dl) [22, 75]. Other studies have showed that an elevated MPO levels, reaching up to 350 ng/ml, in serum plasma, was indicative of a higher risk for cardiovascular events in patients hospitalized for chest pain [76, 77]. A recent study showed a significant correlation between MPO levels and the stage of ovarian cancer, as is the linear trend for MPO with increasing stage [22]. Similarly, there was a significant difference in the level of free iron in serum and tissues obtained from stage I as compared to combined stages II, III, and IV ovarian cancer. There was an overlap between stage I ovarian cancer and inflammation (endometriosis) serum MPO levels, however serum free iron levels were significantly higher in stage I ovarian cancer as compared to inflammation. There was no significant change in free iron levels between the healthy control group, benign gynecologic conditions group, and inflammation group [22].

Due to the overlap of MPO levels in early-stage ovarian cancer and inflammatory conditions, there is a potential for a false positive with MPO alone in patients with cardiovascular, inflammation, and/or asthmatic disorders. It has been reported that MPO heme destruction and iron release is mediated by high levels of both HOCl (a product of MPO) and oxidative stress (i.e. cancer) [22]. The free iron generated by hemoprotein destruction not only contributes to elevation of

serum iron levels, but may also induce oxidative stress, which can promote lipid peroxidation, DNA strand breaks, and modification or degradation of biomolecules [78–80]. Iron reacts with H_2O_2 and catalyzes the generation of highly reactive hydroxyl radicals, which in turn further increases free iron concentrations by the Fenton and Haber–Weiss reaction [81]. Several studies from our laboratories have provided a mechanistic link between oxidative stress, MPO, higher levels of HOCl and higher free iron that could explain the observed accumulation of free iron in epithelial ovarian cancers tissues [53, 82–85]. Utilizing serum iron levels alone as a biomarker is also not sufficient for early detection of ovarian cancer due to many uncontrolled variables, i.e. dietary intake, supplements, effects of other iron-generating enzymes or factors, and more importantly they are not as specific as MPO levels. Specifically, in iron deficiency anemic patients, their free iron levels may become a confounding factor in its utilization for early detection of ovarian cancer. Thus, anemia should be ruled out to eliminate any overlap that would lead to misdiagnosis. The incorporation of iron deficiency anemic patients in a logistic regression model will help determine its overlap with early-stage ovarian cancer. Additionally, currently available clinical studies focused on either biochemical or more recently, genetic markers of iron overload have reported conflicting results regarding the use of iron levels alone for diagnosis [86–89].

Thus, the combination of serum MPO and iron levels should yield a higher power of specificity and sensitivity that should distinguish women with early-stage ovarian cancer from other disorders, specifically inflammation [22]. Additionally, combining serum MPO and iron levels with the best currently existing biomarkers through the creation of a logistic regression model may increase the overall predictive values. Collectively, there is a role for serum MPO and free iron in the pathophysiology of ovarian cancer, which thereby qualifies them to serve as biomarkers for early detection and prognosis of ovarian cancer.

7. Modulation of oxidative stress

Several studies have reported the beneficial effects of modulating the redox status of cancer cells, however few studies have been reported for ovarian cancer [90–92]. Inhibition of pro-oxidant enzymes, such as NAD(P)H oxidase, has been shown to significantly induce apoptosis of cancer cells [93, 94]. We investigated whether NAD(P)H oxidase-mediated generation of intracellular reactive ROS lead to anti-apoptotic activity and thus a growth advantage to EOC cells. Diphenyleneiodonium (DPI) has been used to inhibit ROS production mediated by NAD(P)H oxidase in various cell types [95–97]. Our results showed that NAD(P)H oxidase is over-expressed in EOC tissues and cells as compared to normal ovarian tissues and cells [52]. Indeed, high levels of NAD(P)H oxidase are known to promote tumorigenesis of NIH3T3 mouse fibroblasts and the DU-145 prostate epithelial cells [98].

Inhibition of NAD(P)H oxidase has also been reported to decrease the generation of $O_2^{\bullet-}$, H_2O_2 , as well as other oxidants [93, 94]. Cancer cells are known to manifest enhanced intrinsic oxidative stress and metabolic activity that lead to mitochondrial failure [99, 100]. Indeed, it was previously reported that ovarian tumors are characterized by increased ROS levels as evident from increased $O_2^{\bullet-}$ generated from NAD(P)H oxidase as well as mitochondrial malfunction [101]. The NAD(P)H oxidase redox signaling is controlled by mitochondria, and thus loss of

this control is thought to contribute to tumorigenesis [101]. Others have also shown that inhibition of NAD(P)H oxidase induced apoptosis in cancer cells [102]. Continuous ROS production by the cell and the environment further induces the inhibition of phosphorylation of AKT and subsequent suppression of AKT-mediated phosphorylation of ASK1 on Ser-83, resulting in significant decrease in apoptosis [102–104]. Furthermore, paclitaxel, a chemotherapeutic agent used in the treatment of ovarian cancer and other cancers, induced apoptosis of ovarian cancer cells by negative regulation of AKT–ASK1 phosphorylation signaling [102–104]. On the other hand, activation of AKT by ROS provided protection against apoptosis [102–104].

Data from our laboratory clearly demonstrated that treatment of EOC cells with DPI, which inhibits ROS production mediated by NAD(P)H oxidase, significantly reduced SOD3 and HIF-1 α mRNA and protein levels as early as 30 minutes after treatment with a concomitant increase in apoptosis [52]. The association between increased HIF-1 α expression and decreased cellular apoptosis has also been demonstrated in lung and hepatoma cancer cells [94, 105]. Overexpression of HIF-1 α is thought to decrease apoptosis by the upregulation of anti-apoptotic proteins, Bcl-2 and Bcl-xL and down regulation of pro-apoptotic proteins, BAX and BAK [106]. Inhibition of HIF-1 α by rapamycin increased apoptosis by decreasing the expression of apoptosis inhibitor Bcl-2 in ovarian cancer xenografts [107]. Additionally, inhibition of HIF-1 α by rapamycin enhanced apoptosis through the inhibition of cell survival signals in several other cell lines [107].

Most of the NAD(P)H oxidase-generated $O_2^{\bullet-}$ is utilized to produce H_2O_2 by nonenzymatic or SOD-catalyzed reactions [108–110]. Hydrogen peroxide serves as the precursor of more toxic hydroxyl radicals and thus is extremely destructive to cells and tissues [109–111]. The expression of SOD3 was reported to increase in response to intrinsic oxidative stress in ovarian cancer cells [112]. It has been demonstrated that overexpression of the SOD3 gene significantly suppressed lung cancer metastasis as well as inhibited the growth of B16-F1 melanoma tumors in mice [113, 114]. However, in a somewhat controversial study, it has been shown that inhibition of SOD selectively induced apoptosis of leukemia and ovarian cancer cells [10].

Under hypoxic conditions, SOD3 is overexpressed and has been reported to significantly induce the expression of HIF-1 α in tumors through unknown mechanisms however, steady state levels of $O_2^{\bullet-}$ and the stabilization of HIF-1 α have been proposed to play a role in this mechanism [107, 115]. Therefore, inhibition of NAD(P)H oxidase and the consequent reduction of $O_2^{\bullet-}$ levels may destabilize HIF-1 α , and subsequently increase apoptosis by lowering SOD3 levels. Thus, we conclude that lowering oxidative stress, possibly through the inhibition of NAD(P)H oxidase-generated $O_2^{\bullet-}$, induces apoptosis in ovarian cancer cells and may serve as a potential target for cancer therapy. This effect was attributed to the modulation of key enzymes that are central to controlling the cellular redox balance.

8. Modulation of metabolism

Cancer cells are known to favor anaerobic metabolism, even when oxygen is present and is known as the “Warburg effect” [116, 117]. Aerobic glycolysis is known to decrease ATP yield as well as increase lactate production by cancer cells [116–118]. To compensate for this decrease in

ATP, cancer cells significantly increase glucose uptake through upregulation of glucose receptors [40, 41, 118]. Increased lactate in cancer cells enhances lactic acidosis, which is significantly toxic to the surrounding tissues and can facilitate tumor growth through the stimulation of ECM degradation, angiogenesis, and metastasis [118]. Additionally, aerobic glycolysis in cancer cells activates HIF, an oxygen-sensitive transcription factor that plays an important role in initiation and maintenance of the oncogenic phenotype [118]. In this regard, HIF induces the expression of several glucose transporters and glycolysis enzymes as well as induces the expression of pyruvate dehydrogenate kinase (PDK), an enzyme that stimulates pyruvate entry into the mitochondria for oxidation [41, 118, 119]. Thus, shifting glucose metabolism in cancer cells from glycolysis to glucose oxidation may have therapeutic value [120]. Indeed, inhibiting PDK by dichloroacetate (DCA) has been reported to induce apoptosis in tumor cells and significantly decreased HIF-1 α expression [40]. More importantly DCA is currently in the clinical use for the treatment of hereditary mitochondrial diseases as well as lactic acidosis [41, 121]. The use of DCA at a dose of 35 to 50 mg/kg decreased lactate levels by more than 60% [41, 122]. Dichloroacetate treatment has been shown to significantly induce apoptosis, through the stimulation of caspase-3 activity, in a dose-dependent manner in EOC cells as well as other cancers, such as glioblastoma, endometrial, prostate, and non-small cell lung cancers [40, 123]. Aerobic glycolysis is associated with resistance to apoptosis in cancer cells as many of the enzymes in the glycolysis process are known to modulate gene transcription of apoptotic proteins [40, 41, 69, 124]. Stimulation of pyruvate entry into the mitochondria by DCA, through activation of PDH and inhibition of PDK, is an ideal method to shift aerobic glycolysis to glucose oxidation as inhibiting aerobic glycolysis results in ATP depletion and necrosis, not apoptosis [41, 125].

An additional approach to induce apoptosis in cancer cells is through scavenging high levels of oxidants produced by cancer cells utilizing antioxidants [126]. Deficiency in SOD or inhibition of SOD enzyme activity causes accumulation of O₂^{•-} which is the precursor for several toxic free radicals that are critical to the oncogenic process [127]. Elevated levels of oxidants and free radicals are also known to induce cellular senescence and necrosis, and thus can kill tumor cells [40, 128]. The precise effect of high levels of oxidants and free radicals in cancer cells will depend on the type of cells and tissues, the site of production, and the type and concentration of oxidants [13].

9. Chemotherapy and the acquisition of chemoresistance in EOC cells

Resistance to taxanes and platinum, chemotherapy drugs in current use for ovarian cancer treatment, remains a major obstacle to a successful treatment of ovarian cancer patients [6]. Resistance to chemotherapy not only limits the use of the initial drug but also limits the use of other agents, even those with different mechanisms of action [129]. Chemotherapy drugs exert their actions by the initiation of cell death either directly through the generation of oxidative stress or as an indirect effect of exposure, as observed with several chemotherapeutic agents [130]. The development of chemoresistance to drugs is dependent on several factors that include: influx/efflux of drugs that decrease platinum accumulation in tumor cells, enhanced GSH and GST levels, upregulation of anti-apoptotic proteins such as Bcl-2, loss of tumor necrosis factor receptor ligand which induces apoptosis, increased DNA repair through up-regulation of repair

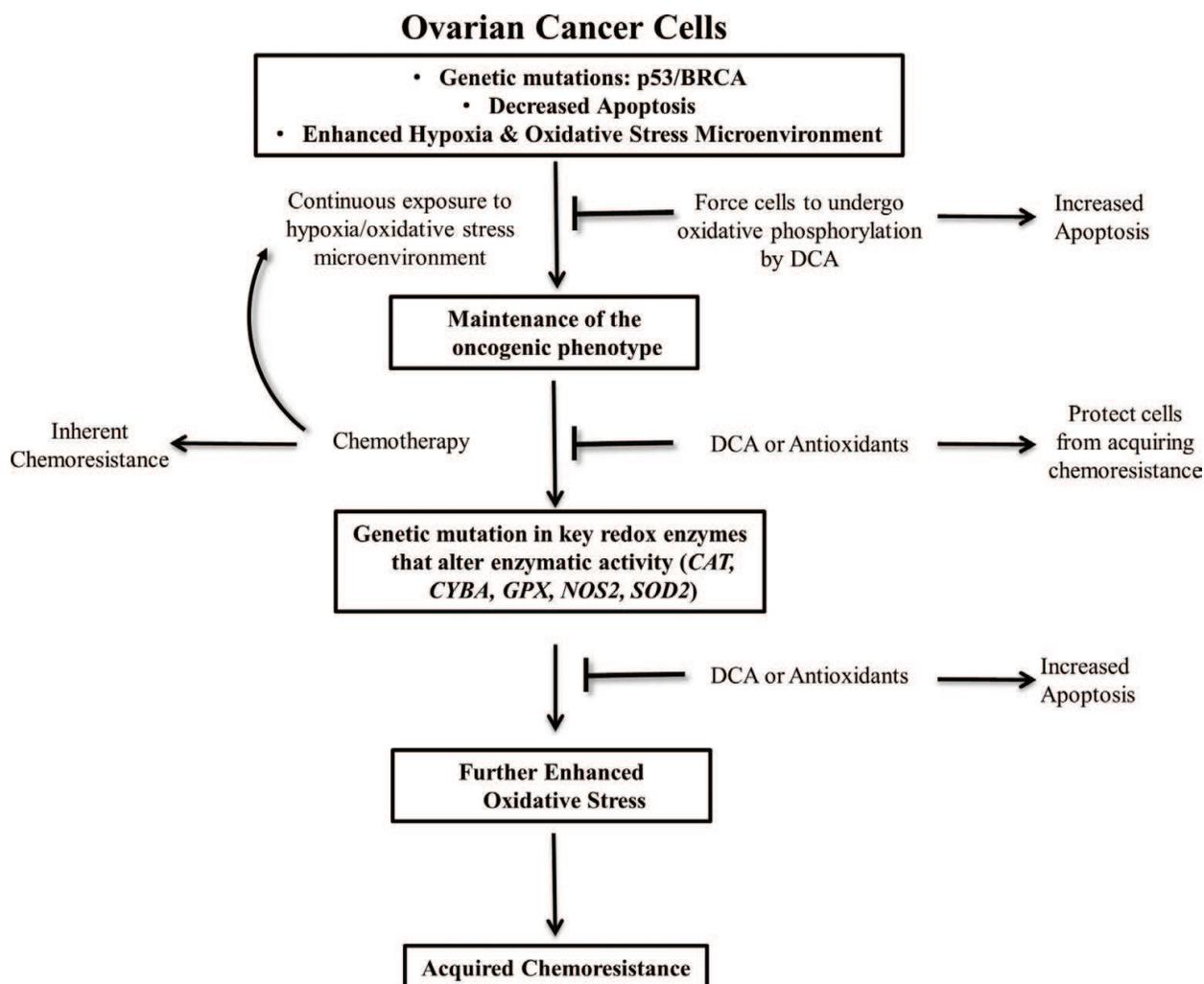


Figure 2. Summary of the role of oxidative stress in the development of sensitive and chemoresistant ovarian cancer [1].

genes, and loss of functional p53 that augments NF- κ B activation [13, 131]. We have previously shown that chemoresistant EOC cells manifested increased iNOS and nitrate/nitrite levels as well as a decrease in GSR expression as compared to sensitive EOC cells, suggesting a further enhancement of the redox state in chemoresistant cells [1, 45]. Additionally, CAT, GPX, and iNOS were shown to be significantly increased while, GSR, SOD, and the NAD(P)H oxidase subunit (p22^{phox}) were decreased in chemoresistant EOC cells as compared to their sensitive counterparts [21]. These findings support a key role for oxidative stress, not only in the development of the oncogenic phenotype, but also in the development of chemoresistance (**Figure 2**).

10. Common polymorphisms in redox enzymes are associated with ovarian cancer

A single nucleotide polymorphism (SNP) occurs as a result of gene point mutations with an estimated frequency of at least one in every 1000 base pairs that are selectively maintained and distributed in populations throughout the human genome [132]. An association

between common SNPs in oxidative DNA repair genes and redox genes with human cancer susceptibility has been established [28]. Common SNPs in the redox enzymes are known to be strongly associated with an altered enzymatic activity in these enzymes, and may explain the enhanced redox state that has been linked to several malignancies, including ovarian cancer [40, 52]. Additionally, it may further explain the observation of significantly decreased apoptosis and increased survival of EOC cells [53]. It is therefore critical to determine the exact effect of common SNPs in various redox enzymes on all process involved in the development of the oncogenic phenotype [21, 46, 133, 134]. Such studies can be linked to other studies focusing on determining the effects of genes involved in carcinogen metabolism (detoxification and/or activation), redox enzymes, and DNA repair pathways [133]. Numerous SNPs associated with change of function have been identified in antioxidant enzymes including *CAT*, *GPX1*, *GSR*, and *SOD2* [21, 134]. Additionally, the association between genetic polymorphisms in genes with anti-tumor activity and those involved in the cell cycle has been reported in ovarian cancer [135, 136]. Recently, several genetic variations have been identified in genome-wide association studies (GWAS), and were found to act as low to moderate penetrant alleles, which contribute to ovarian cancer risk, as well as other diseases [23, 137].

There is now an association of specific SNPs in key oxidant and anti-oxidant enzymes with increased risk and overall survival of ovarian cancer [21, 46]. A common SNP that reduced *CAT* activity (rs1001179) was utilized as a significant predictor of death when present in ovarian cancer patients and was also associated with increased risk for breast cancer [21, 46, 134, 138]. This SNP is also linked to increased risk, survival, and response to adjuvant treatment of cancer patients, including ovarian [46, 139]. Another common SNP that reduced *CYBA* activity (rs4673) was also reported to be associated with an increased risk for ovarian cancer [21, 46]. The mutant genotype of the *CYBA* gene has been shown to both decrease and increase activity of the protein, thereby altering the generation of $O_2^{\bullet-}$ [21, 46]. Moreover, functionally distinct *MPO* polymorphisms, such as (rs2333227) have been linked to relative increased risk for development of ovarian cancer as well as other cancers [21, 44, 46]. Additional SNPs that influenced the risk of EOC have been successfully identified from the GWAS studies including rs3814113 (located at 9p22, near *BNC2*), rs2072590 (located at 2q31, which contains a family of *HOX* genes), rs2665390 (located at 3q25, intronic to *TIPARP*), rs10088218 (located at 8q24, 700 kb downstream of *MYC*), rs8170 (located at 19p13, near *MERIT40*), and rs9303542 (located at 17q21, intronic to *SKAP1*) [21, 46]. Thus, the genetic component of increased ovarian cancer risk may be attributed to SNPs that result in point mutations in the redox genes and potentially other genes [140].

11. Chemoresistance is associated with point mutations in key redox enzymes in EOC cells

To date, the acquisition of chemoresistance in ovarian cancer is not fully understood. The enhanced oxidant state reported in chemoresistant EOC cells may be linked to point mutations in key redox enzymes [21]. Chemoresistant EOC cells manifested increased levels of *CAT*, *GPX*, and *iNOS* and decreased levels of *GSR*, *SOD*, and *NAD(P)H* oxidase as compared to their sensitive counterparts [21]. Interestingly, chemoresistant EOC cells, and not their sensitive counterparts,

manifested specific point mutations that corresponded to known functional SNPs, in key redox enzymes including *SOD2* (rs4880), *NOS2* (rs2297518), and *CYBA* (rs4673) [1]. However, altered enzymatic activity for CAT and GSR observed in chemoresistant EOC cells did not correspond to the specific SNP of interest in those enzymes, indicating involvement of other possible functional SNPs for those enzymes [21]. Coincidentally, chemotherapy treatment induced point mutations that happen to correspond to known functional SNPs in key oxidant enzymes subsequently led to the acquisition of chemoresistance by EOC cells. Indeed, the induction of specific point mutations in *SOD2* or *GPX1* in sensitive EOC cells resulted in a decrease in the sensitivity to chemotherapy of these cells [21]. In fact, the addition of SOD to sensitive EOC cells during chemotherapy treatment synergistically increased the efficacy to chemotherapy [21].

Alternatively, the observed nucleotide switch in response to chemotherapy in EOC cells may be the result of nucleotide substitution, a process that includes transitions, replacement of one purine by the other or that of one pyrimidine by the other, or transversions, replacement of a purine by a pyrimidine or vice versa [21]. Indeed, hydroxyl radicals are known to react with DNA causing the formation of many pyrimidine and purine-derived lesions [21]. The oxidative damage to 8-Oxo-2'-deoxyguanosine, a major product of DNA oxidation, induces genetic alterations in oncogenes and tumor suppressor genes has been involved in tumor initiation and progression [21]. A GC to TA transversion has been reported in the *ras* oncogene and the *p53* tumor suppressor gene in several cancers. However, the GC to TA transversion is not unique to hydroxy-2'-deoxyguanosine, as CC to TT substitutions have been identified as signature mutations for oxidants and free radicals [21].

Moreover, the observed nucleotide switch in response to chemotherapy in EOC cells can be due to the fact that acquisition of chemoresistance generates an entirely different population of cells with a distinct genotype. Hence, chemotherapy kills the bulk of the tumor cells leaving a subtype of cancer cells with ability for repair and renewal, known as cancer stem cells (CSCs) [21]. Indeed, cancer stem cells have been isolated from various types of cancer including leukemia, breast, brain, pancreatic, prostate, ovarian and colon [21]. Interestingly, CSC populations were present in cultures of SKOV-3 EOC cells and have been shown to be chemoresistance in nature [21].

12. Further increasing pro-oxidant enzymes: potential survival mechanism

Apoptosis is a tightly regulated molecular process that removes excess or unwanted cells from organisms. Resistance to apoptosis is a key feature of cancer cells and is involved in the pathogenesis of cancer. We have previously reported that EOC cells have significantly increased levels of NO, which correlated with increased expression in iNOS [54]. We have also reported that 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 [54]. Caspase-3 is known to play a critical role in controlling apoptosis, by participating in a cascade that is triggered in response to proapoptotic signals and culminates in cleavage of a set of

proteins, resulting in disassembly of the cell [141–144]. Caspase-3 was found to be S-nitrosylated on the catalytic-site cysteine in unstimulated human lymphocyte cell lines and denitrosylated upon activation of the Fas apoptotic pathway [145]. Decreased caspase-3 S-nitrosylation was associated with an increase in intracellular caspase activity. Caspase-3 S-nitrosylation/denitrosylation is known to serve as an on/off switch regulating caspase activity during apoptosis in endothelial cells, lymphocytes and trophoblasts [146–149]. The mechanisms underlying S-nitrosothiol (SNO) formation *in vivo* are not well understood.

Myeloperoxidase typically uses H_2O_2 , in combination with chloride to generate hypochlorous acid [55, 150–153]. We, and others, have demonstrated that MPO utilizes NO, produced by iNOS, as a one-electron substrate generating NO^+ , a labile nitrosating species that is rapidly hydrolyzed forming nitrite as end-product [55, 56, 154, 155]. 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 in EOC cells, but also highlights a possible cross-talk between iNOS and MPO. Indeed, we observed that MPO is responsible for the S-nitrosylation of caspase-3, which led to the inhibition of caspase-3 in EOC cells. Silencing MPO gene expression induced apoptosis in EOC cells through a mechanism that involved S-nitrosylation of caspase-3 by MPO.

Molecular alterations that lead to apoptosis can be inhibited by S-nitrosylation of apoptotic proteins such as caspases. Thus, S-nitrosylation conveys a key influence of NO on apoptosis signaling and may act as a key regulator for apoptosis in cancer cells. It has been known that the effects of NO on apoptosis are not only stimulatory but may also be inhibitory. These paradoxical effects of NO on apoptosis seem to be influenced by several factors. It has been suggested that biological conditions, such as the redox state, concentration, exposure time and the combination with O_2 , $O_2^{\bullet-}$ and other molecules, determines the net effect of NO on apoptosis [156]. Also, NO is implicated in both apoptotic and necrotic cell death depending on the NO chemistry and the cellular biological redox state [57, 156]. As described earlier, we have previously demonstrated that the EOC cell lines, SKOV-3 and MDAH-2774, manifested lower apoptosis and had significantly higher levels of NO due to the presence of elevated levels of iNOS [54, 157]. We have also reported significant levels of MPO expression, which was found to be co-localized with iNOS, in both EOC cell lines SKOV-3 and MDAH-2774 [53]. We have demonstrated that 65% of the invasive epithelial ovarian carcinoma specimens tested expressed MPO in the neoplastic cells. The co-localization of MPO and iNOS has been demonstrated by immunohistochemical studies in cytokine-treated human neutrophils and primary granules of activated leukocytes [158]. Both plasma levels and tissue expression of MPO in gynecologic malignancies were previously evaluated and it was found that gynecologic cancer patients had higher plasma MPO compared to control subjects [159]. Using immunostaining, it was also demonstrated that MPO expression was higher in cancer tissues compared to control [159].

We have now characterized chemoresistant EOC cells to manifest an even further increase in pro-oxidant enzymes including MPO, and NO, a surrogate for iNOS activity in conjunction with a further increase in the S-nitrosylation of caspase-3 (*data not published*) and a concurrent decrease in the level of apoptosis [21]. Thus, we hypothesized that the decrease in apoptosis observed in chemoresistant EOC cells is a consequence of a further increase in the degree of S-nitrosylation of caspase-3. Since resistance to apoptosis is a hallmark of tumor

growth, identifying mechanisms of this resistance such as S-nitrosylation may be a key in cancer progression and the development of chemoresistance. S-nitrosylation is reversible and seemingly a specific post-translational modification that regulates the activity of several signaling proteins. S-nitrosylation of the catalytic site cysteine in caspases serves as an on/off switch regulating caspase activity during apoptosis in endothelial cells, lymphocytes, and trophoblasts [147–149]. Targeting MPO may be a potential therapeutic intervention to reverse the resistance to apoptosis in sensitive and chemoresistant EOC cells.

13. Ovarian cancer immunotherapy and oxidative stress

It is well established that tumorigenic cells generate high levels of ROS to activate proximal signaling pathways that promote proliferation, survival and metabolic adaptation while also maintaining a high level of antioxidant activity to prevent buildup of ROS to levels that could induce cell death [160]. Moreover, there is evidence that ROS can act as secondary messengers in immune cells, which can lead to hyperactivation of inflammatory responses resulting in tissue damage and pathology [160]. Ovarian cancer is considered an ideal tumorigenic cancer because ovarian cancer cells have no negative impact on immune cells [161].

Effective immunotherapy for ovarian cancer is currently the focus of several investigations and clinical trials. Current immunotherapies for cancer treatment include therapeutic vaccines, cytokines, immune modulators, immune checkpoint inhibitors, and adoptive T cell transfer [162]. The discovery of a monoclonal antibodies (such as bevacizumab) directed against VEGF have been shown to improve progression free survival compared to cytotoxic chemotherapy alone was a major outcome of these clinical trials [163]. Other monoclonal antibodies currently approved for other cancers such as trastuzumab for breast cancer or cetuximab for colon cancer exhibited limited activity in ovarian cancer [163]. Several clinical trials are ongoing for the utilization of immune checkpoint blockade in ovarian cancer immune therapy [164]. Most recently tested were the programmed death (PD)-1 inhibitors, pembrolizumab and nivolumab, which showed a consistent response rate of 10–20% in phase 2 studies and then failed to improve outcomes in confirmatory trials [164]. Ultimately, larger phase 3 studies are needed to validate these findings for checkpoint inhibitors, particularly with regard to the duration of response seen with these agents. Additionally, the direct intraperitoneal delivery of interleukin (IL)-12, a potent immunostimulatory agent, exhibited some potential therapeutic efficacy in ovarian cancer [165]. Recently, targeting folate receptor alpha, which is found to be expressed in ovarian cancer, has shown promising therapeutic value. The targeting of the folate receptor was achieved by either a blocking monoclonal antibody (farletuzumab) or antibody conjugates of folate analogs, such as vintafolide [166].

14. Summary and conclusion

Oxidative stress has been implicated in the pathogenesis of several malignancies including ovarian cancer. Epithelial ovarian cancer is characterized to manifest a persistent pro-oxidant

state through alteration of the redox balance, which is further enhanced in their chemoresistant counterparts, as summarized in **Figure 2**. Forcing ovarian cancer cells to undergo oxidative phosphorylation rather than glycolysis has been shown to be beneficial for eliminating cells via apoptosis (**Figure 2**). Collectively, there is convincing evidence that indicated a causal relationship between the acquisition of chemoresistance and chemotherapy-induced genetic mutations in key redox enzymes, leading to a further enhanced oxidative stress in chemoresistant EOC cells. This concept was further confirmed by the observation that induction of point mutations in sensitive EOC cells increased their resistance to chemotherapy. Also, a combination of antioxidants with chemotherapy significantly sensitized cells to chemotherapy. Identification of targets for chemoresistance with either biomarker and/or screening potential will have a significant impact for the treatment of this disease.

Acknowledgements

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