THESIS FOR THE DEGREE OF MASTER OF SCIENCE

The mTORC2 Component Rictor Confers Cisplatin Resistance in Human Ovarian Cancer Cells

인간난소암세포에서 mTORC2 요소인 Rictor가 시스플라틴 저항성에 미치는 영향

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CONTENTS

LIST OF FUGURES

LIST OF ABBREVIATIONS

AMPK	AMP-activated protein kinase
ANOVA	Analysis of Variance
BAX	Bcl-associated X-link protein
CDDP	Cisplatin
Chaps	3[(3-cholamidopropyl)dimethyallonio]-1-propanesulfonate hydrate
DDT	DL-Dithiothreitol
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
FBS	Fetal Bovine Serum
FLIP	FLICE-like inhibitory protein
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
ILK	Integrin-linked kinase
IRS-1	Insulin receptor substrate 1
kDa	Kilodalton
MDM2	Murine Double Minute 2
MRI	Magnetic resonance imaging
mTOR	mammalian Target Of Rapamycin

OVCA	Ovarian Cancer
p70S6K1	70-kDa Ribosomal protein S6 kinase 1
PARP	Poly ADP-Ribose polymerase
PKCζ	Protein kinase-C ζ
PMSF	Phenylmethylsulfonyl fluoride
PI3K	Phosphoinositol 3-OH Kinase
PRAS40	Proline-rich protein
PTEN	Phosphatase and Tensin homologue
Ras	Rat sarcoma
SEM	Standard Error of the Mean
Ser	Serine
Smac	Second mitochondria-derived activator of caspases
TEMED	RNase A, *N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamin
Thr	Threonine
TSC2	Tuberous sclerosis complex 2
WCL	Whole cell lysate
XIAP	X-Linked Inhibitor of Apoptosis Protein

CHAPTER 1

GENERAL INTRODUCTION

1. Ovarian Cancer

Epithelial ovarian cancer (OVCA) is the most lethal gynecologic malignancy among women worldwide. The majority of OVCA cases are sporadic with only around 15% attributed to hereditary causes (Romero, I., et al., 2012). The common age range of women suffering from OVCA is between 45-80 years. As the early stages are usually asymptomatic most of the patients are diagnosed at later stages, leading to a low 5-year survival rate of 13% (Guppy et al., 2005). Therefore, it is crucial to take into account the clinical, cellular, and molecular biology of the disease, in order to improve treatment outcomes and move beyond current management and into the age of personalized medicine.

The ovaries are located in the pelvic cavity and contain three major cell types including germ cells, endocrine/interstitial cells, and epithelial cells. These cells function to elicit the production of oocytes and hormones as well as to maintain organ structure (Richards, J. S., et al., 2010). Thus, ovarian cancers are capable of arising from each of these cell types. Germ cell tumors are common in younger women between the age of twenty and thirty, which accounts for 3-5% of ovarian cancers. Sex cord-stromal tumors originate from interstitial and connective tissue, which can occur in women of all ages with an incidence of 7%. Epithelial ovarian cancer is the most common subtype and accounts for 90% of ovarian malignancies (Romero,

I., et al., 2012). There are four histotypes of epithelial ovarian cancer, which are different from most cancers due to development from simple flattened epithelial cells into four distinct major histotypes: serous, endometrioid, mucinous, and clear cell (Fig 1). Moreover, the four histotypes also show different genetic alterations, tumor markers, and chemotherapeutic sensitivity. Clear-cell and mucinous cancers generally do not respond to platinum- and taxane-based chemotherapeutic regimens as well as serous and endometrioid cancers (Bast Junior, C. R., et al., 2009).

A variety of factors influence risk of developing OVCA. Age and family history are the two common factors for cancer development. In OVCA, however, certain diseases, such as Poly-cystic ovary disease and pelvic inflammatory disease are also known to be risk factors. Normal reproductive physiology like ovulation can also cause OVCA due to rupture of the ovarian surface. The healing process and inflammation after ovulation activates cell proliferation. This can cause mistakes in proofreading of DNA and the accumulation of damaged-DNA can eventually lead to the development of OVCA. Conversely, pregnancy, lactation and oral contraceptives, which stop ovulation, have been reported to decrease risk of developing OVCA (Guppy, A. E., et al., 2005).

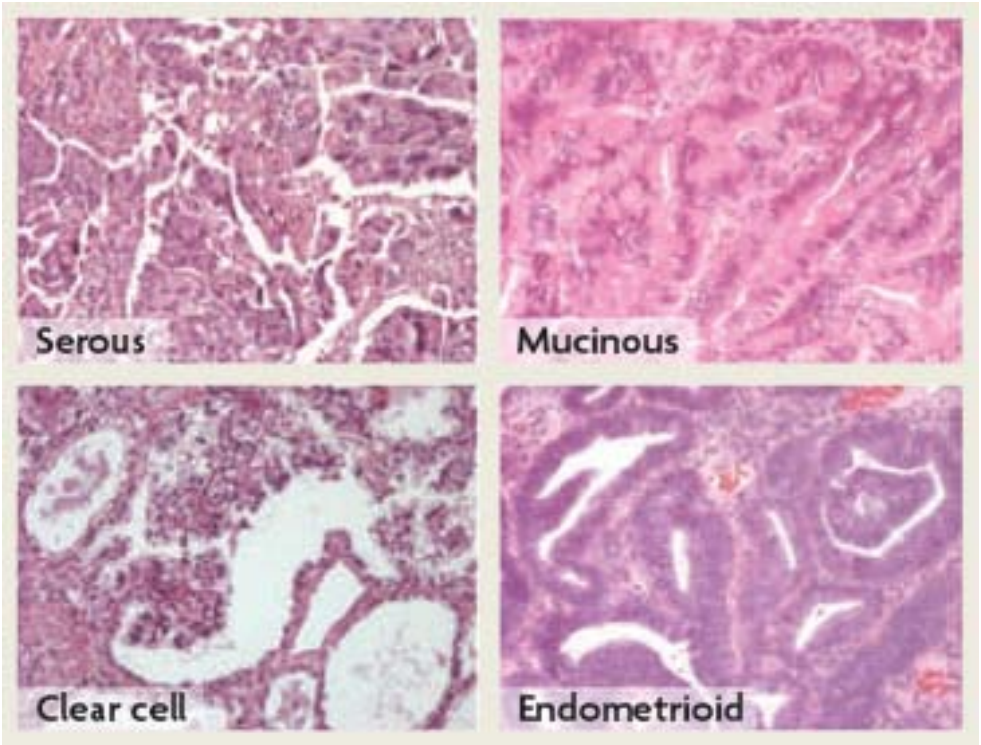

FIGURE 1. Histological features of four subtypes of human epithelial ovarian cancer (modified from Bast Junior, C. R., et al., 2009).

Ovarian cancer is an asymptomatic disease and does not show any clinical signs until advanced stages. The non-specific symptoms include pain, abdominal distension, constipation, and vaginal bleeding. One of the most important signs is a fixed irregular mass in the pelvic cavity. Ascites and pleural effusion may be present in some patients. Diagnosis of OVCA comprises imaging modalities including computed tomography, magnetic resonance imaging (MRI) and pelvic ultrasound which remains the standard pre-operative imaging investigation for OVCA (Guppy, A. E., et al., 2005). The other required diagnostic tests are chest X-rays for examining metastasis and the tumor marker CA125. Serum biomarker CA125 is elevated in more than 80% of patients with epithelial ovarian cancer, and its release and production appear to be related to cellular growth, and is the only well-validated tumor marker for this malignancy (Gadducci, A., et al., 2009) Currently, chemotherapy in combination with surgical debulking is the preferred treatment option. Moreover, targeted drugs and antibodies to personalize therapy have been used for OVCA treatment, yet the impact on patients is only modest. Cisplatin (CDDP) and its derivatives are first-line chemotherapeutic agents, which induce cytotoxic cell death through the formation of DNA-platinum adducts, resulting in DNA damage and activation of apoptotic pathways (Martalou et al., 2001). Despite advances in our understanding of tumor biology, the overall mortality from OVCA remains high.

One of the major hurdles of treatment success in OVCA has been the failure to detect the disease at early stages because OVCA is asymptomatic disease and does not present symptoms until the tumors are at advanced stage. In advance stage, the tumor requires more complex surgical debulking with higher probability of residual tumor cells. Moreover, late stage cancers often contain tumor cells that do not respond to chemotherapeutic challenge. Systemic administration of current chemotherapeutics for OVCA and the high dosages required to penetrate the tumor tissue 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). Despite the use of combination chemotherapy (e.g. cisplatin, taxol, and their derivatives) at low dosages, adverse side effects remain a concern. These therapeutic issues are further complicated by the fact that higher dosages of chemotherapeutic agents are often used to overcome chemoresistance

2. Chemoresistance

The effective treatment of OVCA is often hampered by late diagnosis and the emergence of resistance to chemotherapy. The action of CDDP on inducing apoptosis is to form platinum adducts with DNA, involving intrastrand crosslinks. The adduct formation leads to DNA damage, inhibition of DNA replication, RNA translation, cell cycle arrest at G2 phase and apoptosis (Fig. 2). However, after the first successive rounds of treatment, most recurrent tumors develop a resistant phenotype to CDDP-based therapy. In general, four mechanisms of drug resitance have been documented; (1) defect in drug transport system that the cells have the ability to decrease influx and increase efflux such as ATP- dependent efflux pump, (2) the activation of DNA-repair pathway, which the cell can excise the damaged DNA from the DNA strand, (3) the ability to block apoptosis such as the mutation of p53 or the overexpression of Akt and (4) drug turnover in which the cells have the capability to detoxify the drug entering to the cells by using enzyme such as cytochrome p450 (Kartalou, M., et al., 2001).

Resistance to chemotherapeutics involves complex mechanisms that can stem from dysregulated signaling, enhanced DNA repair, altered cancer cell metabolism (Hsu et al., 2008; Hanahan et al., 2011), drug transport and metabolism (Perego et al., 2003), and the dysregulation of survival factors.

Activated DNA repair pathways can increase excision of adducts from DNA, which results in a defective apoptotic response and CDDP resistance (Kartalou, M., et al., 2001). Decreased intracellular concentration is a consequence of decreased drug uptake or increased reflux which can arise from the development of multidrug resistance genes. In addition, altered expression of regulatory proteins, including FLIP, Xiap and Akt (Sasaki et al., 2000; Fraser et al., 2003; Abedini et al., 2008), involved in pro- and anti-apoptotic pathways are also key factors in chemoresistance (Fraser, M., et al., 2003). These molecular and cellular events alter the overall response of the cell to genotoxic agents like CDDP and influence the cell toward pro-survival decisions.

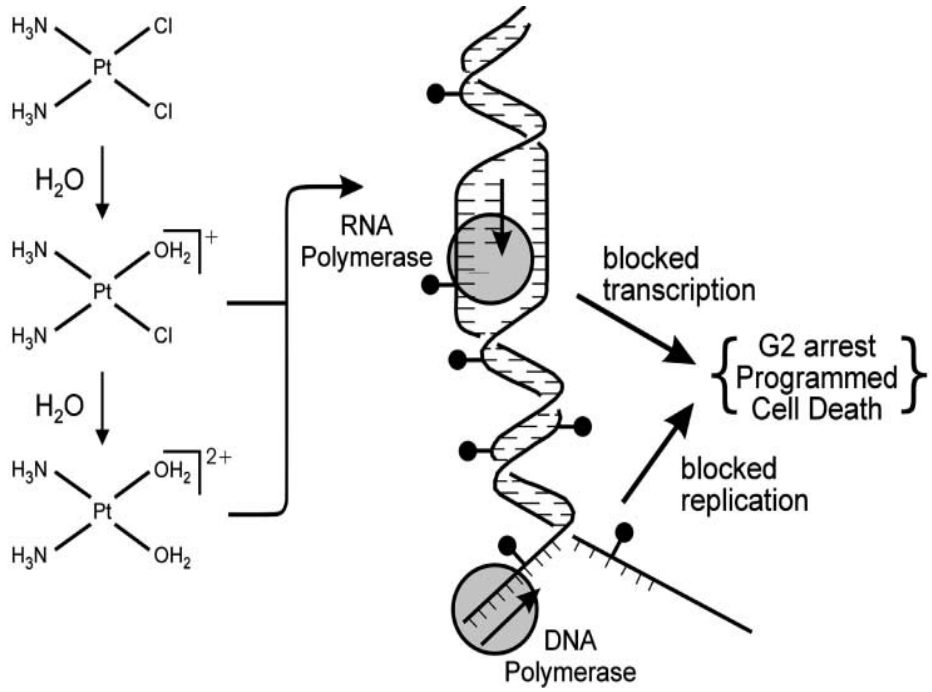

FIGURE 2. Mechanism of cisplatin action. When CDDP enters the cell, chloride ligands will be replaced with water molecules, generating positive charge. This aquated molecule then can bind to DNA and form platinum adducts, resulting in DNA damage, inhibition of DNA replication RNA translation, cell cycle arrest at G2 phase and apoptosis. (Modified from Kartalou, M., et al., 2001)

CHAPTER 2

LITERATURE REVIEW

1. p53 and chemosensitivity

p53 is a tumor suppressor protein that influences downstream effectors of apoptosis through both transcription-dependent and –independent mechanisms (Yang et al., 2006; Fraser et al., 2008). It is normally activated by CDDP via phosphorylation at Ser15 and Ser20, which are essential for its pro-apoptotic properties, and suppression of murine double minute 2 (MDM2) and its ubiquitination and proteasomal degradation (Honda et al., 1997; Moll et al., 2003; Ali et al., 2011). The other major functions of p53, rather than regulation of apoptosis and auto-regulation, are to regulate transcription of genes involve in cell cycle arrest and DNA repair such as p21. Moreover, tumor suppressor p53 is one of the most frequently mutated genes in human OVCA, and could be as high as 50% in OVCA patients (Schuijer et al., 2003). Therefore, loss of p53 can contribute to tumor aggressiveness, poor prognosis and low successful treatment. We have recently demonstrated that loss of p53 function by inactivation or mutation negatively influences apoptosis and chemosensitivity (Fraser et al., 2003; Fraser et al., 2006). Cells lacking functional p53 fail to inhibit mTORC1 in response to DNA damage (Feng et al., 2005). However, the coordination and communication between p53 status and rictor in regulation of chemoresistance is poorly understood.

2. PI3 Kinase-Akt Pathway in chemoresistant ovarian cancer

Phosphoinositide-3 kinase (PI3K) is a heterodimer phospholipid kinase which is activated by several receptor tyrosine kinases in response to growth stimuli. PI3K is composed of regulatory (p85) and a catalytic (p110) subunit, which converts phosphatidylinositol 3,4-bisphosphate (PIP_2) to phosphatidylinositol 3,4,5-triphosphate (PIP_3) leading to activation and further signaling downstream targets containing pleckstrin homology (PH) domain such as Akt, PDK1, PKC, JNK1, and p38 to mediate a variety of cellular responses including cell growth and transformation, differentiation, motility, insulin action, and cell survival (Cheng O. J. et. al., 2002). The tumor suppressor phosphatase and tensin homolog (PTEN) is known to be a protein phosphatase regulating PI3K, to maintain cellular homeostasis, by dephosphorylating PIP_3 to PIP_2 (West K. A. et. al., 2002)

The oncogenic serine/threonine kinase Akt, also known as protein kinase B (PKB), is a major target of the PI3K pathway and is also the only one downstream that has been implicated in malignant transformation (Mende, I. et. al., 2001). Akt is a family of proteins consisting of three members (Akt1, Akt2, and Akt3) which have similar structures. Akt is activated by three major phosphorylation events at the catalytic motif (Thr308), the hydrophobic motif (Ser473) and the turn motif (Thr450), but

only Thr308 and Ser473 residues fulfill crucial and non-overlapping functions in oncogenic activity (Hart et al 2011), while the Thr 450 residue is important for stabilization of Akt content (Wu et al., 2011). The activated Akt will then regulate its downstream effectors, such as Cyclin D1 and Glycogen Synthase Kinase 3, to promote cell proliferation and cell survival (Efeyan. A., et al. 2010).

Aberration of PI3K-Akt pathway has been reported in various cancers (Cheng O. J. et. al., 2002) and its activation promotes a chemoresistant phenotype, frequently detected in as high as 87% of cases in human OVCA (Altomare et al., 2004). In human OVCA, Akt activation and/or over-expression are also a determinant of CDDP sensitivity. Akt activation results in the stabilization of a number of caspase inhibitors such as Xiap and FLIP which can further inhibit caspase-3 and caspase-8, respectively (Dan et al., 2004; abedini et al., 2010), inhibits mitochondrial p53 accumulation and release of death proteins like cytochrome-C, Smac and BAX (Yang et al., 2006; Yang et al., 2008), and attenuates p53 phosphorylation and nuclear function (Fraser et al., 2008). In contrast, Akt inhibition increases p53 phosphorylation (Ser^{15}), which activates p53 function, and CDDP sensitivity (Fraser et al., 2008; Woo et al., 2012).

3. mTOR and ovarian cancer chemoresistance

The mammalian target of rapamycin (mTOR) has emerged as a critical regulatory pathway of cellular metabolism, growth, proliferation, and cell survival. mTOR exists in two functionally distinct complexes, mTORC1 and mTORC2. mTORC1 is sensitive to rapamycin and controls protein synthesis and cellular metabolism, while mTORC2 is involved in cytoskeleton assembly and is also responsible for phosphorylation of Akt at Ser473 allowing full activation and proteasomal degradation (Efeyan. A., et al. 2010) and at Thr 450 to stabilize total Akt content. Conversely, Akt activates mTOR through direct phosphorylation at Ser2448, through which Akt forms its own positive feedback loop to enhance its activation (Nave, B. T., et al., 1999). Signaling pathways for both mTOR complexes interconnect and influence each other via several key mediators, including Akt.

The interplay between Akt and mTOR complexes is essential for their cellular function. After phosphorylation by mTORC2, Akt activates mTORC1 by phosphorylating and inhibiting both tuberous sclerosis complex 2 (TSC2) and proline-rich protein (PRAS40), negative regulators of mTORC1 (Guertin, D. A. et al., 2007). Active mTORC1 elicits a negative feedback loop on Akt activity by influencing the responsiveness of its downstream effector, p70S6K1, to insulin receptor substrate 1 (IRS-1) and rictor, an mTORC2 component. Since mTORC2 was found to be an

activator of Akt by phosphorylating Ser473, Akt activity is both positively and negatively controlled by mTOR. However, it is important to note that phosphorylation of Akt at Ser473 does not require Akt-dependent activation of mTORC1, and Akt cannot promote cell proliferation and oncogenic transformation in mTORC1-null cells (Bhaskar, P. T. et al., 2007). The mTOR signaling pathway is also regulated by other molecular events and stimuli including mitogens, hypoxia, amino acids, and intracellular energy levels (Zoncu, R. A. et al., 2010) One of the most clinical implications of Akt-independent mTOR activity is AMP-activated protein kinase (AMPK), a master metabolic regulator(Memmott, R. M. et al., 2009). AMPK inhibits mTORC1 both directly and indirectly via TSC2 in response to low cellular energy levels. Therefore, reported AMPK activators such as metformin, AICAR and A769662 may be promising therapeutic agents for cancers featuring dysregulation of mTOR signaling pathways.

mTOR signaling dysregulation exists in various cancers including OVCA and is frequently related to tumorigenesis, tumor growth, and metastasis. Aberration of the mTOR signaling pathway confers resistance to CDDP-based chemotherapy and adverse prognosis in OVCA patients (No, J. H. et al., 2011). The role of mTOR in drug resistance is commonly relevant to other common mutational events in cancer such as those occurring in the epidermal growth factor receptor (EGFR), Ras, and Akt. p70S6K1 is the major downstream effector of mTORC1, promoting cancer cell

proliferation, growth, and angiogenesis (Jiang, B. H. et al., 2008). Therefore, mTORC1 is a logical molecular target for overcoming OVCA chemoresistance. Clinical studies in OVCA involving the rapamycin analog, RAD001, have shown it to have positive effects on the sensitization of ovarian tumors to CDDP treatment, prolonging mean survival time in a mouse model (Mabuchi, S., et al., 2007; Mabuchi, S., et al., 2009). BEZ235, a dual PI3K/mTOR inhibitor, has also recapitulated the importance of mTORC1 in regulating cell proliferation in OVCA (Montero, J. C., et al., 2012). Other inhibitors have also been used to demonstrate this concept. Although mTORC1 and dual PI3K/mTOR inhibitors are effective in sensitizing cancer cells to chemotherapy, loosing negative feedback from mTORC1 to Akt and a board effect of dual PI3K/mTOR inhibitor remain major disadvantages. Therefore, development of a specific mTORC2 inhibitor could form an alternative approach for overcoming mTOR-mediated chemoresistance in OVCA since mTORC2 is known to promote cell survival through Akt, and associates with other kinases to promote metastasis and tumor formation in breast and colon cancers, respectively (Roulin, D., et al., 2010).

The plausible molecular targets for the inhibition of mTORC2 function include mammalian stress-activated protein kinase-interacting protein (mSIN1), mTOR-associated protein LST8 (mLST8), and rictor. Those proteins are required for the integrity of the complex, thereby

enhancing tumor development and progression. Rictor also has other roles in cell migration and cancer cell metastasis. Moreover, mTORC2 inhibitors may have an advantage over mTOR catalytic site inhibitors in that they do not perturb the negative feedback loop to IRS-1 as well. This corroborates the possibility that development of an mTOR inhibitor, specifically targeting mTORC2, would provide a great impact on chemoresistant OVCA therapy.

4. Rictor and cancer

Rictor is an essential component of the complex mTORC2, and is required for full function (Sarbassov et al., 2004). Over-expression of rictor increases mTORC2 activity and promotes cell growth and motility (Masri et al., 2007). Conversely, rictor down-regulation suppresses cell proliferation and tumor formation in certain cancers (Fu et al., 2009; Roulin et al., 2010; Back et al., 2012). Rictor also interacts with the integrin-linked kinase (ILK) to promote cancer cell survival through Akt phosphorylation at Ser473, and with PKCζ for cancer cell invasion and metastasis (McDonald et al., 2008; Zhang et al., 2010). Rictor is required for prostate cancer development induced by PTEN loss (Guertin et al., 2009). Targeting rictor induces cell cycle arrest at G1 phase and decreases cyclin D1 expression in breast, colon and prostate cancer cells (Hietakangas et al., 2008; Roulin et al., 2010). Moreover, mTORC2 down-regulation facilitates chemotherapeutic drug-

induced apoptosis in breast cancer cells (Li et al., 2012). However, the role of rictor in CDDP resistance in OVCA remains unknown.

CHAPTER 3

THE mTORC2 COMPONENT RICTOR CONFERS CISPLATIN RESISTANCE IN HUMAN OVARIAN CANCER CELLS

1. Abstract

Resistance to cisplatin (CDDP: cis-diamminedichloroplatinum)-based therapy is a major cause of treatment failure in human ovarian cancer (OVCA). A better under-standing of the mechanisms of CDDP resistance will offer new insights for novel therapeutic strategies for this deadly disease. Akt and p53 are determinants of CDDP sensitivity. Rictor is a protein kinase component of mTOR complex 2, which is required for Akt phosphorylation (Ser473) and full activation. However, the precise role of rictor and the relationship between rictor and p53 in cisplatin resistance remains poorly understood. Here, using sensitive wild-type p53 (OV2008 and A2780s), resistant wild-type p53 (C13* and OVCAR433), and p53 compromised (A2780cp, OCC1, and SKOV-3) OVCA cells, we have demonstrated that (i) rictor is a determinant of CDDP resistance in human OVCA cells; (ii) CDDP down-regulates rictor content by caspase-3 cleavage and proteasomal degradation; (iii) rictor down-regulation sensitizes chemo-resistant OVCA cells to CDDP-induced apoptosis in a p53-dependent manner; (iv) rictor suppresses CDDP-induced apoptosis and confers resistance by activating and stabilizing Akt. These findings extend current knowledge on the molecular and cellular basis of cisplatin resistance and provide a rational basis for Rictor as a potential therapeutic target for chemoresistant OVCA.

Key Words: Rictor, mTORC2, ovarian cancer, Chemoresistance

Student ID: 2011-24114

2. Introduction

The mammalian target of rapamycin (mTOR) pathway has emerged as a critical regulator of cellular metabolism, growth, proliferation and survival. Its aberration, which is present in up to 50% (Altomare et al., 2004) of OVCA patients, has been shown to confer resistance to CDDP-based treatment and is associated with an adverse prognosis (Jaing et al., 2008; Zhang et al., 2009; Peng et al., 2010; No et al., 2011). The mTOR pathway involves two signaling complexes: mTORC1 and mTORC2. mTORC1 is sensitive to rapamycin and controls protein synthesis and cellular metabolism. p70S6K1 is the major downstream effector of mTORC1, promoting cancer cell proliferation, growth, and angiogenesis (Liu, L. Z. et al. 2008). These regulations lead mTOC1 to being a molecular target for overcoming chemoresistance in cancers. mTORC2 is essential for cell viability (Sparks et al., 2010) and is also known for its role in the phosphorylation of Akt at Ser473 allowing full activation and proteasomal degradation (Sarbassov et al., 2005; Bhaskar et al., 2007; Wu et al., 2011). Activated Akt then stimulated downstream targets to promote cell survival.

Although clinical studies in OVCA involving the rapamycin analog have shown positive effects on the sensitization of ovarian tumors to CDDP treatment, prolonging mean survival time in a mouse model, (Mabuchi, S. et al. 2007; Mabuchi, S. et al. 2009) loosing negative feedback from mTORC1

to Akt remains major disadvantages. Therefore, development of a specific mTORC2 inhibitor could form an alternative approach for overcoming mTOR-mediated chemoresistance in OVCA. One of the plausible molecular targets for the inhibition of mTORC2 function could be the rapamycin-insensitive companion of mTOR (Rictor).

Rictor is an essential component of the complex mTORC2, and is required for full function (Sarbassov et al., 2004). Over-expression of rictor increases mTORC2 activity and promotes cell growth and motility (Masri et al., 2007). Conversely, rictor down-regulation suppresses cell proliferation and tumor formation in certain cancers (Fu et al., 2009; Roulin et al., 2010; Back et al., 2012). Rictor also interacts with the integrin-linked kinase (ILK) to promote cancer cell survival through Akt phosphorylation at Ser473, and with PKCζ for cancer cell invasion and metastasis (McDonald et al., 2008; Zhang et al., 2010). Rictor is required for prostate cancer development induced by PTEN loss (Guertin et al., 2009). Targeting rictor induces cell cycle arrest at G1 phase and decreases cyclin D1 expression in breast, colon and prostate cancer cells (Hietakangas et al., 2008; Roulin et al., 2010). Moreover, mTORC2 down-regulation facilitates chemotherapeutic drug-induced apoptosis in breast cancer cells (Li et al., 2012). However, the role of rictor in CDDP resistance in OVCA remains unknown.

We hypothesize that rictor confers CDDP resistance and its down-regulation sensitizes chemoresistant OVCA cells to CDDP treatment by facilitating proteasomal Akt degradation and in a manner dependent upon p53 status. Our overall objective is to better understand the cellular and molecular mechanisms of chemoresistance in human OVCA and, specifically, to examine the role and regulation of the mTORC2 component Rictor and its association with p53 in relation to chemoresistance.

In the present study, we have investigated the relationship between rictor, Akt and p53 in OVCA cell lines in the context of CDDP resistance. We have demonstrated for the first time that (a) rictor stabilization confers CDDP resistance; (b) rictor knock-down facilitates CDDP-induced apoptosis which is p53-dependent; (c) rictor contributes to CDDP resistance in human OVCA in part by activating and stabilizing Akt; (d) caspase-3 activation and proteasomal degradation are responsible for rictor processing during CDDP-induced apoptosis. These findings will extend current knowledge on the molecular and cellular basis of cisplatin resistance and form a rational basis for targeting rictor as a novel therapeutic strategy in overcoming CDDP resistance in human OVCA, particularly for those with wt-p53 status. In addition, targeting rictor may potentiate effective therapeutic outcomes, since this is likely to not perturb the negative feedback pathway between Akt and mTORC1.

3. Materials and Methods

Reagents

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). CDDP, dimethyl sulfoxide (DMSO), Hoechst 33248, aprotinin, sodium orthovanadate (Na_3VO_4), phenylmethylsulfonyl fluoride (PMSF) were from Sigma-Aldrich (St. Louis, MO, USA). Rabbit polyclonal antibodies: anti-phospho-Ser^{473}-Akt, anti-phospho-Ser^{450}-Akt, anti-Akt, anti-phospho-Ser^{15}-p53, anti-PARP and Rabbit monoclonal anti-rictor antibody were purchased from Cell Signaling Technology (Beverly, CA, USA). Mouse monoclonal anti-p53 and anti-GAPDH anti-body was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Abcam (Cambridge, MA, USA), respectively. Goat anti-mouse and anti-rabbit secondary antibodies were from Bio-Rad Laboratories (Hercules, CA, USA). Rictor and p53 siRNA were purchased from Cell Signaling Technology (Beverly, CA, USA). Control siRNA was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Lipofectamine 2000, RNase A, *N*,*N*,*N'*,*N'*-tetramethyl-ethane-1,2-diamine (TEMED), TRIzol and ROX dye were from Invitrogen (Carlsbad, CA, USA). RNeasy Mini Kit was from Qiagen (Valencia, CA, USA). Adenovirus containing wt-p53 gene and eGFP gene were from Vector Biolab (Philadelphia, PA,

USA). Epoxomycin, lactacystin and sucrose were from EMD Chemical (Gibbstown, NJ, USA). Z-DEVD-FMK and Z-VAD-FMK were from Tocris Bioscience (Ellisville, MO, USA). Rictor and GAPDH RNA primer were from Bioneer (Daejeon, Korea) and human recombinant active caspase 3 was from BioVision (Mountain View, CA, USA). PIPES, DL-Dithiothreitol (DDT), Ethylenediaminetetraacetic Acid (EDTA) and 3[(3-cholamidopropyl)dimethyallonio]-1-propanesulfonate hydrate (Chaps) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Cell lines and culture

CDDP sensitive [OV2008 and A2780s (wt-p53)] and resistant [C13* (wt-p53), OVCAR-433 (wt-p53), A2780cp (mutant-p53), OCC-1 (mutant-p53), and SKOV3 (p53 null)] human OVCA cell lines were generously provided by Drs. Rakesh Goel and Barbara Vanderhyden (Ottawa Hospital Cancer Center, Ottawa, ON, Canada). The cells were maintained in RPMI 1640 and DMEM/F-12 at 37°C and 5% CO_2 as previously reported Abedini et al., 2010; Ali et al., 2011). OV2008 cells and their resistant counterparts C13* cells were derived from ovarian endometrioid adenocarcinoma with squamous differentiation. OCC-1, A2780s and A2780cp cells originated from undifferentiated ovarian carcinoma tumors. SKOV3 cells were isolated from clear cell carcinoma origin (Shaw et al., 2004) and OVCAR433 cells

were derived from serous cystadenocarcinomas of the ovary (Woo et al., 2012). Following the indicated treatments, the cells were harvested for analysis.

Protein extraction and western Analysis

The procedures for Protein extraction and Western blotting analysis were performed as previously described (Abedini et al., 2010; Ali et al., 2011). Membranes were incubated at 4°C, if no further indications are provide, with anti-rictor (1:100), phospho-Ser^{473}-Akt (1:1000), phospho-Ser^{450}-Akt (1:1000), p53 (1:5000), phospho-Ser^{15}-p53 (1:1000), PARP (1:2000) and 1 h at room temperature for anti-GAPDH (1:10,000) and HRP-conjugated rabbit or mouse secondary antibodies (1:5000-1:10,000). Band densities were analyzed for quantification by ChemiDOC^{TM} XRS+ (Bio-Rad Laboratories; Hercules, CA, USA)

Assessment of nuclear morphology with Hoechst 33258 staining

The procedure was performed as previously described (Abedini et al., 2010; Ali et al., 2011). A minimum of 400 cells per treatment group were counted and the counter was blinded for the counting to avoid experimental bias.

siRNA transfection

OVCA cells were transfected with rictor siRNA (0-100 nM; 48 h), p53 siRNA (100 nM; 48 h), or control siRNA (0-100 nM; 48 h) and treated thereafter with CDDP (0-10 μM; 24 h), as previously described (Abedini et al., 2010), and harvested for further analysis.

Adenoviral infection

A2780cp and SKOV3 cells were infected with adenoviral wt-p53 (MOI = 10; 24 h; GFP as control) as previously described (Abedini et al., 2010).

In Vitro caspase-3 activity assay

In vitro caspase-3 activity assay was performed with whole cell lysates of OV2008. Whole cell lysates (50 ug) were incubated (30^{o}C, 2h) in Pipes assay buffer [1,4-piperazinediethanesulfonic acid (Pipes, 20 mM), NaCl (100 mM), dithiothreitol (DDT, 10mM), EDTA (1 mM), 3-[(3-cholamindopropyl)-dimethylammoniol]-1-propanesulfonic acid (Chaps, 0.1%, w/v), sucrose (10%, w/v), pH 7.2] containing recombinant active caspase-3 (0-20 □g/ml). The samples were boiled in Laemmli sample buffer (10 min), centrifuged, and supernatant proteins resolved on 8% SDS-PAGE as previously described (Woo et al., 2012).

Statistical analysis

Results are expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was performed by pair T-test, one-way or two-way ANOVA, depending upon experiment, by using SigmaPlot® 12(Systat Solfwere; Suite, IL, USA). Differences between multiple experimental groups were determined by the Bonferroni post-hoc test. Statistical significance was inferred at $P < 0.05$.

4. Results

4.1 CDDP down-regulates Rictor content and induces apoptosis in chemosensitive but not resistant

To determine whether rictor content is altered during CDDP treatment in OVCA, chemosensitive OVCA (OV2008 and A2780s) and chemoresistant (C13*, A2780cp*, OVCAR433, OCC1 and SKOV-3) OVCA cell lines were treated with CDDP (0-10 μM; 24 h) and rictor content was assessed by immunoblotting (Fig. 3). CDDP significantly down-regulated intact rictor content (200 kDa) and induced apoptosis in chemosensitive (OV2008, ***P<0.001; and A2780s, **P<0.01), but not in chemoresistant OVCA irrespective of CDDP concentration (C13*, A2780cp, OVCAR433, OCC1 and SKOV-3; p>0.05). These results demonstrate that rictor is not subjected to down-regulation by CDDP in

chemoresistant OVCA cells, a phenomenon that may contribute to the resistant phenotype.

To better understand how rictor content in OVCA cells is regulated by CDDP *in vitro* and to determine if the downregulation in rictor content could be a result of suppression at the transcriptional level, rictor mRNA abundance in chemosensitive OVCA cells (OV2008 and A2780s) treated with CDDP (0 - 10 μM; 0 - 24h) was analyzed by quantitative real-time PCR. No statistical difference in rictor mRNA abundance between the CDDP treatment and control group was observed in both cell lines (data not shown).

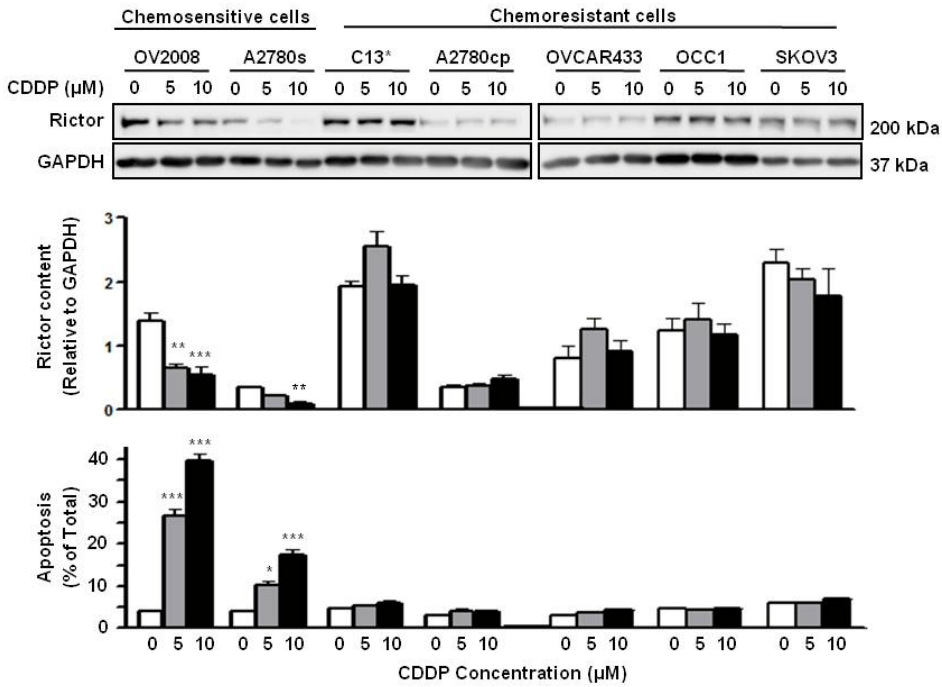

FIGURE 3. CDDP down-regulates rictor content and induces apoptosis in chemosensitive but not resistant OVCA cells *in vitro*. Rictor protein expression is down-regulated in chemosensitive (OV2008 and A2780s) but not chemoresistant OVCA (C13* and A2780cp*) during CDDP treatment (0 - 10 μM CDDP; 24 h). Decreased rictor content in OV2008 and A2780s following CDDP treatment was associated with increased apoptosis. Rictor protein content in chemoresistant OVCA (OVCAR433, OCC1 and SKOV3) was not affected by CDDP. Rictor content was normalized against GAPDH (loading control). A representative Western blot from three independent experiments is shown. Results are presented as mean ± SEM of three independent experiments.*$p<0.05$, **$p<0.01$, ***$p<0.001$, (vs CTL in sensitive cells).

4.2 CDDP induces rictor processing and chemosensitivity in a caspase-3 and proteasome-dependent manner.

To determine if CDDP-induced rictor down-regulation could be due to post-translational processing, we first investigated the possibility of caspase-dependent cleavage of rictor in OV2008 and A2780s when treated with the pan-caspase inhibitor Z-VAD FMK (10 μM) before (30 min) and during CDDP challenge (0 - 10 μM; 24 h). OV2008 cells treated with CDDP alone exhibited an intact rictor (200 kDa) and two immunoreactive cleaved products which migrated at 160 kDa and 130 kDa (Fig 4). CDDP decreased intact rictor content and the 160 kDa protein but markedly increased the levels of 130 kDa band. Although treatment of A2780s with CDDP also resulted in down-regulation of intact rictor (200 kDa), the level of the 160 kDa protein was markedly elevated while that of the 130 kDa was not significant affected. However, cleaved rictor was significantly rescued in both cell lines ($P<0.05$ Fig. 4) Pretreatment of the cells with the caspase inhibitor significantly attenuated the CDDP-induced changes in intact and cleaved rictor contents in both sensitive cells ($P<0.05$ and $P<0.01$ in OV2008 and A2780s, respectively, Fig. 4), suggesting that CDDP down-regulates rictor in part by increased caspase activity and that cleavage at different caspase consensus sites may be involved. In addition, pretreatment of the OVCA cells with the specific caspase-3 inhibitor Z-DEVD-FMK produced similar results, indicating that caspase-3 is involved in CDDP-

induced rictor processing. Interestingly, while CDDP induced apoptosis in both chemosensitive cell lines, pretreatment of the cells with either the pan-caspase or specific caspase-3 inhibitor attenuated this response, suggesting that CDDP induces rictor processing by caspase-3 and dysregulation of this process confers chemoresistance in OVCA cells.

To determine whether proteasomal degradation plays a role in CDDP-induced rictor down-regulation, OV2008 cells were cultured [30 min pre-treatment; 24 h during treatment with CDDP (0 - 10 μM)] with the proteasome inhibitors epoxomycin (10 nM) and lactacystin (4 μM). Whereas CDDP alone significantly down-regulated intact rictor and induced apoptosis, as expected, the presence of the inhibitors completely blocked CDDP-induced rictor down-regulation and significantly, but not completely attenuated apoptosis, as evidenced by PARP cleavage and nuclear morphology ($P<0.001$; Fig 5: A).

We then investigated further if rictor processing by caspase-3 activity and proteasomal degradation occurs in separate pathways. OV2008 cells were cultured [30 min pre-treatment; 24 h during treatment with CDDP (0 - 10 μM)] with proteasome inhibitors and/or specific caspase-3 inhibitor. The same results were observed when either inhibitor was present. However, no additional effect was observed when both inhibitors were used together (Fig.5: B).

In addition, to provide further evidence that rictor processing induced by CDDP treatment is caspase-3 dependent, whole cell lysates from OV2008 cells were used to perform an *in vitro* caspase-3 activity assay. The *in vitro* data was in agreement to that obtained from the cell line experiment (Fig.5: C). Taken together, these results indicate that CDDP down-regulates intact rictor at the protein level via caspase-3 cleavage and proteasomal degradation.

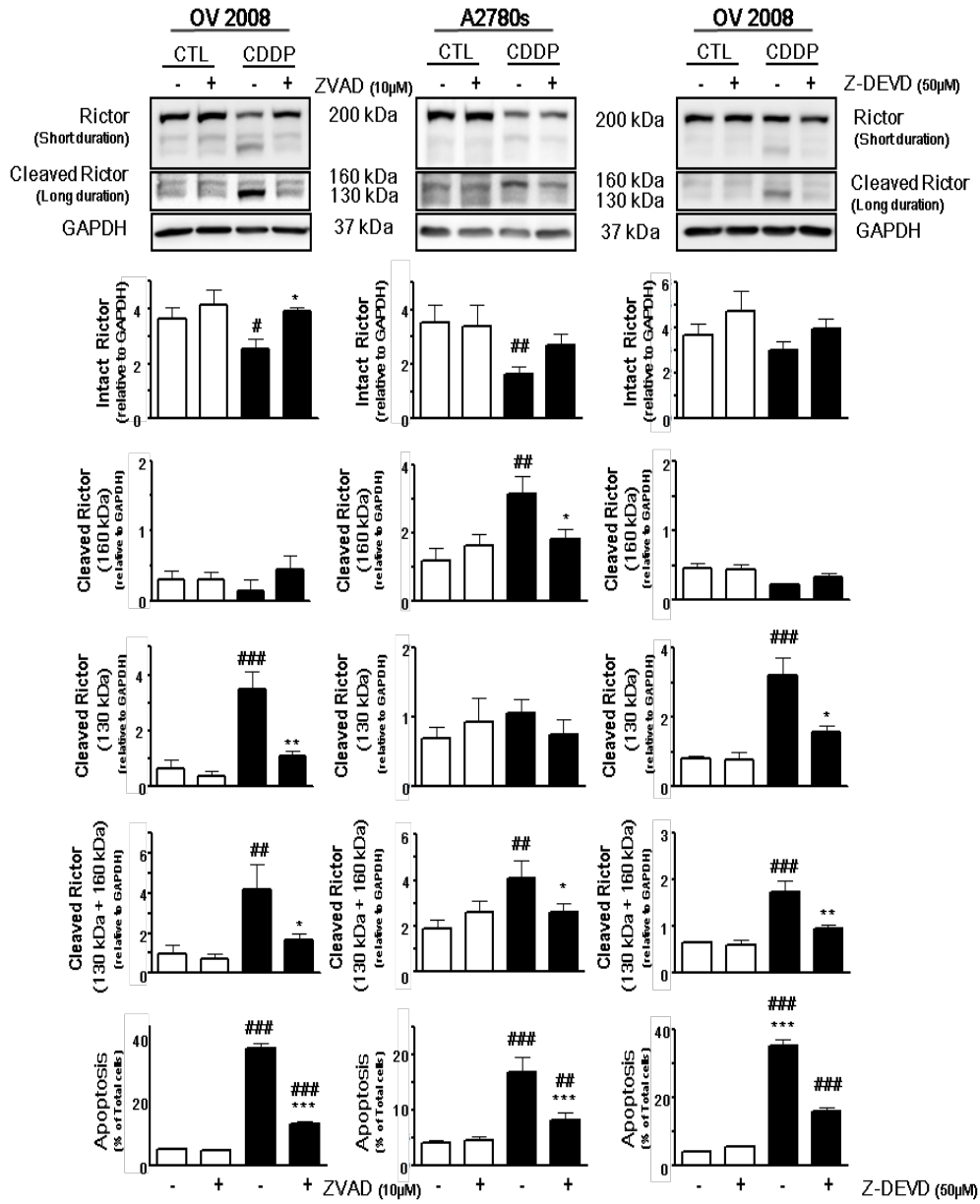

FIGURE 4. CDDP-induced rictor downregulation in CDDP-sensitive cells involves caspase-3-mediated cleavage. OV2008 and A2780s were pretreated with Z-VAD FMK (10 μM) and Z-DEVD FMK (50 μM) for 30 minutes before and during CDDP challenge (0 - 10 μM; 24 h) and rictor content and apoptosis were

assessed. OV2008 cells treated with CDDP alone exhibited an intact rictor (200 kDa) and two cleaved products (160 kDa and 130 kDa). CDDP decreased intact rictor content and 160 kDa protein but markedly increased levels of the 130 kDa band. Treatment of A2780s with CDDP resulted in down-regulation of intact rictor but increased the level of the 160 kDa protein and had no effect on the 130 kDa protein. Pre-treatment of the cells with the pan-caspase inhibitor (Z-VAD) or the specific caspase-3 inhibitor (Z-DEVD) significantly attenuated the CDDP-induced changes in intact and cleaved rictor contents in both sensitive cells, and CDDP-induced apoptosis was significantly but not completely attenuated by the presence of the inhibitors in both chemosensitive cell lines. Results are presented as mean ± SEM. (n=3 and n=5 in OV2008 and in A2780s, respectively) *$p<0.05$, **$p<0.01$, ***$p<0.001$ (vs respective controls of inhibitor), ##$p<0.01$, ###$p<0.001$ (vs respective controls of CDDP).

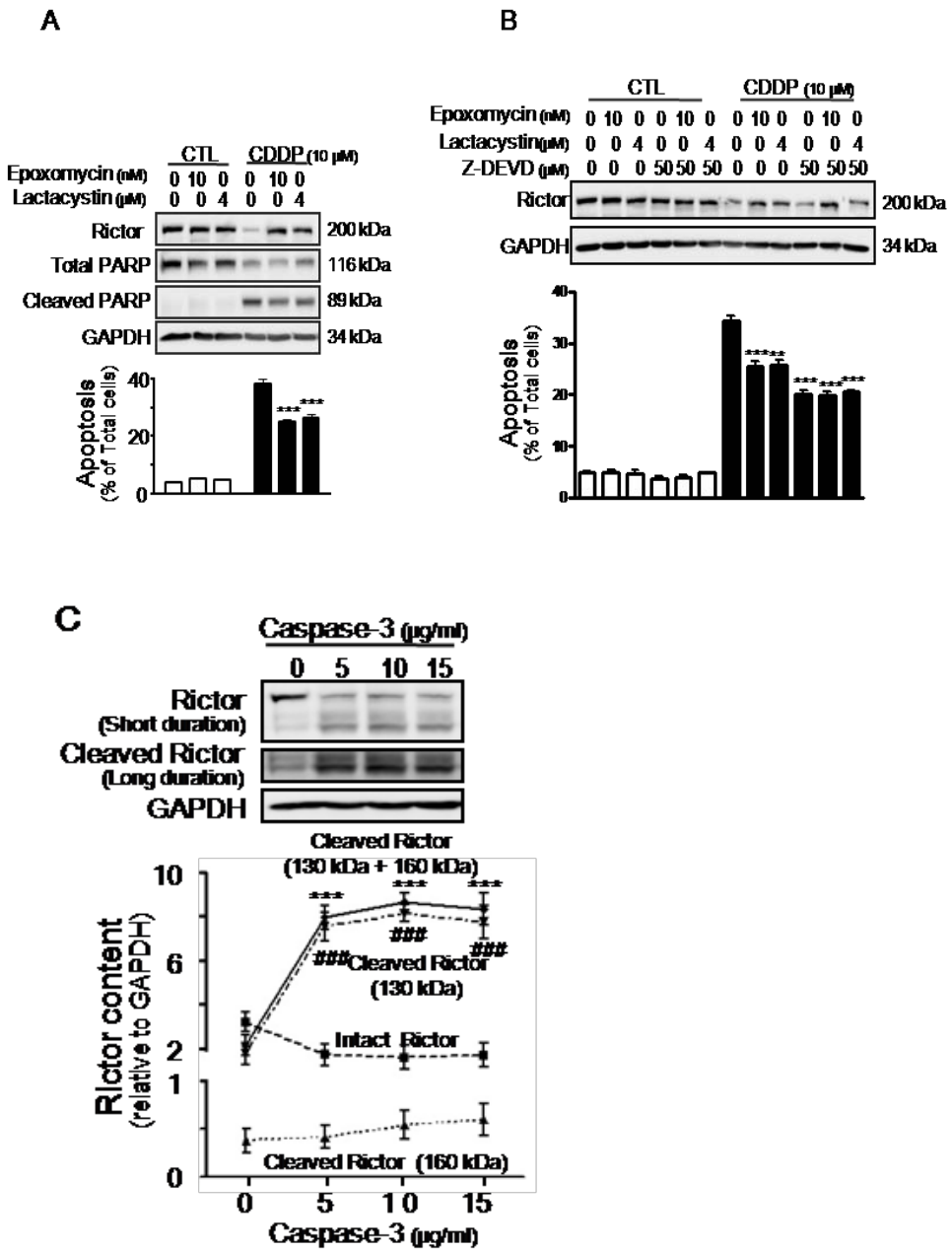

FIGURE 5. Proteasome-mediated degradation and caspase-3 activity are responsible for CDDP-induced rictor down-regulation in chemosensitive OVCA. **A.** OV2008 cells were pretreated (30 min) with the proteasomal inhibitors [epoxomycin (10 nM) and lacytasystin (4 μM) and

subjected to CDDP challenge (0 - 10 μM; 24 h). Rictor content and apoptosis were analyzed by Western blotting and nuclear morphology assessment. Both epoxomicin and lactacystin effectively blocked CDDP-induced rictor degradation ($P<0.001$) but only partially attenuated apoptosis. **B.** OV2008 cells were cultured in the same conditions as in A, but pretreated with proteasome inhibitor and/or Z-DEVD (50 μM). No synergistic effect between the two inhibitors was observed. **C.** Incubation of OV2008 whole cell lysate (30 min, 30^{o}C) with recombinant active caspase-3 (5 -20 μg/ml) resulted in Rictor cleavage as evidenced by decreased intact rictor content (200 kDa) and increased in the cleaved forms (130 kDa and 160 kDa). Results are presented as mean ± SEM of three independent experiments.*$p<0.05$, **$p<0.01$, ***$p<0.001$ (vs respective controls).

4.3 Rictor knockdown sensitizes chemoresistant OVCA to CDDP-induced apoptosis.

To establish the role of rictor in OVCA chemoresistance, rictor expression in a wt-p53 chemoresistant OVCA cell line (C13*) was silenced by siRNA (0, 50 and 100 nM; 48 h) prior to treatment with CDDP (10 μM; 24 h), and apoptosis was assessed. Intact rictor and its cleaved forms were significantly down-regulated by siRNA in a concentration-dependent manner. A significant decrease in intact rictor and cleaved rictor were observed at 50 nM ($p<0.05$) and to an approximate 30% reduction at 100 nM, irrespective of the presence of CDDP. These findings not only confirmed that the 160kDa and 130 kDa immunoreactive bands were indeed cleaved products of rictor, but also demonstrated that rictor knockdown induced apoptosis ($p<0.05$) as well as enhanced CDDP-induced apoptosis in a concentration-dependent manner ($p<0.001$) (Fig 6). These results suggest that rictor is an important determinant of CDDP resistance in OVCA.

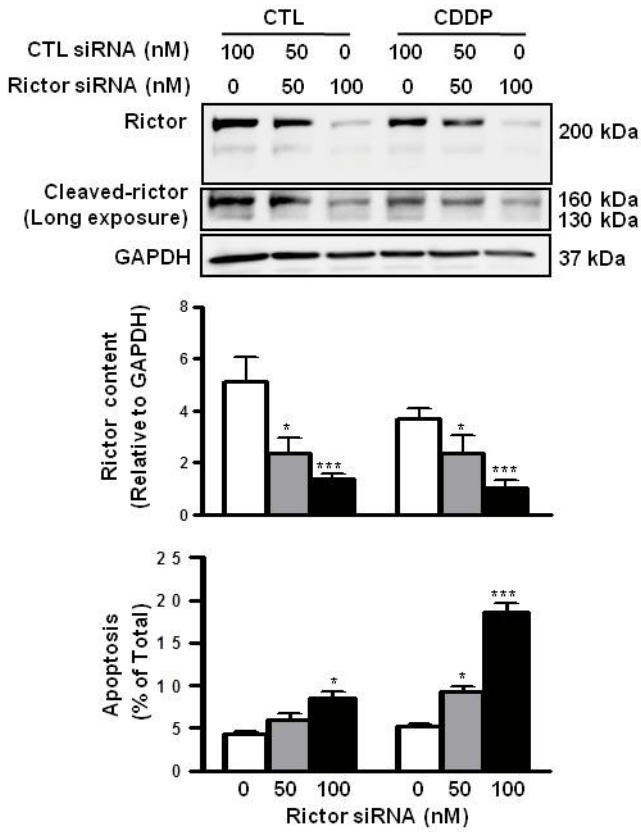

FIGURE 6. Rictor knockdown sensitizes chemoresistant OVCA cells to CDDP-induced apoptosis. C13* cells were transfected with rictor siRNA (0-100 nM; 48 h) and cultured with or without CDDP (10 μM; 24 h). Rictor knockdown significantly enhanced CDDP-induced apoptosis in C13* cells in a concentration-dependent manner. Results are expressed as mean ± SEM of three independent experiments. *$p<0.05$, ***$p<0.001$ (vs respective CTL siRNA).

4.4 Apoptotic response to CDDP following rictor down-regulation is dependent on p53 status

The tumor suppressor p53 is an important mediator of CDDP-induced apoptosis (Yang et al., 2006; Abedini et al., 2008). Since close to 50% of OVCA patients carry *TP53* gene mutation(s) (Schuijer et al., 2003), it is of interest to determine if CDDP-induced apoptosis in chemoresistant cells following rictor knockdown is dependent on a functional p53. To investigate this possibility, rictor expression in chemoresistant OVCA cell lines with varying p53 status (wt-p53, C13* and OVCAR433; p53-mutant, OCC1 and A2780cp; p53-null, SKOV3) were silenced with rictor siRNA (100 nM siRNA, 48 h) prior to treatment with CDDP (0-10 μM, 24 h). Rictor knock-down significantly sensitized chemoresistant wt-p53 cells (C13* and OVCAR433, Fig 7A; $P<0.001$) but not p53-deficient cells (OCC1, Fig 7A; A2780cp and SKOV3, Fig 7B) to CDDP-induced apoptosis, suggesting that a functional p53 might be needed for CDDP-induced apoptosis in the chemoresistant OVCA cells with rictor knockdown. To further investigate this hypothesis, CDDP-resistant OVCA cell lines harboring a p53 mutation (A2780cp) and a p53-null line (SKOV-3) were treated with rictor siRNA (0-100 nM; 48 h), followed by reconstitution of wt-p53 via adenoviral infection (0-10 MOI; 24 h) and CDDP treatment (0-10 μM CDDP; 24 h). As shown in Fig. 7B, while rictor knockdown alone did not significantly increase CDDP sensitivity in the absence of wt-p53,

wt-p53 reconstitution significantly enhanced the effects of rictor-knockdown on CDDP-induced phospho-p53 Ser15 content and apoptosis in both p53-deficient cell lines ($P<0.001$).

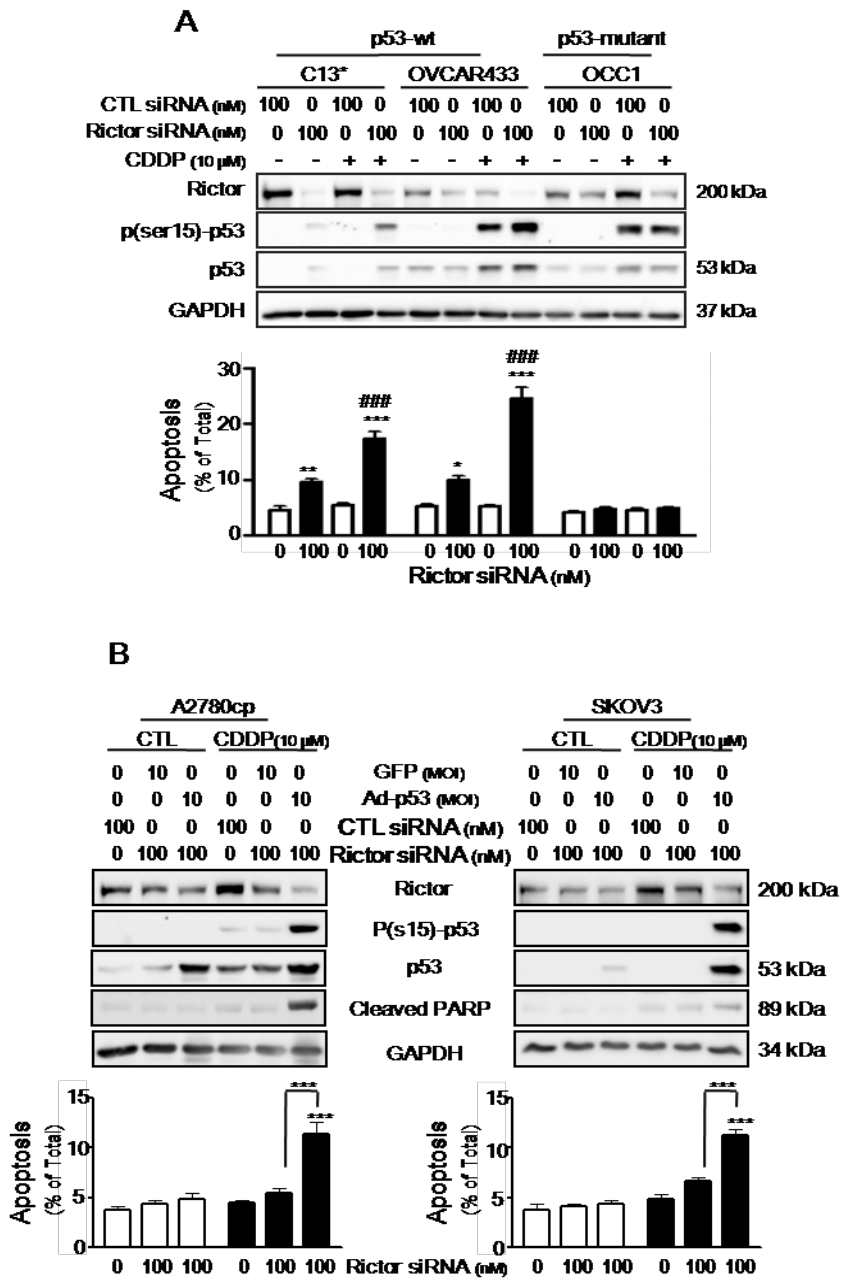

FIGURE 7. The apoptotic response to CDDP following rictor down-regulation is dependent on p53 status. Chemoresistant OVCA cell lines with varying p53 status (wt-p53, C13* and OVCAR433; p53-mutant, OCC1

and A2780cp; p53-null, SKOV3) were transfected with rictor siRNA (100 nM siRNA, 48 h) with or without adenoviral wt-p53 infection (0-10 MOI; 24 h; p53-deficient cells) and CDDP treatment (0-10 μM CDDP; 24 h). Rictor, p-p53 ser15, p53, PARP and GAPDH content, as well as apoptosis were assessed. Rictor knock-down significantly sensitized chemoresistant wt-p53 cells (**A**, C13* and OVCAR433; $P<0.001$) but not p53-difficient cells (**A**, OCC1; **B**, A2780cp and SKOV3) to CDDP-induced apoptosis. Reconstitution in A2780cp and SKOV-3 of wt-p53 significantly enhanced CDDP-induced apoptosis ($P<0.001$). Results are presented as mean ± SEM of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (vs respective CTL siRNA), ###$p<0.05$ (vs respective controls CDDP).

4.5 Rictor down-regulation sensitized chemoresistant cells to CDDP-induced apoptosis by facilitating Akt proteasomal degradation

Rictor is known to be required for mTORC2 to phosphorylate Akt at Ser473. To examine if and how Akt is relevant to this phenomenon, chemoresistant OVCA cells (C13*) rictor expression was silenced by siRNA (100 nM; 48 h) prior to pre-treatment with epoxomycin (12.5 nM; 30 min) and treatment with CDDP (10 μM; 24 h), and apoptosis was assessed. Rictor knockdown alone increased phospho-Akt (Ser473) content but decreased the levels of phospho-Akt Ser450 (Fig. 8), a phosphorylation site known to be regulated in Akt stability (Wu et al.,). The increase in Akt phosphorylation at Ser473 following rictor silencing without the proteasomal inhibitor was accompanied by Akt down-regulation ($P<0.05$ in treatment group) and apoptosis induction irrespective of the presence of CDDP, two responses attenuated by the presence of the proteasome inhibitor epoxomycin. (Fig 8) Moreover, the ratio between phospho-Akt at Ser473 and total Akt was also markedly increased in rictor knockdown with CDDP-treatment and significantly decreased when epoxomicin was added ($P<0.001$ and $p<0.05$; with and without epoxomycin, respectively). These results suggest that rictor plays a role in Akt stabilization and CDDP resistance in human OVCA and its knockdown promotes Akt proteasomal degradation and enhances CDDP sensitivity.

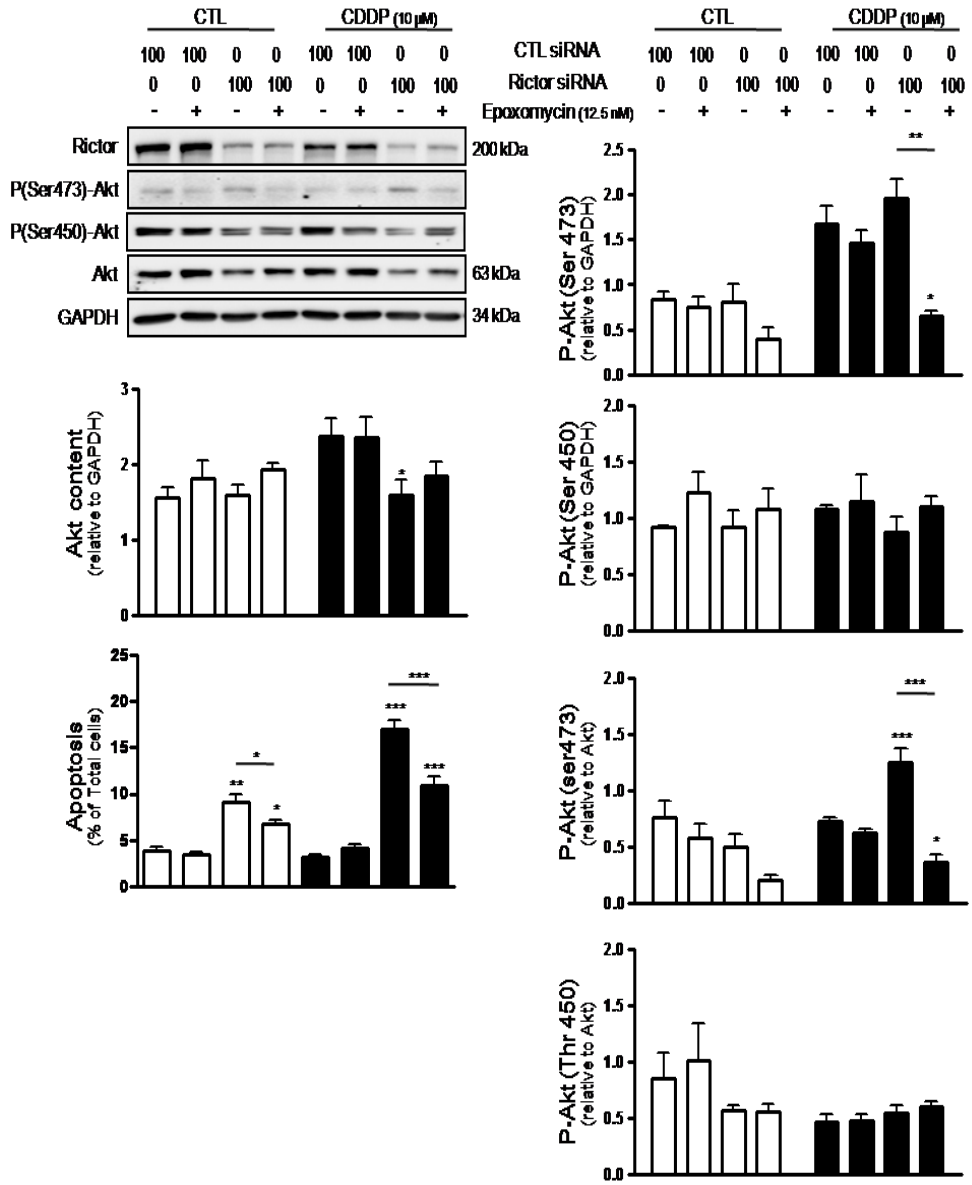

FIGURE 8. Rictor down-regulation sensitizes chemoresistant cells to CDDP-induced apoptosis by facilitating Akt proteasomal degradation. Chemoresistant OVCA cells (C13*) were transfected with rictor siRNA (100 nM siRNA, 48 h) and pretreated with epoxomycin (0-12.5 nM) 30 minutes prior to CDDP treatment (0-10 μM CDDP; 24 h). Rictor, Akt,

phospho- Akt (Thr450 and Ser473), and GAPDH content, as well as apoptosis were assessed. Rictor knock-down significantly enhanced CDDP-induced apoptosis in chemoresistant OVCA cells ($P<0.01$ and $P<0.001$, respectively) and this effect was rescued by epoxomycin ($P<0.05$ and $p<0.001$, respectively) Total-Akt was significantly decreased during rictor silencing with CDDP treatment and a massive increase in the ratio between phospho-Akt (Ser473) and total-Akt was also observed, which significantly decreased when epoxomycin was added ($P<0.001$ and $P<0.05$, respectively) Results are presented as mean ± SEM of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (vs respective CTL siRNA), ###$p<0.05$ (vs respective controls).

5. Discussion

Chemoresistance is a major therapeutic hurdle in ovarian cancer and its cellular mechanism is complex and poorly understood. Although the mTOR signaling pathway is known to promote cell proliferation and survival, whether rictor is involved in the control of chemosensitivity is unknown. In the present study, we have investigated the role of rictor in CDDP resistance using CDDP-sensitive and resistant OVCA cell lines with different p53 status. We have demonstrated for the first time that rictor is a determinant of CDDP resistance in OVCA. CDDP down-regulates rictor content in chemosensitive cells by caspase-3 and proteasome-dependent degradation, but no similar effect was observed in resistant cells. In addition, we have shown that rictor suppresses CDDP-induced apoptosis and confers resistance by activating and stabilizing Akt. Silencing rictor sensitizes chemoresistant OVCA cells to CDDP-induced apoptosis in cells harboring wild type-p53, but not in p53-compromised cells unless reconstituted with a functional p53. Taken together, these data support the hypothesis that rictor down-regulation sensitizes chemoresistant OVCA cells to CDDP treatment by facilitating Akt-dependent proteasomal degradation, in a manner dependent upon p53 status.

The mTOR signaling pathway is known to promote cellular metabolism, growth, proliferation and cell survival, and its dysregulation is frequently associated with tumorigenesis, tumor growth and metastasis (Jaing et al., 2008). The role of the mTOR pathway in various cancers has been extensively explored, particularly for mTORC1. The role of mTORC2, however, remains poorly understood (Guertin et al., 2007). mTORC2 is known to play essential roles in cell survival and metastasis in a number of cancers, but in normal cells, the importance of mTORC2 activity is less clearly defined (Sparks et al., 2010). Rictor is a critical component of the mTORC2 complex and its function is to maintain complex integrity. It is known to interact with other kinases for the promotion of cell survival, but the role of rictor in chemosensitivity has not been defined to date. In this study, we have examined the role of rictor and its relation to CDDP-resistance and found that rictor is down-regulated by CDDP in the chemosensitive response and its stabilization contributes to CDDP-resistance in human OVCA cells. A previous study has shown that mTORC2 activity is elevated in gliomas, both in cell lines and primary tumors, and over-expression of rictor promotes cell growth and cell motility (Masri et al., 2007). In contrast, other studies have reported that down-regulation of rictor attenuates cell proliferation and autophagy (Fu et al. 2009; Back et al., 2012). Our present investigation shows that down-regulation of rictor occurs concomitantly with CDDP-induced apoptosis in sensitive OVCA cells in a

concentration-dependent manner, and rictor content appears to have an influence on cell fate. This observation is further supported with results using various chemoresistant OVCA cell lines with different p53 status, in that the failure of CDDP to down-regulate rictor content allows the cells to survive CDDP treatment, irrespective of concentration. Higher levels of rictor expression in the resistant counterpart of the genetically-matched cell lines (OV2008 and C13*) was also observed, suggesting that rictor is a pro-survival protein and its stabilization confers CDDP-resistance.

The role of rictor in cell survival has been previously demonstrated, yet only one study has addressed the notion that rictor and other proteins in the mTOR pathway are down-regulated via the proteasome-ubiquitin pathway after chemo-therapeutic challenge (Fu et al., 2009). Our results showing that CDDP induces rictor down-regulation via the proteasome are in agreement with this finding. Moreover, we have also illustrated and added to the current knowledge that caspase-3 is required for rictor processing induced by CDDP treatment in chemosensitive human OVCA cells. Taken together, these results suggest that rictor confers CDDP resistance in human OVCA and CDDP-induced rictor down-regulation is dependent upon both caspase-3 activity and proteasomal degradation in chemosensitive, but not in chemoresistant OVCA cell lines.

The mechanism of CDDP resistance in human OVCA is multi-factorial and involves the dysregulation of multiple pathways controlling

DNA repair and apoptosis (Kartalou et al., 2001). p53 is a tumor suppressor playing a crucial role in apoptosis via both transcription-dependent and -independent mechanisms (Yang et al., 2006; Woo et al., 2012). It is also one of the most frequently mutated genes in human OVCA, and could be as high as 50% in OVCA patients (Schuijer et al., 2003). Loss or mutation of p53 can have an enormous impact on tumor aggressiveness, prognosis and successful implemention of treatment (Kmet et al., 2003). Previous studies have shown that rictor plays a role in cancer development, survival and aggressiveness (McDonald et al., 2008; Guertin et al., 2009; Zhang et al., 2010) and targeting rictor attenuates colon and breast cancer cell proliferation by inducing G1 phase arrest and decreasing cyclin D1 expression (Hietakangas et al., 2008; Roulin et al., 2010). Since p53 status is a major concern in human OVCA particularly in the context of chemoresistance, we have investigated the relationship between rictor and the tumor suppressor p53. Our investigation further addresses the role of rictor in CDDP resistance and shows that rictor knockdown not only promotes apoptosis, but also enhances CDDP-induced apoptosis in human OVCA cells. This result correlates with a recent study showing that targeting of rictor prevents cell migration and promotes apoptosis in breast cancer (Li et al., 2012). Our current study also show that sensitizing chemoresistant OVCA cells by silencing rictor appears to depend upon p53 status. This notion is further supported by the observation that a

combination of rictor knockdown and reconstitution of wt-p53 enhances apoptosis in p53-compromised cells when induced by CDDP treatment, an outcome that does not eventuate without wt-p53. The latter response is concomitant with an increase in p53 activation through phosphorylation at the ser15 residue, which changes p53 structure and inhibits the p53-MDM2 interaction, thereby stabilizing p53 (Moll et al., 2003). This could be the reason for p53 stabilization observed in A2780cp and SKOV-3 in the presence of phospho-p53 (ser15). Taken together, these observations suggest that rictor knockdown induces apoptosis and enhances CDDP-induced apoptosis in a p53-dependent manner.

The Akt signaling pathway is a determinant of chemoresistance and its activation promotes a chemoresistant phenotype, frequently detected in as high as 87% of cases in human OVCA (Altomare et al., 2004). Akt is an oncoprotein playing a pivotal role in cancer cell survival, aggressive behavior and apoptosis, and we and others have previously reported that Akt promotes CDDP resistance by inhibiting both intrinsic and extrinsic apoptotic pathways, and perturbing p53 function and localization (Yang et al., 2006; Fraser et al., 2008; Abedini et al., 2010; Woo et al., 2012). Activation of Akt requires three major phosphorylation events at the catalytic motif (Thr308), the hydrophobic motif (Ser473) and the turn motif (Thr450), but only Thr308 and Ser473 residues fulfill crucial and non-overlapping functions in oncogenic activity (Hart et al 2011), while the Thr

450 residue is important for stabilization of Akt content (Wu et al., 2011).

The interplay between Akt and mTOR complexes is essential for their cellular function and rictor is known to be required for phosphorylation of Akt at both Thr450 and Ser473, allowing full activation and degradation (Bhaskar et al., 2007; Ikenoue et al., 2008; Wu et al., 2011). In the present study, we have illustrated that rictor contributes to CDDP resistance in human OVCA, in part, by activating and stabilizing Akt. Silencing rictor sensitizes human OVCA cells to CDDP-induced apoptosis through facilitating Akt proteasomal degradation. Although these findings are in contrast to previous studies showing that rictor knockdown attenuates Akt phosphorylation at Ser473 leading to a loss of Akt function (Guertin et al., 2009; Roulin et al., 2010; Li et al., 2012), mTORC2 disruption has been reported to destabilize Akt due to its ability to phosphorylate Akt at Ser 473 but not at Thr450, thus leading to preferential degradation and inactivation These notions are supported by the further observation that Akt and phospho-Akt (Thr450) content were decreased, consistent with an increase in phospho-Akt (Ser473), when rictor expression was silenced. This phenomenon could be rescued by a proteasomal inhibitor. Although significantly increased levels of phospho-Akt (Ser473) were not observed after rictor silencing with CDDP treatment, that total-Akt content was decreased significantly could be a reason. This observation was further buttressed by the massive increase in the ratio between phospho-Akt

(Ser473) and total-Akt when treated with CDDP with rictor knockdown. Conversely, phospho-Akt (Thr450) was much higher in content in the cells without rictor knockdown, resulting in Akt stabilization and promoting CDDP resistance.

Another observation is that phospho-Akt (Thr450) content was intimately related to total-Akt content. This could indicate that phosphorylation of Akt at Ser473 is a predominant mechanism steering Akt stability and degradation in human OVCA. Rictor induced and enhanced CDDP-induced apoptosis which was also attenuated by Akt stabilization following epoxomycin treatment. Taken together, our findings suggest that rictor is indispensible for mTORC2 to stabilize and activate Akt and expands the current knowledge that rictor knockdown not only attenuates Akt function, but also facilitates proteasomal degradation.

CHAPTER 4

CONCLUSION AND FUTURE DIRECTIONS

1. Conclusion

In summary, we have demonstrated for the first time that CDDP-induced rictor down-regulation involves caspase-3 cleavage and proteasomal degradation, and that rictor confers chemoresistance by promoting Akt activation and stabilization in OVCA. This occurs in a p53-dependent manner. To facilitate our future investigation on the precise role of rictor in CDDP resistance in OVCA cells, we have proposed a hypothetical model (Figure 9). In chemosensitive cells, CDDP induces rictor processing resulting in instability of mTORC2. This phenomenon consequently facilitates Akt proteasomal degradation by phosphorylating Akt mainly at Ser473, a site known to promote Akt proteasomal degradation, and induces apoptosis. However, in chemoresistant cells, the absence of rictor processing promotes Akt activation and stabilization, thereby contributing to CDDP resistance. However, precisely how mTORC2 regulates Akt phosphorylation and its site-specificity in the control of chemosensitivity in ovarian cancer cells and whether DNA-dependent protein kinase is also involved, remains to be determined. Moreover, the mechanism (s) by which caspase-mediated processing and proteasomal degradation of rictor is regulated and the interdependence of these processes await further investigation. Establishing the role of rictor in chemoresistance may justify the targeting of rictor as a novel therapeutic strategy in

overcoming CDDP resistance in human OVCA, particularly for those with wt-p53 status. Targeting rictor may potentiate effective therapeutic outcomes, since this is likely to not perturb the negative feedback pathway between Akt and mTORC1.

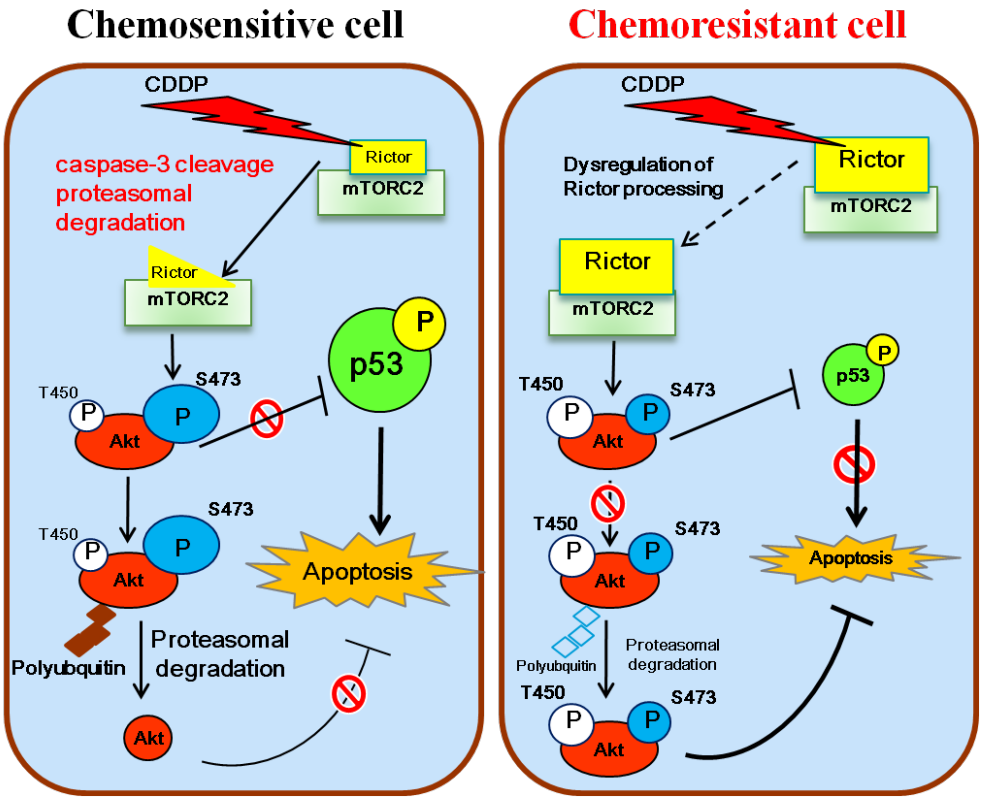

FIGURE 9. A hypothetical model illustrating the role of rictor in regulation of CDDP sensitivity in OVCA cells. In chemosensitive cells, rictor level is low and tightly regulated by CDDP, which leads to mTORC2 instability. In response to CDDP, Akt is mainly phosphorylated at Ser473 and down-regulated by proteasomal degradation, and apoptosis is induced. In chemoresistant cells, however, high expression and dysregulation of rictor processing promote Akt activation and stabilization, and inhibit apoptosis, resulting in CDDP resistance in OVCA cells.

2. Future directions

Although we have established the role of rictor in chemoresistance and demonstrated the mechanisms underlying these phenomena, our findings need to be verified *in vivo* to support the validity of this hypothesis. Animal model needs to be applied to demonstrate whether these phenomena will be consistent *in vivo* system. Clinical samples from OVCA patients who are responsive and non-responsive to cisplatin-based therapy should be assessed for differences in rictor content, thus supporting an involvement of rictor in chemoresitance in OVCA patients. We will also examine the p53 and Akt status in these tumour to determine if an association between these cellular intermediates and chemosensitity. If the clinical data correlates with our current *in vitro* results, we could further develop a small molecule to target rictor *in vivo* and clinical study. This could not only validate our hypothesis but also utilize rictor as a molecular target for overcoming chemoresistance in human OVCA especially for patients containing wt-p53.

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ABSTRACT IN KOREAN

초록

시스플라틴에 기반한 치료요법에 대한 저항성은 인간의 난소암 치료에 있어서 주요한 실패 원인으로 작용한다. 시스플라틴 저항성의 메커니즘에 대한 더 나은 이해는 이러한 치명적인 질병을 위한 새로운 치료 전략들에 있어서 새로운 통찰을 가져올 것이다. Akt 와 P53는 시스플라틴의 민감도의 결정요인들이다. Rictor는 Akt 의 인산화 (세린기 473) 및 이를 최대로 활성화 시키는데 있어서 필요한 mTOR 복합체2의 단백질 키나아제 요소이다. 그러나 시스플라틴 저항성에 있어서 rictor의 정확한 역할과 rictor와 p53 사이의 관계에 대한 이해도가 부족한 실정이다. 본 연구에서는 민감한 야생형 p53 (OV2008과 A2780s), 저항성이 있는 야생형 p53 (C13*과 OVCAR433), 그리고 p53 기능이 결여된 (A2780cp, OCC1, and SKOV-3) 인간의 난소암 세포들을 이용하여 (1) rictor가 인간난소암세포의 시스플라틴 저항성에 한 요인이라는 것 (2) 시스플라틴이 caspase-3의 절단 및 프로테오좀의 분해에 의하여 rictor의 양을 하양조절시킨다는 것 (3) rictor의 하양조절은 화학적 내성을 지닌 인간난소암 세포들을p53 의존적하에 시스플라틴에 유도된 세포소멸로 민감화시킨다는 것 (4) rictor가 시스플라틴

에 유도된 세포소멸을 억제시키고, Akt 를 활성화 및 안정화를 시킴으로써 저항성을 높인다는 것을 입증하였다. 이러한 결과들은 현재의 시스플라틴 저항성에 대한분자 및 세포수준에서의지식 수준을 높이고, 화학내성을 지닌 인간 난소암에 잠재적 치료의 표적을 위한 Rictor의 이용에 관한 합리적 기반을 제공한다.

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