The Role and Regulation of the Phosphatase PPM1D in Chemoresistant Gynecological Cancers

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Abstract

Cisplatin (CDDP; *cis*-diamminedichloroplatinum) resistance presents a major impediment in the treatment of several gynecologic solid tumors, including ovarian and cervical tumors. p53, a critical regulator of cellular apoptosis, is a determinant of CDDP sensitivity. In our study, we have observed that the dysregulation of p53 regulators, checkpoint kinase 1 (Chk1) and protein phosphatase magnesium-dependent 1 (PPM1D), significantly reduced CDDP responsiveness in human cancer cells. Isogenic wt-p53 CDDP-sensitive (OV2008) and -resistant (C13*) cervical cancer cells, and isogenic wt-p53 CDDP-sensitive (A2780s) and p53 mutant resistant (A2780cp) ovarian cancer cells, along with CDDP-resistant ovarian cancer cell lines (OCC-1 and OVCAR-3, mutant p53; SKOV-3, p53 null) were used to elucidate the mechanisms of p53 regulation in human gynecologic cancer cells. We have complemented our study with a xenograft model (A2780s) and a tissue microarray of human ovarian tumors to validate our *in vitro* observations.

We have demonstrated that CDDP differentially regulated the p53 activator Chk1 in sensitive and resistant cancer cells; it enhances Chk1 activation in sensitive but not resistant cells. This differential regulation also extended to PPM1D, whereby CDDP enhanced PPM1D content in resistant but not sensitive cells. PPM1D knockdown sensitized resistant cells to CDDP, which was associated with up-regulation of Chk1 and p53 activations, while PPM1D over-expression had the opposite effect. We have also shown that CDDP sensitivity in response to PPM1D down-regulation was p53-dependent. Moreover, CDDP promotes PPM1D nuclear localization in resistant cells and nuclear exclusion in sensitive cells and xenograft tumors. Enhanced PPM1D expression in human ovarian tumors is significantly associated with tumor aggression.

Dysregulation of the oncogene Akt has been implicated in a variety of human malignancies, including ovarian cancer. We have demonstrated that Akt regulates PPM1D stability, since activated Akt over-expression in sensitive cells rescued PPM1D from CDDP-induced proteasomal degradation and Akt down-regulation in resistant cells lead to PPM1D de-stabilization and down-regulation. We have shown for the first time that PPM1D is downstream of Akt through which it can modulate CDDP sensitivity in human cancer cells. These findings extend the current knowledge on the molecular basis of CDDP resistance in gynecological cancers and may help in developing effective therapeutic strategies.

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AIF	Apoptosis inducing factor
Akt	v-akt murine thymoma viral oncogene homolog
Apaf-1	Apoptotic protease activating factor-1
ARF	Alternate open reading frame
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia mutated and Rad3-Related
ATRIP	ATR interacting protein
BAD	Bcl-2 antagonist of cell death
Bak	Bcl-2 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
BH3	Bcl-2 homology domain 3
Bid	BH3-interacting domain death agonist
Bim	Bcl-2 interacting mediator of cell death
BRAF	B-raf murine sarcoma viral oncogene homolog
BRCA 1	Breast cancer susceptibility gene 1
BRCA 2	Breast cancer susceptibility gene 2
CA-125	Cancer antigen 125
CDC25	Cell cycle division 25
CDC25A	Cell cycle division 25A

CDC25B	Cell cycle division 25B
CDC25C	Cell cycle division 25C
CDDP	Cis-diamminedichloroplatinum (II)
cDNA	Complementary deoxyribonucleic acid
CECA	Cervical Cancer
Chk1	Checkpoint kinase 1
Chk2	Checkpoint kinase 2
CREB	Cyclic AMP response element binding protein
Ctr1	Copper transporter homolog 1
DDR	DNA damage response
DIABLO	Direct IAP binding protein with low pI
DMEM	Dulbecco's modified Eagle Medium
DNA	Deoxyribonucleic acid
DN-Akt	Dominant negative Akt
DOX	Doxycycline
DR 4	Death receptor 4
DR 5	Death receptor 5
ERα	Estrogen receptor α
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1
FasL	Fas ligand
FIGO	International Federation of Gynecology and Obstetrics
FITC	Fluorescein isothiocyanate

FLICE	Fas-associated death domain-like interleukin 1 β-converting enzyme
FLIP	FLICE-like inhibitory protein
FOXO-1	Forkhead transcription factor 1
FSH	Follicle stimulating hormone
G_1 phase	Gap 1 phase of the cell cycle
G_2/M	Gap 2/Mitosis phase of the cell cycle
γH2AX	Gamma histone 2AX
GAPDH	Glyceraldehyde phosphate dehydrogenase
CDK1	Cyclin dependent kinase 1
CDK2	Cyclin dependent kinase 2
GSH	Glutathione
HA	Hemagglutinin
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis protein
IgG	Immunoglobulin G
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
IKKα	Inhibitor of kappa B kinase α
IR	Ionizing radiation
JNK	c-Jun N-terminal kinase
kD	Kilodalton
KRAS	Kirsten rat sarcoma viral oncogene homolog
LH	Luteinizing hormone

LMP	Low malignant potential tumors
MAPK	Mitogen-activated protein kinase
MDM2	Murine double-minute-2
MDM4	Murine double-minute-4
MDR	Multidrug resistance
MDR1	Multidrug resistance protein 1
MOI	Multiplicity of infection
MOMP	Mitochondrial outer membrane permeabilization
mRNA	Messenger ribonucleic Acid
mTOR	Mammalian target of rapamycin
mTORC2	Mammalian target of rapamycin complex 2
NER	Nucleotide excision repair
NF-κB	Nuclear factor kappa B
NLS	Nuclear localization signal
NOXA	NADPH oxidase activator
OSE	Ovarian surface epithelium
OVCA	Ovarian cancer
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (adenosine diphosphate) polymerase
PBS	Phosphate buffered saline
PDK-1	Phosphotidylinositol-dependent protein kinase-1
PI3K	Phosphoinositide-3 kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate

PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKB	Protein kinase B
PH	Pleckstrin homology domain
PMSF	Phenylmethylsulfonyl fluoride
PP2Cδ	Protein phosphatase type 2C delta
PPM1D	Protein phosphatase magnesium-dependent 1D
PFS	Progression-free survival
PTEN	Phosphatase and tensin homolog
PUMA	p53-upregulated mediator of apoptosis
Q	Glutamine
qPCR	Quantitative polymerase chain reaction
RAD51	Rad51 homolog (S. Cerevisiae)
RPMI	Roswell Park Memorial Institute
RTK	Receptor tyrosine kinase
rtTA	Reverse tetracycline-controlled transactivator
S	Serine
S phase	Synthesis phase of the cell cycle
SDS	Sodium dodecylsulfate
SEM	Standard error of mean
SMAC	Second mitochondria-derived activator of caspases
STR	Short tandem repeat
T	Threonine
TBS	Tris-buffered saline

TBS-T	TBS-Tween
TNFR	Tumor necrosis factor receptor family
TNFR1	Tumor necrosis factor receptor 1
TP53	Tumor suppressive protein 53 gene
TVUS	Transvaginal ultrasound
UNG2	Uracil DNA glycosylase 2
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
Wip1	Wild type p53 inducible phosphatase 1
XIAP	X-linked inhibitor of apoptosis protein
XPA	Xeroderma pigmentosum complementation group A
XPC	Xeroderma pigmentosum complementation group C

Dedication

I dedicate this thesis to:

- ***My dear departed father, Yassen H. Ali***

  Thank you for being the constant source of inspiration, encouragement, advice, and guidance. I wish I had more time to express my deepest gratitude and love to you. I am sure you are in a better place now and would be proud of what I have become. I will always miss you.

- ***My dear mother, Narjes H. Al-Dhaher***

  No one can ask for a more compassionate and altruistic mother like you. No sacrifice has been too great for you to see us achieve our goals and prepare us for the life ahead, and for that I am deeply grateful. Thank you for your continuing support throughout my life and during the past 6 years of my studies. I hope I make you proud. I love you Mom.

- ***My dear and only brother, Dr. Bshar Y. Ali***

  Thank you for setting an excellent example to follow. Growing up, I have always looked up to you and wanted to follow in your footsteps dear brother. You have taught me that with hard work and perseverance one can achieve the impossible. I am eternally indebted to you for all what you have done and continue to do for me.

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1. Chapter 1 - Introduction

1.1 Ovarian Cancer

1.1.1 Incidence and Mortality

Ovarian cancer (OVCA) is the most lethal of all gynecological malignancies and the fifth most frequent cause of cancer-related death in women. It ranks second among all gynecological cancers in the number of new cases and the first in the number of deaths each year. In Canada, it is estimated that 2600 women will be diagnosed with OVCA in 2013, while more than half that number (1700 women; 65% mortality rate) will die from it. 1 in 68 Canadian women is expected to develop OVCA in her life time, while 1 in 95 women will die from it according to 2007 estimates[1].

1.1.2 Risk Factors of Ovarian Cancer

A risk factor for the development of cancer is defined as any substance or condition that increases cancer risk. The overwhelming majority of OVCA cases are diagnosed late due primarily to asymptomatic presentation of the disease and the lack of proven and reliable screening methods for early OVCA detection. To this day, the underlying molecular mechanisms of ovarian oncogenesis remain poorly understood. While family history is the most important risk factor for the development of familial OVCA which represents 5-10 % of all OVCA, other factors including age, number of lifetime ovulations, genetic mutations in tumor suppressors and oncogenes, gonadotropins, infertility, and exposure to environmental pollutants, may contribute to sporadic OVCA, which accounts for 90-95% of all cases of OVCA[2].

1.1.2.1 Family History

The most significant risk factor for OVCA is a family history of the disease. The life-time risk of OVCA dramatically increases depending on the prevalence of affected first- and second-degree relatives from both sides of the family. Hereditary OVCA is frequently associated with germline mutations of breast cancer susceptibility genes 1 and 2 (*BRCA 1* and *2*) with approximately 90% of familial OVCA attributed to *BRCA1/2* mutations. BRCA1/2 proteins participate in multiple functions within the cell, most important of which is the recognition and repair of DNA double strand breaks. This is achieved through the regulation of repair through homologous recombination by forming a DNA repair foci with the homologous recombination repair protein RAD51. Therefore, the loss of BRCA1/2 function results in genomic instability which contributes to neoplastic transformation[3,4].

1.1.2.2 Ovulation

The ovarian surface is surrounded by a single layer of epithelial cells, the ovarian surface epithelium (OSE). The incessant ovulation hypothesis suggests that the repeated rupture of the ovarian surface during ovulation and its ensuing repair through the proliferation of the surface epithelium increases the probability of the occurrence of replicative genetic errors. Due to the frequency of OSE rupture/repair cycles, the potential of neoplastic transformation increases[5]. This hypothesis is supported by the observations that parity, lactation, and the use of oral contraceptives, all of which suppress ovulation, lead to a decreased risk of OVCA. Furthermore, early menarche, late menopause, and nulliparity increase the risk of developing OVCA[2,6].

1.1.2.3 Gonadotropins

The ovary is a gonadotropic-responsive organ, stimulated by the pituitary gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), which promote follicular development, ovulation, and corpus luteum formation. Interestingly, OVCA develops most frequently in peri- or postmenopausal women when serum gonadotropin levels are high. Moreover, excessive FSH and LH production has been shown to promote OSE proliferation through the induction of estrogen production which may contribute to an increase in genetic mutations, the malignant transformation of OSE, and the development of OVCA[7].

1.1.3 Ovarian Cancer Screening Tests

There are currently two available screening tests for the detection of OVCA. The transvaginal ultrasound (TVUS) test employs ultrasound waves for the detection of lumps in the vagina, uterus, ovaries, and fallopian tubes through the use of an ultrasound wand. While TVUS provides a convenient method for doctors to detect abnormal masses in the ovaries, it cannot distinguish between cancerous tumors and benign cysts and an exploratory laparotomy is often needed to confirm the malignancy of the mass[8].

The CA-125 blood test checks for protein levels of CA-125 that is often associated with OVCA, and its decrease is useful for monitoring the progress of therapy and tumor recurrence. However, CA-125 test is not an accurate screening method to detect OVCA since CA-125 elevation can be caused by other medical conditions. Moreover, not all women with OVCA exhibit elevated levels of CA-125[8]. TVUS and the CA-125 test can be inaccurate and highly unreliable as screening methods for OVCA since they often lead to further unnecessary testing

and exploratory surgeries. For early detection of OVCA, more reliable methods need to be developed to decrease the high mortality rate of OVCA.

1.1.4 Ovarian Cancer Staging

Staging of OVCA is a crucial prognostic factor and is essential in determining the course of treatment. According to the International Federation of Gynecology and Obstetrics (FIGO), OVCA is based on surgical findings and is divided into four stages:

Stage I: Tumor is limited to one or both ovaries.

Stage II: Tumor involves one or both ovaries with pelvic extension, and the presence of ascites fluid containing malignant cells in peritoneal washes.

Stage III: Tumor involves one or both ovaries with peritoneal implants outside the pelvis or the tumor is limited to the pelvis with variable degree of malignant extension to lymph nodes, small bowel, or omentum, and the presence of ascites fluid containing malignant cells in peritoneal washes.

Stage IV: Tumor involves one or both ovaries with distant metastases, significant accumulation of ascites fluid, and the presence of parenchymal liver metastasis.

OVCA is often diagnosed when it has already metastasized beyond the ovaries (at stages III or IV), when it is associated with poor prognosis and reduced therapeutic outcome[9].

1.1.5 Ovarian Cancer Histological Classification

Ovarian cancer comprises a heterogeneous grouping of malignancies. Although OVCA was clinically considered to be one disease, cumulative evidence suggests that the various

subtypes have different natural behaviors, histological characteristics, prognoses, responsiveness to therapy, and cell type of origin[10]. OVCA arises from the cells that comprise the ovaries: germ cells (oocytes); stromal/sex-cord cells and follicular cells (theca and granulosa responsible for steroid production essential for follicular growth and development); and a single layer of epithelial cells lining the ovaries and experiences periodic rupture/repair cycles during ovulation[1,8].

1.1.5.1 Germ Cell Tumors

Germ cell tumors arise from the cells that give rise to the ova and comprise a small proportion of OVCA (~ 5 %). Although a small percentage are cancerous and have the potential to become life threatening, most germ cell malignancies are benign and occur often in teenagers and young females. The most common germ cell tumors are dysgerminomas, teratomas, and endodermal sinus tumors. The majority of germ cell malignancies are curable with high rate of maintenance of patient fertility[1].

1.1.5.2 Stromal/Sex-Cord Tumors

Ovarian stromal/sex-cord tumors originate from cells that comprise the connective tissue that holds the ovary together and follicular cells which produce and secrete female sex hormones. These tumors are quite rare, the majority of which are commonly considered to be low-grade and are detectable at an early stage. The most common types are granulosa cell tumors and granulosa-theca tumors, and comprise ~ 5% of OVCA[1].

1.1.5.3 Epithelial Ovarian Tumors

The overwhelming majority of OVCA (~ 90 %) originates from the surface epithelium of the ovaries or the distal fallopian tube and occurs predominantly in peri- and postmenopausal women[1]. Epithelial tumors are further classified according to the predominant pattern of differentiation of the tumor cells. Serous carcinomas are by far the most common primary ovarian epithelial malignancy (> 70 %) with the majority of tumors diagnosed at advanced stages (III and IV). Serous carcinomas are usually large and bilateral, and often invade through the ovarian capsule and grow on the surface of the ovary. They exhibit a mixture of cystic, papillary, and solid growth architecture. Foci of necrosis and hemorrhage are common in serous carcinoma[9]. There are two distinct types of serous carcinomas. Although termed low grade and high grade ovarian serous carcinomas, these are not two grades of the same malignancy, but rather two distinct tumor types with different underlying molecular profile, pathogenesis, prognosis, behavior, and responsiveness to therapy. Low grade carcinomas are less common and are thought to arise from serous borderline tumors. They are slow growing and compatible with prolonged survival, but exhibit poor response to platinum-based chemotherapeutics which incorporate into the DNA of highly proliferating cells. The genetic mutations of both oncogenes *BRAF* and *KRAS* are important early molecular events in the development of low grade serous tumors[10].

High grade serous carcinomas are highly aggressive and respond initially to platinum-based chemotherapeutics, but present with a high incidence of recurrence and resistance to further therapeutic intervention. There is growing evidence that suggests that some high grade serous tumors do not actually originate from the ovarian surface epithelium, but rather from the epithelial lining of the distal fimbrial segments of the fallopian tube, which lie in very close

proximity to the ovaries[11-13]. This notion is still highly debated in the literature and in need of further confirmation[14,15]. *TP53* mutation is a common early event in the pathogenesis of high grade serous carcinomas[10,16].

The majority of epithelial ovarian tumors of the endometrioid subtype is diagnosed at low stage and arise due to deletion or inactivating mutations of the tumor suppressor PTEN, and/or activating mutations of PIK3CA which encodes the catalytic PI3K subunit p110[17]. They are usually well differentiated and commonly originate from precursor adenofibromas. The tumors are usually solid masses due primarily to their cystic nature. They are typically characterized by the presence of stromal elements, epithelial elements, or a combination of both that histologically resembles the structure of endometrial adenocarcinomas. Benign and borderline ovarian endometrioid carcinomas are quite rare[9].

Mucinous ovarian carcinomas are typically the largest of OVCA tumors and are predominantly solid. Mucinous carcinomas are usually cystic neoplasms with a thin smooth wall filled with watery fluid and the majority arises due to activating mutations of oncogenic KRAS[18]. Borderline and invasive mucinous tumors often contain both papillae and solid areas. Necrosis and hemorrhage are more common in invasive than borderline and benign mucinous tumors. It is quite common to find areas of benign, borderline, and malignant characteristics contained alongside each other in the same tumor mass[9,10].

Clear cell ovarian carcinomas are characterized by the presence of clear cytoplasm rich in glycogen, histologically resembling in appearance clear cell renal cell carcinomas and predominantly arise due to PTEN deletion and/or PI3KCA activating mutations[17,18]. The majority of clear cell carcinomas (> 50 %) originate from ovarian endometriotic cysts which are clearly identifiable in foci adjacent to the tumor mass. Clear cell tumors are predominantly solid

and are associated with cystic masses protruding into the lumen with abundant stromal component[9,10].

Transitional cell/Brenner tumors, undifferentiated tumors, and mixed epithelial tumors are rarer in incidence and comprise the rest of the epithelial ovarian carcinoma classification.

1.2 Cervical Cancer

Cervical cancer (CECA) is the second most common gynecological malignancy affecting women. The lifetime risk of developing CECA is 1 in 145, with an estimated 1450 women that will be diagnosed this year and 380 will die from it in Canada[1]. CECA is usually detected at an early stage with periodic pap tests, which are able to detect abnormal changes in cervical cells, such as cervical dysplasia that are considered preneoplastic and can be excised by cone biopsies, or partial/total hysterectomy depending on the extent of spread of the disease[19]. The most important risk factor for the development of CECA is the infection with the human papilloma virus (HPV). The HPV oncogene E6 binds to and causes the degradation of the tumor suppressor p53, while the E7 oncogene binds to and inhibits the activation of the tumors suppressor Rb, which collectively inhibits the apoptotic potential of cervical cells and enhances their proliferation and eventual neoplastic transformation[19,20].

1.3 Gynecological Cancer Chemoresistance

For the past four decades, despite considerable resources that have been invested to combat OVCA, significant reductions in cancer incidence and deaths have not materialized. The major obstacles in OVCA treatment has been the failure to detect the disease at early stages due to the lack of presentable symptoms until the tumors are too advanced to be contained by

available therapeutic options and have metastasized to secondary target tissues (omentum, liver, bladder, spleen, gastrointestinal tract, etc.)[1]. Even in the absence of metastasis, late detection presents two complications: a need for more complex surgical techniques for removing the bulk of the tumor mass with higher probability of residual tumor cells which exhibit greater heterogeneity; late stage cancers often contain tumor cells that do not respond to chemotherapeutic challenge, and hence, are inherently resistant to the available therapeutics due to the existence of cancer stem cells and higher mutational frequencies in heterogeneous subpopulations within the tumor[21].

The conventional course of therapy for OVCA includes surgical de-bulking of the tumor mass followed by adjuvant chemotherapy, such as a combination of cisplatin and paclitaxel. Most patients are responsive to chemotherapeutic treatment at first, however 40-50% of patients who achieve a remission after first-line chemotherapy will experience a recurrence of cancer within 3 years, and the recurrent tumors are characteristically aggressive, disseminate to secondary target tissues, and acquire resistance to conventional chemotherapeutics[8].

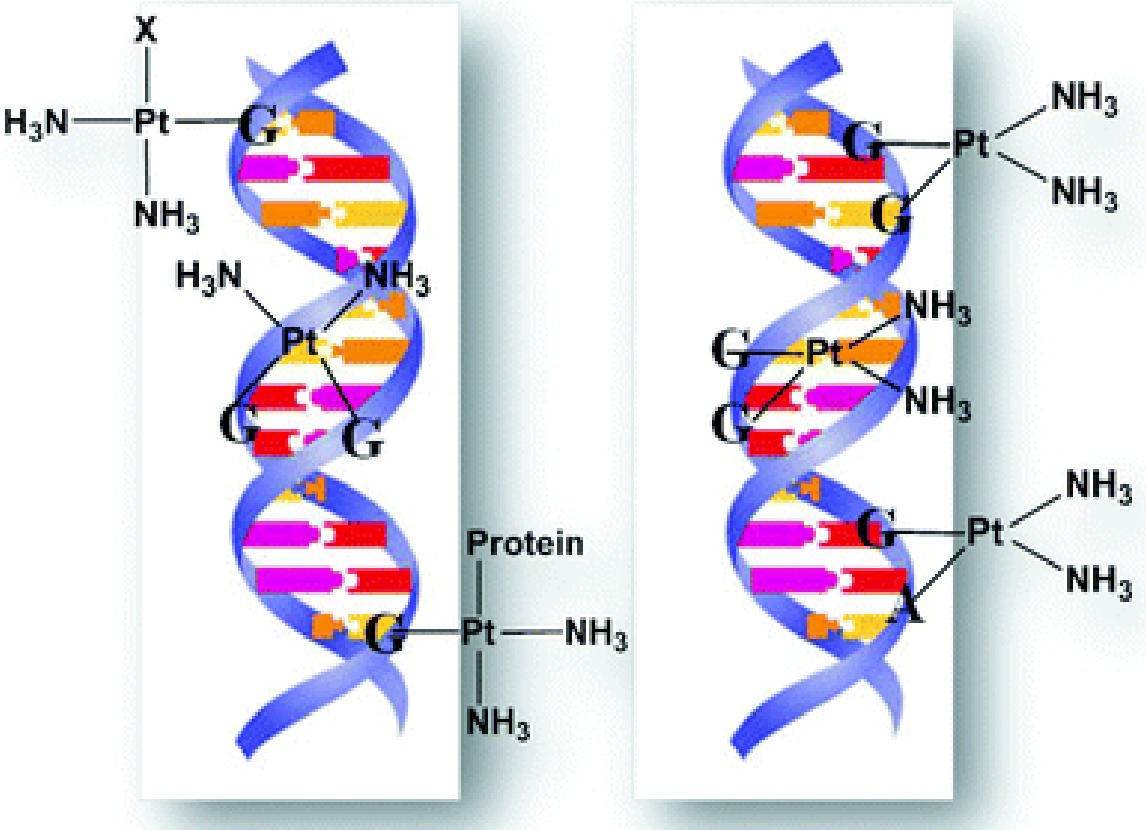
Systemic administration of current chemotherapeutics for OVCA often results in non-specific toxicity. The side effects range from moderate (alopecia, nausea, and erythema) to severe (neuropathy, nephrotoxicity, ototoxicity, myelotoxicity, hemolytic anemia, and compromised immunity leading to higher rate of infections).

The conventional course of therapy for CECA is a combination of external beam radiation and cisplatin[1,8]. The recurrence rate of CECA for stages I and II are quite low (10-20%), and it increases to 50-70% for stages III and IV[1,8].

1.3.1 Cisplatin

Cisplatin (CDDP; *cis*-diamminedichloroplatinum) and its derivatives (carboplatin and oxaliplatin) are the most widely used platinum-based chemotherapeutic agents in the treatment of OVCA. Platinating chemotherapeutics are used to treat a variety of solid tumors, including OVCA. Their primary mechanism of action is to cause DNA damaging events through irreversible intercalation of DNA strands by creating inter- and intra-strand platinum-DNA adducts, thereby activating the DNA damage response and the subsequent induction of the apoptotic machinery[22]. CDDP consists of two chloride ions and two amino groups connected to a central platinum ion in a *cis* configuration. Inside the cells, CDDP donates its chloride ions and becomes positively charged, thereby enabling it to bind to negatively charged molecules such as DNA. The vast majority of CDDP-DNA adducts are intra-strand 1,2-ApG, 1,2-GpG, and 1,3-GpXpG crosslinks (Figure 1.1), which cause distortion of the DNA helix and initiate the DNA damage responses[23-25].

Figure 1.1: Formation of CDDP-DNA adducts (*modified from Bugarčić et al., 2012[26]*): Following the hydrolyzation of the chloride ions, positively charged CDDP covalently binds purinic DNA nucleotides (G and A), forming intra- and inter-strand DNA crosslinks which cause distortion of DNA helices. The most prevalent are intra-strand crosslinks between adjacent guanosine nucleotides.


X
H3N
Pt
G
NH3
H3N
NH3
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Protein
G
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Pt
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NH3
X

1.3.2 Molecular Mechanisms of Cisplatin Resistance

Drug resistance is a major obstacle to the successful treatment of human malignancies. CDDP resistance is multifactorial and can develop due to several functional changes that occur in cancer cells, including increased repair capability to CDDP-induced DNA damage[27], decreased drug accumulation[28,29], enhanced drug efflux[30], enhanced drug detoxification[31], and reduced apoptotic potential in response to CDDP-induced DNA damage[32].

Enhanced DNA repair in response to CDDP-induced damage is one of the major factors in conferring CDDP resistance in OVCA and CECA cells. DNA lesions are primarily repaired by the nucleotide excision repair (NER) mechanism[24,33]. Damaged nucleotides are excised from DNA on both sides of the lesion, followed by DNA synthesis to restore genetic integrity. NER is a complex mechanism which involves a large number of proteins, including the excision repair cross-complementing 1 (ERCC1), a single-strand DNA endonuclease responsible for the removal of bulky DNA lesions. ERCC1 expression has been associated with poor CDDP responsiveness[34,35].

Reduced CDDP accumulation plays an important role in determining resistance to CDDP. Several CDDP resistant cancer cells exhibit consistent reductions in the accumulation of CDDP[36]. CDDP was believed to enter cells primarily by passive diffusion across the plasma membrane, mainly because the uptake of CDDP is relatively slow[37]. However, recent reports point to Ctr1, a highly conserved ATPase copper transporter, as a key player in CDDP cellular uptake[38-40]. Ctr1 functions as a major platinum drug transporter and its knockout leads to platinum resistant phenotypes due to reduced uptake of CDDP, carboplatin, and oxaliplatin *in vitro* and *in vivo*[40].

The membrane-associated multidrug resistance (MDR) proteins are implicated in drug resistance due to the control of drug efflux, thereby limiting drug availability and access to its

target molecules. The MDR1 protein product p-glycoprotein has been implicated in CDDP resistance by decreasing its intracellular concentration, and its level is inversely associated with chemotherapeutic responsiveness[41,42].

Beside the control of DNA repair and drug transport, drug detoxification is another major factor in determining the effectiveness of CDDP treatment. Glutathione (GSH), an important molecule involved in detoxification of many anticancer drugs, has high affinity to CDDP and forms GSH-CDDP adducts, leading to CDDP transport outside the cell[43]. It has been demonstrated that elevated intracellular GSH content is correlated with CDDP resistance in several OVCA cell lines[44].

A key determinant of CDDP resistance is the decreased potential to induce apoptosis in response to CDDP-induced DNA damage. This is achieved through acquired genetic mutations which alter the activities of cellular proto-oncogenes and tumor suppressors, such as p53, Akt, pro- and anti-apoptotic Bcl-2 family member, and the inhibitor of apoptosis (IAP) proteins[45-48].

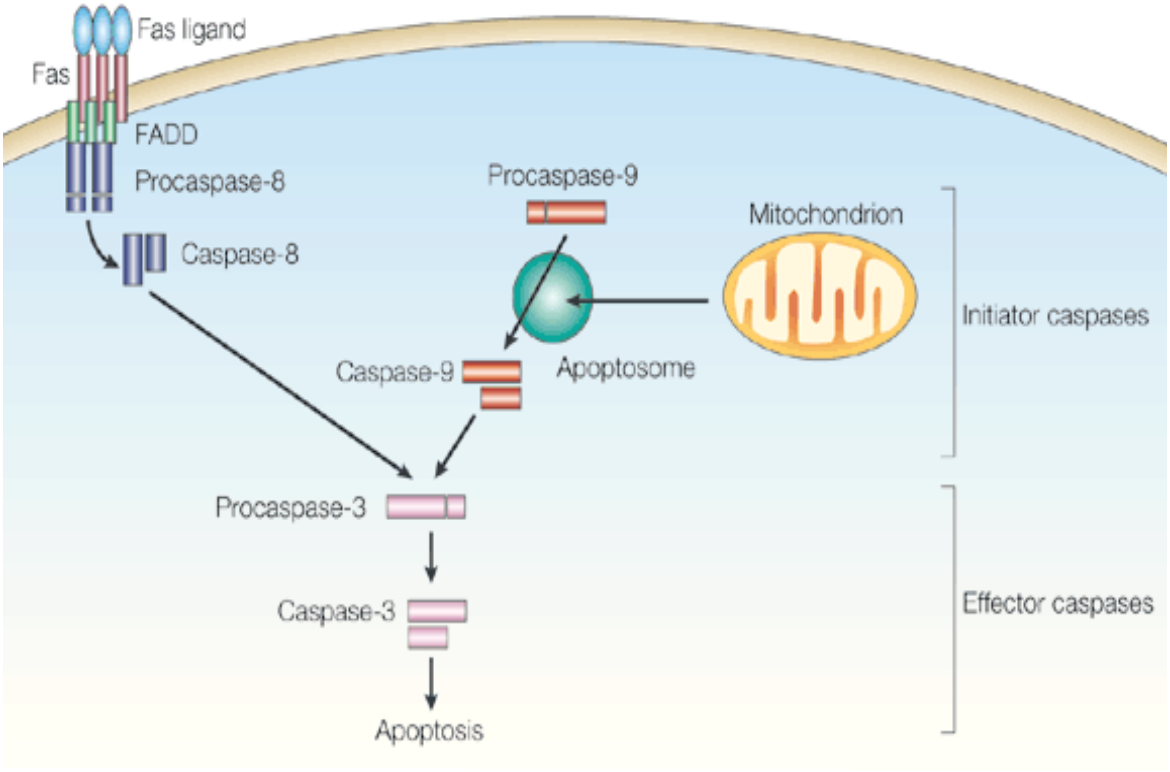
1.4 Apoptosis in Ovarian Cancer

Apoptosis is a form of programmed cell death which can be caused by a wide range of physiological and non-physiological stimuli. It is a strictly controlled event that is characterized by cell shrinkage, chromatin condensation, membrane blebbing, DNA fragmentation, loss of mitochondrial membrane potential, and nucleic acid and protein degradation[49,50]. Apoptosis is a naturally occurring physiological process critical in tissue development and homeostasis, and the elimination of genetically unstable cells[51-54].

Apoptosis is primarily initiated by two pathways: the intrinsic pathway which is initiated by the loss of mitochondria membrane potential and the subsequent release of the mitochondrial

death proteins, such as cytochrome c, apoptotic peptidase activating factor 1 (Apaf-1), apoptosis inducing factor (AIF), and second mitochondria-derived activator of apoptosis/Diablo homolog (smac/DIABLO)[55-58]; the extrinsic pathway which is initiated by the membrane-associated death receptors belonging to the tumor necrosis factor receptor family (TNFR), such as Fas and TNFR1[59,60]. Both pathways converge on and sequentially activate a cascade of a family of cysteine-aspartic acid proteases, caspases (Figure 1.2), which are responsible for the execution of apoptosis[61].

Figure 1.2: The activation of the caspase cascade (*Adapted from Holcik and Korneluk, 2001*[62]*).* The extrinsic apoptotic pathway is initiated by the stimulation and activation of death receptors (i.e. Fas) by the binding of an external death signal (i.e. FasL), leading to the oligomerization of the death receptor and the recruitment and activation of the initiator caspase, caspase 8, which then cleaves and activates the apoptotic executioner, caspase 3. The intrinsic apoptotic pathway is initiated by the release of the mitochondrial death proteins following the loss of mitochondrial membrane potential and subsequent permeabilization. Procaspase 9, Apaf-1 and cytochrome c form a complex, the apoptosome, leading to the proteolytic activation of caspase 9 and subsequently caspase 3 and the induction of caspase-mediated apoptosis.


Fas ligand
Fas
FADD
Procaspase-8
Caspase-8
Procaspase-9
Mitochondrion
Apoptosome
Caspase-9
Initiator caspases
Procaspase-3
Caspase-3
Effector caspases
Apoptosis

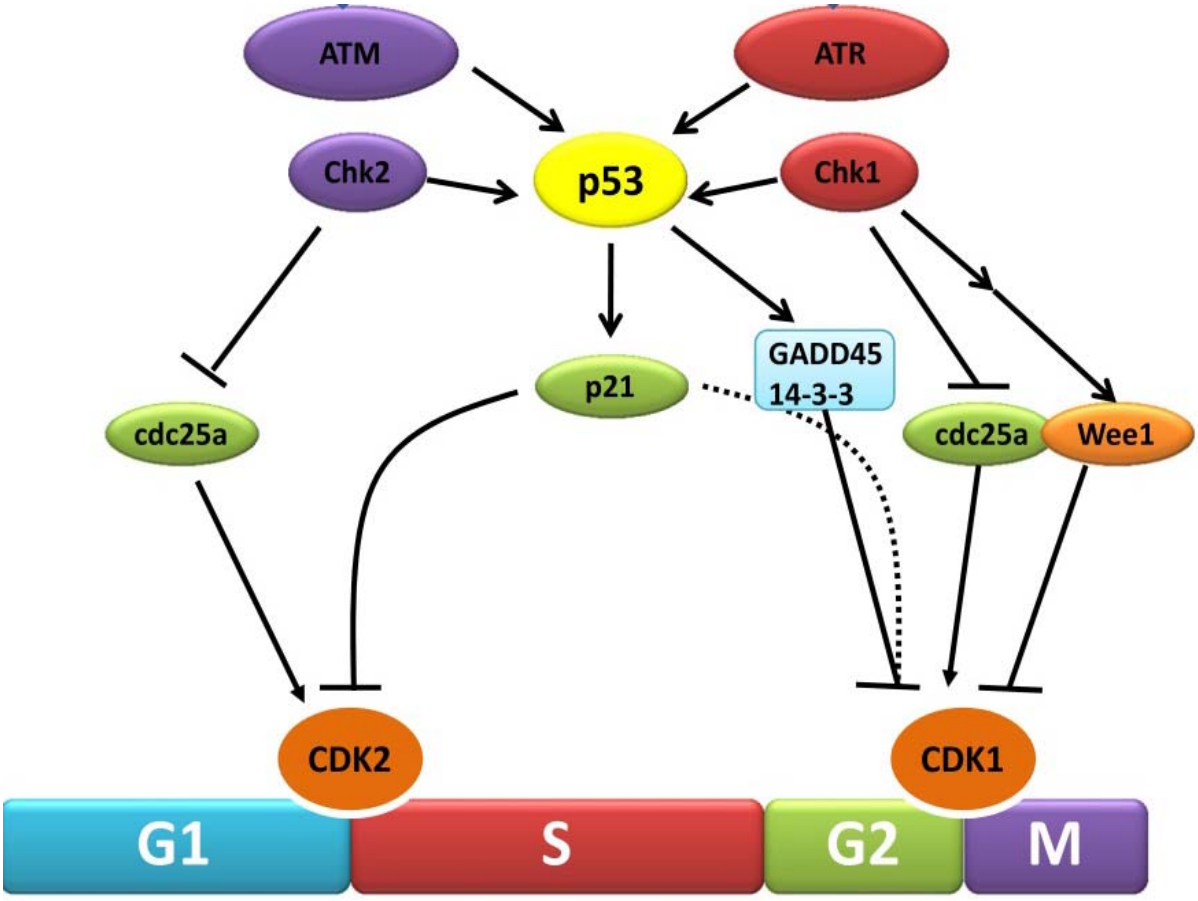
1.4.1 Molecular Determinants of Apoptosis

Several studies have shown that different proteins are involved in the regulation of apoptotic signaling pathways, such as Fas-associated death domain-like interleukin-1b-converting enzyme (FLICE)-like inhibitory protein (FLIP)[63-65], X-linked inhibitor of apoptosis (XIAP)[47,66,67], Bcl-2 family members[50,68], p53[46,48], Akt[69,70], PPM1D[71,72], and Chk1[71,73]. The proteins which are most relevant to this thesis will be discussed in greater detail in this section.

1.4.1.1 p53

The tumor suppressor p53 is a transcription factor which plays an important role within the cell as guardian of genomic integrity. It is involved in the regulation of cellular proliferation and apoptosis through the control of several molecular pathways. p53 elicits its actions via transcription-dependent and –independent mechanisms[74,75]. It has been well established that a functional p53 signaling pathway is necessary to sensitize cancer cells to DNA-damaging chemotherapeutic agents, such as CDDP[46,48,64,65,69,74,76,77]. p53 is activated in response to genomic insults by the DNA damage sensors ataxia talengiectasia mutated protein (ATM) and ataxia talengiectasia mutated and Rad3-related protein (ATR) and their downstream effectors checkpoint kinases 1 and 2 (Chk1 and Chk2). In turn, p53 maintains a sustained cell cycle arrest by up-regulating the expression of the cyclin-dependent kinase inhibitor p21, which arrests the cell cycle at the G_1 phase[78], and 14-3-3σ, which sequesters CDC25C phosphatase in the cytoplasm and promotes G_2/M cycle arrest (Figure 1.3)[79,80].

Figure 1.3: The DNA damage response and checkpoint regulation (*Modified from Lopez-Contreras and Fernandez-Capetillo, 2012[81]*). DNA double strand breaks activate ATM, which in turn activates both Chk2 and p53, ultimately leading to the inhibition of CDK2 and the regulation of G_1/S transition. DNA single strand breaks activate ATR, which in turn activate both Chk1 and p53, ultimately inhibiting CDK1 and leading to G_2/M cell cycle arrest.


ATM
ATR
Chk2
p53
Chk1
p21
GADD45
14-3-3
cdc25a
cdc25a
Wee1
CDK2
CDK1
G1
S
G2
M

Furthermore, p53 induces the expression of several pro-apoptotic proteins which act on the mitochondria and cause the release of mitochondrial death proteins. These include p53 upregulated modulator of apoptosis (PUMA), NADPH oxidase activator (NOXA)[48,82,83], Bcl-2 family members Bax and Bid[84,85], and Apaf-1[58]. Mitochondrial outer membrane permeabilization (MOMP) is a crucial step in the apoptotic process which is directly governed by pro-apoptotic Bcl-2 family members including Bax and Bak, and BH3-only proteins, Bid, Bim and PUMA[86,87] Under cellular stress, p53 also directly translocates to the mitochondria and participates in the induction of apoptosis both by interacting with pro-apoptotic Bcl-2 family members to induce MOMP, and by forming an inhibitory complex with Bcl-2 and Bcl-XL to neutralize their anti-apoptotic effects[88]. Interestingly, the p53 DNA binding domain interacts directly with the interface of Bcl-2 protein. p53 mutants are incapable of Bcl-2 complex formation and are therefore defective in mitochondrial permeabilization[89]. In response to CDDP, p53 is targeted to the mitochondria to induce the release of mitochondrial death proteins, resulting in caspase activation[47,75,90].

p53 also up-regulates the expression of the death receptors Fas, DR4, and DR5[91-93]. p53 facilitates the ubiquitination and proteasomal degradation of the anti-apoptotic FLIP by promoting its interaction with the E3 ubiquitin ligase Itch[64]. These few examples of p53-regulated genes and gene products belong to a growing list that emphasizes the multi-factorial role of the p53 network in tumor growth and differentiation. Mutations and/or functional inactivation of *TP53* are a hallmark of many human malignancies, including OVCA and CECA[94,95].

1.4.1.2 Chk1

Cells have mechanisms that ensure the order and fidelity of cell cycle events to maintain genomic stability. Once DNA is damaged or replication is inhibited, cells respond by the activation of an evolutionarily conserved signal transduction pathway that delays cell cycle progression to allow the repair of the damaged DNA. The damage is recognized by protein sensors, which trigger the activation of several kinases that lead to cell cycle arrest. ATR plays an important role in the cellular response to DNA damage. It is phosphorylated by the tumor suppressor $p14^{ARF}$ at Ser^{428} following DNA damage and then recruited to sites of single-stranded DNA breaks and stalled replication forks by the ATR interacting protein (ATRIP) and the single strand-DNA binding replication protein A (RPA)[96,97]. ATR then phosphorylates and activates a number of proteins which lead to cell cycle arrest. ATR preferentially phosphorylates serine (S) or threonine (T) residues followed by glutamine (Q)[98,99].

Chk1 is an important downstream target of ATR. It is a serine/threonine kinase which acts as a G_2 cell cycle checkpoint regulator. Evidence of G_2 regulation by Chk1 in human cells comes from studies which found that the use of potent Chk1 inhibitors UCN-01 and SB-218078 abrogates G_2/M checkpoint function[100-102]. Chk1 contains several SQ/TQ residues, including Ser317 and Ser345, which are phosphorylated by ATR and are essential phosphorylation sites for optimal Chk1 kinase activity[103]. Being a Chk1 substrate, p53 is phosphorylated by activated Chk1 at serine 15 and serine 20, which leads to its stabilization due to the inability of MDM2 to bind to it and mark it for proteolysis through its E3 ubiquitin ligase activity[104,105].

One of the well-known substrates of Chk1 is the family of CDC25 phosphatases, whose phosphorylation leads to the suppression of cyclin-CDK complexes and ultimately causes cell cycle arrest. Chk1 was found to target CDC25A for ubiquitin-mediated proteasomal degradation

which leads to the suppression of CDK2 activity and the subsequent S phase arrest[106,107]. Chk1 also induces the cytoplasmic sequestration of both CDC25B and CDC25C through the binding of the 14-3-3 proteins, ultimately resulting in failure to activate CDK 1 and the subsequent cell cycle arrest at the G_2/M phase[108-110]. It has been demonstrated that in p53-deficient tumors, G_2/M cell cycle arrest is primarily achieved through Chk1 activation due to the lack of functional p53[111]. By using a combination of Chk1 inhibitors and a DNA damaging agent, S/G_2 arrest was abrogated in p53-deficient cancer cells, sparing healthy cells with functional p53. This causes the cells to enter mitosis without having the chance to repair the damaged DNA which ultimately causes cell death through mitotic catastrophe. It has also been demonstrated that in radiation resistant stem-like glioma cells containing wild-type p53, the mechanism of resistance implicated the Chk1 pathway[112]. Those stem-like cells within gliomas are resistant to radiation to a certain extent due to elevated DNA damage response and swift repair. The inhibition of Chk1 significantly sensitized these cells to the DNA damaging effect of radiation and lead to increased cell death. Although these are interesting targeting mechanisms for p53-deficient and radiation resistant cancer cells, whether Chk1 inhibition in chemoresistant, p53-proficient cancer cells would sensitize them to chemotherapeutic agents, such as CDDP, is not known.

1.4.1.3 PPM1D

Protein phosphatase magnesium-dependent 1 D (PPM1D), also known as wild-type p53 inducible phosphatase 1 (Wip1) and protein phosphatase type 2C delta (PP2Cδ), is a member of the type 2C phosphatase family, specifically belonging to the magnesium-dependent sub-family of PPM1 phosphatases. It was first identified as a p53-induced phosphatase in response to ultraviolet (UV) and ionizing radiation (IR)[113-115]. While PPM1D is induced by p53 in response

to DNA damage, recent evidence has revealed that PPM1D is also regulated by several transcription factors including cyclic AMP response element binding protein (CREB)[115], NF-κB[116], estrogen receptor α (ERα)[117], E2F1[118] and c-Jun[119]. PPM1D preferentially dephosphorylates phosphoproteins containing SQ/TQ or TXY motifs[113,120-122].

Under normal physiological conditions, PPM1D restores cellular homeostasis following a genomic insult resulting in DNA damage by cooperating with p53 to induce G_2/M cell cycle arrest, thereby allowing ample time for repair of the damaged DNA before the resumption of the cell cycle[123-125]. However, PPM1D amplification and/or enhanced stabilization allow for sustained inhibition of DNA damage response proteins and numerous tumor suppressors. PPM1D over-expression has been implicated in a variety of human malignancies, including OVCA, and its level is directly related to poor overall prognosis and reduced therapeutic outcome[126-136]. PPM1D has been found to enhance mammary transformation in a breast cancer-susceptible animal model[137]. Moreover, PPM1D-null mice exhibit a lower incidence of spontaneously occurring tumors[138] and resistance to oncogene-induced transformation[137,139,140]. The primary function of PPM1D in tumorigenesis is the attenuation of DNA damage and apoptotic responses following a genomic insult. This occurs through the regulation of the ATM/ATR and p53 pathways. ATM has been found to be directly dephosphorylated by PPM1D at Ser^{1981}, which significantly down-regulates ATM activity and downstream signaling[140,141]. PPM1D also targets proteins downstream of ATM. Chk2 is dephosphorylated by PPM1D at its DNA damage-induced, ATM-dependent phosphorylation site (Thr^{68}), leading to decreased Chk2 kinase activity[142-145]. PPM1D also dephosphorylates MDM2 (Ser^{395}) and MDM4 (Ser^{403}), enhancing their stability, interaction and cooperative proteasomal degradation of p53[146-148]. Although PPM1D has not been shown to target ATR directly, it does dephosphorylate its

downstream targets. It has been demonstrated that PPM1D directly dephosphorylates Chk1 at its ATR-dependent phosphorylation site (Ser^{345}), and in fact PPM1D is a more crucial factor than ATR in regulating Chk1 activation in response to CDDP and ultimately chemosensitivity of OVCA cells[77,121,149]. PPM1D also dephosphorylates and inactivates gamma histone 2AX (γH2AX) at Ser^{139}, thereby inhibiting the formation of DNA damage foci, and recruitment and subsequent formation of DNA repair complexes[150-152]. PPM1D regulates both base excision repair (BER) and nucleoside excision repair (NER) by dephosphorylating uracil DNA glycosylase 2 (UNG2) at Ser^{6} [122,153], as well as excision repair proteins xeroderma pigmentosum complementation group A (XPA; Ser^{196}) and complementation group C (XPC; Ser^{892})[154,155], respectively.

PPM1D expression is induced by p53 and forms a negative feedback loop by dephosphorylating p53 at Ser^{15}, a site important for its pro-apoptotic activity. PPM1D knock-down sensitizes resistant cancer cells to CDDP primarily by enhancing p53 activation via Ser^{15} phosphorylation[48,77,121]. However, the role of PPM1D in regulating p53 function goes beyond direct regulation, whereby PPM1D indirectly regulates p53 activation and stability. As mentioned previously, PPM1D regulates the activation of ATM, Chk1 and Chk2, known regulators of p53 activation, as well as MDM2 and MDM4, regulators of p53 stabilization. Moreover, PPM1D deactivates p38 mitogen-activated protein kinase (p38 MAPK) and down-regulates the expression of its downstream effectors $p16^{Ink4a}$ and $p14^{ARF}$, which are vital tumor suppressors and important regulators of p53 activity[120,139,156]. PPM1D ultimately inhibits DNA repair, cell cycle checkpoints, and cellular apoptosis, thereby promoting proliferation and passage of corrupted genome. By these means, PPM1D enhances oncogenic transformation, cellular proliferation, and tumor growth.

PPM1D has two functional isoforms: full-length PPM1D (PPM1D605) and a shorter isoform, PPM1D430. PPM1D605 is ubiquitously expressed, while PPM1D430 is exclusively expressed in leukocytes and testes where it plays an important role in lymphocyte maturation and spermatogenesis, respectively[157]. Both isoforms retain phosphatase activity and are able to dephosphorylate similar target proteins. PPM1D605 has two putative nuclear localization signal (NLS) domains and is assumed to be a strictly nuclear phosphatase, while PPM1D430 contains only one NLS and exhibits both nuclear and cytoplasmic localizations. However, whether PPM1D605 cellular localization is differentially regulated in CDDP-sensitive and –resistant OVCA and CECA cells is unknown.

1.4.1.4 The PI3K/Akt survival pathway

A hallmark of OVCA is the over-expression and/or activation of the phosphoinositide-3 kinase (PI3K)/Akt survival pathway. Improper activation of this pathway is associated with tumorigenesis in several tissue types[158-162]. It has been demonstrated that Akt activation enhances the survival of OVCA cells and promotes chemoresistance through attenuating p53 pro-apoptotic signaling[46-48,65,75].

PI3K is a phospholipid kinase which phosphorylates the 3' hydroxyl group of the inositol ring of phosphoinositide lipids. It is composed of a catalytic subunit (p110) and a regulatory subunit (p85)[163]. PI3K is activated in response to growth factors by several receptor tyrosine kinases (RTKs) which interact with the p85 subunit and activate the p110 catalytic subunit. Activated PI3K phosphorylates the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to form phosphatidylinositol 3,4,5-triphosphate (PIP_3), which recruits PI3K cytosolic effectors to the plasma membrane for further activation and downstream signaling. This process is regulated

by the tumor suppressor phosphatase and tensin homolog (PTEN), which dephosphorylates PIP_3 to PIP_2, effectively shutting down the PI3K signaling cascade[164].

One of the main downstream effectors of the PI3K pathway is the potent oncogenic serine/threonine kinase Akt, also known as protein kinase B (PKB). The Akt family consists of three members (Akt1, Akt2, and Akt3) that have high sequence homology and share similar domain structures including an N-terminal pleckstrin homology (PH) domain necessary for protein-protein interaction, a central catalytic domain important for Akt kinase activity and a C-terminal regulatory domain[165]. Akt is activated through recruitment to the plasma membrane by PIP_3[166,167], followed by phosphorylation of Thr^{308} by the phosphoinositide-dependent kinase 1 (PDK1)[168,169], and Ser^{473} by the mammalian target of rapamycin complex 2 (mTORC2)[170,171].

Activated Akt plays an important role in the regulation of several important molecular pathways including cell survival, proliferation, and apoptosis. Akt phosphorylates the pro-apoptotic Bcl-2 family member BAD on its inhibitory Ser^{136} site, leading to decreased binding with Bcl-XL and thus inhibiting the release of mitochondrial death proteins[172]. Akt also inhibits the activity of the initiator caspase caspase-9 through phosphorylation of Ser^{196}, thereby inhibiting the cleavage of both procaspase-3 and procaspase-7 and their subsequent activation[173]. Akt phosphorylates and inhibits the forkhead transcription factor 1 (FOXO-1) through phosphorylation of Thr^{32} and Ser^{253}, facilitating its binding to 14-3-3 proteins and cytoplasmic sequestration, causing decreased expression of FOXO-1 targets, including the pro-apoptotic Bcl-2 family member Bim and Fas ligand[174]. Akt activates the inhibitor of κB kinases (IKK) through phosphorylation of IKKα on Thr^{23}, leading to IκB phosphorylation and degradation, and subsequent activation of the transcription factor NF-κB. NF-κB, in turn, promotes the transcription of several pro-survival genes, including PPM1D[116,175,176]. Akt relieves cell cycle

arrest at both the G_1 and G_2/M phases, resulting in increased proliferation. This is achieved through Akt-dependent phosphorylation and inhibition of p21 (Thr^{145}) and down-regulation of p27, which relieves the G_1 checkpoint[177-179], and Chk1 inhibitory phosphorylation (Ser^{280}), inhibiting G_2/M cell cycle arrest[180]. Akt activates mTOR through direct phosphorylation at Ser^{2448}, resulting in the activation of mRNA translational machinery[181]. Akt also stabilizes the caspase-3 inhibitor XIAP by phosphorylating Ser^{87}, preventing XIAP auto-ubiquitination and subsequent proteasomal degradation[182].

Akt regulates p53 protein stability and activation through phosphorylation of MDM2 on Ser^{166} and Ser^{186}, facilitating its nuclear translocation and thereby enhancing p53 ubiquitination and proteasomal degradation[183,184]. On the other hand, p53 can conversely regulate Akt activity by promoting the transcription of the PIP_3 inhibitor PTEN, resulting in enhanced p53 protein level and activation as well as negative regulation of PI3K expression[185,186]. However, whether and how Akt plays a role in the regulation of PPM1D protein stability in response to DNA damage in OVCA and CECA cells is unknown. This may highlight a possible new mechanism through which Akt regulates the activity of several tumor suppressors and disrupts the DNA damage response to enhance cellular survival, proliferation and chemoresistance through its stabilization of PPM1D, and thus warrants further investigation.

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Chapter 2 – Objectives and Hypotheses

The main challenges in OVCA treatment are the failure of early detection of the disease and the development of chemoresistance. Despite the considerable resources and efforts expended in the fight against OVCA in the past decades, it is still the number one cause of death among women with gynecological malignancies. Resistance to drug-induced cell death hampers the effects of chemotherapeutics in the treatment of human cancers. CDDP resistance in OVCA and CECA is a multifactorial event that severely reduces the chances of a successful therapeutic outcome for the patient. It has been demonstrated by our group and others that the underlying molecular mechanisms of CDDP resistance in OVCA and CECA are quite complicated and involve the disruption of the delicate balance between cell survival and apoptotic pathways, and by factors such as p53, Akt, XIAP, AIF, and FLIP[36,38,54,55,59,64,66].

The protein phosphatase PPM1D has recently been discovered as a tumorigenic protein in a variety of human cancers and its level is directly correlated to reduced therapeutic responsiveness and poor prognosis[115-125]. The main tumorigenic function of PPM1D is to allow uncontrolled cancer cell proliferation and to inhibit cell death. This is achieved through the regulation of Chk1, p53, and a host of cell cycle and fate regulators such as DDR proteins, tumor suppressors, and oncogenes[110,129,135,145]. However, the role and regulation of PPM1D in human gynecological cancers and its contribution to CDDP resistance are unknown. A better understanding of the mechanisms by which PPM1D may regulate CDDP sensitivity in OVCA and CECA cells may provide an insight into the underlying causes of CDDP resistance and may potentially help in the development of effective therapeutics in the treatment of resistant OVCA and CECA.

2.1 Overall Objective

The overall objective of this study is to improve our understanding of cellular and molecular mechanism (s) of CDDP sensitivity and to study the possible role and regulation of Chk1 and its modifiers in chemoresistance in gynecological malignancies.

2.2 Overall Hypothesis

We hypothesize that Chk1 activation is a determinant of chemosensitivity in gynecological cancer cells and it is required for p53-induced apoptosis following CDDP challenge. Furthermore, PPMID plays an important role in the regulation of chemosensitivity in gynecological cancer cells through Akt-mediated Chk1 and p53 dephosphorylation by PPM1D, leading to decreased CDDP sensitivity in OVCA cells.

2.3 Specific Hypotheses

1. Chk1 is required for p53-induced apoptosis of CECA cells following CDDP challenge;
2. PPM1D regulates CDDP sensitivity in CECA cells primarily by attenuating Chk1 and p53 activation;
3. Akt enhances PPM1D stability in resistant OVCA cells, thereby contributing to resistance to CDDP-induced apoptosis;
4. CDDP induces PPM1D down-regulation through proteasomal degradation;
5. CDDP enhances PPM1D activity in resistant CECA cells by increasing its nuclear localization and interaction with its nuclear targets, while it decreases PPM1D activity in sensitive OVCA and CECA cells by enhancing its nuclear exclusion;

6. PPM1D expression in human ovarian tumors is indicative of tumor chemoresistance.

2.4 Specific Objectives

1. Compare the level and activation of Chk1 in sensitive and resistant CECA cells and determine if this is related to resistance to CDDP-induced apoptosis;
2. Examine the role of Chk1 in regulating p53-mediated CDDP sensitivity and its regulation by the p53-induced phosphatase, PPM1D;
3. Establish the mechanism of CDDP-induced PPM1D down-regulation in sensitive OVCA cells;
4. Establish the role of Akt in promoting PPM1D stability and its influence on CDDP sensitivity;
5. Examine the influence of CDDP on PPM1D nuclear localization in OVCA and its impact on CDDP sensitivity;
6. Determine PPM1D expression in human OVCA cells and tumors.

Preface:

Re: Oncogene 31(17):2175-86, 2012

The authors declare that at the time of publication of this manuscript, the chemosensitive OV2008 cells and their resistant counterparts C13* were considered ovarian cancer cell lines of endometrioid origin. However, after the publication of this manuscript, the results of cell authentication (see Appendix 1) have correctly identified the cell lines as the cervical cell line ME-180.

Chapter 3 – Oncogene 31(17):2175-86, 2012

The oncogenic phosphatase PPM1D confers cisplatin resistance in ovarian carcinoma cells by attenuating checkpoint kinase 1 and p53 activation.

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Running Title: PPMID confers cisplatin resistance in ovarian cancer cells

Key words: Cisplatin, Chemoresistance, Apoptosis, Ovarian cancer, PPM1D, Chk1, p53.

Contribution of Co-Authors.

All experimental work and the writing of the manuscript were carried out by Ahmed Y. Ali. All studies were conducted under the supervision of Dr. Benjamin K. Tsang. Dr. Mohammad R. Abedini was involved in the experimental design, consulted on the writing and editing of the manuscript, and also contributed to the editing of the final manuscript.

Abstract

Cisplatin (CDDP: *cis*-diamminedichloroplatinum) resistance is a major hurdle in the treatment of human ovarian cancer (OVCA). A better understanding of the mechanisms of CDDP resistance can greatly improve therapeutic outcome for patients. p53, a determinant of CDDP sensitivity in OVCA, is activated by Chk1 in response to DNA damage. Although the oncogenic phosphatase Protein Phosphatase Magnesium-dependent 1 (PPM1D) can deactivate both p53 and Chk1 through site-specific dephosphorylation, whether PPM1D plays a role in CDDP resistance is unknown. Here, using pair-matched wt-p53 CDDP-sensitive (OV2008) and –resistant (C13*) cells, and p53-compromised CDDP-resistant cells (A2780cp, OCC-1, OVCAR-3, and SKOV3), we have demonstrated (i) the existence of site-specific differences in phospho-Ser-Checkpoint kinase 1 (Chk1) content between sensitive and resistant cells in response to CDDP; (ii) PPM1D, but not phosphoinositide-3-kinase-related kinase (ATR), is important in the regulation of CDDP-induced Chk1 activation and OVCA cell chemosensitivity; (iii) PPM1D down-regulation sensitizes resistant cells to CDDP primarily by activating Chk1 and p53. Our findings establish for the first time that PPM1D confers CDDP resistance in OVCA cells through attenuating CDDP-induced, Chk1-mediated, p53-dependent apoptosis. These findings extend the current knowledge on the molecular and cellular basis of CDDP resistance and offer the rationale for PPMID as a potential target for treatment of chemoresistant OVCA.

Introduction

Ovarian cancer (OVCA) is the most common gynecological malignancies and the fifth most frequent cause of cancer death in women. It ranks second among gynecological cancers in the number of new cases and the first in the number of deaths each year (Canadian Cancer Society, 2009). Cisplatin (CDDP: *cis*-diamminedichloroplatinum) and its derivatives are the most widely used chemotherapeutic agents in the treatment of OVCA. It is a DNA damaging agent, which creates DNA adducts, causing the activation of several pathways that culminate in apoptosis (Perego et al., 1996).

Drug resistance is a major obstacle to successful treatment of human malignancies. CDDP resistance is multi-factorial and may result from functional changes in OVCA cells, such as enhanced DNA damage repair, decreased drug accumulation and altered metabolism, and loss of apoptotic capacity (Mistry et al., 1992; Jekunen et al., 1994; Perego et al., 1996; Perego et al., 2003). However, the precise mechanisms of CDDP resistance in human OVCA remain unknown.

The phosphoinositide-3-kinase-related kinase, ATR, plays an important role in the DNA damage response. ATR activates several proteins, leading to cell cycle arrest and allowing repair of damaged DNA. ATR preferentially phosphorylates serine (S) or threonine (T) residues followed by glutamine (Q) (Capasso et al., 2002; Traven & Heierhorst, 2005). Checkpoint kinase 1 (Chk1), a serine/threonine kinase and important downstream target of ATR, is a G_2/M cell cycle checkpoint regulator (Graves et al., 2000; Busby et al., 2000). Chk1 contains several SQ/TQ residues, including Ser^{317} and Ser^{345}, which are phosphorylated by ATR and are essential for optimal Chk1 kinase activity

(Zhao & Piwnica-Worms, 2001). However, the exact role of Chk1 in CDDP resistance in human OVCA is unclear.

The tumor suppressor p53 is a transcription factor, activated by CDDP through a Ser^{15} and Ser^{20} phosphorylation-dependent mechanism, which increases its pro-apoptotic properties (Ko & Prives, 1996; Levine, 1997; Shieh et al., 1997; Shieh et al., 1999; Shieh et al., 2000; Damia et al., 2001; Yang et al., 2006). p53 is phosphorylated by activated Chk1 at Ser^{15} and Ser^{20}, suppressing its binding to MDM2 and its ubiquitination and proteasomal degradation (Honda et al., 1997; Qu et al., 2005). Moreover, p53 phosphorylation results in its activation, causing the transcription of several genes, the products of which are anti-proliferative, such as p21 which inhibits CDK1 and ultimately causes cell cycle arrest at the G_1 phase (Shieh et al., 2000; Jurvansuu et al., 2007).

One of the transcriptional targets of p53 is the type 2C serine/threonine phosphatase Protein Phosphatase Magnesium-dependent 1 (PPM1D), also known as Wild-type p53 induced phosphatase 1 (Wip1), which preferentially targets phospho-serine and phospho-threonine residues in the SQ/TQ motifs and phospho-threonine residues in the TXY motifs (Lu et al., 2004; Yamaguchi et al., 2007; Lu et al., 2008; Chuman et al., 2008). PPM1D expression is known to be induced by p53 in response to genotoxic stress (Choi et al., 2000) and it dephosphorylates both phospho-p53 at Ser^{15} (Lu et al., 2007) and phospho-Chk1 at Ser^{345} (Lu et al., 2005), significantly reducing Chk1 kinase activity (Capasso et al., 2002). It has been demonstrated that p38 MAP kinase, which activates p53 during genotoxic stress, is dephosphorylated by PPM1D thereby negatively regulating the activation of p53 (Bulavin et al., 2002; Rauta et al., 2006). Recent studies have shown that PPM1D also interacts with several proteins

involved in numerous cellular processes: MDM2 and MDMX in the DNA damage response (Lu et al., 2008; Zhang et al., 2009); UNG2 in base excision repair (Lu et al., 2004); and NF-κB in the inflammatory response (Chew et al., 2009). Moreover, PPM1D is over-expressed in a panel of ovarian clear cell adenocarcinomas (Hirasawa et al., 2003; Tan et al., 2009). Down-regulation of PPM1D or the inhibition of its activity in PPM1D-overexpressing cells alone was sufficient to decrease their survival even in the absence of genotoxic stress, suggesting that the cell survival is dependent on PPM1D expression and phosphatase activity (Tan et al., 2009). However, the role of PPM1D in CDDP chemoresistance in OVCA cells, and if it is related to p53 and Chk1 regulation is not well understood.

In the present study we have examined the interaction between PPM1D, Chk1, and p53 and how PPMID regulates CDDP sensitivity in OVCA cells. We have demonstrated for the first time that PPM1D contributes to CDDP resistance in human OVCA cells by directly targeting Chk1 and p53, diminishing their activation and thereby reducing p53-dependent, CDDP-induced apoptosis.

Results

CDDP increases phospho-Ser ^{345}-Chk1 content in sensitive but not resistant OVCA cells

To examine whether Chk1 activation is dysregulated in resistant OVCA cells, we carried out time-course (0-10 μM CDDP; 0-6h) and concentration-response (0-10 μM; 6 h) experiments with OV2008 cells and its resistant counterpart C13*. CDDP significantly increased phospho-Ser^{345}-Chk1 content in OV2008 but not C13* cells (Fig. 1A).

Analysis of phospho-Ser^{345}-Chk1 content indicates significant effects of CDDP ($P < 0.001$), duration of culture ($P < 0.001$) and cell type ($P < 0.001$). There was a highly significant, time-dependent increase in phospho-Ser^{345}-Chk1 level in OV2008 cells ($P < 0.001$, Fig 1A) as well as a significant interaction between CDDP and culture duration ($P < 0.001$), suggesting that the magnitude of CDDP-induced phospho-Ser^{345}-Chk1 content is dependent on the treatment duration. There were highly significant effects of cell line ($P < 0.001$), CDDP treatment ($P < 0.001$), and interaction between the two factors ($P < 0.001$, Fig. 1A). In contrast, no difference in Chk1 content was detected (Fig**.** 1A), suggesting that the increased phospho-Ser^{345}-Chk1 content in OV2008 was not due to increased substrate. In addition, the increased phospho-Ser^{345}-Chk1 level in OV2008 but not C13* cells in response to CDDP appeared to be substrate site-specific, since phospho-Ser^{317}-Chk1 content in response to CDDP treatment was not significantly different between the two cell lines, irrespective of the duration of culture and concentration (Fig. 1A). Analysis of Chk1 phosphorylation on Ser^{317} in response to CDDP indicates a significant time-dependent increase of phospho-Ser^{317}-Chk1 level in both OV2008 and C13* cells following CDDP treatment ($P < 0.001$, $P < 0.001$ respectively, Fig. 1A), suggesting phospho-Ser^{317}-Chk1 level may not be an important factor in the control of CDDP sensitivity.

Changes in CDDP-induced phospho-Ser^{345}-Chk1 content in OVCA cells is not associated with dysregulated ATR expression and activation

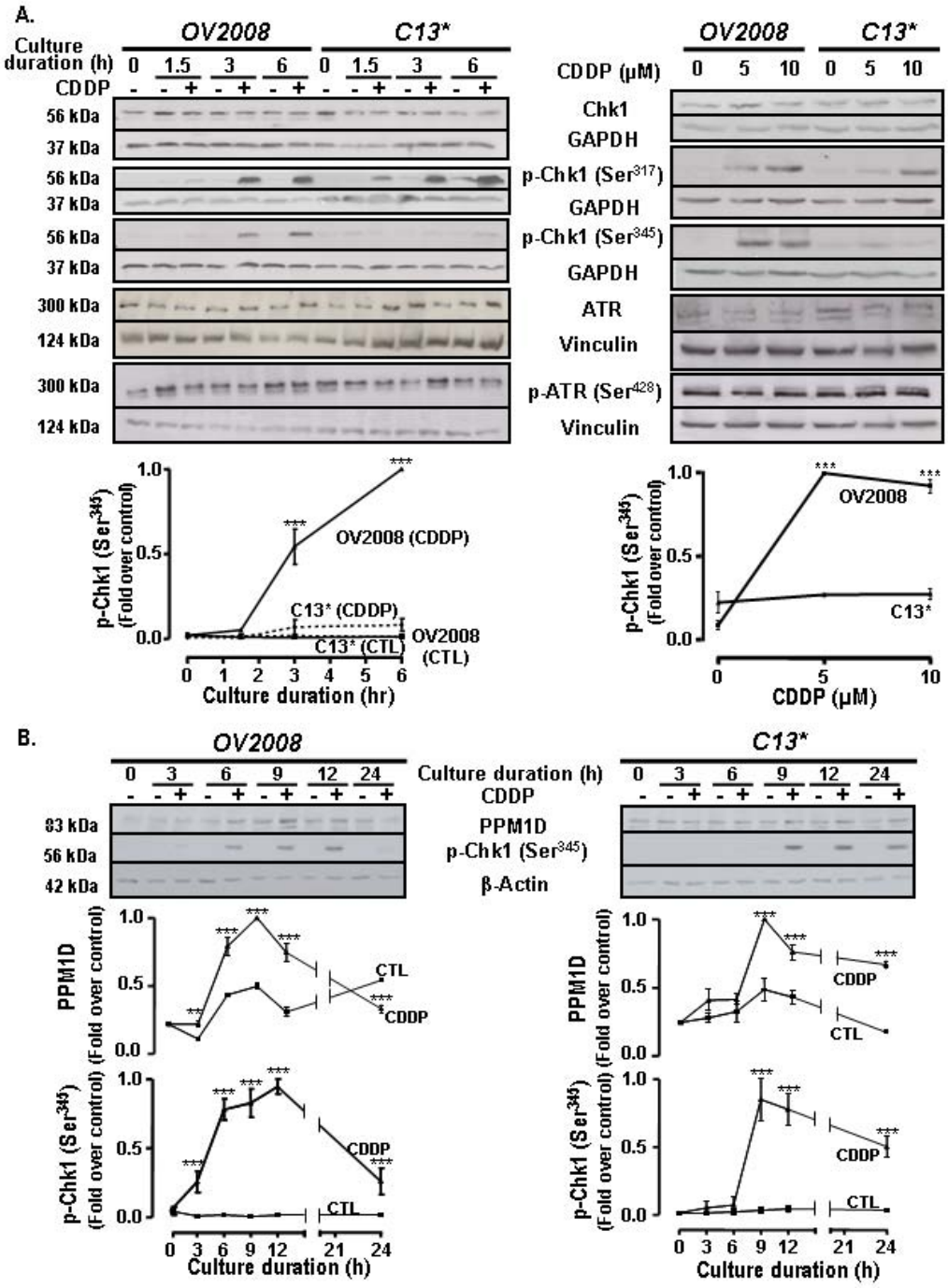
To investigate if the low level of phosphorylated Chk1 (Ser^{345}) in C13* cells is associated with abrogated ATR activation, total and phospho-Ser^{428}-ATR contents

following CDDP treatment were assessed in time-course and concentration-response studies by Western analyses. Unlike the increase in phospho-Ser^{345}-Chk1content in response to CDDP in OV2008 but not in C13* cells, ATR and phospho-Ser^{428}-ATR contents were not significantly effected in either cell line. No significant effects of culture duration, cell line, and CDDP treatment, as well as their interactions (Fig. 1A).

Up-regulation of PPM1D content by CDDP in OVCA cells is accompanied by increased phospho-Ser ^{345}-Chk1 level

PPM1D is known to dephosphorylate Chk1 at Ser^{345} (Lu et al., 2005). However, whether PPM1D-mediated Chk1 dephosphorylation is involved in CDDP chemoresistance has not been reported. To determine the expression of PPM1D in OVCA cells and its response to CDDP, we performed a time-course experiment (0-10 μM CDDP; 0-24 h) with OV2008 and C13* cells. In OV2008 cells, PPM1D content steadily increased following CDDP treatment starting at 3 hours ($P < 0.001$), reaching a maximum at 9 hours ($P < 0.001$) when compared to the control. Thereafter, PPM1D level started to decline and it was significantly lower than the control at 24 hours ($P < 0.001$). Phospho-Ser^{345}-Chk1 level followed a similar pattern, reaching a maximum at 12 hours after CDDP treatment ($P < 0.001$) and then sharply declined. In C13* cells, PPM1D levels sharply increased at 9 hours ($P < 0.001$). PPM1D level slightly decreased afterwards but by 24 hours, it was significantly higher than control ($P < 0.001$). Again, Phospho-Ser^{345}-Chk1 level followed a similar pattern, reaching a maximum level at 9 hours ($P < 0.001$) and steadily declined thereafter (Fig. 1B).

Figure 1: CDDP induced biphasic expression of phospho-Ser^{345}-Chk1 and PPM1D content in sensitive OVCA cells. A) CDDP increased phospho-Ser^{345}-Chk1 in OV2008 but not C13* cells ($P < 0.001$; $n = 3$). The expression of phospho-Ser^{317}-Chk1 in response to CDDP was enhanced in both cell types, while total Chk1, ATR, and phospho-Ser^{428}-ATR levels remained unchanged irrespective of CDDP treatment or cell type. OV2008 and C13* cells were cultured with CDDP (*left panel*: 10 μM, 0-6h; *right panel*: 0-10 μM, 6h) and assessed for Chk1, phospho-Ser^{317}-Chk1, phospho-Ser^{345}-Chk1, ATR, phspho-Ser^{428}-ATR, GAPDH, and vinculin contents by Western blotting. GAPDH and vinculin served as loading controls. B) Delay in phospho-Ser^{345}-Chk1 up-regulation in response to CDDP in chemoresistant cells is accompanied by up-regulation of PPM1D. OV2008 and C13* were cultured with CDDP (10 μM, 0-24h) and PPM1D, phospho-Ser^{345}-Chk1, and β-Actin were assessed. β-Actin was the loading control ($n = 3$).


A.
OV2008
C13*
Culture duration (h)
0 1.5 3 6 0 1.5 3 6
CDDP
- - + - + - + - - + - + - +
56 kDa
37 kDa
56 kDa
37 kDa
56 kDa
37 kDa
300 kDa
124 kDa
300 kDa
124 kDa
OV2008
C13*
CDDP (μM)
0 5 10 0 5 10
Chk1
GAPDH
p-Chk1 (Ser317)
GAPDH
p-Chk1 (Ser345)
GAPDH
ATR
Vinculin
p-ATR (Ser428)
Vinculin
p-Chk1 (Ser345) (Fold over control)
OV2008 (CDDP)
C13* (CDDP)
OV2008 (CTL)
C13* (CTL)
Culture duration (hr)
OV2008
C13*
CDDP (μM)
B.
OV2008
C13*
Culture duration (h)
0 3 6 9 12 24
CDDP
83 kDa
56 kDa
42 kDa
PPM1D
p-Chk1 (Ser345)
β-Actin
PPM1D (Fold over control)
p-Chk1 (Ser345) (Fold over control)
CTL
CDDP
Culture duration (h)

PPM1D is involved in the regulation of Chk1-mediated CDDP sensitivity

To assess the role of PPM1D in the regulation of CDDP sensitivity, PPM1D in OV2008 and C13* cells was knocked down by siRNA (0-400 nM; 24h) prior to CDDP challenge (0-10 μM; 12-24h), and phospho-Ser^{345}-Chk1 content and apoptosis were determined. PPM1D down-regulation significantly increased CDDP sensitivity in both OV2008 and C13* cells in a concentration-dependent manner, which was confirmed by PARP cleavage ($P < 0.001$, Fig. 2A). Contrary to our expectation, however, phospho-Ser^{345}-Chk1 content decreased in C13* cells following PPM1D knockdown. Since Ser^{345}-Chk1 phosphorylation might have occurred at an earlier time point following PPM1D knockdown, we examined the acute effects of CDDP on phospho-Ser^{345}-Chk1 content (0-10 μM; 0-6 h) following PPM1D down-regulation in C13* cells. PPM1D knockdown resulted in a significant increase of phospho-Ser^{345}-Chk1 level following 6 hours of CDDP challenge ($P < 0.001$, Fig. 3A). These results indicated that enhancement of Chk1 phosphorylation at an earlier time point might be associated with the sensitization of C13* cells to CDDP seen at 24 hours. Furthermore, PPM1D over-expression in OV2008 cells with PPM1D cDNA or empty vector followed by CDDP challenge (10 μM; 12 h) resulted in a significant reduction in CDDP-induced apoptosis compared to the control ($P < 0.001$) which was accompanied with significant decrease in phospho-Ser^{345}-Chk1 and phospho-Ser^{15}-p53 levels ($P < 0.001$, Fig. 2B).

To investigate whether the sensitization of C13* cells to CDDP following PPM1D knockdown is in some extent due to Chk1 activation, we knocked down both Chk1 (0-10 nM; 48h) and PPM1D (0-400 nM; 24h) in C13* cells followed by CDDP treatment (0-10 μM; 24h). PPM1D knockdown significantly sensitized C13* cells to

CDDP-induced apoptosis compared to the control ($P < 0.001$), while the double knockdown of both PPM1D and Chk1 significantly reduced CDDP-induced apoptosis ($P < 0.001$), but did not completely abolish CDDP sensitivity (Fig. 3B).

To assess if Chk1 deficiency affects CDDP sensitivity and cell cycle checkpoint, we performed flow cytometric analyses of CDDP response (0-10 μM; 24 h) of Chk1-deficient MDA-MB-ΔChk1 breast cancer cells compared to their parental cell line. Chk1 deficiency caused decreased sensitivity to CDDP, indicated by lack of increase in MDA-MB-231ΔChk1 cells in the sub-G_1 fraction compared to the control, and loss of cell cycle checkpoint, indicated by absence of cell cycle arrest in the G_2/M phase observed in the wt-MDA-MB-231 cells in response to CDDP. Furthermore, Western analyses revealed that MDA-MB-231ΔChk1 cells had a significantly reduced phospho-Ser^{15}-p53 content following CDDP treatment compared to wt-MDA-MB-231 cells (Fig. 3C).

Figure 2: Involvement of PPM1D in the regulation of CDDP sensitivity in OVCA cells. A) siRNA-mediated PPM1D down-regulation sensitized C13* cells, as evident by apoptotic cell count and PARP cleavage, and enhanced sensitivity of OV2008 to CDDP-induced apoptosis in a concentration-dependent manner. OV2008 and C13* cells were incubated with PPM1D siRNA or control siRNA (0-400, 24h), treated with CDDP (*left panel*: 0-10 μM, 12h; *right panel*: 0-10 μM, 24h) and PPM1D, phospho-Ser^{345}-Chk1, phospho-Ser^{317}-Chk1, and β-Actin contents and apoptosis were assessed (n = 4). B) Over-expression of PPM1D in OV2008 significantly decreased CDDP-induced apoptosis ($P < 0.001$), which was associated with decreased phospho-Ser^{345}-Chk1 and phospho-Ser^{15}-p53 contents ($P < 0.01$). OV2008 cells were transfected (1μg, 24h) with PPM1D cDNA or empty pCMV6-XL5 vector, treated with CDDP (0-10 μM, 24h) and PPM1D, phospho-Ser^{345}-Chk1, phospho-Ser^{15}-p53, β-Actin contents and apoptosis were assessed (n = 3).

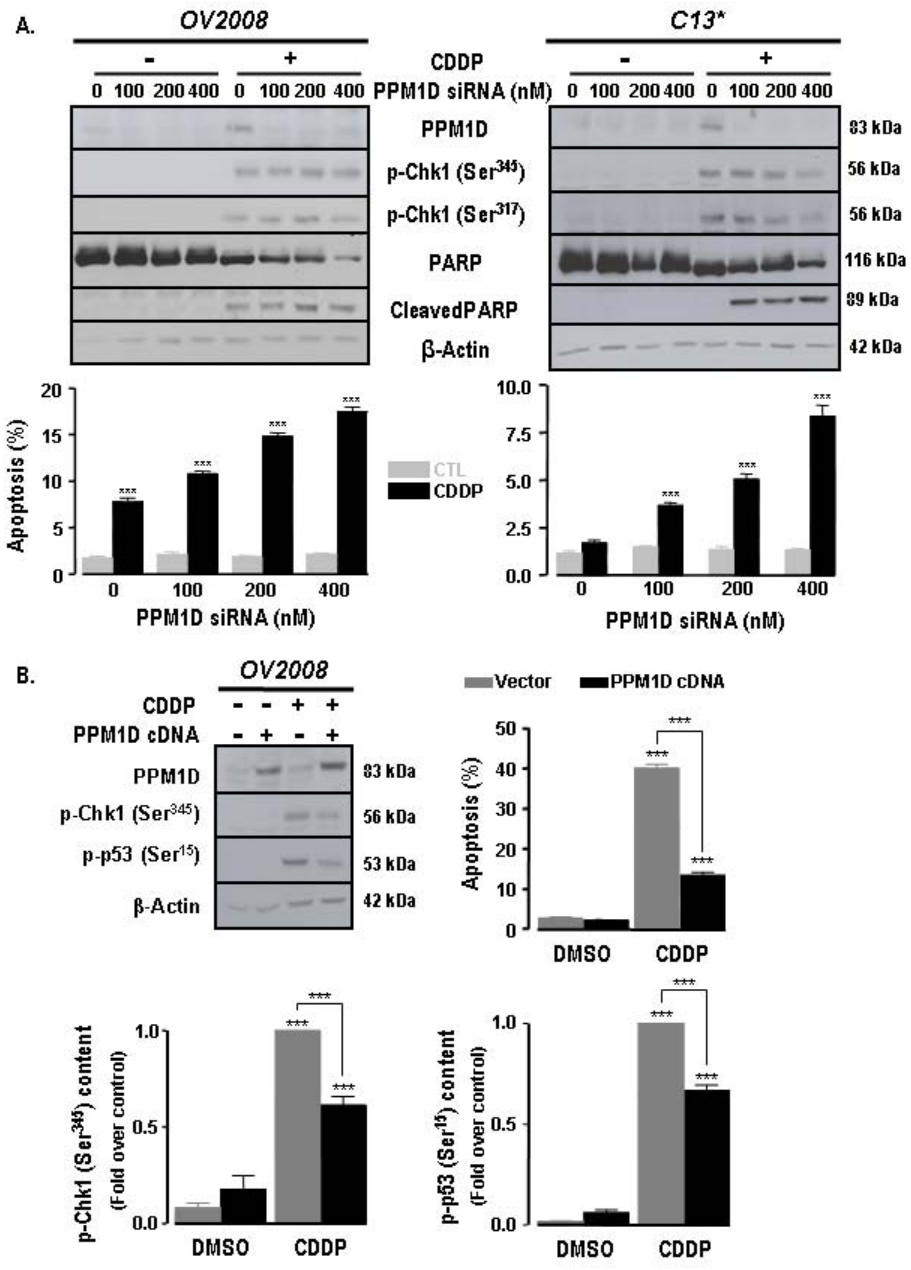
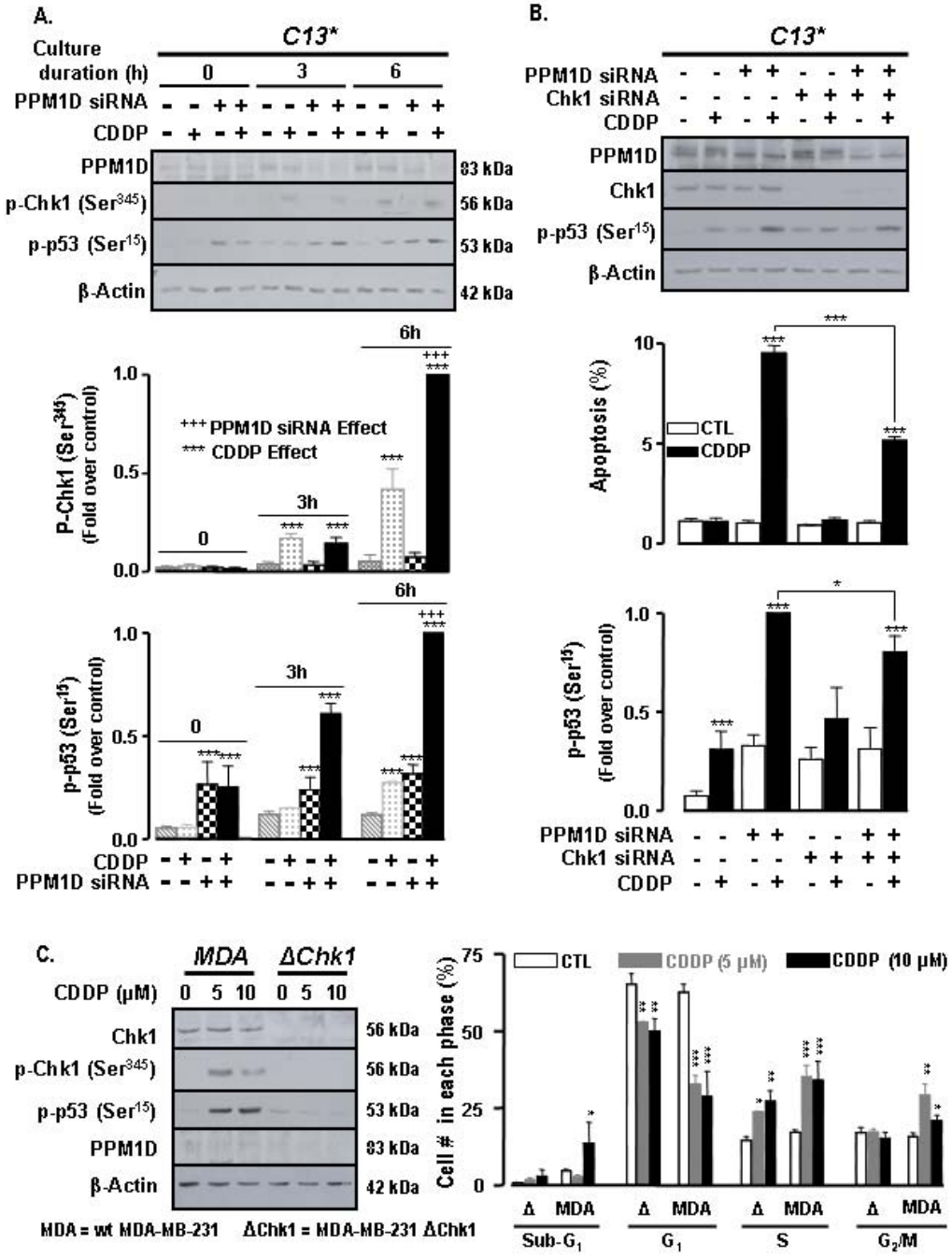

A.
OV2008
C13*
CDDP
PPM1D siRNA (nM)
0 100 200 400
PPM1D
p-Chk1 (Ser345)
p-Chk1 (Ser317)
PARP
CleavedPARP
β-Actin
83 kDa
56 kDa
116 kDa
89 kDa
42 kDa
Apoptosis (%)
CTL
CDDP
PPM1D siRNA (nM)
B.
OV2008
PPM1D cDNA
Vector
p-p53 (Ser15)
53 kDa
DMSO
p-Chk1 (Ser345) content
(Fold over control)
p-p53 (Ser15) content

Figure 3: CDDP-induced, Chk1-mediated apoptosis is attenuated by PPM1D. A) PPM1D knockdown in C13* cells significantly up-regulated phospho-Ser^{345}-Chk1 and phospho-Ser^{15}-p53 contents ($P < 0.001$). C13* cells were incubated with PPM1D siRNA or control siRNA (0-400 nM, 24h), treated with CDDP (0-10 μM, 0-6h) and PPM1D, phospho-Ser^{345}-Chk1, phospho-Ser^{15}-p53, β-Actin contents were assessed (n = 3). B) PPM1D knockdown in C13* cells significantly enhanced CDDP-induced apoptosis ($P < 0.001$), while the concomitant knockdown of PPM1D and Chk1 significantly, but partially, reduced CDDP sensitivity ($P < 0.001$), which was associated with significant reduction in phospho-Ser^{15}-p53 content ($P < 0.05$). C13* cells were treated with Chk1 siRNA (0-10 nM, 48h) and/or PPM1D siRNA (0-400 nM, 24h), treated with CDDP (0-10 μM, 24h) and PPM1D, Chk1, phospho-Ser^{15}-p53, β-Actin contents and apoptosis were assessed (n = 3). C) Chk1-deficient MDA-MB-231ΔChk1breast cancer cells exhibits reduced phospho-Ser^{15}-p53 content in response to CDDP, loss of cell cycle checkpoint, and decreased CDDP sensitivity. MDA-MB-231ΔChk1 and wt-MDA-MB-231 were treated with CDDP (0-10 μM, 24h), fixed and incubated with RNase A (50 μg/ml) and propidium iodide (50 μg/ml), analyzed for cell cycle progression (flow cytometry). Chk1, phospho-Ser^{345}-Chk1, phospho-Ser^{15}-p53, PPM1D, β-Actin contents were assessed (WB) (n = 3).


A.
C13*
Culture duration (h)
0
3
6
PPM1D siRNA
CDDP
PPM1D
p-Chk1 (Ser345)
p-p53 (Ser15)
β-Actin
83 kDa
56 kDa
53 kDa
42 kDa
P-Chk1 (Ser345) (Fold over control)
+++ PPM1D siRNA Effect
*** CDDP Effect
p-p53 (Ser15) (Fold over control)
B.
C13*
Chk1 siRNA
Chk1
Apoptosis (%)
CTL
CDDP
C.
MDA
ΔChk1
CDDP (μM)
0 5 10 0 5 10
Chk1
p-Chk1 (Ser345)
p-p53 (Ser15)
PPM1D
β-Actin
56 kDa
56 kDa
53 kDa
83 kDa
42 kDa
MDA = wt MDA-MB-231 ΔChk1 = MDA-MB-231 ΔChk1
CTL
CDDP (5 μM)
CDDP (10 μM)
Cell # in each phase (%)
Δ MDA
Sub-G1
G1
S
G2/M

Involvement of PPM1D in CDDP-induced, p53-mediated regulation of chemosensitivity in OVCA cells

To investigate whether the sensitization of C13* cells to CDDP following PPM1D knockdown is mediated by Chk1 but ultimately p53-dependent, we knocked down p53 (0-100 nM; 48h), Chk1 (0-10 nM; 48h) and PPM1D (0-400 nM; 24h) in C13* cells followed by CDDP treatment (0-10 μM; 24h). PPM1D knockdown significantly sensitized C13* cells to CDDP-induced apoptosis compared to the control ($P < 0.001$), while the double knockdown of both PPM1D and Chk1 significantly reduced CDDP-induced apoptosis ($P < 0.001$), but did not completely abolish CDDP sensitivity. The triple knockdown of PPM1D, Chk1, and p53 significantly reduced CDDP-induced apoptosis ($P < 0.001$), and restored CDDP resistance in C13* cells (Fig. 4). To further explore this question and demonstrate that the observed phenomena are not cell line-specific, we reconstituted wt-p53 with adenoviral p53 or LacZ as control (0-10 MOI; 4h) in several p53-compromised, CDDP-resistant OVCA cell lines (p53 mutant: A2780cp, OCC-1, OVCAR-3; p53 null: SKOV3) followed by PPM1D down-regulation (0-400 nM; 24h) and CDDP treatment (10 μM; 24h). p53 reconstitution significantly sensitized resistant OVCA cells to CDDP-induced apoptosis ($P < 0.001$), and PPM1D down-regulation further enhanced CDDP sensitivity in A2780cp and OCC-1 cells ($P < 0.01$), as well as OVCAR-3 and SKOV3 cells ($P < 0.05$). However, PPM1D knockdown alone did not increase CDDP sensitivity, and enhanced CDDP sensitivity was only observed in the presence of wt-p53 (Fig. 5).

Figure 4: Involvement of PPM1D in CDDP-induced, p53-mediated regulation of chemosensitivity in OVCA cells. PPM1D knockdown significantly enhanced CDDP-induced apoptosis in C13* cells ($P < 0.001$), while knockdown of PPM1D and Chk1 partially but significantly, reduced CDDP sensitivity ($P < 0.001$), and the concomitant knockdown of PPM1D, Chk1, and p53 abolished CDDP-induced apoptosis ($P < 0.001$). C13* cells were treated with p53 siRNA (0-100 nM, 48h), Chk1 siRNA (0-10 nM, 48h) and/or PPM1D siRNA (0-400 nM, 24h), treated with CDDP (0-10 μM, 24h) and PPM1D, Chk1, phospho-Ser^{345}-Chk1, p53, phospho-Ser^{15}-p53, β-Actin contents and apoptosis were assessed ($n = 3$).

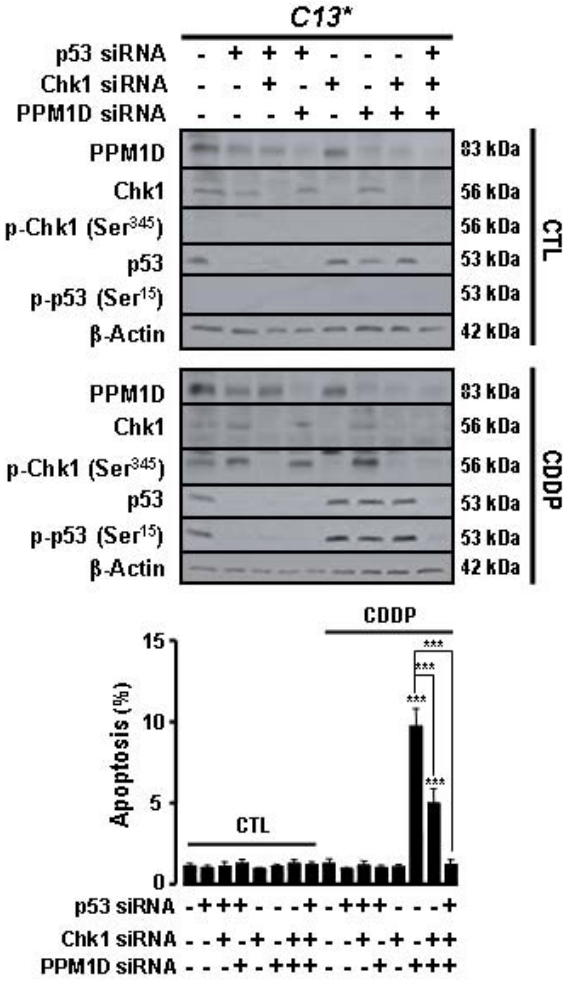
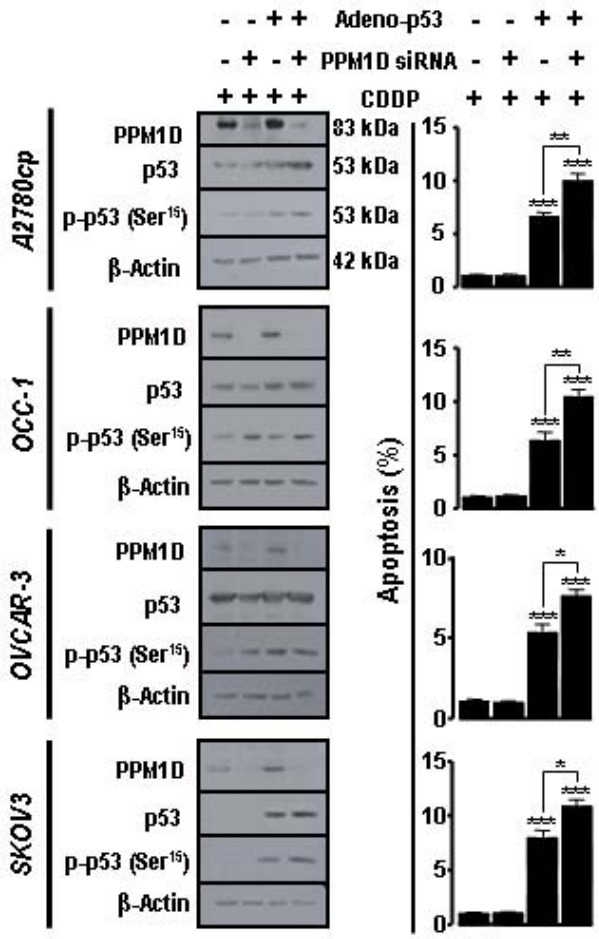

C13*
p53 siRNA - + + + - - - +
Chk1 siRNA - - + - + - + +
PPM1D siRNA - - - + - + + +
PPM1D
Chk1
p-Chk1 (Ser345)
p53
p-p53 (Ser15)
β-Actin
83 kDa
56 kDa
56 kDa
53 kDa
53 kDa
42 kDa
CTL
CDDP
Apoptosis (%)
15
10
5
0
CTL
CDDP
***
p53 siRNA - + + + - - - + - + + + - - - +
Chk1 siRNA - - + - + - + + - - + - + - + +
PPM1D siRNA - - - + - + + + - - - + - + + +

Figure 5: PPM1D down-regulation sensitized chemoresistant p53-compromised OVCA cells only when wt-p53 is reconstituted. PPM1D knockdown did not sensitize A2780cp (p53 mutant), OCC-1 (p53 mutant), OVCAR-3 (p53 mutant), and SKOV3 (p53 null) cells to CDDP-induced apoptosis. However, while wt-p53 reconstitution significantly sensitized resistant OVCA cells to CDDP ($P < 0.001$), combined PPM1D down-regulation and p53 reconstitution significantly enhanced CDDP-induced apoptosis ($P < 0.01$ for A2780cp and OCC-1 cells; $P < 0.05$ for OVCAR-3 and SKOV3), which was associated with an increase in phospho-Ser^{15}-p53 content. A2780cp, OCC-1, OVCAR-3, and SKOV3 cells were infected with adenoviral p53 (0-10 MOI; 5 h), treated with PPM1D siRNA (0-400 nM, 24h) and cultured with CDDP (10 μM, 24h). PPM1D, p53, phospho-Ser^{15}-p53, β-Actin contents and apoptosis were assessed ($n = 3$).


Adeno-p53
PPM1D siRNA
CDDP
A2780cp
OCC-1
OVCAR-3
SKOV3
PPM1D
p53
p-p53 (Ser15)
β-Actin
83 kDa
53 kDa
53 kDa
42 kDa
Apoptosis (%)
15
10
5
0
**
***
*

Effects of p53 and Chk1 down-regulation on CDDP-induced cell cycle regulation in chemoresistant C13* cells

To investigate the cell cycle regulation by CDDP in C13* cells and how this regulation might be altered following p53 or Chk1 down-regulation, we conducted flow cytometric analyses of C13* cells in the presence or absence of CDDP. CDDP treatment significantly decreased the cell population in the G_1 phase, but increased the population in both the S and G_2/M phases. p53 silencing did not alter the CDDP responses. Since p53 activation in response to CDDP is reduced in resistant C13* cells (Fig. 3A), we did not expect any significant changes in the cell cycle in response to p53 down-regulation and following CDDP treatment. However, Chk1 down-regulation caused a significant decrease in S phase ($P < 0.05$) and G_2/M phase ($P < 0.01$) in response to CDDP. None of the treatments altered the cell population in the sub-G_1 phase, indicating no sensitization of resistant C13* cells to CDDP regardless of siRNA treatment (Fig. 6). While CDDP treatment enhanced cell cycle arrest of C13* cells at the S and G_2/M phases, Chk1 down-regulation significantly but partially rescued CDDP-induced cell cycle arrest, suggesting a possible involvement of a compensatory pathway (e.g. ATM/Chk2 pathway) in the DNA damage response (Fig. S 1A).

Figure 6: Effects of p53 and Chk1 down-regulation in chemoresistant C13* cells on CDDP-induced cell cycle regulation. The down-regulation of p53 did not alter CDDP-induced cell cycle regulation in C13* cells, however, Chk1 knockdown significantly but partially decreased CDDP-induced cell cycle arrest at the S and G_2/M phases($P < 0.05$ and $P < 0.01$, respectively). C13* cells were incubated for 24h with CTL siRNA (100 nM), p53 siRNA (100 nM), or Chk1 siRNA (10 nM) and then treated with CDDP (0-10 μM, 24h), fixed and incubated with RNase A (50 μg/ml) and propidium iodide (50 μg/ml), analyzed for cell cycle progression (flow cytometry). Cell cycle phases: B = sub-G_1; C = G_1; D = S; E = G_2/M. (n = 3).

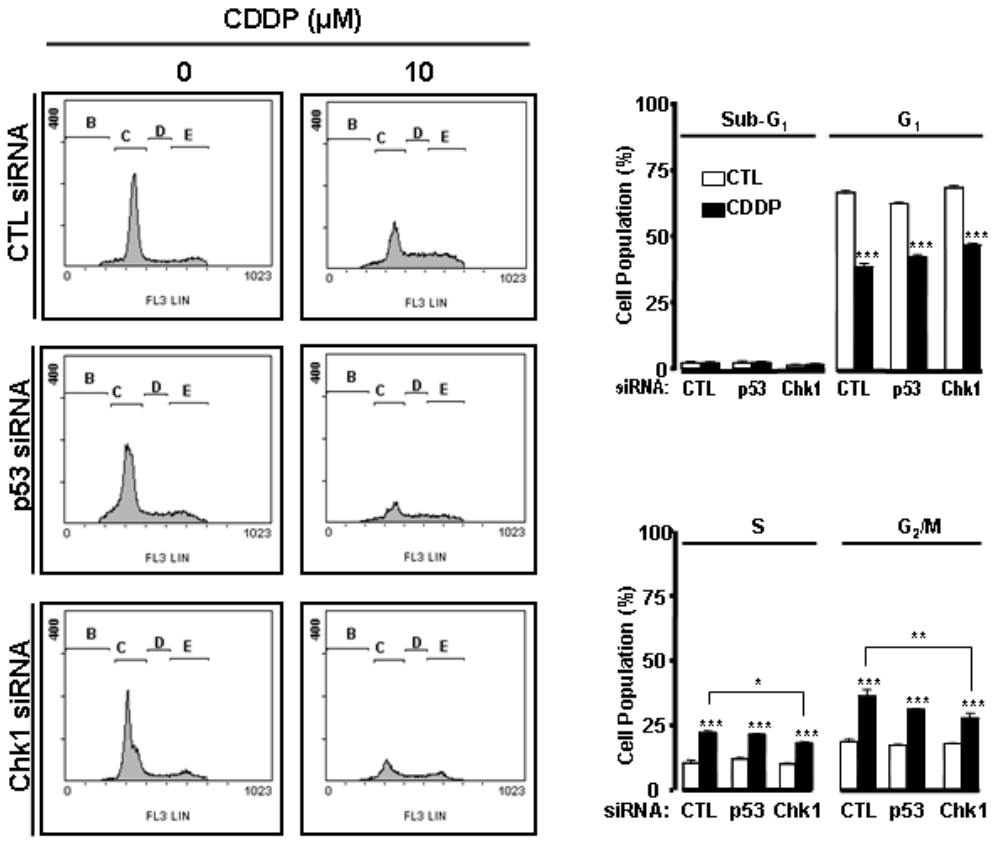
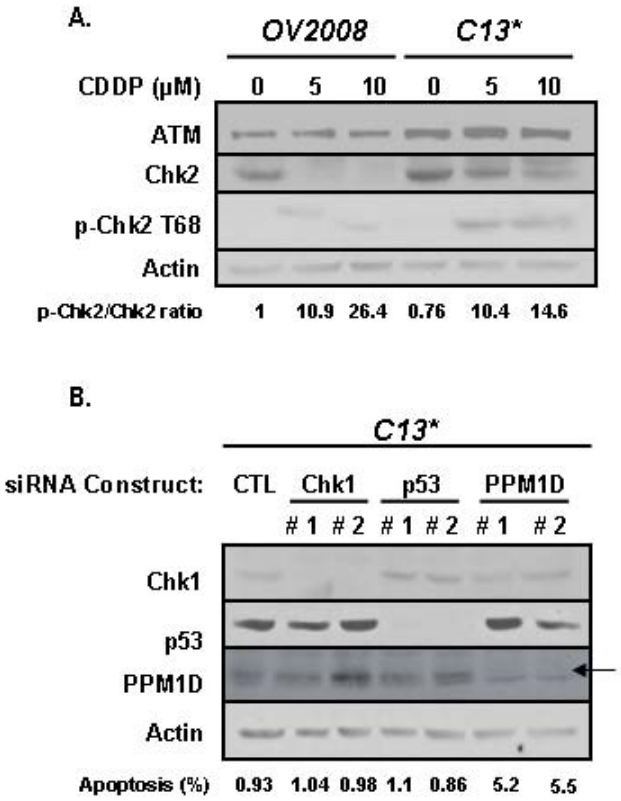

CDDP (μM)
0
10
CTL siRNA
p53 siRNA
Chk1 siRNA
B
C
D
E
FL3 LIN
Sub-G1
G1
CTL
CDDP
Cell Population (%)
siRNA: CTL p53 Chk1 CTL p53 Chk1
S
G2/M

Figure S1: CDDP-induced expression of ATM, Chk2, phospho-Thr^{68}-Chk2 in OV2008 and C13* cells, validation of Chk1, p53, and PPM1D siRNA construct specificities, and confirmation of PPM1D molecular size. A) ATM was expressed in both cell lines, however at a higher level in C13* cells, and its level was not altered by CDDP treatment. Total Chk2 level was significantly reduced in OV2008 by both concentration of CDDP ($P < 0.001$), and there was a concentration-dependent decrease in C13* cells following CDDP treatment ($P < 0.01$). Phospho-Thr^{68}-Chk2 increased in a dose-dependent manner in OV2008 and C13* cells following CDDP challenge ($P < 0.001$). OV2008 and C13* cells were cultured with CDDP (0-10 μM, 24h) and assessed for ATM, Chk2, phospho-Thr68-Chk2, and β-Actin contents by Western blotting ($n = 3$). B) Validation of the specificities of the siRNA constructs used in the study. Chk1 siRNA constructs: # 1: Applied Biosystems (10 nM, 24h); # 2: Qiagen (100 nM, 24h), p53 siRNA constructs: # 1: Cell Signaling (100 nM, 24h); # 2: Qiagen (100 nM, 24h), PPM1D siRNA constructs: # 1: Santa Cruz (100 nM, 24h); # 2: Qiagen (100 nM, 24h); C) Full-length PPM1D protein was confirmed to be 83 kDa, not 62 as previously reported. C13* cells were incubated for 24 h with the following constructs: lane 1: Scramble siRNA; lane 2: PPM1D siRNA (200 nM); lane 3: Empty vector; lane 4: PPM1D cDNA (2 μg). The arrow indicates the band corresponding to PPM1D.


A.
OV2008
C13*
CDDP (μM)
0 5 10 0 5 10
ATM
Chk2
p-Chk2 T68
Actin
p-Chk2/Chk2 ratio
1 10.9 26.4 0.76 10.4 14.6
B.
C13*
siRNA Construct:
CTL
Chk1
p53
PPM1D
1 # 2 # 1 # 2 # 1 # 2
Chk1
p53
PPM1D
Actin
Apoptosis (%)
0.93 1.04 0.98 1.1 0.86 5.2 5.5
C.
C13*
1 2 3 4
95 kDa
72 kDa
55 kDa

Discussion

In the present study, we examined the molecular basis of CDDP resistance using a pair-matched CDDP-sensitive (OV2008) and -resistant (C13*) OVCA cells and p53-compromised CDDP resistant OVCA cells (A2780cp, OCC-1, OVCAR-3, and SKOV3). We have demonstrated (i) the existence of site-specific differences in phospho-Ser-Chk1 content between sensitive and resistant cell lines in response to CDDP; (ii) PPM1D, but not ATR, is important in the regulation of CDDP-induced Chk1 activation and OVCA cell chemosensitivity; (iii) PPM1D down-regulation sensitizes resistant OVCA cells to CDDP primarily by activating Chk1 and p53. Our findings establish for the first time that PPM1D confers CDDP resistance in OVCA cells through attenuating CDDP-induced, Chk1-mediated, p53-dependent apoptosis.

Although a major hurdle in the treatment of human OVCA is chemoresistance, its underlying molecular mechanism is complex and not well understood (Sasaki et al., 2000; Yang et al., 2006; Yang et al., 2008; Fraser et al., 2008; Abedini et al., 2008; Abedini et al., 2010). We have previously shown that CDDP induces apoptosis in sensitive human OVCA cells, but not their resistant counterparts (Sasaki et al., 2000; Abedini et al., 2008; Abedini et al., 2010) and that CDDP-induced apoptosis in sensitive OVCA cells is dependent on p53 activation, particularly p53 phosphorylation on Ser^{15} and Ser^{20} (Fraser et al., 2008). It is believed that CDDP-induced DNA damage leads to activation of the DNA damage pathway, particularly the DNA damage sensor ATR. ATR in turn activates its effector Chk1 through phosphorylation of serine residue 317, which is required to achieve ATR-mediated Ser^{345} phosphorylation (Wilsker et al., 2008). Ser^{345} phosphorylation is an essential phosphorylation site for Chk1 nuclear localization and

retention. If the DNA damage is too serious to be repaired, ATR activates p53 through phosphorylation of Ser^{15} and Ser^{20} directly and indirectly through Chk1 (Graves et al., 2000; Capasso et al., 2002; Jiang et al., 2003; Yazlovitskaya & Persons, 2003; Qu et al., 2005; Ouyang et al., 2009; Rong et al., 2010). It has also been established that the p53-responsive phosphatase PPM1D modulates the action of ATR by direct dephosphorylation of phospho-Ser^{15} of p53 and phospho-Ser^{345} of Chk1 (Choi et al., 2000; Lu et al., 2004; Lu et al., 2005; Yamaguchi et al., 2007; Lu et al., 2008). However, the impact of p53 and Chk1 deactivation by PPM1D on CDDP sensitivity in human OVCA has not been studied. In the present study we provide a novel mechanism of CDDP chemoresistance in human OVCA involving the oncogenic phosphatase PPM1D, Chk1, and p53, furthering the current knowledge and adding to the previously illustrated underlying mechanisms of CDDP resistance in human OVCA.

Chk1 could play both pro- and anti-apoptotic roles in resistant cancer cells, depending on their p53 status (Bunch & Eastman, 1996; Shao et al., 1997; Sugiyama et al., 2000; Playle et al., 2002; Carrassa et al., 2004; Chen et al., 2006; Reinhardt et al., 2007; Wagner & Karnitz, 2009; Pan et al., 2009). Our working hypothesis assumes Chk1 is pro-apoptotic and acts in a p53-dependent manner. The dimorphic nature of Chk1 (pro- or anti-apoptotic) depends primarily on the presence of a functional p53 signaling pathway, a key mediator of its pro-apoptotic activity. However, in the studies where Chk1 inhibition lead to sensitization to several cytotoxic agents, the cell lines used had aberrant p53 signaling due to deficiency or mutation (Bunch & Eastman, 1996; Shao et al., 1997; Sugiyama et al., 2000; Playle et al., 2002; Carrassa et al., 2004; Chen et al., 2006; Reinhardt et al., 2007; Wagner & Karnitz, 2009; Pan et al., 2009). Our data suggest

that the delay in Chk1 activation (Ser^{345} phosphorylation) in response to CDDP observed in resistant OVCA cells, which cannot be substantiated by aberrant upstream signaling (ATR activation and Ser^{317} phosphorylation), is associated with CDDP resistance and synchronized temporally with PPM1D accumulation. Our findings appear not to be in accord with the findings that Ser^{345} phosphorylation in Chk1 is dependent on the activation of Chk1 at the Ser^{317} site in colorectal cancer cells following hydroxyurea treatment or ionizing radiation (Wilsker et al., 2008). Our data show that ATR activation and phospho-Ser^{317}-Chk1 up-regulation appeared not to be associated with increased phospho-Ser^{345}-Chk1 content in resistant C13* cells in response to CDDP. Thus, in the context of CDDP chemoresistance in OVCA cells, PPM1D but not ATR or phospho-Ser^{317}-Chk1, is important in the regulation of phospho-Ser^{345}-Chk1 level. While the reason (s) for these apparent differences is not immediately clear, the possibility that this reflects variations in cancer cell type, genetic background or treatment remains to be investigated.

Moreover, we did not observe any sensitization of resistant OVCA cells to CDDP following siRNA-mediated Chk1 down-regulation, while Chk1 knockout lead to a loss of G_2/M checkpoint in MDA-MB-231ΔChk1 breast cancer cells, a response associated with the virtual absence of p53 activation (Ser^{15} phosphorylation) and decreased CDDP sensitivity. Moreover, Chk1 down-regulation in resistant OVCA cells significantly abrogated CDDP-induced G_2/M cell cycle arrest. Our findings strongly support the hypothesis that Chk1 confers CDDP sensitivity in our cells which contain a functional p53.

Under normal physiological conditions, PPM1D plays a crucial role in restoring cellular homeostasis through promoting cell cycle re-entry following DNA repair by deactivating several DNA damage sensors, stress sensors, and checkpoint proteins, including ATR, ATM, p38 MAPK, Chk1, Chk2, and p53 (Lu et al., 2005; Lu et al., 2008). However, in pathophysiological conditions such as cancer, we believe that PPM1D dampens the DNA damage response through sustained inhibition of its targets, thus promoting cell survival and resistance to chemotherapy. This certainly holds true in our system, since down-regulation of PPM1D sensitized resistant OVCA cells to CDDP and further enhanced CDDP-induced apoptosis in sensitive OVCA cells. Moreover, over-expression of PPM1D in sensitive OVCA cells reduced CDDP sensitivity, which was associated with decreased Chk1 and p53 activation. PPM1D regulates several activators of p53 in the DNA damage pathway other than Chk1. It could be argued that down-regulation of PPM1D in resistant OVCA cells alleviates the inhibition on those kinases along with Chk1, facilitating p53-dependent, CDDP-induced apoptosis. However, our data clearly demonstrate that Chk1 plays a significant part in sensitizing resistant OVCA cells to CDDP following PPM1D down-regulation.

Chk1 activates p53 in response to DNA damage through direct phosphorylation of Ser^{15} and Ser^{20} (Qu et al., 1997; Shieh et al., 2000), thus protecting p53 from MDM2-mediated proteasomal degradation (Honda et al., 1997; Lakin & Jackson, 1999). Our data suggest that p53 activity is critical in mediating Chk1 pro-apoptotic signal. While partial but significant decrease in CDDP sensitivity of wt-p53 resistant OVCA cells following down-regulation of both PPM1D and Chk1, down-regulation of PPM1D, Chk1, and p53 led to complete suppression of CDDP-induced apoptosis in these cells. Moreover, the

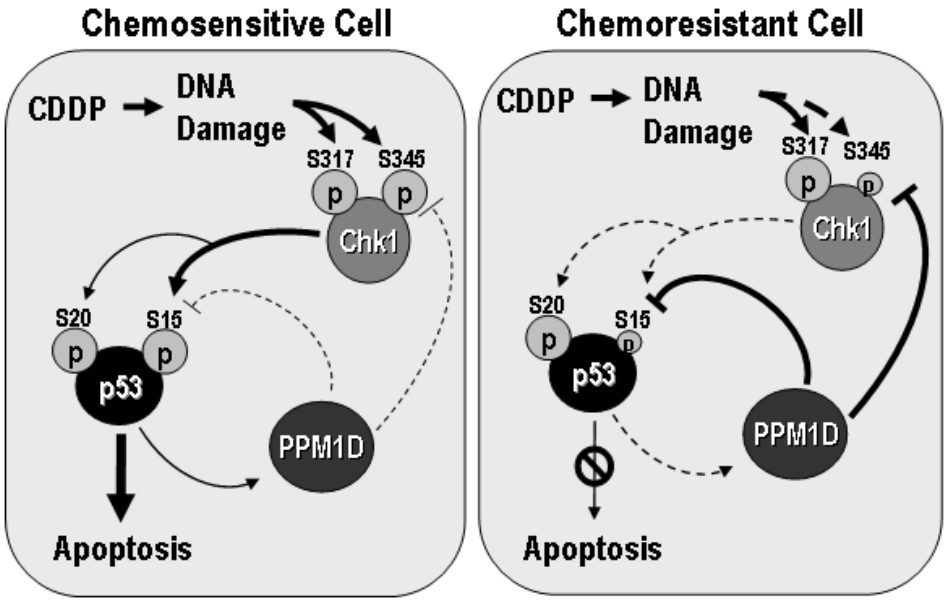
down-regulation of PPM1D in p53-compromised resistant OVCA cells significantly enhanced CDDP-induced apoptosis only following wt-p53 reconstitution. Thus, facilitation of CDDP-induced apoptosis by PPMID down-regulation may involve activation of both Chk1 and p53, and may ultimately be dependent on p53 status.

Both p53 and Chk1 can regulate the cell cycle at different phases, where p53 controls the G_1 checkpoint through the transcriptional regulation of the CDK inhibitor p21 (Shieh et al., 2000; Jurvansuu et al., 2007), and Chk1 regulates the critical transition from the G_2 to the M phase through phosphorylation-dependent inactivation of CDC25 phosphatases (Graves et al., 2000; Busby et al., 2000). Our findings suggest that, p53 activation in response to CDDP is reduced in chemoresistant C13* cells and p53 knockdown failed to significantly influence CDDP-induced cell cycle regulation. In contrast, Chk1 down-regulation significantly but incompletely abrogated CDDP-induced G_2/M cell cycle arrest. Given the complexity of the DNA damage response and its involvement of several proteins, we can not exclude that other DNA damage pathways which converge on the G_2/M checkpoint (i.e. ATM/Chk2 pathway) could be at play. This may explain the partial abrogation of CDDP-induced G_2/M checkpoint following Chk1 knockdown.

In summary, the present studies have established a novel role for PPM1D in the regulation of CDDP-induced apoptosis and chemosensitivity in human OVCA cells. We have demonstrated that PPMID, through dephosphorylation and inactivation of Chk1 and p53, confers CDDP chemoresistance. To facilitate our future investigation on the precise role of PPM1D in CDDP resistance in OVCA cells, we proposed a hypothetical model (Fig. 7). In sensitive OVCA cells, CDDP induces Chk1 and p53 activation, while PPM1D

level is tightly regulated. However, in chemoresistant cells sustained PPM1D expression suppresses the activity of both Chk1 and p53, thereby contributing to CDDP resistance in OVCA cells. It is worthy to mention that the expression of PPM1D in response to CDDP may be induced by factors other than p53. Our data identify PPM1D as a potential novel therapeutic target in the treatment of chemoresistant human OVCA. However, while cell lines provide a convenient way to examine the molecular mechanisms involved in CDDP sensitivity, our findings have to be verified in an *in vivo* model to confirm the validity of this hypothesis.

Figure 7: Hypothetical model illustrating the role of PPM1D in the regulation of CDDP sensitivity in OVCA cells. In chemosensitive cells, PPM1D level is tightly regulated to allow for the activation of Chk1 and p53 and CDDP-induced apoptosis. In chemoresistant cells, however, sustained PPM1D expression hinders the activity of both Chk1 and p53, thereby contributing to CDDP resistance in OVCA cells.


Chemosensitive Cell
CDDP
DNA Damage
S317
S345
p
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Chk1
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p53
PPM1D
Apoptosis
Chemoresistant Cell
CDDP
DNA Damage
S317
S345
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p53
PPM1D
Apoptosis

Materials and Methods

Reagents

CDDP, Hoechst 33258, aprotinin, sodium orthovanadate (Na_3VO_4), phenylmethylsulfonyl fluoride (PMSF), and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (St. Louis, MO, USA). RPMI 1640 and DMEM/F12 culture media, fetal bovine serum (FBS), non-essential amino acids, penicillin, streptomycin, and amphotericin B were from Life Technologies (Carlsbad, CA, USA). Rabbit polyclonal antibodies: anti-Chk1, anti-phospho-Ser^{317}-Chk1, anti-ATR, anti-phospho-Ser^{15}-p53, PARP, and rabbit monoclonal anti-phospho-Ser^{345}-Chk1 antibody were from Cell Signaling Technology (Beverly, CA, USA). Mouse monoclonal p53 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies: anti-pospho-Ser^{428}-ATR, anti-PPM1D, and mouse monoclonal antibodies were: anti-GAPDH from Abcam (Cambridge, MA, USA) and anti-β-Actin from Cedarlane Laboratories (Burlington, ON, Canada). Goat anti-mouse and goat anti-rabbit secondary antibodies were from Bio-Rad Laboratories (Hercules, CA, USA). Control siRNA was from Dharmacon (Lafayette, CO, USA). siRNA constructs were: Chk1; Applied Biosystems (Foster City, CA, USA) and Qiagen (Valencia, CA, USA), PPM1D; Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Qiagen (Valencia, CA, USA), p53; Cell Signaling Technology (Beverley, CA, USA) and Qiagen (Valencia, CA, USA). pCMV6-XL5 vector containing wt-PPM1D cDNA clone and empty pCMV6-XL5 vector were from Origene (Rockville, MD, USA). Propidium iodide was from Roche Applied Sciences (Penzberg, Germany). Lipofectamine, Lipofectamine 2000, Lipofectamine Plus

transfection reagents, RNase A, and *N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine (TEMED) were from Invitrogen (Carlsbad, CA, USA). wt-p53 and LacZ adenovirus were synthesized at the University of Ottawa Adenoviral Core Facility (Ottawa, ON, Canada).

Cell lines and culture

CDDP sensitive [OV2008 (wt-p53)] and resistant [C13* (wt-p53), A2780cp (p53 mutant), OCC-1 (p53 mutant), OVCAR-3 (p53 mutant), and SKOV3 (p53 null)] human OVCA cell lines, gifts from Drs. Rakesh Goel and Barbara Vanderhyden (Ottawa Regional Cancer Center, Ottawa, ON, Canada), were cultured as previously reported (Abedini et al., 2008; Abedini et al., 2010). OV2008 cells and their resistant counterparts C13* cells are of ovarian endometrioid adenocarcinoma origin with squamous differentiation. A2780cp and OCC-1 cells are from undifferentiated ovarian carcinoma tumors. SKOV3 cells are of clear cell carcinoma origin (Shaw et al., 2004). None of the cells used in this study over-express PPM1D. Human breast cancer cell lines (wt-MDA-MB-231 and MDA-MB-231ΔChk1; generously provided by Dr. Allan Eastman, Dartmouth Medical School, Hanover, NH, USA) were cultured as previously reported (Zhang et al., 2008). Following the indicated treatments, the cells were harvested for analysis.

Protein extraction and Western blot analysis

The procedures were performed as previously described (Abedini et al., 2008; Abedini et al., 2010). Unless indicated otherwise, membranes were incubated overnight at 4°C, with anti- Chk1 (1:500), phospho-Ser^{317}-Chk1 (1:1000), phospho-Ser^{345}-Chk1

(1:500), ATR (1:500), phospho-Ser^{428}-ATR (1:500), PPM1D (1:500), p53 (1:5000), phospho-Ser^{15}-p53 (1:1000), and 1h at room temperature for anti-GAPDH (1:10,000), β–Actin (1:10,000), vinculin (1:10,000), and HRP-conjugated rabbit or mouse secondary antibodies (1:2000-1:10,000), and band densities were analyzed (Scion Image software; Scion Corporation Inc., Frederick, MD, USA).

Hoechst 33258 staining

The procedure was performed as previously described (Abedini et al., 2008; Abedini et al., 2010).

RNA Interference

Cells were transfected with PPM1D siRNA (0-400 nM; 24h), Chk1 siRNA (10 nM; 48h), p53 siRNA (100 nM; 48h), or control siRNA (0-510 nM; 48h) were treated with CDDP (0-10 μM; 24h) as previously described (Abedini et al., 2008; Abedini et al., 2010), and harvested for further analysis.

Transient cDNA transfection

OV2008 were transfected with 1 μg of PPM1D cDNA amplified from wt-PPM1D cDNA clone supplied in pCMV6-XL5 expression vector, as previously described (Yang et al., 2006). Empty pCMV6-XL5 vector served as a control. Following transfection (24h), the cells were treated with 10 μM CDDP or vehicle (DMSO) and harvested for analyses.

Flow Cytometry

Cells (MDA-MB-231, MDA-MB-231ΔChk1 and C13* cells) were cultured in 60 mm dishes overnight, and then treated accordingly. Cells were washed with chilled 1X PBS and centrifuged (200 x *g*, 5 min). They were fixed by 70% ethanol on ice for 30 min and washed with chilled 1X PBS, centrifuged, and then suspended in 200 µl of 1X PBS. The cells were incubated in the dark at room temperature for 30 min with 10 µl of RNase A (50 µg/ml) and propidium iodide (50 µg/ml). Cell cycle analysis was performed using Coulter Epics XL/MCL Flow Cytometer Centre from Beckman Coulter Inc. (Brea, CA, USA) according to the manufacturer's instructions.

Adenoviral infection

A2780cp, OCC-1, OVCAR-3, and SKOV3 cells were infected with adenoviral wt-p53 (MOI = 10, 5h) or LacZ (as control) as previously described (Abedini et al., 2008; Abedini et al., 2010).

Statistical Analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was carried out by one-way, two-way or three-way ANOVA (where appropriate) using PRISM software (Versions 3.0 and 5.0; GraphPad, San Diego, CA, USA). Differences between multiple experimental groups were determined by the Bonferroni post-hoc test. Statistical significance was inferred at $P < 0.05$.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Chapter 4 – Manuscript in preparation

Akt confers cisplatin chemoresistance in human gynecological carcinoma cells by modulating PPM1D stability.

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Running Title: Akt confers cancer cell cisplatin resistance through PPM1D

Key words: Cisplatin chemoresistance, Apoptosis, Ovarian cancer, Akt, PPM1D

Contribution of Co-Authors.

Figures 1, 2, 3 and 8, as well as the preparation of the manuscript were carried out by Ahmed Y. Ali. Figures 4 and 5 were completed by Dr. Ji-Young Kim. Figures 6, 7, and Table 1 were completed by Jean-François Pelletier under the supervision of Dr. Dimcho R. Bachvarov. All studies were conducted under the supervision of Dr. Benjamin K. Tsang.

Abstract

Ovarian cancer (OVCA) and cervical cancer (CECA) are lethal gynecological malignancies. Platinum derivatives such as cisplatin (CDDP) are the first line chemotherapeutics and their resistance is a major hurdle to successful treatment. Understanding the molecular dysregulation underlying chemoresistance is important in developing rational therapeutic strategies. We have established that Protein Phosphatase Magnesium-dependent 1 D (PPM1D) confers CDDP resistance in gynecological cancer cells by deactivating p53. However, whether CDDP regulates intra-cellular PPM1D localization and whether this regulation is different between chemosensitive and chemoresistant cancer cells is unknown. Moreover, whether Akt regulates PPM1D in the context of CDDP resistance has not been studied. The objective of this study was to determine the role of PPM1D in gynecological cancer cell chemoresistance and its regulation by Akt. To this end, we have demonstrated that: (a) CDDP induced PPM1D down-regulation through proteasomal degradation in sensitive CECA cells; (b) CDDP induced PPM1D nuclear localization in resistant CECA cells, and nuclear exclusion in sensitive CECA cells and OVCA xenografts; (c) Over-expression of active Akt in sensitive CECA cells stabilized PPM1D content through inhibition of CDDP-induced PPM1D down-regulation; (d) Inhibition of Akt activity in resistant OVCA cells leads to decrease PPM1D stability and CDDP-induced down-regulation in resistant CECA cells; and (e) PPM1D is highly expressed in human ovarian tumor subtypes and in a tissue microarray panel of human ovarian tumors. In conclusion, we have established that PPM1D plays an important role in promoting CDDP resistance and as a novel

downstream target of Akt, PPM1D mediates its action in conferring CDDP resistance in gynecological cancer cells.

Introduction

Ovarian cancer (OVCA) and cervical cancer (CECA) are the most lethal gynecological malignancies. Although OVCA ranks first in the number of deaths each year, due primarily to its late diagnosis and the development of chemoresistance[1], CECA is more frequent and less deadly due to early detection. Surgical de-bulking of the tumor mass followed by adjuvant chemotherapy is the conventional course of therapy for OVCA, whereas a combination of radiation and chemotherapy is the preferred treatment regimen for CECA. Platinum derivatives, including Cisplatin (CDDP; *cis*-diamminedichloroplatinum) in combination with paclitaxel, are first-line chemotherapeutic agents in the treatment of these gynecologic cancers. CDDP induces apoptosis by creating inter- and intra-strand DNA adducts through irreversible intercalation, thereby inducing both the DNA damage and the apoptotic responses[2]. The majority of patients respond to chemotherapy at first, however recurrence of the disease is a common event and tumors are usually more aggressive, metastasize to secondary target tissues, and acquire resistance to conventional chemotherapeutics. Drug resistance is a multi-factorial problem and is characterized by gene mutations, increased DNA repair, reduced drug efficacy, and enhanced drug clearance and detoxification. However, the underlying molecular mechanism of chemoresistance is complex and not well understood. We have previously shown that CDDP induces apoptosis only in sensitive CECA cells, but not their resistant counterparts[2-8].

Protein Phosphatase Magnesium-dependent 1 D (PPM1D), also known as wild-type p53 inducible phosphatase, is a member of the magnesium/manganese-dependent sub-family of PPM1 phosphatases, belonging to the larger family of type 2C

phosphatases. It was first identified as a phosphatase induced by ultraviolet (UV) and ionizing radiation (IR) in a p53-dependent manner[9-11]. PPM1D expression has also been shown to be regulated by several other transcription factors including estrogen receptor α (ERα)[12], cyclic AMP response element binding protein (CREB)[11], E2F1[13,] NF-κB[14], and c-Jun[15]. PPM1D has been shown to preferentially targets phosphoproteins containing SQ/TQ or TXY motifs for dephosphorylation[10,16-18]. We have previously demonstrated that PPM1D is differentially regulated by CDDP between chemosensitive and chemoresistant CECA cells, whereby PPM1D was down-regulated by CDDP in sensitive but not in resistant cells[8]. However, the mechanism by which PPM1D is down-regulated in response to CDDP in these cancer cells is unclear.

A main function of PPM1D is the inhibition of both the DNA damage and apoptotic responses following a DNA-damaging insult. This is achieved by the regulation of the ataxia talengiectasia mutated (ATM)/ATM and Rad3 related (ATR) and p53 pathways. PPM1D directly dephosphorylates ATM[19,20] and its downstream target checkpoint kinase 2 (Chk2; Thr^{68})[21-23,] significantly reducing their activities. Although PPM1D has not been shown to target ATR directly, it does dephosphorylate downstream targets of ATR. We have previously demonstrated that PPM1D directly dephosphorylates checkpoint kinase 1 (Chk1) at its ATR-dependent phosphorylation site (Ser^{345})[8]. Through these means, PPM1D effectively promotes cell cycle progression to inhibit DNA damage repair. PPM1D regulates apoptosis primarily by targeting the tumor suppressor p53 both directly and indirectly. We and others have shown that PPM1D directly dephosphorylates p53 (Ser^{15})[8,16], significantly reducing its pro-apoptotic activity. Furthermore, PPM1D dephosphorylates murine double minute 2 (MDM2) and 4 (MDM4), enhancing their

stability, interaction and the cooperative ubiquitination of p53 and its subsequent proteasomal degradation[24-27].

PPM1D protein contains two nuclear localization signals[28] and is assumed to be a strictly nuclear phosphatase since all the known targets of PPM1D to date are nuclear proteins. It is safe to assume then that PPM1D cytoplasmic sequestration and nuclear exclusion will significantly attenuate PPM1D phosphatase activity. However, whether CDDP regulates PPM1D intra-cellular localization and if this regulation is different between chemosensitive and chemoresistant cancer cells, has yet to be investigated.

Over-expression and/or activation of the phosphoinositide-3 kinase (PI3K)/Akt survival pathway have been associated with carcinogenesis in several tissue types[29-33]. One of the main downstream effectors of PI3K is the potent oncogenic serine/threonine kinase Akt, also known as protein kinase B (PKB). Akt is activated by phosphorylation of Thr^{308} by the phosphoinositide-dependent kinase 1 (PDK1)[34,35], and Ser^{473} by the mammalian target of rapamycin complex 2 (mTORC2)[36,37]. Activated Akt plays an important role in the regulation of several important molecular pathways, including those of cell survival, proliferation and apoptosis. We have shown that Akt activation enhances the survival of OVCA cells and promotes chemoresistance through attenuating p53 pro-apoptotic signaling[4,6,38-40]. However, whether Akt regulates PPM1D in the context of CDDP resistance has not been studied.

In the present study we have examined the mechanism of PPM1D processing in OVCA and CECA cells, PPM1D intra-cellular localization *in vitro* and *in vivo* in response to CDDP, and finally we have demonstrated the role Akt plays in regulating PPMID stability and how this regulation influences CDDP sensitivity. We have

demonstrated for the first time that intra-cellular PPM1D localization is differentially regulated by CDDP between sensitive and resistant cancer cell lines and tumors. Furthermore, PPM1D is a downstream target of Akt and both of them cooperatively regulate CDDP sensitivity in gynecological cancer cells.

Results

Influence of CDDP on PPM1D content in chemosensitive and resistant OVCA cells.

We have previously reported that CDDP significantly decreased PPM1D contents in sensitive cells but up-regulated this response in their resistant counterparts[8]. However, the exact molecular mechanism involved in this process remained unknown. To test the notion that CDDP-induced PPM1D down-regulation in chemosensitive cells is a consequence of increased degradation in addition to or instead of decreased synthesis, sensitive (A2780s) cells and their resistant counterparts (A2780cp) were pre-treated with cycloheximide (CHX) to block PPM1D synthesis (2 μg/ml, 2 h), followed by CDDP treatment (10 μM, 3-24 h). PPM1D content decreased in a time-dependent manner in sensitive but not in resistant OVCA cells (Fig. 1A), suggesting that CDDP-induced PPM1D down-regulation in sensitive cells was due to enhanced degradation.

Akt confers resistance by promoting PPM1D stability

To investigate whether Akt regulates PPM1D content in OVCA cells, resistant A2780cp and OCC-1 OVCA cells were infected with adeno-HA-DN-Akt or adeno-LacZ (control) [MOI = 80, 24h] to down-regulate Akt activity, and then treated with CDDP (10 μM, 24 h). PPM1D content and mRNA abundance were enhanced by CDDP in both

resistant cell lines. However, the down-regulation of Akt activity significantly decreased CDDP-induced PPM1D protein content ($p < 0.001$) but not PPM1D mRNA abundance, suggesting that Akt stabilizes PPM1D through regulating it at the protein level rather than controlling its transcription (Fig. 1B).

To investigate if Akt down-regulation decreases PPM1D protein stability and reduces its half-life in OVCA cells, chemoresistant A2780cp and OCC-1 cells were infected with Adeno-HA-DN-Akt or LacZ (MOI = 80, 24 h) and then treated with CHX to inhibit PPM1D synthesis (2 μg/ml, 2 h). CHX treatment significantly reduced basal PPM1D content in A2780cp and OCC-1 cells ($p < 0.01$). While Akt down-regulation alone significantly reduced PPM1D content ($p < 0.01$, A2780cp; $p < 0.05$, OCC-1), the addition of CHX caused a further decrease in PPM1D content ($p < 0.001$), suggesting that Akt stabilizes PPM1D content in resistant OVCA cells (Fig. 1C). To further elucidate the effects of Akt down-regulation on PPM1D half-life in chemoresistant OVCA cells, we performed a pulse-chase analysis in A2780cp cells (Fig. 1D). As per our earlier observations, basal PPM1D content was significantly reduced by the down-regulation of Akt activity, which accelerated its degradation and significantly reduced its half-life when compared to the control, suggesting that Akt is involved in maintaining PPM1D stability and content in chemoresistant OVCA cells (Fig. 1D).

To investigate the commonality between chemosensitive and chemoresistant OVCA and CECA cells in their response to CDDP with regards to PPM1D expression, sensitive (OV2008) and resistant (C13*) CECA cells and sensitive (A2780s) and resistant (A2780cp) OVCA cells were cultured with CDDP (10 μM, 24 h). While CDDP down-regulated PPM1D content in both chemosensitive OVCA and CECA cells, PPM1D was

up-regulated in their chemoresistant counterparts (Fig. 2A), highlighting the similarity in CDDP-induced PPM1D regulation in OVCA and CECA cells

To confirm that Akt regulates PPM1D content, chemosensitive CECA cells (OV2008) were infected with a constitutively activated Akt2 (adeno-HA-AAkt2; meristoylated) or a control (adeno-LacZ) (MOI = 40; 24 h) and then cultured with CDDP (10 μM, 24 h). While CDDP treatment significantly down-regulated PPM1D content as expected ($p < 0.05$), over-expression of activated Akt2 rescued PPM1D from CDDP-induced down-regulation (Fig. 2B), suggesting that Akt stabilizes PPM1D protein content in CECA and OVCA cells.

We have previously demonstrated that CDDP treatment significantly down-regulated PPM1D content in sensitive OV2008 cells[8]. To investigate the mechanism of CDDP-induced PPM1D down-regulation in chemosensitive OVCA cells, we have explored the possible involvement of the proteasomal pathway. Chemosensitive OV2008 cells were pre-treated with the proteasomal inhibitor epoxomicin (12.5 nM; 1h) or the vehicle (DMSO) and then challenged with CDDP (10 μM; 24 h). The administration of CDDP resulted in a marked decrease in PPM1D content as has been previously observed (Fig. 2C), while the pre-treatment with epoxomicin resulted in the rescue of PPM1D content from CDDP-induced down-regulation, suggesting that PPM1D is processed through the proteasomal pathway in sensitive cancer cells in response to CDDP treatment as previously reported[41,42].

Figure 1: Akt regulates PPM1D expression level and increases its content in chemoresistant cells challenged with CDDP. ***(A) CDDP decreases PPM1D content in sensitive but not resistant OVCA cells.*** A2780cp and A2780s cells were pre-treated with CHX (2 μg/ml, 2 h), were incubated with CDDP (10 μM) and then harvested at the indicated time points (n = 3). ***(B) Akt down-regulation in resistant OVCA cells significantly reduced CDDP-induced PPM1D content, but not mRNA abundance.*** A2780cp and OCC-1 cells were incubated with Adeno-HA-DN-Akt or Adeno-LacZ (MOI = 80, 24 h), treated with CDDP (0-10 μM, 24 h; n = 3). ***(C) Akt stabilizes PPM1D content in resistant OVCA cells.*** A2780cp and OCC-1 cells were pre-treated with CHX (2 μg/ml, 2 h) and incubated then with Adeno- HA-DN-Akt or LacZ (MOI = 80, 24 h) and harvested at 24h (n = 3). ***(D) PPM1D half-life in resistant OVCA cells significantly decreased in response to the down-regulation of Akt activity.*** Chemoresistant A2780cp cells were infected with adeno-DN-Akt (80 MOI; 24h) or adeno-LacZ as a control. Cells were then pulsed with 3H-leucine (50 μCi/ml; 1h). PPM1D was immunoprecipitated and radioactively labeled PPM1D was detected by liquid scintillation counting (n = 3). To calculate PPM1D half-life, normalized data were used to calculate the time at which 50% of radioactively-labeled PPM1D has been degraded. PPM1D immunoprecipitation was validated by Western blotting.

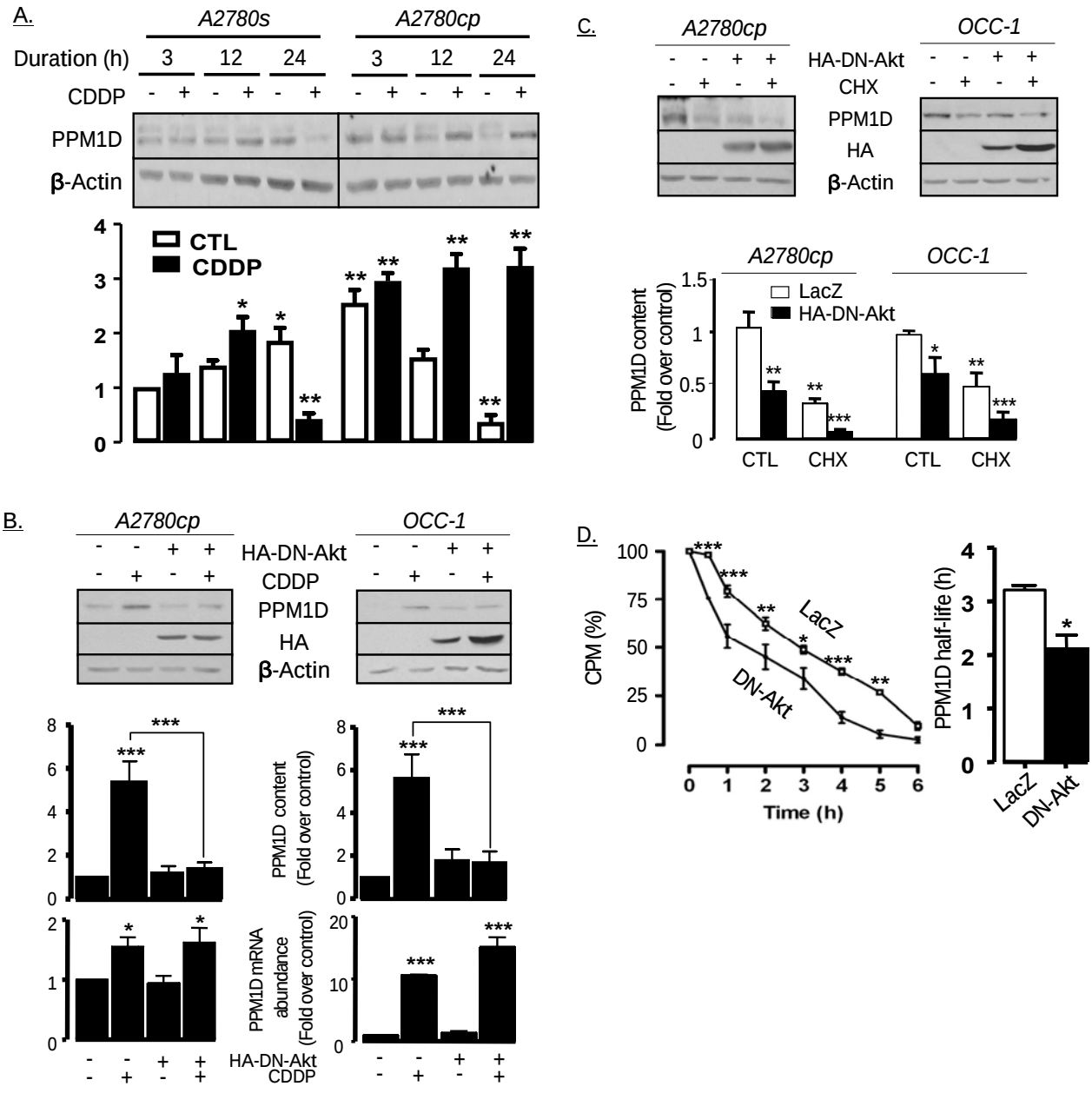
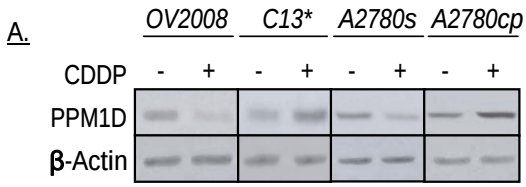
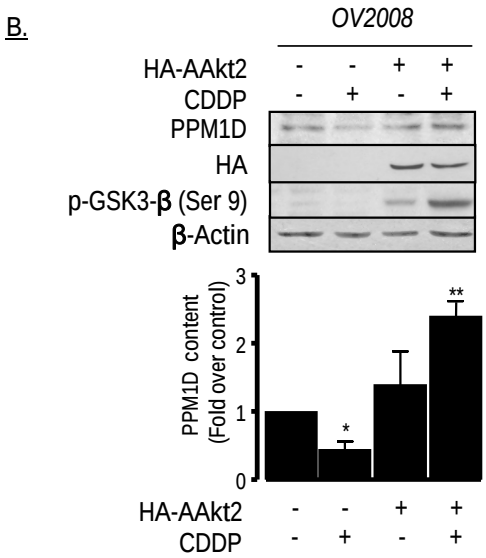
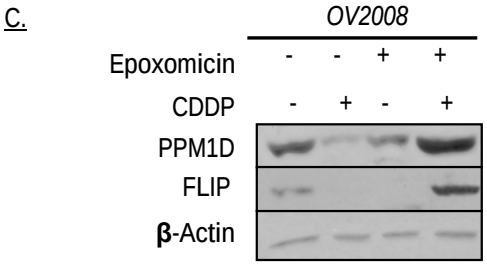

A.
A2780s
A2780cp
Duration (h)
3
12
24
CDDP
PPM1D
β-Actin
CTL
CDDP
PPM1D content (Fold over control)
B.
HA-DN-Akt
HA
PPM1D mRNA abundance (Fold over control)
C.
CHX
OCC-1
LacZ
CTL
CHX
D.
CPM (%)
Time (h)
LacZ
DN-Akt
PPM1D half-life (h)

Figure 2: Regulation of PPM1D content in OVCA and CECA cells in response to CDDP.

(A) CDDP exhibited common response in PPM1D regulation in OVCA and CECA cells. Sensitive (OV2008) and resistant (C13*) CECA cells and sensitive (A2780s) and resistant (A2780cp) OVCA cells were cultured with CDDP (10 μM, 24 h; n = 3). ***(B) Akt activation attenuated CDDP-induced PPM1D down-regulation in chemosensitive CECA cells.*** OV2008 cells were infected with Adeno-HA-AAkt2 (myristoylated; MOI = 40; 24 h) and then treated with CDDP (10 μM; 24 h; n = 3). Phospho-GSK3-β (Ser 9), a known phosphorylation target of Akt, served as a positive control for Akt activity. ***(C) CDDP decreases PPM1D content in chemosensitive CECA cells through proteasomal degradation.*** OV2008 cells were incubated with CDDP (10 μM; 24h) in the presence or absence of epoxomicin (12.5 nM, 1h). FLIP served as positive control.


A.
OV2008
C13*
A2780s
A2780cp
CDDP - + - + - + - +
PPM1D
β-Actin


B.
OV2008
HA-AAkt2 - - + +
CDDP - + - +
PPM1D
HA
p-GSK3-β (Ser 9)
β-Actin
PPM1D content (Fold over control)
0
1
2
3
*
**
HA-AAkt2 - - + +
CDDP - + - +


C.
OV2008
Epoxomicin - - + +
CDDP - + - +
PPM1D
FLIP
β-Actin

Differential regulation of intra-cellular PPM1D localization in chemosensitive and chemoresistant CECA cells and in OVCA tumors.

To investigate intra-cellular PPM1D localization in chemosensitive and chemoresistant CECA cells in response to CDDP, OV2008 and C13* cells were cultured for 24 h in the absence or presence of CDDP (10 μM) and were immunostained for PPM1D. In sensitive OV2008 cells at rest, PPM1D was distributed diffusely and evenly throughout the cells. However, CDDP treatment led to significant punctuated PPM1D cytoplasmic localization ($p < 0.01$) and PPM1D nuclear exclusion ($p < 0.05$). Similarly, PPM1D exhibited diffused localization with no significant clustering in resistant C13* in the absence of CDDP. In contrast to the sensitive cells, however, PPM1D exhibited significant nuclear localization in resistant C13* cells following CDDP treatment (Fig. 3; $p < 0.001$).

PPM1D regulates p53 and Chk1 activation in the nucleus[8,24,43]. However, whether and how the function of PPM1D is regulated by its intra-cellular localization is not known. To investigate PPM1D expression and nuclear localization, we have performed a xenograft model of chemosensitive A2780s cells. In line with our earlier *in vitro* observations, CDDP down-regulated PPM1D expression and significantly decreased nuclear PPM1D localization (Fig. 4A; $p < 0.001$), suggesting the decreased availability at the nucleus may be responsible for the CDDP responsiveness of the cells. Moreover, CDDP treatment led to a decrease in tumor volume (Fig. 4B), which was associated with a significant decrease in the cellular mitotic rate (Fig. 4C; $p < 0.05$) and a significant enhancement of apoptosis (Fig. 4Cand D; $p < 0.05$).

Figure 3: CDDP differentially regulates PPM1D intra-cellular localization *in vitro*. *CDDP facilitates PPM1D cytoplasmic localization (punctated) and nuclear exclusion in sensitive cells (OV2008) but induces nuclear PPM1D accumulation in their resistant counterpart (C13*).* OV2008 and C13* cells were cultured for 24 h in the absence and presence of CDDP (10 μM) and were immunostained for PPM1D (400X magnification; n = 3). The first (OV2008) and the third row (C13*) of images were exposed under the same conditions to convey the relative abundance of PPM1D in each cell line, while the second row (OV2008*) of images was overexposed to illustrate PPM1D intra-cellular localization.

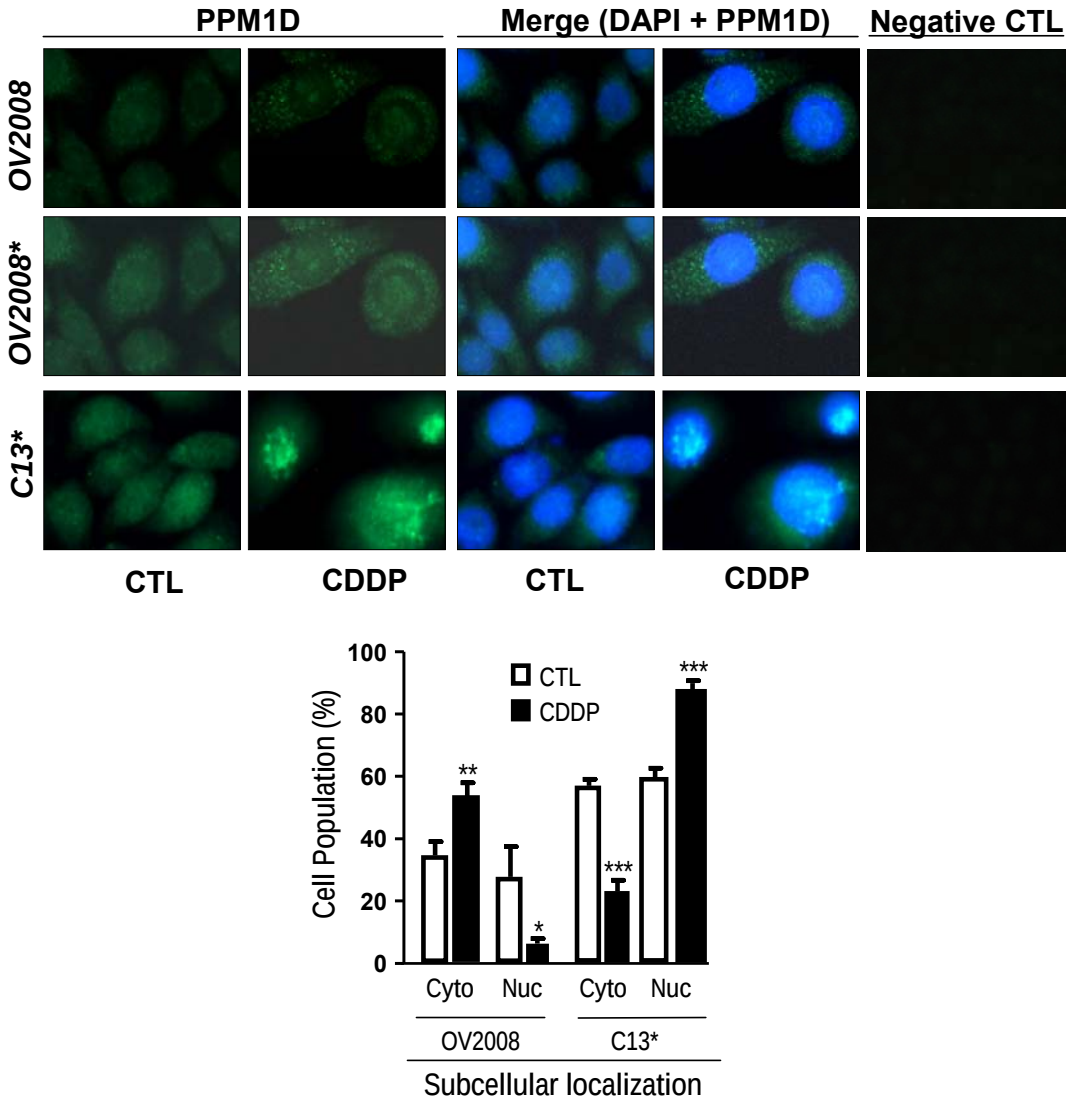
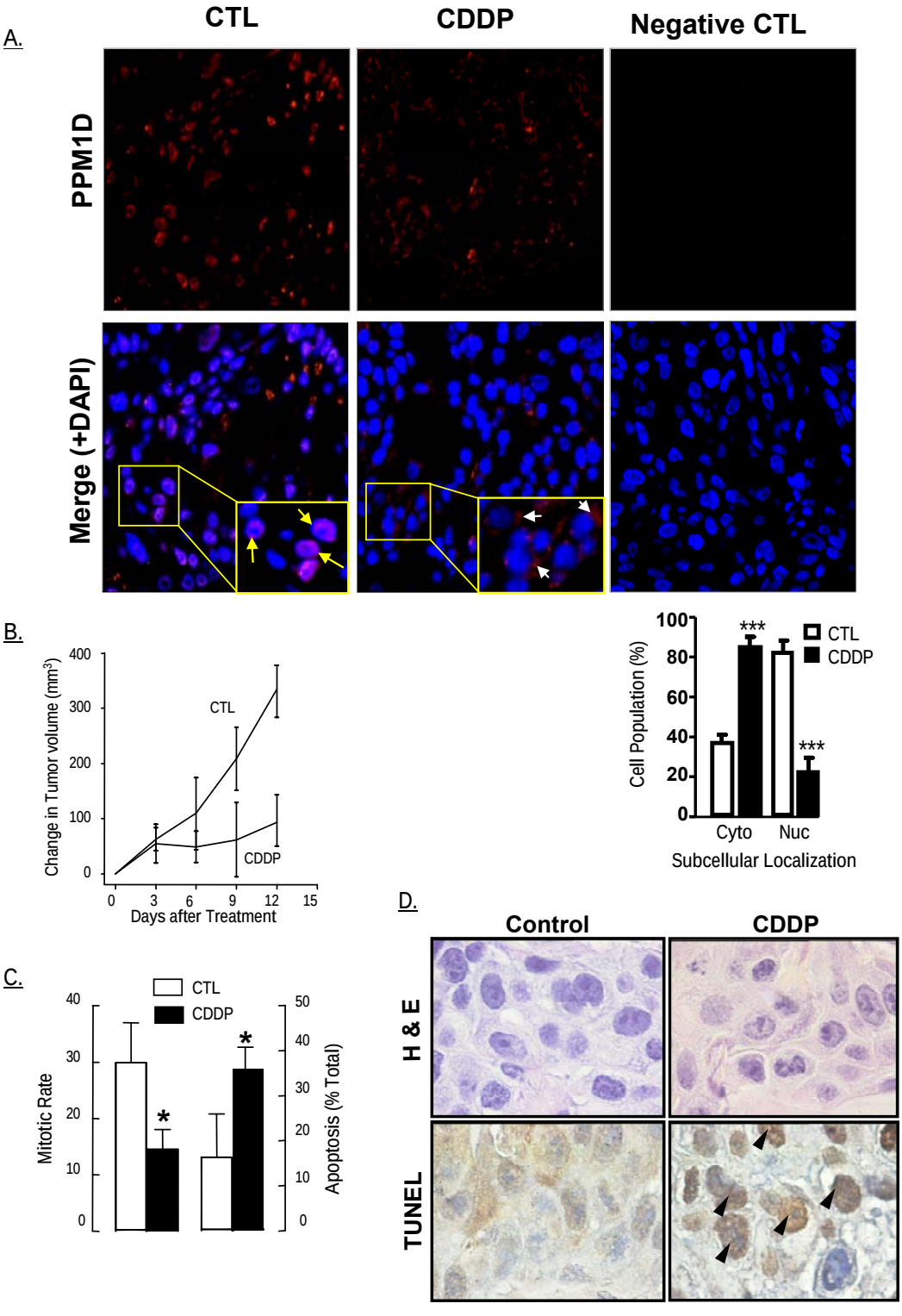

PPM1D
Merge (DAPI + PPM1D)
Negative CTL
OV2008
OV2008*
C13*
CTL
CDDP
CTL
CDDP
Cell Population (%)
100
80
60
40
20
0
CTL
CDDP
**
*
***
***
Cyto
Nuc
Cyto
Nuc
OV2008
C13*
Subcellular localization

Figure 4: CDDP differentially regulates PPM1D intra-cellular localization *in vivo*.

(A) CDDP decreases PPM1D content in a xenograft model of chemosensitive A2780s cells and leads to its nuclear exclusion. Primary antibody (anti-PPM1D) and normal rabbit IgG (negative control; 1:50, 24 h) were used with the secondary antibody (rabbit IgG-Alexa 594, 1:100, 2 h). Yellow arrows indicate nuclear PPM1D and white arrows indicate cytoplasmic PPM1D. ***(B) CDDP-treated mice exhibited decreased tumor volume.*** Change in tumor volume = volume at day after treatment – volume at day 0. ***(C) Tumors of CDDP-treated mice exhibited decreased mitotic activity and increased apoptosis.*** The mitotic rate is defined as the total number of mitotic cells (p-histone 3 positive) in magnification (400X) fields in each sample. Apoptosis is expressed as the percentage of total cells that were TUNEL-positive. > 500 cells were counted in tumors from each mouse (mean ± SD). ***(D)*** Representative H & E and TUNEL staining.


A.
CTL
CDDP
Negative CTL
PPM1D
Merge (+DAPI)
B.
Change in Tumor volume (mm3)
CTL
CDDP
Days after Treatment
Cell Population (%)
CTL
CDDP
***
***
Cyto
Nuc
Subcellular Localization
C.
CTL
CDDP
Mitotic Rate
Apoptosis (% Total)
*
*
D.
Control
CDDP
H & E
TUNEL

Regulation of PPM1D expression in ovarian tumors in vivo

We have examined a representative panel of human ovarian tumors from several histological subtypes (serous, endometrioid, and clear cell). PPM1D exhibited high level of expression in all the subtypes of the human ovarian carcinoma examined (Fig. 5). Moreover, the expression of PPM1D appeared higher in malignant glandular region than stromal region in human ovarian serous, endometrioid, and clear cell carcinomas, suggesting the involvement of PPM1D in ovarian malignancy.

Furthermore, assessment of PPM1D expression in a panel of human serous ovarian tumors by a tissue microarray indicates that while PPM1D expression was significantly lower in normal non-ovarian tissue ($p < 0.001$) compared to normal ovarian tissue, PPM1D was significantly expressed in both primary serous tumors ($p < 0.05$) and metastatic serous tumors ($p < 0.01$) compared to normal ovarian tissue and low malignant potential tumor samples (Fig. 6), demonstrating a positive relationship between PPM1D expression level and tumor aggression. However, we did not observe any significant differences between the levels of PPM1D expression and patient progression-free survival (PFS; Fig. 7), which do not correlate with our *in vitro* observations that emphasized the role of PPM1D as an important factor in promoting CDDP chemoresistance. There were only 99 out of 130 patients that had been followed-up to determine disease recurrence (Table 1). In the chemoresistant group (PFS = 0-6), there were only 43 patients out of the 99 follow-ups that fit in this category, while the chemosensitive group (PFS = > 25) had much less patients (21/99). The sample size of those two groups may not be sufficient enough to draw a solid conclusion regarding the role of PPM1D as a determinant of CDDP chemoresistance in OVCA, and that may

possibly explain the discrepancy between our *in vitro* and *in vivo* observations. This highlights the necessity of studying a larger sample size of tumors to demonstrate the importance of PPM1D in CDDP chemoresistance in OVCA.

Figure 5: Expression of PPM1D in human serous, endometrioid and clear cells ovarian carcinoma tumors. ***PPM1D exhibits high expression level in human ovarian serous, endometrioid, and clear cells carcinomas.*** Ovarian carcinoma tissues were immunostained for PPM1D (400X magnification). "a" indicates malignant glandular region; "b" indicates stromal regions.

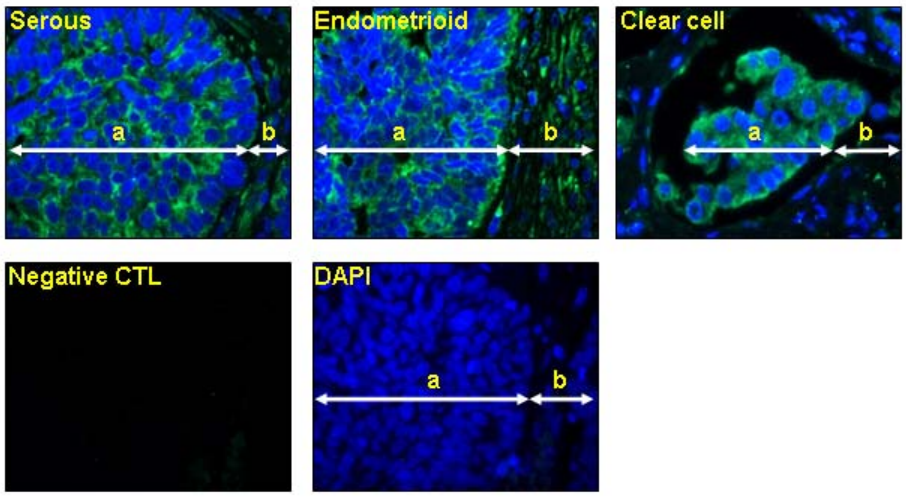
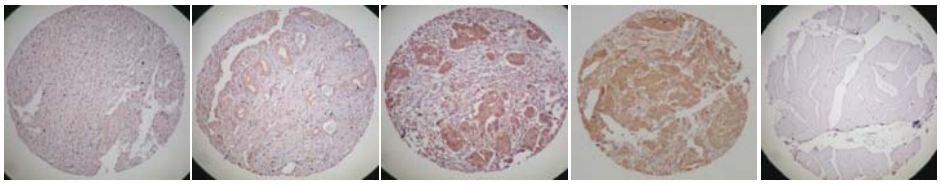

Serous
a
b
Endometrioid
a
b
Clear cell
a
b
Negative CTL
DAPI
a
b

Figure 6: Expression of PPM1D in human ovarian serous tumors (low malignant potential tumors, primary tumors, and metastatic tumors) versus normal ovarian and non-ovarian tissues. ***PPM1D is significantly expressed in primary and metastatic human ovarian serous tumors compared to low malignant potential tumors and normal ovarian and non-ovarian tissues.*** Tissues were immunostained for PPM1D using a tissue microarray developed in-house by DB. The boxes represent the variation in PPM1D expression in the tumor samples examined. The black line represents the median of PPM1D expression, while the whiskers represent the variation in scoring PPM1D expression between the three independent observers. The number of subjects in each experimental groups are indicated in parentheses: Normal ovarian tissues (13), low malignant potential tumors (13), High-grade serous tumors (52), Metastatic tumors (52), Normal non-ovarian tissues (10). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$. Statistical analysis was performed using the Mann-Whitney U test.



Normal Ovarian Tissue | Low Malignant Potential Tumor | Primary Tumor | Metastatic Tumor | Normal Non-Ovarian Tissue

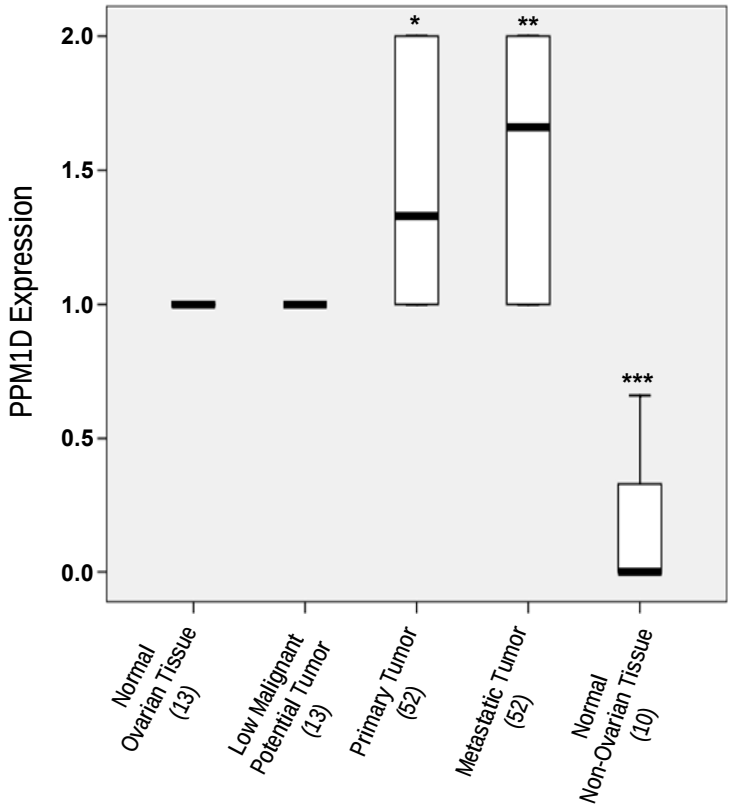
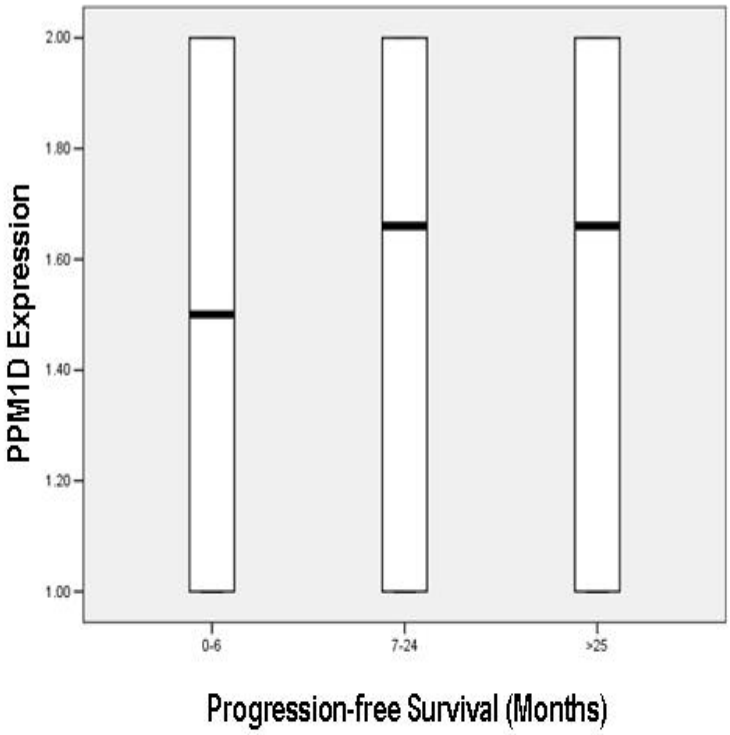


Figure 7: Comparison of PPM1D expression in human ovarian serous tumors from patients with progression-free survival (PFS) of 0-6 months (chemoresistant), 7-24 months, and > 25 months (chemosensitive).

The tables illustrate the standard deviation and the median for each respective group, and the p-values for statistical comparison of PPM1D expression between the different groups, using the Mann-Whitney U test.



PFS (Months)	0-6	7-24	>25
Standard deviation (SD)	0.459	0.441	0.451
Median	1.5	1.66	1.66

Group Comparisons	p-value
0-6 vs. 7-24	0.284
0-6 vs. >25	0.557
7-24 vs. >25	0.809

Table 1:

Table 1 illustrates the clinical characteristics of the patients recruited for the TMA study. The majority of patients were in the 51-60 age group, with a median age of 64 for all patients. The tumors were primarily high-grade and metastatic serous ovarian tumors (80 %), with 10% each of normal ovarian tissue and low malignant potential serous ovarian tumors (LMP). Most of the tumors used were stage III and IV. Large number of patients had a recurrent disease and progression-free survival (PFS) of 6 months or less, while the majority of patients had a recurrence within the first year.

Variables	*Range*	*N/Total*	*%*
Age (Years)	< 50	18/130	13.8
	51-60	66/130	50.8
	> 61	46/130	35.4
Median Age	64		
Ovarian Tissue/Tumor Type	Normal	13/130	10
	LMP	13/130	10
	High-Grade	52/130	40
	Metastatic	52/130	40
Stage	III	69/130	53.1
	IV	30/130	23.1
PFS (Months)	0-6	43/99	43.4
	7-24	35/99	35.4
	> 25	21/99	21.2

Discussion

In the present study, we have examined the molecular basis of CDDP resistance using pair-matched isogenic CDDP-sensitive OVCA (A2780s) and CECA (OV2008) cell lines and their resistant counterpart (A2780cp and C13*, respectively) and a p53-compromised CDDP resistant OVCA cell line (OCC-1). We have demonstrated that (i) CDDP induced PPM1D down-regulation through proteasomal degradation in sensitive cancer cells; (ii) CDDP induced PPM1D nuclear localization in resistant cells, and PPM1D nuclear exclusion in sensitive cancer cells and OVCA xenografts; (iii) Inhibition of Akt function in resistant cells leads to decrease PPM1D stability and CDDP-induced down-regulation, whereas over-expression of active Akt in sensitive cells stabilized PPM1D content through inhibition of CDDP-induced PPM1D degradation; (iv) PPM1D is highly expressed in human ovarian tumor subtypes (serous, endometrioid, and clear cell) and in primary and metastatic human ovarian tumors. Our findings establish for the first time that Akt confers CDDP resistance in cancer cells in part through stabilization of PPM1D.

Under normal conditions, PPM1D restores cellular homeostasis following DNA-damage by cooperating with p53 to induce G_2/M cell cycle arrest, thereby allowing ample time for repair of the damaged DNA[44-46]. However, PPM1D amplification and/or enhanced stabilization allow for sustained inhibition of DNA damage response proteins and numerous tumor suppressors, including ATM, Chk1 and 2, and p53. PPM1D over-expression has been implicated in a variety of human malignancies, including OVCA, and its level is directly related to poor prognosis and reduced therapeutic outcome[47-57].

We have demonstrated that in response to protein synthesis inhibition, PPM1D content decreased following CDDP challenge, a response more prominent in the sensitive cells. However, CDDP caused PPM1D stabilization and accumulation in resistant cells, in line with our earlier observations[8]. These findings suggest that CDDP-induced PPM1D down-regulation in sensitive cells was due primarily to PPM1D destabilization and enhanced degradation rather than diminished protein synthesis. Moreover, PPM1D enhanced stability in resistant cells in response to CDDP could be due to an inherent mechanism associated with chemoresistance that is regulating PPM1D stability. We have previously demonstrated that Akt is a determinant of CDDP sensitivity in the chemoresistant cell lines used in this study[4,6,38-40,58] and we have shown that Akt regulates the activities of several cell survival proteins, including the X-linked inhibitor of apoptosis (XIAP). XIAP exhibited similar differential expression profile induced by CDDP as PPM1D between sensitive and resistant cancer cells and that was due to XIAP proteasomal degradation in sensitive cells and Akt-mediated stabilization in resistant cells[2]. We have corroborated that PPM1D is down-regulated by CDDP in chemosensitive cells through proteasomal degradation.

A hallmark of chemoresistant cancers is the over-expression and/or activation of the PI3K/Akt survival pathway. Improper activation of this pathway has been associated with chemoresistance in several tissue types, including OVCA[29-33]. In the present study, we have observed that Akt plays an important role in the regulation of PPM1D protein stability and enhancement of its content in response to DNA damage in OVCA cells, and thus chemosensitivity. We have demonstrated that Akt confers CDDP resistance in part through the regulation of PPM1D protein stability, preventing its proteasomal

degradation and consequently increasing its half-life. However, since PPM1D protein sequence does not contain a consensus Akt phosphorylation site, it is unlikely that PPM1D is a direct Akt target. The mechanism by which Akt enhances PPM1D stability is unknown, and the mediator(s) that facilitate Akt-mediated PPM1D regulation remain to be investigated. Nonetheless, to our knowledge, this is the first demonstration of an association between Akt and PPM1D in the promotion of cell survival and chemoresistance. Our observations suggest a possible new mechanism by which Akt can attenuate the DNA damage response to enhance cellular survival, proliferation and chemoresistance through positive regulation of its newly discovered effector, PPM1D.

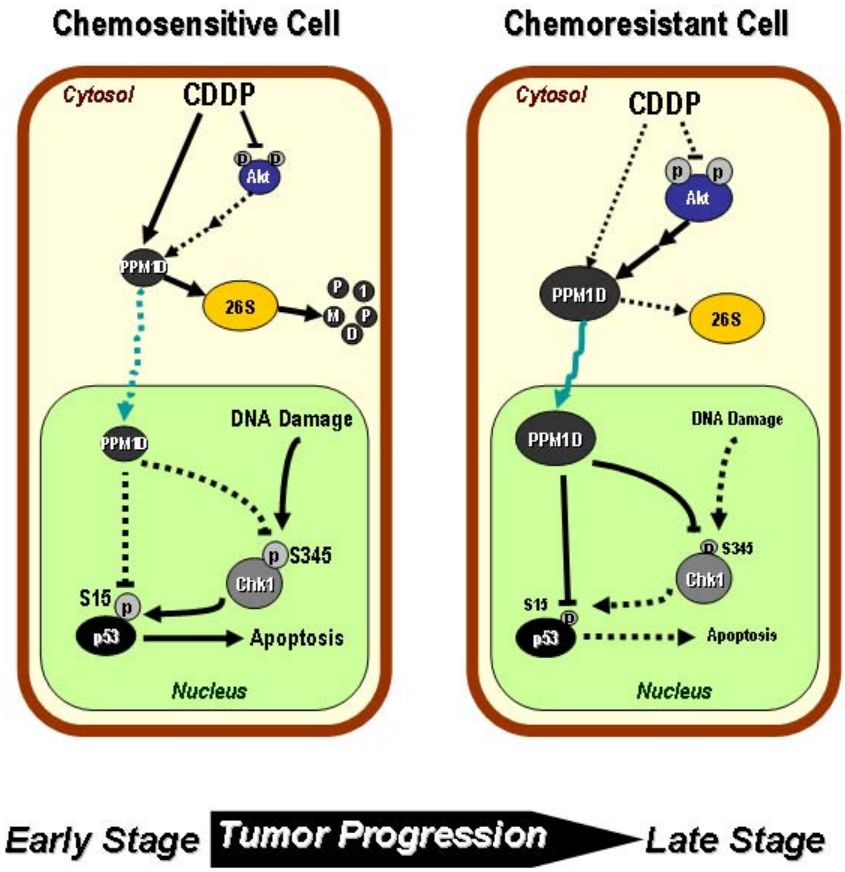
In the present studies, we also investigated the regulation of intra-cellular PPM1D localization by CDDP in sensitive and resistant cells. PPM1D is a nuclear phosphatase[28] and its nuclear exclusion may have negative repercussion on its activity. Using ovarian xenograft tumors developed from chemosensitive OVCA cells, we observed CDDP-induced PPM1D down-regulation and its nuclear exclusion compared to the control tumors, consistent with decreased availability in the nucleus where PPM1D targets are located. This latter notion is also supported by our current *in vitro* studies demonstrating nuclear localization of PPM1D in resistant but not sensitive cells in response to CDDP treatment, suggesting that differential intra-cellular PPM1D localization may contribute to CDDP chemoresistance by enhancing its nuclear availability in chemoresistant cells to act on its targets (e.g. p53) and subsequently inhibit the apoptotic process. While we have demonstrated CDDP-induced differential intra-cellular PPM1D localization, the exact mechanisms by which intra-cellular PPM1D transportation is regulated, and if Akt plays a role in these processes have yet to be elucidated.

Using a panel of human ovarian tumors, we have also demonstrated in the present study that PPM1D was highly expressed in the different histological subtypes, with higher expression in malignant glandular region than in stromal region, suggesting a possible involvement of PPM1D in ovarian tumorigenesis. This notion is supported by our findings in our tissue microarray panels showing that PPM1D was highly expressed in primary tumors when compared to normal ovarian and non-ovarian tissues, supporting the involvement of PPM1D in ovarian carcinogenesis. Furthermore, late stage OVCA tumors frequently develop a chemoresistant phenotype, where PPM1D could potentially act as a critical driver of CDDP chemoresistance through negative regulation of p53, DDR proteins, and ultimately cell death. Lastly, PPM1D may be involved in ovarian tumor progression and metastasis since it is expressed at significantly higher levels in metastatic tumors compared to high grade primary tumors.

In summary, the present studies have established a novel regulation mechanism of PPM1D stability by Akt, which modulates CDDP-induced apoptosis and chemosensitivity in human OVCA cells; however the exact molecular mechanism has yet to be elucidated. Moreover, we have discovered a new facet of PPM1D regulation through the control of its intra-cellular localization and its availability to dephosphorylate its targets. The mechanism of regulating PPM1D localization and the possible consequences of this regulation are not known and will be the subject of our future studies. To facilitate future investigations on the precise mechanisms of Akt-mediated PPM1D regulation as well as its intra-cellular localization, and how this will ultimately impact CDDP resistance in OVCA cells, we proposed a hypothetical model (Fig. 8). In sensitive cancer cells, CDDP induces PPM1D nuclear exclusion and proteasomal

degradation, thereby allowing CDDP-induced Chk1 and p53 activation, and ultimately the initiation of the apoptotic response. However, Akt activation and over-expression stabilizes PPM1D in chemoresistant cells, leading to sustained PPM1D expression and nuclear localization, which suppresses the activity of both Chk1 and p53, thereby contributing to CDDP resistance in these cancer cells.

Figure 8: Hypothetical model illustrating the role of PPM1D in the regulation of CDDP sensitivity in cancer cells and its regulation by Akt in response to CDDP. In sensitive cells, CDDP induces PPM1D nuclear exclusion and proteasomal degradation, thereby allowing CDDP-induced Chk1 and p53 activation, and ultimately the initiation of the apoptotic response. However, in chemoresistant cells Akt activation and over-expression stabilizes PPM1D, leading to sustained PPM1D expression and nuclear localization, which suppresses the activity of both Chk1 and p53, thereby contributing to CDDP resistance in cancer cells.


Chemosensitive Cell
Chemoresistant Cell
Cytosol
CDDP
Akt
PPM1D
26S
DNA Damage
S345
Chk1
S15
p53
Apoptosis
Nucleus
Early Stage
Tumor Progression
Late Stage

Materials and Methods

Reagents

Chemicals and reagents used in the current studies and their sources (in parenthesis) were as follows: CDDP, dimethyl sulfoxide, sodium orthovanadate, aprotinin, phenylmethylsulfonyl fluoride, CHX, L-leucine, PBS, polyoxyethylene-sorbitan monolaurate, and Hoechst 33258 (Sigma-Aldrich, St. Louis, MO, USA); Fetal Bovine serum), dialyzed FBS, DMEM/F12 and RPMI1640 culture media, non-essential amino acids, streptomycin, penicillin, and amphotericin B (Life Technologies, Carlsbad, CA, USA); Leucine-deficient RPM1 1640 culture media (US Biological, Swampscott, MA, USA); toluene, methanol, isopropanol, hydrogen peroxide 34.5-38%, 10mM citrate buffer pH 6.0, and citric acid monohydrate (LabMat, Québec, QC, Canada); Epoxomicin (EMD Chemicals, Gibbstown, NJ, USA); PPM1D and β-actin primers (Origene, Rockville, MD, USA); RNeasy Plus microkit, Omniscript reverse transcriptase kit, Sensiscript RTkit, and QuantiTect SYBR Green PCR kit (Qiagen, Mississauga, ON, Canada); Rabbit polyclonal antibodies: anti-Akt and anti-phospho-GSK-3β Ser 9 (Cell Signaling Technology, Beverly, CA, USA), anti-PPM1D (WB; Abcam, Cambridge, MA, USA), and anti-PPM1D (ICC, IHC; clone H-300; Santa Cruz Biotechnologies; Dallas, TX, USA); mouse monoclonal antibodies: anti-β-Actin (Cedarlane Laboratories, Burlington, ON, Canada), anti-PPM1D (IP; clone F-10, Santa Cruz Biotechnologies), and anti-FLIP (Alexis Biochemicals; San Diego, CA, USA); rat monoclonal anti-HA (Roche Diagnostics, Mannheim, Gremany); HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies (Bio-Rad Laboratories; Hercules, CA, USA); HRP-conjugated goat anti-rat, and normal mouse IgG (Santa Cruz Biotechnologies); antibody

diluent with background reducing components, Envision+ dual link system-HRP, and DAB chromogen substrate pack, biotinylated secondary antibody, and streptavidin complex (Dako, Carpinteria, CA, USA); Hematoxylin 560 MX and Mounting medium MM24 (Leica Microsystems, Concord, ON, Canada); Tissue Arrayer (Beecher Instruments, Sun Prairie, WI, USA); *N*,*N*,*N*′,*N*′-tetramethyl-ethane-1,2-diamine, Dynabeads protein G immunoprecipitation kit, and Alexa Fluor 488 (green) and Alexa Fluor 594 (red) goat anti-rabbit antibodies (Invitrogen, Carlsbad, CA, USA); Pierce immunoprecipitation cell lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA); Matrigel matrix (BD Biosciences, Franklin Lakes, NJ, USA). Severe Combined Immunodeficiency (SCID) mice (Charles River Laboratories, Wilmington, MA, USA); Isoflurane (Abbot Laboratories; North Chicago, IL, USA); [^{3}H]-L-leucine (Moravek Biochemicals and Radiochemicals, Brea, CA, USA); HA-tagged, triple-A mutated (K179A, T308A, S473A) kinase-dead DN-Akt, meristoylated Akt, and LacZ adenoviral constructs were synthesized at the University of Ottawa Adenoviral Core Facility (Ottawa, ON, Canada).

Cell lines and culture

CDDP sensitive [OV2008, A2780s (wt-p53)] and resistant [C13* (wt-p53), A2780cp (p53 mutant), OCC-1 (p53 mutant)] human cancer cell lines, provided by Drs. Rakesh Goel and Barbara Vanderhyden (Ottawa Hospital Research Institute; Ottawa, ON, Canada), were cultured as previously reported[5,6]. OV2008 cells and their resistant counterparts C13* cells are of cervical carcinoma origin with squamous differentiation. A2780s, A2780cp, and OCC-1 cells were derived from undifferentiated ovarian

carcinoma tumors[59] (See cell line authentication in Appendix 1 showing genetic profiles). Following the indicated treatments, the cells were harvested for analysis.

Protein extraction and Western blot analysis

Protein extraction and Western blotting were performed as previously described[5,6]. Unless otherwise indicated, membranes were incubated overnight at 4°C with anti-PPM1D (1:500), anti-Akt (1:1000), anti-HA (1:5000), anti-phospho-GSK-3β (1:1000), and anti-FLIP (1:1000) antibodies, and 1h at room temperature for anti-β–Actin (1:10,000) and anti-rabbit or anti-mouse secondary antibodies (1:2000 - 1:10,000), and band densities were analyzed (Scion Image software; Scion Corporation Inc., Frederick, MD, USA).

Real-time quantitative PCR

Relative differences in PPM1D mRNA levels in experimental groups were determined by real-time quantitative-PCR (RT-qPCR), using PPM1D and β–Actin primers (Origene). Total RNA was reverse transcribed followed by PCR as described previously[60].

Adenoviral infection

A2780cp and OCC-1 cells were infected with adenoviral HA-DN-Akt (MOI = 80, 24h), and OV2008 cells were infected with adenoviral HA-AAkt2 (meristoylated; MOI = 40, 24h) as previously described[5,6]. Adenoviral LacZ served as a control.

Pulse-chase assay

The experiment was conducted as previously described[61]. A2780cp cells were infected with adeno-DN-Akt (MOI = 80; 24h) or adeno-LacZ as a control, then pulsed with leucine-deficient RPMI1640 media supplemented with dialyzed FBS (24h). The cells were then pulsed with leucine-deficient RPMI1640 media supplemented with ^{3}H-leucine (50 μCi/ml, 1h). The cells were then incubated with the chase solution (RPMI1640 media supplemented with 5% cold leucine) for the indicated time points. PPM1D was immunoprecipitated and the radioactivity was measured by liquid scintillation counting.

Immunoprecipitation

PPM1D protein was immunoprecipitated using the Dynabeads protein G immunoprecipitation kit as previously described[62].

Immunocytochemistry and Immunohistochemistry

PPM1D expression in sensitive (OV2008) and resistant (C13*) cells**,** xenograft tumors of A2780s cells, as well as in human ovarian tumor samples and tissue microarrays was performed as previously described[63].

Xenograft model

Intraperitoneal xenograft model has been performed as previously described[64]. All procedures involving animals and their care were approved by the Institutional Animal Care Committee of The Ottawa Hospital Research Institute in accordance with

institutional guidelines. Briefly, $6x10^6$ A2780cp cells, mixed with 1X PBS:matrigel (1:1) solution, were injected into 5- to 7-week-old female SCID mice. Eighteen days following injection, the experimental group was injected every other day with CDDP (3 mg/kg; s.c., 5 animals/group), while the control group received placebo. The animals were sacrificed 3 weeks after drug administration.

Tissue microarray

Tissue microarray was constructed, as previously described[65]. Briefly, 0.6 mm cores (3 cores/tumor) were placed in a recipient paraffin block. Immunohistochemistry was performed on 4 μm thick sections, as previously described[65,66]. Sections were incubated with anti-PPM1D antibody (1:75 dilution) at room temperature. PPM1D protein expression was assessed by semi-quantitative scoring of the intensity of staining and recorded as absent (0), weak (1), moderate (2) or strong (3) by three independent observers.

Statistical Analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was carried out by one-way, two-way, three-way ANOVA, and Mann-Whitney U tests (where appropriate) using PRISM software (Versions 5.0; GraphPad, San Diego, CA, USA). Differences between multiple experimental groups were determined by the Bonferroni post-hoc test. Statistical significance was inferred at $P < 0.05$.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

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Chapter 5 - General Discussion

5.1 Overview and significance

OVCA is the most lethal of all gynecological malignancies, primarily due to late diagnosis and the development of chemoresistance. The conventional course of therapy for OVCA includes surgical de-bulking of the tumor mass followed by adjuvant chemotherapy[1]. Most patients are responsive to chemotherapy at first, however recurrent ovarian tumors are more aggressive, metastasize to secondary target tissues, and acquire resistance to conventional chemotherapeutics. Platinum derivatives are first-line chemotherapeutic agents in the treatment of OVCA[2]. Resistance to available chemotherapeutics is a major hurdle in the treatment of OVCA. Drug resistance is a multi-factorial problem and is characterized by acquired genetic mutations, which can lead to dysregulation of the balance between cellular survival pathways and apoptosis-inducing tumor suppressors, enhanced drug clearance and detoxification, reduced drug accumulation, and increased DNA repair[3-8].

The current thesis addresses a number of regulatory mechanisms that may help clarify the picture of CDDP chemoresistance in gynecological malignancies. The original aim of this thesis was to study the role of Chk1 in gynecological cancer cell chemoresistance through the regulation of p53 activation. Our studies were then extended to examine the role of the newly described p53-induced phosphatase PPM1D and its regulation of the activation of both p53 and Chk1 in response to CDDP. We have also examined the differential regulation of PPM1D intra-cellular localization by CDDP in chemosensitive and chemoresistant cells. Finally, we have examined the importance of Akt in the regulation of PPM1D and their contribution to the chemoresistant

phenotype of gynecological cancer cells, thereby attenuating CDDP sensitivity and gynecological cancer cells apoptotic capacity. Our studies identified PPM1D as a critical executor of Akt-mediated CDDP resistance in ovarian and cervical cancers and a potential therapeutic target important in developing targeted treatment strategies to combat drug chemoresistance that may ultimately improve drug responsiveness and clinical outcomes for cancer patients with chemoresistant tumors.

5.2 CDDP and DNA Damage Response

CDDP-induced DNA damage causes the activation of the DNA damage response (DDR) to salvage the DNA and commence its repair. DNA damage is detected by the well-known DNA damage sensors ATM and ATR, which respond to double-strand and single-strand DNA breaks, respectively[9,10]. They subsequently signal to their downstream effectors, Chk1 and Chk2, to cause a replication stall through cell cycle arrest and allow ample time for DNA repair[10]. However, in the presence of irreparable DNA damage, such as the case with CDDP-induced DNA adducts, the focus shifts from DNA repair to the execution of programmed cell death to ensure the destruction of the damaged cells and inhibit the propagation of the corrupted genome, thereby decreasing the possibility of neoplastic transformation and subsequent tumorigenesis. In such case, the DDR pathway converges on the executioner of apoptosis, the tumor suppressor p53. It has been demonstrated that ATR activates its effector Chk1 through phosphorylation of serine residues 317 and 345, with Ser^{345} being an essential phosphorylation site for Chk1 nuclear localization and retention, and optimal kinase activity[11]. ATR also activates p53 through phosphorylation of Ser^{15} and Ser^{20} directly and indirectly through Chk1[12-18]. We

have demonstrated that CDDP caused a differential activation of Chk1 in chemosensitive and resistant cancer cells, which could not be accounted for by dysregulated ATR expression and activation (Figure 3.1A). The down-regulation of activated Chk1 in resistant cells and its up-regulation in sensitive cells suggest the existence of a distinct molecular mechanism regulating CDDP-induced Chk1 phosphorylation, the subsequent activation of p53, and CDDP sensitivity in chemoresistant cells operating independently from ATR-mediated Chk1 and p53 regulation in response to DNA damage.

Cells must ensure the preservation of genomic integrity in order to guarantee the accurate propagation of genetic information following cellular division. The tumor suppressor p53 is a key protein that acts as a transcription factor for a host of genes involved in cell cycle regulation, DNA repair, and apoptosis in order to maintain the integrity of the genome[19-21]. p53 can also induce apoptosis in a transcription-independent manner through initiating the caspase cascade by direct activation of the mitochondrial apoptotic pathway and the regulation of cytochrome c release through the modulation of pro-apoptotic Bcl-2 family members[22-24].

TP53 mutation is a common event in the oncogenesis of many cancers[25-27] and correlates with resistance to chemotherapeutic agents[28-30]. It has been demonstrated that p53 is activated by CDDP through a phosphorylation-dependent mechanism of serine residues 15 and 20, which increase its pro-apoptotic properties[31-34]. Our laboratory has demonstrated that the existence of a functional p53 pathway and the capacity of p53 activation are indispensable for mediating the apoptotic effects of CDDP in gynecological cancer cells[24,29,35-40]. Furthermore, we have demonstrated the importance of p53 in the sensitization of chemoresistant cancer cells to CDDP, whereby the down-

regulation of p53 completely abolished CDDP sensitivity (Figure 3.4), and the recapitulation of wt-p53 into p53-compromised resistant OVCA cells sensitized them to CDDP (Figure 3.5).

PPM1D works to re-establish cellular homeostasis following genomic insults and the subsequent repair of the DNA damage through the activation of the DDR pathway. The latter is achieved through the restoration of baseline activities of several tumor suppressors and DDR proteins. However, in pathophysiological conditions such as cancer, persistent PPM1D activity due to over-expression and/or enhanced stabilization may sustain the inhibition of the DDR pathway and, most significantly, the tumor suppressor p53, which may eventually result in the development of a chemoresistant phenotype. We have demonstrated this notion where CDDP caused the down-regulation of PPM1D in chemosensitive cells, but sustained PPM1D content in their chemoresistant counterparts (Figure 3.1B). Moreover, PPM1D down-regulation sensitized chemoresistant cancer cells to CDDP-induced apoptosis (Figure 3.2A), while its over-expression in sensitive cancer cells led to diminished responsiveness to CDDP accompanied by significant decrease in both Chk1 and p53 activations (Figure 3.2B).

Full-length PPM1D is assumed to be a strictly nuclear phosphatase since it contains two nuclear localization signals[41], and all of its known targets are nuclear proteins[42-46]. Therefore it has been assumed that PPM1D nuclear exclusion would significantly affect its ability to function, and the opposite would be true. We have observed that CDDP treatment lead to differential intra-cellular PPM1D localization. CDDP caused significant PPM1D nuclear localization in chemoresistant CECA cells, suggesting enhanced nuclear availability and possible interaction with its target proteins

(Figure 4.3). Furthermore, CDDP treatment lead to PPM1D nuclear exclusion in chemosensitive CECA cells *in vitro* and in a xenograft model of sensitive OVCA cells, which may lead to decreased PPM1D interaction with its target proteins and, hence, diminished activity (Figures 4.3, and 4.4). We have also shown that PPM1D is highly expressed in a panel of serous, endometrioid, and clear cell human ovarian tumors, and its expression was correlated with tumor aggression and metastatic potential in tissue microarray of human serous ovarian tumors (Figures 4.5 and 4.6). Our observations confirm the importance of PPM1D as a determinant of CDDP chemoresistance in gynecological cancer cells and a potential marker for overall prognosis and chemotherapeutic responsiveness.

Aberration of the PI3K/Akt survival pathway contributes in the oncogenesis of several human malignancies, including OVCA and CECA. It has been demonstrated that the activation and/or amplification of Akt, in particular Akt2, is associated with the development of OVCA and poor responsiveness to chemotherapy[47-51]. Our laboratory has demonstrated that Akt is a determinant of CDDP chemoresistance in OVCA and CECA cells, whereby the stable transfection of sensitive OVCA cells with activated Akt2 (A2780s-Akt2) fail to undergo CDDP-induced apoptosis and the suppression of Akt function by dominant-negative Akt expression also sensitizes chemoresistant cells to CDDP[24,36,40,52,53]. As previously mentioned, Akt regulates a host of both pro- and anti-apoptotic proteins to display its oncogenic characteristics and attenuate chemotherapeutic responsiveness. Most significantly, Akt regulates p53 cellular content by causing its de-stabilization and ubiquitin-mediated proteasomal degradation through the positive regulation of the E3 ubiquitin ligase MDM2[53-55]. We have shown that while CDDP treatment enhanced

PPM1D content in chemoresistant OVCA cells, Akt down-regulation significantly reduced PPM1D protein level, but not its mRNA abundance (Figure 4.1B). Furthermore, the down-regulation of Akt activity in chemoresistant OVCA cells resulted in the decrease of PPM1D protein stability and half-life (Figure 4.1C and 4.1D). And finally, while CDDP decreased PPM1D content in chemosensitive cancer cells, forced expression of activated Akt led to the stabilization of PPM1D protein and enhanced its content (Figure 4.2B). We have established a new mechanism by which Akt can exert its pro-survival functions. We have demonstrated that Akt can regulate the stability of PPM1D in OVCA cells, thereby attenuating both the apoptotic and DDR pathways. To our knowledge, this is the first demonstration where a connection between Akt and PPM1D has been illustrated in the regulation of OVCA cell sensitivity to chemotherapeutic agents such as CDDP. However, the exact molecular mechanism of Akt-mediated PPM1D regulation remains unknown and has yet to be illustrated.

5.3 Common Pathways in Chemoresistance

The mechanisms of CDDP resistance are complex and involve multiple parallel and inter-connected cellular signaling networks that work in counterbalancing CDDP influence and aiding in the development of a chemoresistant tumor phenotype. However as complex as it is, the underlying molecular mechanisms of CDDP resistance in human malignancies share numerous critical commonalities, which highlights the advantages of the use of targeted therapies developed for specific neoplasms to be applied to other human malignancies sharing similar CDDP chemoresistant profile. Our laboratory has worked extensively on delineating the molecular mechanisms underlying CDDP

chemoresistance employing several *in vitro* models comprised mainly of ovarian carcinoma cell lines and a pair-matched CDDP-sensitive and –resistant cervical carcinoma cell line. We have found several general mechanisms of CDDP resistance which exist in the chemoresistant cell lines such as the dysregulation of the p53 and Akt signaling pathways, which confirms the universality of the observed phenomena.

5.4 Experimental Advantages for Utilizing Isogenic Cancer Cell line Pairs

The present thesis had the distinct benefit of using matched pairs of chemosensitive parental cell lines (OV2008 and A2780s) with their isogenic resistant counterparts (C13* and A2780cp), which were developed by extended exposure to increasing concentrations of CDDP. The use of these cell lines facilitated our investigation of the molecular mechanisms underlying CDDP chemoresistance in cancer cells. The resistant cell lines have an identical genetic background as their parental sensitive lines; therefore any differences between them at the molecular level may potentially be responsible for the development of the chemoresistant phenotype, without any confounding differences in drug responsiveness due to the use of cell lines with differing genetic profiles. The utilization of these isogenic cell line pairs allowed us to draw crucial conclusions from the observed differences between the chemosensitive and chemoresistant cells with regards to CDDP responsiveness, CDDP effect on the expression of our genes of interest and their protein products, and their subsequent molecular manipulations (up- and down-regulation). The observed differences in responses were not due to random differences in cell-specific characteristics, which make

the collected findings more relevant and applicable for the study of chemoresistance in OVCA cells.

5.5 Future Directions

5.5.1 Xenograft Models

Although we have used exploratory xenograft models to determine PPM1D expression and intra-cellular localization (Figures 4.4), further studies are needed to fully illustrate the role of PPM1D in CDDP chemoresistance in gynecological cancers. Xenografts of CDDP-sensitive and -resistant OVCA cell lines in female Swiss nude mice will be used to study the involvement of PPM1D in the regulation of apoptosis in chemosensitive and resistant human ovarian tumors *in vivo*. We will use chemosensitive A2780s cells constitutively expressing PPM1D (A2780s-His-PPM1D) or control vector (A2780s-His CTL) into nude mice (See Table 5.1). The influence of CDDP on tumor volume and apoptosis will be assessed. We expect that CDDP will induce apoptosis and growth suppression of tumors developed from the A2780s-His CTL, but not A2780s-His-PPM1D cells, suggesting that PPM1D over-expression leads to CDDP resistance, as shown *in vitro.*

To determine if PPM1D down-regulation would activate p53 and Chk1 and sensitize resistant OVCA tumors to CDDP, the Tet-On system will be used to generate the cell lines stably expressing the reverse tetracycline-controlled transactivator (rtTA) under control of the CMV promoter (p53). The gene of interest can be induced by the addition of doxycycline in drinking water. Under this system, gene expression can be induced *only* in the appropriate cells, and can be maintained over time through the doxycycline

administration. Nude mice harbouring A2780cp-[His-CTL]-[HA-p53-Tet] and A2780cp-[His-shPPM1D]-[HA-p53-Tet] cells (See Table 5.1) will be treated with CDDP with or without doxycycline, and tumor progression (volume) and animal survival (Kaplan-Meier plot) will be assessed. At sacrifice, tumor tissues will be collected for assessment of P-p53 (S15) and P-Chk1 (S345) contents. Moreover, to determine Akt involvement, we plan to include Akt down-regulation as illustrated in Table 5.1. P-p53 (S15) and P-Chk1 (S345) contents and apoptosis will be assessed.

We will examine the time- and dose-response effects of doxycycline on HA-p53 expression and its influence on CDDP-induced apoptosis/tumor regression. If Akt down-regulation is effective in overcoming resistance and if this depends on p53, we expect that DN-Akt will have no effect on these responses in the p53 mutant cells unless wt-p53 is reconstituted.

Although cancer cell lines provide convenient means to study many aspects of cancer biology and to test the effectiveness of cancer therapeutics, successive passages of cancer cells allows for the introduction of mutations that may alter their genetic profile to the point that they may have lost the characteristics of the tumors they were derived from. Therefore, the use of OVCA cell lines in the development of xenograft models, while convenient, may not be appropriate to recapitulate the disease[56]. For this reason, we will attempt to establish human tumor xenografts models as previously demonstrated[57]. Primary and metastatic ovarian tumors will be obtained from the Ottawa Ovarian Tumour Bank in collaboration with Dr. B. Vanderhyden. The tumors will be enzymatically digested to produce a single cell suspension and then injected under the ovarian bursa into female Swiss nude mice to mimic the tumor microenvironment. To ensure tumor

propagation *in vivo*, xenograft tumors will be excised, enzymatically processed, purified of murine cell component, and then transplanted into new hosts as previously described[57]. Tumor histology will be examined by a qualified pathologist to verify the maintenance of tumor integrity with successive transplantations. The influence of CDDP on tumor progression (volume) and cellular apoptosis (TUNEL assay) will be evaluated. PPM1D expression in primary and metastatic tumors will be assessed to validate our earlier observations and determine PPM1D involvement in tumor aggression.

Table 5.1: Construction of Tet-ON cell lines**.** A2780s (p53 wt, CDDP sensitive) and A2780cp (p53 mutated, CDDP resistant) cell lines will be stably transfected using the Tet-On system and/or constitutively stably transfected with PPM1D (His-PPM1D), shPPM1D (His-shPPM1D), and/or dominant negative Akt (Flag-DN-Akt) in order to modify their p53 status and/or regulation.

	Cell definition	Vector constitutively expressed in Cells	Inducible Tet-On system	Expected CDDP Sensitivity
1	A2780s-[His-PPM1D]	His-PPM1D	None	resistant
2	A2780s-[His-CTL]	Empty vector	None	sensitive
3	A2780cp-[His-shPPM1D]-[Flag-DN-Akt]-[HA-p53-Tet]	Flag-DN-Akt	HA-p53	sensitive
4	A2780cp-[His-CTL]-[Flag-CTL]-[HA-p53-Tet]	Flag-empty vector	HA-p53	resistant
5	A2780cp-[His-shPPM1D]-[Flag-CTL]-[HA-p53-Tet]	His-shPPM1D Flag-empty vector	HA-p53	sensitive
6	A2780cp-[His-CTL]-[Flag-DN-Akt]-[HA-p53-Tet]	His-empty vector Flag- DN-Akt	HA-p53	sensitive

5.5.2 Assessment of Clinical Samples

While established cell lines provide a convenient method for the study of molecular mechanisms involved in tumorigenesis and chemotherapeutic resistance, their clinical relevance and applicability to the human disease should be established. Specifically, continuous passage of cell lines may change the features of the original tumors. To address this concern, the above studies will be extended to include primary cultured human OVCA cells from ascites fluids before and after chemotherapy and solid tumors from patients with stage III/IV OVCA after surgical de-bulking. These cells will be treated with CDDP *in vitro,* and PPM1D, p53 content, total and P-Akt will be evaluated. The above studies will determine if the observations on OVCA cell lines are clinically relevant. While these studies will be correlative, they will provide important clinical relevance for the above findings.

5.6 Conclusions

The current thesis provides new and significant contributions regarding the cellular and molecular mechanisms of CDDP resistance in OVCA cells. It is the first to demonstrate that PPM1D, by regulating Chk1 and p53 activities and through its regulation by Akt, is a critical mediator of OVCA cell sensitivity to CDDP. The cellular status of Akt and p53, the interactions between them, and the net effect of these interactions will ultimately influence the outcome of treatment with chemotherapeutics. Our findings improve the current understanding of cell fate regulation in OVCA cells, and further the existing knowledge of the etiology of chemoresistance in these cells. A better

understanding of the cellular and molecular mechanisms of chemoresistance may someday improve the treatment regimen for this disease.

Chapter 6 – References

6.1 General Introduction

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Appendix 1: Cell line Short Tandem Repeat (STR) profiles used for cell authentication. The STR profiles have been generated using the Human Identification Application (AmpFLSTR Identifiler®) which employs 16 genetic markers. The numbers for each marker represent the STR copy number in each genetic marker examined. Genomic DNA extraction was performed using the DNeasy® Blood and Tissue kit from Qiagen (Catalogue No. 69504). A minimum of 10 ng/µl of genomic DNA from each cell line was used to generate the STR profiles. The cell line STR profiles were generated at The Genetic Analysis Centre, The Sick Children's Hospital, Toronto, Ontario, Canada.

Designation	Amelogenin	CSF1PO	D13S317	D16S539	D5S818	D7S820
OV2008	XX	11,11	11,12	12,13	12,12	9,10
C13	XX	11,11	11,12	12,13	12,12	9,9
SKOV3	XX	11,11	8,11	12,12	11,11	13,14
A2780cp	XX	11,12	13,13	11,14,15	11,12,13	10,10
A2780s	XX	10,11	12,13	11,13	11,12	10,10
Designation	vWA	FGA	D3S1358	D8S1179	D18S51	D21S11
OV2008	15,17	23,23	16,16	14,14	12,12	30,31
C13	15,17	23,23	16,16	14,14	12,12	30,31
SKOV3	17,18	24,25	14,14	14,15	16,17	30,31.2
A2780cp	15,16	19,23,24	14,16	15,16,17	16,19	28,29
A2780s	15,16	19,24	14,16	15,17	16,18	28,28

Designation	TH01	TPOX	D19S433	D2S1338
OV2008	8,9.3	8,10	13,15.2	18,18
C13	8,9.3	10,10	15.2,15.2	18,18
SKOV3	9,9.3	8,11	14,14.2	18,23
A2780cp	6,6	8,10	12,12	21,22
A2780s	6,6	8,10	12,12	21,22