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p53 promotes chemoresponsiveness by regulating Hexokinase II gene transcription and metabolic reprogramming in epithelial ovarian cancer

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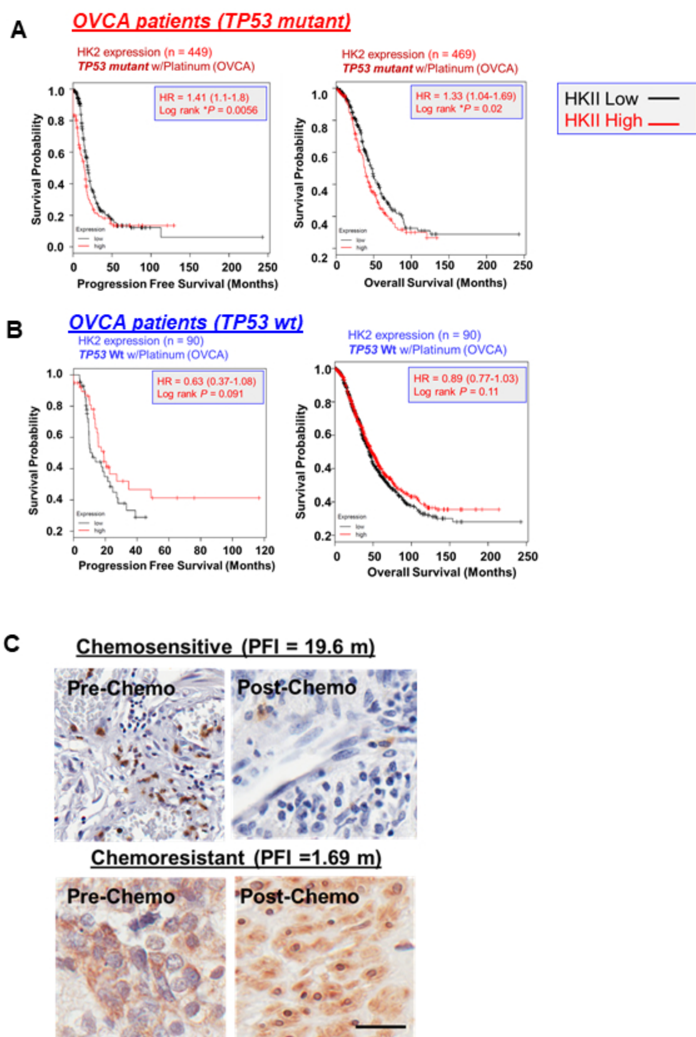

Fig 1. Effects of HKII expression in the clinical outcome of OVCA patients in TP53 status dependent manner.

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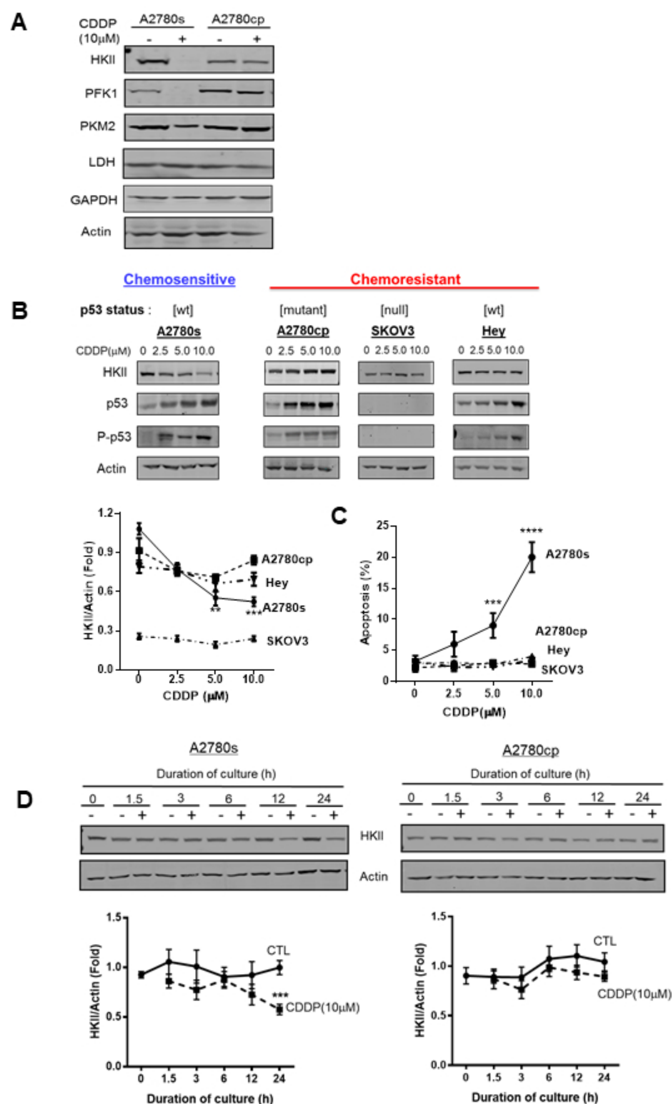

Fig 2. CDDP-induced apoptosis in OVCA is associated with decreased HKII protein content.

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Fig 3

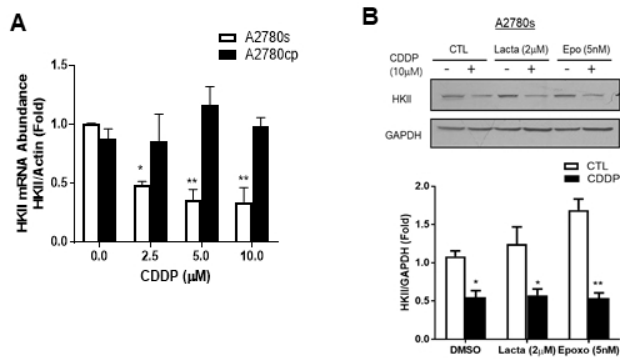

Fig 3. CDDP transcriptionally decreases HKII protein content in chemosensitive OVCA.

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Fig 4

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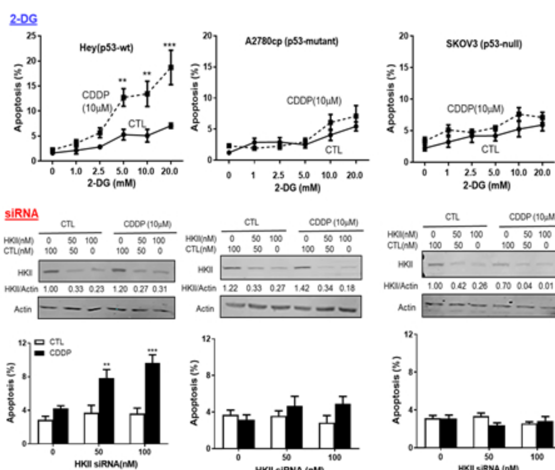

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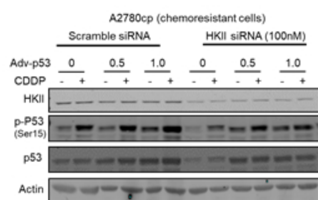

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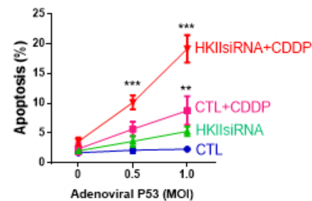

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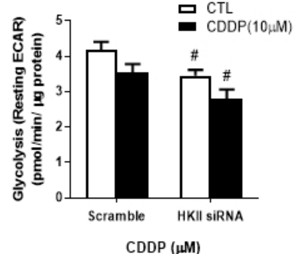

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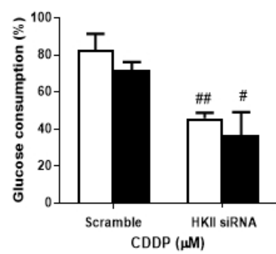

Fig 4. p53 is required for CDDP induced apoptosis in HKII down-regulated cells.

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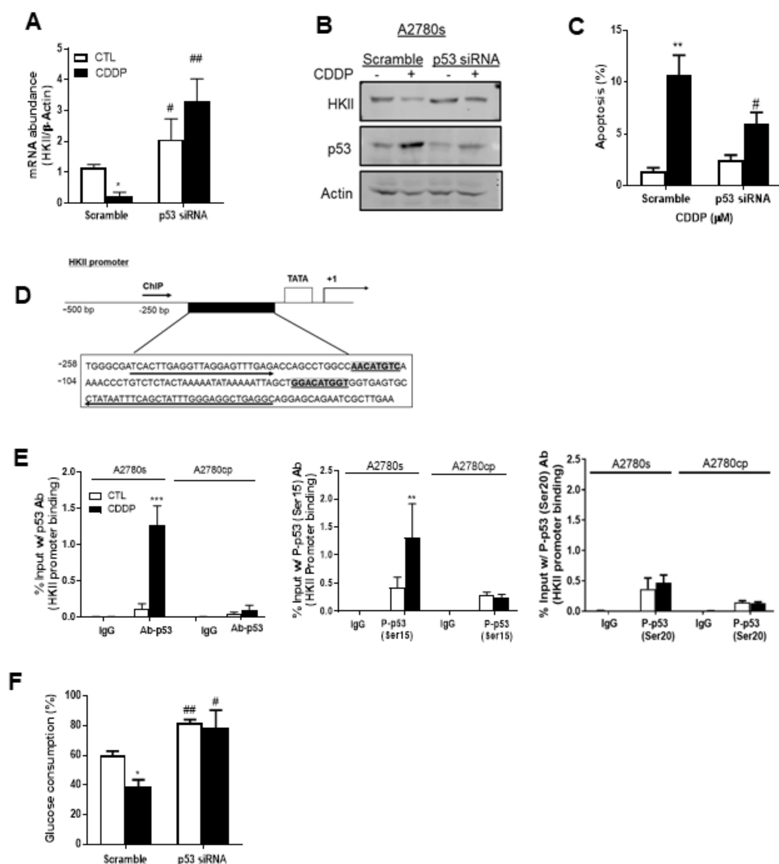

Fig 5. p53 transcriptionally regulates HKII via promoter binding.

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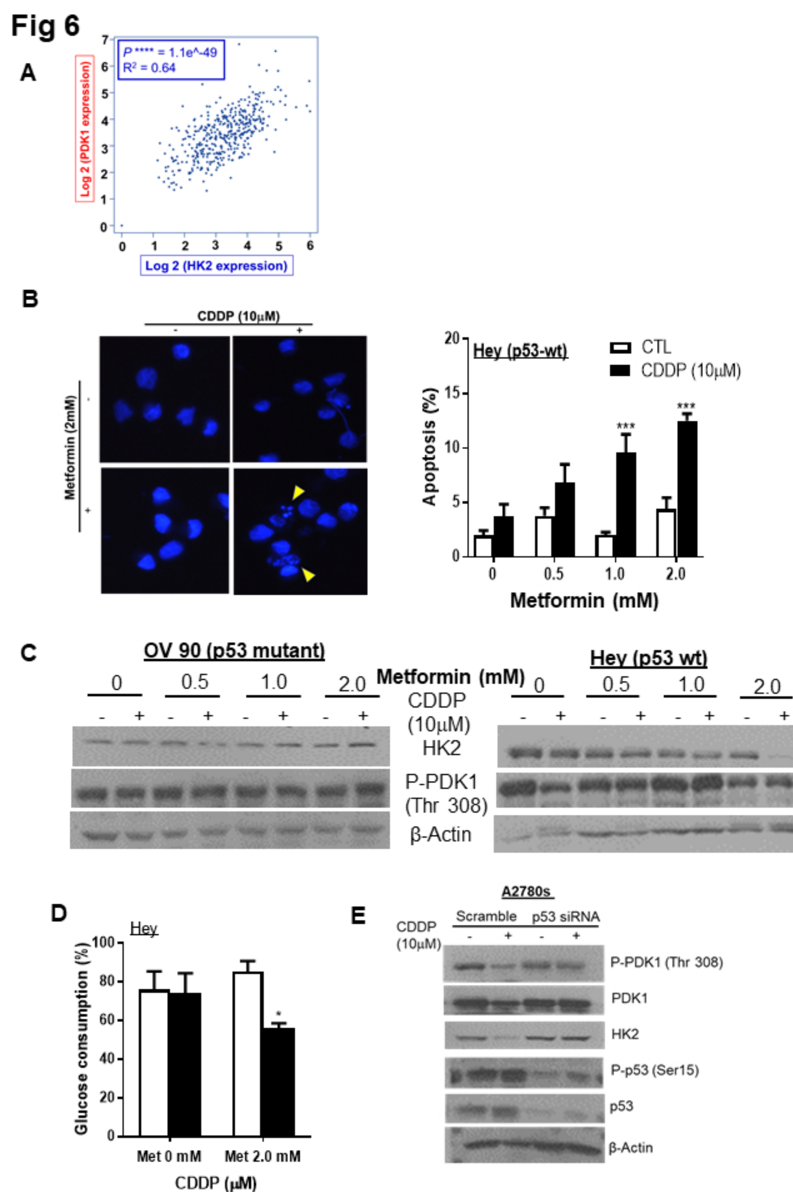

Fig 6. PDK1 correlates with HKII and affects chemoresponsiveness to metformin in OVCA cells in p53 dependent manner.

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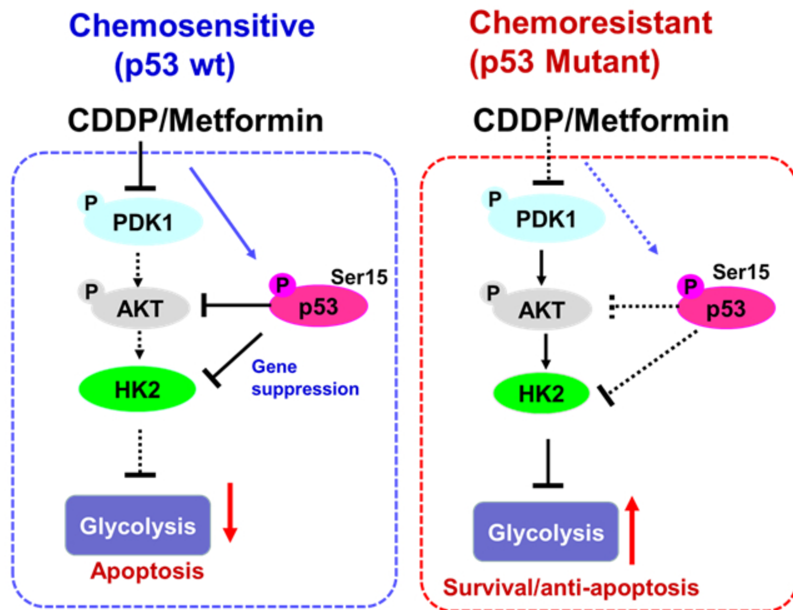

Fig 7. Hypothetical model illustrating the regulatory loop of PDK1-HKII-p53 and controlling role in cellular metabolism and chemosensitivity in OVCA.

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p53 promotes chemoresponsiveness by regulating Hexokinase II gene transcription and metabolic reprogramming in epithelial ovarian cancer

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ABSTRACT

Metabolic reprogramming (Warburg effect) is a hallmark of cancer, yet the association between metabolic change and chemoresistance remains elusive. Hexokinase II (HKII) is a key metabolic enzyme and is upregulated in multiple cancers. In this study, we examined the impact of targeting metabolism via silencing of HKII could be a point of therapeutic intervention in chemoresistant ovarian cancer (OVCA). In addition, the regulatory molecular mechanism of tumor metabolism was examined using gain- and loss-in function approaches in epithelial OVCA cell lines of various histologic subtypes. We demonstrated that Cisplatin (CDDP)-induced p53-mediated HKII down-regulation is a determinant of chemosensitivity in OVCA. Silencing of HKII sensitized chemoresistant OVCA cells to apoptosis in p53-dependent manner. As a negative regulator, p53 suppressed HKII transcription by promoter binding and decreased glycolysis. Pyruvate dehydrogenase kinase-1 (PDK1) is a key regulator of cell proliferation involved in Akt signaling axis. Our Gene Expression Profiling Interactive (GEPIA) analysis and molecular study also revealed that PDK1 as an upstream activator strongly correlates with HKII expression and affects its mediated metabolism. Finally, we demonstrated that the clinically approved drug metformin sensitizes chemoresistant OVCA cells to CDDP via HKII-PDK1 pathway. Collectively, our data implicate that p53-HKII-PDK1 axis is a central regulatory component of metabolism in conferring chemoresistance in OVCA.

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1 INTRODUCTION

Ovarian Cancer (OVCA) is the fifth leading cause of cancer deaths in women [1], with a high mortality rate of 30-50%. Major hurdles in the successful therapy in OVCA include late diagnosis and chemoresistance [2,3]. Analogs of Cis-Diammine dichloroplatinum (II) (Cisplatin, CDDP) are commonly used chemotherapeutic agents for OVCA; however, 70% of patients in advanced stage experience chemoresistance during treatment. Platinum responsiveness is defined as follows: (a) platinum sensitive: patients showed no relapse or progression within 6 months after the last cycle of the chemotherapy; (b) platinum resistance (acquired resistance): patient responds initially but relapsed or progressed within 6 months upon the termination of last cycle chemotherapy, and (c) platinum refractory: patient shows neither therapeutic effect nor progressive disease during therapy [4,5].

The underlying mechanism of chemoresistance is multifactorial and may involve defects in apoptosis, dysregulated metabolism, and detoxification of cytotoxic drugs [6-8]. Despite extensive researches, the development of chemoresistance remains unresolved. Thus, the pursuit of a better understanding of the molecular mechanisms underpinning chemoresistant OVCA and the development of more effective strategies are required.

Metabolic reprogramming enables cancer cells to fulfill their high proliferative and survival potentials [9]. The high rate of aerobic glycolysis is a distinctive character of cancer cells (Warburg effect), contributing to malignant transformation [10,11]. Accumulating evidence suggests that many glycolytic enzymes including hexokinase 2 (HKII), Phosphofructokinase (PFK), and Pyruvate kinase muscle isozyme 2 (PKM2) are associated with chemoresistance and cell survival. These enzymes are regulated by multiple oncogenes (e.g. Akt, mTOR) and key

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tumour suppressors (e.g. p53 and PTEN), and a defect of tumor suppressors often leads to the impaired metabolic control and growth of cancer cells.

Targeting a metabolic enzyme that is specifically for OVCA but not the corresponding normal cells are a critical consideration in the development of therapeutic inhibitor [9,12]. The key glycolytic enzyme, HKII is responsible for the conversion of glucose to glucose-6-phosphate, first committed step in glycolysis. HKII is highly expressed in multiple cancers, associated with tumorigenesis [13-15]. In OVCA, high expression of HKII is associated with poor progression free survival (PFS)[15].

TP53 encodes p53, a key tumor suppressor protein responsible for apoptosis, cell cycle, DNA repairs and the control of oncogenic metabolic reprogramming [16]. *TP53* is frequently mutated in various subtypes of epithelial OVCA (e.g. high grade, low grade, endometrioid, clear cells, and mucinous) and are prevalent (> 90%) in most common aggressive sub-type of high grade serous (HGS) [5]. *TP53* mutation is associated with development of chemoresistance, tumour progression, metastasis, and adverse clinical outcome in OVCA [17]. The majority of p53 mutation are missense mutation caused by a single residue change in amino acid [18] and is mostly occurred in the conserved DNA binding domain, causing an inactive conformation with target genes and results in decreased transcription [19].

p53 has been suggested to regulate the cellular metabolism in controlling excessive growth of the cancer by shifting glycolysis to oxidative phosphorylation [16], but there is no concrete evidence in support of this notion. Activation of p53 occurs through its site-specific phosphorylation, which attenuates its binding to the oncoprotein mouse double minute 2 (MDM2) [20,21], which ubiquitinates and facilitates the proteasomal degradation of p53. We previously reported that among various phosphorylation sites of p53 (Ser 15, 20, 37 and 46), CDDP

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specifically increased phosphorylation Ser 15 and Ser 20 in chemosensitive, but not in chemoresistant OVCA cells, suggesting aberrant phosphorylation of these latter sites is critical for determining chemosensitivity [7]. Still, the mechanistic role of p53 in regulating HKII and metabolism is not fully understood and needs to be further examined.

Pyruvate dehydrogenase kinase (PDK1) is a serine/threonine kinase which acts as a key activator in the loop of lipid kinase phosphoinositide 3-kinase (PI3K)-protein kinase B (AKT)[22,23]. PDK1 is involved in cell survival, cell cycle control, protein synthesis, and glucose metabolism whereas it suppresses apoptotic machinery through activating Akt pathway[22]. Therefore, PDK is highlighted as a potential candidate for therapeutic intervention via targeting metabolism.

The aim of this study was to examine whether HKII depletion sensitizes OVCA cells to apoptosis and how p53 regulates HKII and its mediated aerobic glycolysis in the context of cellular metabolism and cell survival. Using HGS ovarian tumour sections, primary OVCA cells and OVCA cell lines of various histologic subtype with wild-type and mutant p53, we demonstrated the expression HKII in OVCA and investigated underlying regulatory molecular mechanism by which activated p53 regulates HKII gene transcription, controlling tumour metabolism and eliciting chemosensitivity. In addition, we also examined whether PDK1 acts as an upstream activator of HKII and cell survival. We propose that the PDK1-HKII- pathway contributes to chemoresistance whereas p53, in turn, suppresses this cascade, attenuating cancer metabolism and cell survival. These findings highlight the role of PDK1-HKII axis as a potential therapeutic target for chemotherapy of OVCA.

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2 MATERIALS AND METHODS

2. 1 Reagents & Chemicals

Metformin and CDDP were purchased from Sigma-Aldrich (MO, USA). Glucose colorimetric assay kit and XFe96 well plates were purchased from Biovision (CA, USA) and Agilent (CA, USA), respectively. Hexokinase activity assay kit was purchased from Abcam (MA, USA). Adenoviral constructs containing LacZ, and p53-GFP adenoviruses were purchased from Applied Biological Materials (BC, Canada) and amplified in the laboratory of Dr. Robin Parks at Ottawa Hospital Research Institute (OHRI). Information on antibodies used in the present study is described in Supplemental Table1.

2.2 Cell Lines and culture

CDDP sensitive [A2780s (wild type-p53)] and resistant [A2780cp (mutant-p53), Hey (wild type-p53), SKOV3 (null-p53), OVCAR3 (mutant-p53) human OVCA cell lines were gifts from Drs. Rakesh Goel and Barbara Vanderhyden (OHRI, Canada). OV-90 cell is primary OVCA cell culture collected from the patient. It is HGS subtype harboring mutant p53 [p.Ser215Arg (c.643A>C)] and was a gift from Dr. Anne-Marie Mes-Masson at Centre de recherche du Centre hospitalier de l'Université de Montréal and Institut du cancer de Montréal, Canada (CRCHUM). Cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin/streptomycin (10,000 U/mL), Amphotericin B at 37°C with 5% CO_2. Detailed information on the cell lines, including chemosensitivity and p53 mutational status, is indicated in Supplemental Table 2.

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2.3 Immunohistochemistry (IHC) tumor sections

Under IRB-approved protocols of collaborating institution, Dr. Yong Sang Song lab at Seoul National University Hospital (IRB No.:H-1711-142-904), formalin-fixed-paraffin-embedded (FFPE) ovarian tumor sections were collected and assessed (High grade serous subtype with advanced stage III or IV of ovarian cancer). The stage, histology, and tumor grades were determined using criteria with the International Federation of Gynecology and Obstetrics (FIGO) classification. Pre-chemotherapy and post-chemotherapy ovarian tumor sections were obtained at primary and secondary cytoreductive surgery, respectively.

2.4 Apoptosis (Hoechst staining)

Apoptosis was assessed morphologically, using Hoechst nuclear staining as previously described [24]. At least 400 cells were counted for each treatment group, and the process was blinded to avoid experimental bias.

2.5 Western blot

Protein extraction and Western blot analyses were performed as previously described [25]. Unless indicated otherwise, membranes were incubated overnight at 4°C with primary antibodies. On the second day, the membrane was washed with TTBS and incubated with fluorescence conjugated goat anti-rabbit or anti-mouse secondary antibodies, followed by quantification and analysis using LI-COR (Odyssey Imager, Nebraska, USA).

2.6 Adenoviral infection

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165 Cells were infected with adenoviral constructs (Adv) containing wild type (wt)-p53
166 (multiplicity of infection, MOI =0.5 - 1.0, 12h) and Lac Z (MOI = 0.5-1.0, 12h) as previously
167 described [26]. Adv-LacZ served as a control. Total MOI was maintained constant for all
168 treatment groups between Adv and LacZ. Adenovirus infection efficiency was > 80%, based on
169 GFP expression.
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171 **2.7 siRNA transfection**

172 OVCA cells were transfected using Lipofectamine 2000 (16-24 h), following the
173 manufacturer's protocols as previously described [27]. Scrambled siRNA was used as a negative
174 control.
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176 **2.8 Quantitative real time-PCR (qPCR)**

177 Quantitative real-time PCR was conducted as previously described [28]. Total RNAs
178 were isolated with TRIzol (Thermo Fisher Scientific), followed by the synthesis of cDNA
179 primers. mRNA abundances of target genes were analyzed with quantitative real-time PCR
180 (qPCR) using SYBR Green I master mix at Light Cycler 480 machine (Roche Life Science). The
181 data were analyzed by the $2^{-\Delta\Delta CT}$ method and normalized by expression of GAPDH or actin as
182 housekeeping genes. Primer information is described in Supplemental Table 3.
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184 **2.9 Extracellular Flux assays (Seahorse)**

185 Metabolic measurements of the extracellular acidification rate (ECAR) were performed
186 on OVCA cell lines using the Seahorse XFe96 Extracellular Flux Analyzer (Agilent, USA), as
187 previously described [29]. Briefly, 20,000 cells/well were seeded on XFe96 cell culture

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3 188 microplate 1 day before each experiment and the culture medium was replaced with glucose-free
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6 189 DMEM for ECAR and incubated in a non-CO_2 incubator (37°C, 1 h).
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8 190 The glycolytic stress test for the assessment of ECAR (basal glycolysis, glycolytic
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10 191 capacity, and glycolytic reserve) was performed following the sequential addition of glucose (10
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12 192 mM), oligomycin (1.0 μM), and 2-Deoxy-D-Glucose (DG; 50 mM) in an XFe96 flux analyzer.
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15 193 ECAR were measured over a 3-min period and the values were normalized to the protein
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17 194 concentration (determined by the Bradford assay).
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22 196 **2.10 Glucose colorimetric assay**
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24 197 Glucose consumption was measured using the colorimetric glucose assay kit (Bio vision).
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27 198 The amount of glucose in the media and standard were detected as per manufacturer's instruction
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29 199 Cells were seeded in 12 well and treated as indicated. Spent media from cell cultures was
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31 200 collected and diluted (1:100) in assay buffer and cell viability were determined using Trypan
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34 201 blue assay. Glucose consumption was determined by subtracting the amount of glucose in each
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36 202 sample from the total amount of glucose in the blank media (without cells). The detection was
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38 203 performed using a microplate reader (absorbance at 570 nm).
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43 205 **2.11 ChIP assay**
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45 206 Chromatin immunoprecipitation (ChIP) assay was performed as previously described
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48 207 [30,31]. Briefly, cells were cross-linked, lysed, and sonicated as 150-250 bp size using an ultra
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50 208 sonicator (Peak PW: 140.0, Duty Factor: 10.0, Cycle Burst: 200; Covaris S220, MA, USA).
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53 209 After sonication, the lysate was centrifuged and diluted with dilution buffer with strong 5%
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55 210 volume for input. After preclearing, immunoprecipitation was conducted with a mixture of
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Protein A/G dynabeads and a mixture of 2-3 μg antibodies: 2μg of p53, 3μg of phosphorylated (P)-p53 Ser 15 or Ser 20, and 1μg of normal rabbit IgG (4°C, overnight). Immunoprecipitates were washed and heated for reverse crosslinking. DNA fragments were then purified and subjected to qPCR, using primers listed in Supplemental Table 3. Data are presented as an enrichment of the precipitated target sequence as compared with input DNA.

2.12 Kaplan Meier Curves and Clinical Correlations

Kaplan Meier curves were drawn using the Kaplan Meier plotter (http://kmplot.com/ovar/) using cohort of The Cancer Genome Atlas (TCGA) based on microarray data. Clinical correlations between target genes and HK2 expression (RNA sequencing data) in OVCA in the TGCA database was determined using the data sets of Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/) tool.

2.13 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 using Prism (version 7.0; Graph Pad, San Diego, CA) or Sigma Plot (version 12. Systat Software, Chicago, IL). Differences between multiple experimental groups were determined by Bonferroni post-hoc test. Statistical significance was inferred at $P < 0.05$.

3 RESULTS

3.1 HKII expression is associated with the clinical outcome of OVCA in p53 dependent manner

Substantial studies have shown that p53 regulates metabolic pathways including glycolysis [32] and that its mutation is frequently found in epithelial OVCA. However, the regulatory role of p53 on metabolic enzymes and tumor metabolism in OVCA has been limitedly addressed. Hence, we first investigated whether the expression level of HKII is related to the clinical outcome of OVCA patients harbouring either *TP53* wild type (wt) or *TP53* mutant (HGS and endometriod subtypes) by analyzing the microarray data from the cancer genome atlas (TCGA) collaborative dataset (*TP53* wt, n = 90 ; *TP53* mutant, n = 492; KM plot.com). These patients were treated with platinum containing chemotherapeutic agents such as CDDP. Kaplan Meier curves revealed that low HKII expression significantly prolonged progression free survival (PFS, $^{**}P = 0.006$, Fig 1A) and overall survival (OS, $^{*}P = 0.02$, Fig 1A) in p53 mutant OVCA patients than patients with high HKII expression. However, HKII expression level does not affect the clinical outcome in *TP53-wt* patients (Fig 1B), suggesting that *TP53* status may be a pivotal factor in the modulatory influence of HKII in tumour progression and clinical outcome.

We also determined clinicopathological expression pattern of HKII in OVCA patients. Progression free interval (PFI) is a common indicator of chemo-responsiveness (the duration from the termination of chemotherapy to relapse [3]) and is generally defined by a cut-off of 6 months (m) period: chemosensitivity (PFI ≥ 6 m) and chemoresistance (PFI < 6 m). To determine the association between PFI and the expression pattern and localization of HKII, human OVCA tumour sections were examined by immunohistochemistry (IHC) (Fig 1C). HKII

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3 255 expression (Brown color) was elevated in chemoresistant OVCA tissues compared to
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6 256 chemosensitive OVCA (Fig 1C). In addition, CDDP treatment promoted nuclear localization of
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8 257 HKII and lowered its expression (Fig 1C, PFI = 19.6 m) in chemosensitive samples compared
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10 258 with that of chemoresistant OVCA regardless of treatment (Fig 1C, PFI = 1.69 m)
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13 259 **3.2 CDDP-induced apoptosis is associated with decreased HKII protein content in OVCA**
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16 260 Based on previous analysis, we observed that HKII is an important metabolic enzyme which
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18 261 affects the clinical outcome of OVCA patients. However, there are other enzymes in the
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21 262 glycolytic pathway, including PFK1 and PKM2. Using *in vitro* approaches, we first identified
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23 263 which metabolic enzyme(s) in the glycolysis pathway may be mostly associated with
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26 264 chemoresistance. Paired endometrioid subtype of epithelial OVCA, chemosensitive A2780s
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28 265 (wildtype p53) and its counterpart chemoresistant A2780cp (mutant p53) cells, were cultured
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30 266 with or without CDDP (10 μM, 24 h) and protein expression of various metabolic enzymes in the
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33 267 glycolytic pathway was determined by Western blotting (WB). CDDP markedly decreased the
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35 268 protein content of HKII and PFK1 and to a lesser extent of pyruvate kinase muscle (PKM) 2,
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37 269 while without significant effect on the levels of lactate dehydrogenase (LDH) or glyceraldehyde
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39 270 3-phosphate dehydrogenase (GAPDH) in A2780s cells. In contrast, none of these glycolytic
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41 271 enzymes in the chemoresistant counterpart (A2780cp) were affected by CDDP, implying that
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44 272 HKII and PFK1 may be strong candidates involved in both cellular metabolism which may be
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46 273 possibly associated with p53 status and chemoresistance (Fig 2A).
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49 274 Using endometroid OVCA cells (chemosensitive A2780s; p53-wt) and its resistant
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51 275 counterpart (A2780cp; p53-mutant), and other resistant variants OVCA cell lines with varied p53
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54 276 status and different subtypes of epithelial OVCA (Hey, high grade serous, p53-wt; SKOV3, high
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56 277 grade mucinous, p53-null; Supplemental Table 2), we then examined the influence of CDDP (0-
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3 278 10 μM, 24 h) on HKII protein content and apoptosis. CDDP decreased HKII protein content in a
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5 279 concentration-dependent manner (Fig 2B, ** $P < 0.01$), with a significant decrease detectable in
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7 280 chemosensitive A2780s at 5 μM CDDP, but not in A2780cp cells and other resistant variants
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9 281 with mutant p53 status. A significant increase in CDDP-induced apoptosis was also observed
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11 282 starting at 5 μM in chemosensitive cells but not in chemoresistant cells (Fig 2C, ****$P < 0.0001$).
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13 283 Time-course experiments indicate that CDDP slightly, although not significantly, decreased
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15 284 HKII protein content in chemosensitive A2780s cells at 12 hours, but the effect was more
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17 285 pronounced and significant at 24 hours (Fig 2D, ***$P < 0.001$). However, irrespective of the
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19 286 culture duration (0-24 h), CDDP (10 μM) had no effect on HKII protein content in A2780cp
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21 287 cells.

288 3.3 CDDP regulates HKII by altering its transcript levels, but not proteasomal 289 degradation pathway in chemosensitive OVCA

290 To investigate how CDDP decreases HKII content in a p53-dependent manner, we
291 examined whether this response is associated with either change in HKII mRNA abundance or
292 proteasomal degradation (Fig 3A). With qPCR analysis, we observed that HKII mRNA
293 abundance was suppressed by CDDP in a concentration-dependent manner in A2780s but not in
294 A2780cp (Fig 3A; * $P < 0.05$ & ** $P < 0.01$). To determine whether the CDDP-induced changes
295 in HKII protein levels in chemosensitive cells were also due to increased proteasomal
296 degradation, chemosensitive A2780s cells were treated with the proteasomal degradation
297 inhibitors Lactacystin (2 μM) and epoxomicin (5 nM) 3 h prior to and during CDDP treatment.
298 Although CDDP markedly decreased HKII protein content (Fig 3B, * $P < 0.05$, CTL vs. CDDP)
299 as previously shown, this response was not affected by the presence of either inhibitor,

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300 suggesting that CDDP-induced changes in HKII protein level could be caused by down-
301 regulation of mRNA, likely at the transcriptional level.

302 ***3.4* p53 is required for CDDP-induced apoptosis in HKII knockdown cells**

303 To further understand the role of HKII in chemoresistance, we examined the effect of
304 HKII depletion on the apoptotic response in chemoresistant OVCA cells. Various chemoresistant
305 cells, A2780cp (p53-mutant), Hey (p53-wt), and SKOV3 (p53-null) were treated with either
306 pharmacologic HKII inhibitor (2-deoxy-D-glucose: 2-DG) or HKII siRNA. 2-DG treatment
307 markedly sensitized Hey cells (p53-wt) to CDDP-induced apoptosis starting from 12 % (5 mM)
308 (Fig 4A; **$P < 0.01$) and maximally up to 20% (20 mM) (Upper Panel, Fig 4A; ***$P < 0.001$),
309 but not in other p53 defective chemoresistant cells (A2780cp, p53-mutant; SKOV3, p53-null).
310 Consistent with previous findings, HKII siRNA facilitates CDDP-induced apoptosis as 8% (50
311 nM) and increased up 12 % (100 nM) (Bottom Panel, Fig 4A; ***$P < 0.001$) of p53-wt Hey cells,
312 but not in other p53 defective chemoresistant cells. To confirm whether p53 is required for the
313 induction of apoptosis in HKII depleted cells, chemoresistant A2780cp cells were re-constituted
314 with *TP53-wt* [adenoviral constructs (Adv)-p53; Multiplicity of Infection (MOI) = 0 - 1.0, 12 h)
315 followed by HKII knock-down and CDDP treatment (10μM, 24h, Fig 4B). In HKII-knockdown
316 OVCA cells, infection of A2780cp with Adv-p53 markedly enhanced apoptosis rate to 10 %
317 (MOI = 0.5, 12 h) and maximally up to 20 % at highest MOI (MOI = 1.0, 12h) in response to
318 CDDP (Fig 4C; ***$P < 0.001$).

319 We also investigated the role of HKII in glycolytic metabolism in OVCA cells. As
320 expected, HKII knockdown significantly decreased glycolysis (ECAR) and glucose consumption
321 compared with control cells (Fig. 4D and 4E; #$P < 0.05$). However, HKII knockdown did not
322 show any significant difference in glycolysis between control and CDDP treatment (10μM, 24h).

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Collectively, these data suggest that HKII knockdown alone is not sufficient, but functional p53 is required for induction of apoptosis and metabolic regulation in chemoresistant OVCA cells.

3.5 p53 regulates HKII gene transcription and aerobic glycolysis

We previously showed that CDDP promotes phosphorylation of p53 (Ser15 & 20) and DNA binding affinity of phosphorylated (P)-p53 in OVCA cells [7,28]. Based on that, the role of *TP53* in transcriptional regulation of HKII is determined. To examine if p53/P-p53 transcriptionally represses HKII and HKII-mediated glycolysis in OVCA cells, p53 was knocked down in chemosensitive A2780s cells, which were then treated with CDDP (10 μM, 24 h), and HKII mRNA and protein levels were assessed. CDDP significantly decreased HKII mRNA abundance and protein contents, but this down-regulation was largely attenuated in p53-knocked down cells (Fig 5A and 5B; $^{*}P < 0.05$). In addition, p53 knockdown significantly suppressed CDDP-induced apoptosis (Fig 5C, # $P < 0.05$).

As a transcription factor, p53 binds to a target promoter with specific consensus DNA binding (Pu-Pu-Pu-C-A/T-T/A-G-Pu-Pu-Pu)[33]. We then hypothesized that p53 suppresses HKII expression by directly binding to its promoter (Fig 5D). Thus, we performed chromatin immunoprecipitation (ChIP) assays in A2780s and A2780cp cells treated with CDDP (10 μM, 24 h) followed by qPCR. p53 binding to the HK promoter was prominently increased in CDDP-treated chemosensitive A2780s cells (~20 fold) compared with control. Conversely, this response was largely compromised in chemoresistant cells, irrespective of CDDP (Fig 5E; $^{***}P < 0.001$). Our data demonstrated that P-p53 (Ser15), but not P-p53 (Ser20), is involved promoter binding and the transcriptional regulation of HKII (Fig 5E; Ser15: $^{**}P < 0.01$; Ser 20: $P > 0.05$). In addition, CDDP induced downregulation of glucose consumption, but it was recovered in p53-depleted groups irrespective of CDDP treatment (Fig 5F; # $P < 0.05$). Collectively, this evidence

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346 supports the notion that p53 is required for transcriptional repression of HKII and its mediated
347 glycolysis.

348 3.6 PDK1 correlates with HKII and affects chemoresponsiveness to metformin in OVCA 349 cells in p53 dependent manner.

350 We previously showed that p53 is a negative regulator of HK2 in terms of mRNA
351 abundance and protein contents. Conversely, GEPIA analysis using RNA sequencing data
352 (http://gepia.cancer-pku.cn/) revealed a significant strong clinical correlation between
353 expression of PDK1 and HKII (R2 = 0.64, Fig 6A), suggesting that PDK1 may positively
354 influence on HKII function.

355 Previous data revealed that pharmacologic inhibition of HKII with 2-DG sensitized
356 chemoresistant OVCA cells to CDDP-induced apoptosis. Due to the reported major adverse
357 effects of 2-DG (e.g. Hypoglycemia, cardiac side effect) [34] and the termination of its clinical
358 usage [35], we examined the influence of the clinically approved drug metformin on HK2 and P-
359 PDK1 content and CDDP-induced apoptosis in two high grade serous cells lines (Hey, p53-wt;
360 OV90 cells, p53-mutant) *in vitro*. Metformin has been shown to competitively inhibit HKI and
361 HKII, mimicking of its enzymatic product glucose 6-phosphate [36]. The presence of metformin
362 (2.0 mM) significantly enhanced apoptotic rate in chemoresistant Hey cells (14 %) induced by
363 CDDP (10 μM, 24h) (*** $P < 0.001$, Fig 6B).

364 In addition, we observed that metformin decreased HKII and activated form of
365 phosphorylated PDK1 (P-PDK1) in Hey cells but not in p53 mutant OV-90 cells, suggesting that
366 p53 is required for facilitating the suppression of HKII and P-PDK1 induced by metformin and

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3 367 CDDP (Fig 6C). In addition, metformin also significantly decreased glucose consumption level
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6 368 in Hey cells treated together with CDDP (10 μM, 24h) (*$P < 0.05$, Fig 6D).
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9 369 We then examined the role of p53 in the regulation of the HK2-PDK1 signalling pathway.
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11 370 p53 in chemosensitive A2780s cells was knocked down using p53 siRNA (100 nM, 24h) and the
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13 371 cells were then treated with CDDP (10 μM, 24h). While CDDP decreased both HKII and P-
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16 372 PDK-1 (Thr 308) in chemosensitive A2780s cells, these responses were attenuated by p53
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18 373 knock-down (Fig 6E), suggesting that p53 is a key regulator of HKII and PDK signaling cascade.
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20 374 Collectively, these data suggest that PDK1-HKII- axis is a critical signaling cascade for cell
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23 375 survival and elevated metabolism, which is regulated by p53.
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377 # 4 DISCUSSION

378 Metabolic adaptation is a distinctive characteristic of cancer, and is associated with
379 progression and tumorigenesis. However, the role of metabolic reprogramming in
380 chemoresistance is not fully understood. p53 is a key tumor suppressor governing cell
381 proliferation, apoptosis, and metabolism. In the present study, we have established a key role of
382 p53 and PDK1 as a regulator of the glycolysis pathway engaged in controlling chemoresistance
383 in OVCA. Our study showed for the first time, that CDDP- activated P-p53(Ser15) regulates
384 HKII transcription via promoter binding and elicits CDDP-induced apoptosis in chemosensitive
385 OVCA cells. However, these responses are suppressed in chemoresistant cells harboring mutant
386 p53, leading to elevated glycolysis and cell survival.

387 Among various metabolic enzymes, HKII is considered a promising therapeutic target
388 and is highly associated with tumorigenesis and cell survival in multiple cancers (e.g. lung
389 cancer, glioblastoma, and breast cancer) [13,37,38]. Interestingly, while systematic HKII
390 deletion in mice does not cause adverse physiological consequences, it prevents the development
391 of cancer [13]. In clinical pathological expression study of human ovarian tumour sections, high
392 HKII protein expression has been reported to be more closely associated with the PFS compared
393 to OS [15]. Yet, our data support that there are other molecular mechanisms regulating HKII and
394 its expression.

395 The present studies demonstrate an important role of p53 and thus its mutational status in
396 the regulation of HKII and chemosensitivity. Our Kaplan-Meier analysis with a larger population
397 cohort from TCGA data has shown that patients with an ovarian tumour harboring p53 mutations
398 and low HKII expression showed both prolonged PFS and OS than those with higher HKII. Yet,

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the expression of HKII does not influence the clinical outcome of OVCA patients with wild type p53, suggesting the presence of functional p53 is required in the regulation of HKII and for better clinical outcome. Our *in vitro* results showed that high expression of HKII is not a sole determinant of chemoresistance since both chemosensitive and chemoresistant OVCA cells have similar levels of HKII protein expression. Instead, p53-dependent CDDP-induced HKII down-regulation seems also to be important. Previous studies suggested that other glycolytic enzymes, including PFK1 and PKM2, are also involved in the regulation of chemosensitivity in cancer [39,40]. Although our studies do not exclude the possible involvement of these enzymes in chemoresistance in OVCA, CDDP-induced HKII down-regulation seems to be an important determinant for CDDP responsiveness.

Emerging evidence indicates that PDK1 is required for metabolic adaptation. Dupuy et al. reported that PDK 1 is required for adaptation of metabolic phenotype toward glycolysis under the condition of hypoxic tumour microenvironments (such as limited oxygen)[41]. PDK1 activates Akt through phosphorylation of Thr 308 sites [42] and Akt has been shown to activate HKII via promoting mitochondrial HKII binding [43]. Interestingly, our results showed that PDK1 and phosphorylation of PDK1 affect protein expression of HKII, which in turn, promotes glycolysis and cell survival of chemoresistant cells. However, the precise mechanism how P-PDK1 activates HKII remains to be determined

Metformin, a clinically approved drug for the treatment of diabetes, has been reported to inhibit binding of HKII to mitochondrial and its enzymatic activity by mimicking HKII substrate, G-6-P [36]. metformin has also been reported as an AMPK activator in OVCA, possibly by inducing energy stress through inhibition of complex I of the respiratory chain in mitochondria. This leads to a change in the ATP-to-AMP ratio and canonical AMPK activation. It inhibits cell

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growth through suppressing mTOR signalling [44,45]. In this context, it is possible that metformin inhibits HKII-PDK1 loop via suppressing AMPK/mTOR [46]. However, this study is limited by the use of metformin alone. To demonstrate AMPK/mTOR signaling modulates HKII-PDK11, future studies should use other direct AMPK activators (e.g. A-769662 and salicylate), to exclude the involvement of mitochondrial activities. In the present studies, neither the combination of metformin and CDDP nor 2-DG and CDDP increased apoptosis in chemoresistant p53 deficient OVCA cells. Interestingly, reconstitution of a functional p53 was able to support the apoptotic response. Similarly, Lengyel et al. reported that they failed to elicit an apoptotic response, but prevented tumor growth, cell cycle and cell proliferation with combined treatment with metformin and Paclitaxel [47]. Notably, we found that metformin decreases HKII-PDK1 loop in the presence of p53, but not in OVCA with p53 mutations. Our findings support a rationale for optimizing personalized therapies that CDDP treatment with metformin may be effective in treating chemoresistant OVCA.

Additional studies are needed to determine whether the role of metabolism in chemoresistance in OVCA is histologic subtype-dependent [48]. The majority of high grade serous subtype showed acquired resistance whereas other subtypes such as high grade mucinous and clear cell carcinoma often showed refractory phenotype, as shown in neither change in progression of cancer or responsiveness to chemotherapy. Our present studies were conducted with cell lines of multiple subtypes and showed that status of p53 appeared more important in regulation of metabolism independent of subtype. The association of metabolic character, subtype specificity and p53 require further investigation.

Here, we report a regulatory role of P-p53(Ser15) by promoter binding in HKII transcription in OVCA. Wang et al. previously showed that p53 and PTEN regulate the

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expression of HKII in prostate cancer cells [38]. Moreover, we showed that CDDP-induced P-p53(Ser15) but not P-p53(Ser20) is involved in the suppression of HKII transcription, and is associated with CDDP responsiveness. This is consistent with our previous finding that P-p53 (Ser15) is more closely involved in DNA binding affinity and DNA transcriptional activation [7]. Our studies indicated that the mutation status of *TP53* alone is not a sufficient determinant for chemosensitivity in OVCA, but other oncogenic molecules (e.g., Akt, PI3K, PDK1) may also be involved in the control of cell metabolism and survival in OVCA. These findings highlight the role of PDK1-HKII as a potential therapeutic target and provide a potential therapeutic strategy for chemotherapy of OVCA

To facilitate future investigation into the molecular mechanism and cellular metabolism of chemoresistant OVCA, we propose the following hypothetical model (Fig 7). In chemosensitive cells, P-p53 (Ser15) suppresses HKII transcription and its mediated metabolism, eliciting apoptosis in responses CDDP and metformin. In contrast, in chemoresistant cells, PDK1 as an upstream activator of Akt and HKII promotes the cell survival and metabolic upregulation, suppressing CDDP-induced apoptosis. Collectively, we propose that PDK1-HKII-p53 constitutes the regulatory loop in the regulation of cellular metabolism and chemosensitivity in OVCA.

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CYH and BKT designed the experiments; CYH, DAP, and SGL performed research; MEH provided new reagents/analysis. CYH, DAP, MEH, DWC and BKT wrote and reviewed the paper.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

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491 # FIGURE LEGENDS

492 **Fig 1. Effects of HKII expression in the clinical outcome of OVCA patients in *TP53* status**
493 **dependent manner.** Kaplan-Meier survival curves of progression free survival and overall
494 survival were analyzed comparing the low and high HKII expression groups in OVCA patients
495 harboring **(A)** *TP53* mutant and **(B)** *TP53* Wild type (WT). High grade serous (HGS) and
496 endometrioid sub-types of ovarian cancer (OVCA) patients treated with platinum containing
497 chemotherapeutic agents are analyzed using microarray analysis (Affymetrix ID: 202934) in
498 TCGA population using Kaplan Meier plotter (KM plot.com). Kaplan-Meier curves were
499 analyzed by log rank *P* method. **(C)** Representative images of Immunohistochemistry (IHC)
500 staining showing HKII expression in chemosensitive and chemoresistant HGS ovarian tumor
501 sections (Brown: HKII; Blue: DAPI, nucleus). Each pair of sections are matched pre- and post-
502 chemotherapy from same OVCA patients (n = 10 pairs). Pictures were obtained as (x400)
503 magnification using histoscanner.

504 **Fig 2. CDDP-induced apoptosis in OVCA is associated with decreased HKII protein**
505 **content. (A)** Paired chemosensitive A2780s (p53-wild type, wt) and its resistant counterpart
506 A2780cp (p53-mutant) OVCA cells were cultured with or without CDDP (10μM, 24 h; DMSO
507 as vehicle) and protein contents of HKII, PFK1, PKM2, LDH, GAPDH, and actin (loading
508 control) were measured using western blot (WB). **(B)** Chemosensitive A2780s and variant
509 chemoresistant OVCA cells [A2780cp (p53-mutant), Hey (p53-wt), and SKOV3 (p53-null)]
510 were cultured in different concentration of CDDP (0-10 μM; 24 h) followed by WB of HKII, p53,
511 P-p53(Ser15) and actin. **(C)** Apoptosis was measured by Hoechst staining using cells treated as
512 indicated. **(D)** A2780s and A2780cp cells were cultured with different CDDP culture duration

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3 513 (10µM, 0-24h) followed by WB of HKII and actin. (*** $P < 0.001$, **** $P < 0.0001$ vs. CTL).
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6 514 Control, CTL, Error bars denote ± SEM (n = 4).
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10 516 **Fig 3. CDDP transcriptionally decreases HKII protein content in chemosensitive OVCA.**
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13 517 **(A)** A2780s and A2780cp cells were cultured with different concentrations of CDDP (0-10µM,
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15 518 0-24h) followed by qPCR of HKII and actin (as loading control). **(B)** A2780s cells were pre-
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17 519 treated with proteasomal inhibitors, 2µM Lactacystin (Lacta) and 5nM Epoxomomycin (Epo) for
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19 520 3h followed by CDDP treatment (10µM, 24 h). The protein content of HKII and GAPDH
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21 521 (loading control) were assessed by WB. For all experiments described, DMSO was used as a
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23 522 vehicle control (CTL). Error bars denote ± SEM (n = 4)
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28 524 **Fig 4. p53 is required for CDDP induced apoptosis in HKII down-regulated cells.**
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30 525 **(A)** Hey (p53-wt), A2780cp (p53-mutant), and SKOV3 (p53-null) OVCA cells were pre-treated
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32 526 with the glycolysis inhibitor 2-deoxy-D-glucose (2-DG, 0 - 20 mM, 6 h; Upper panel) or
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34 527 transfected with 0 ~100 nM of HKII siRNA (16 h; Bottom panel), treated with CDDP (10 µM,
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36 528 24h) followed by assessment of apoptosis with Hoechst nuclear staining. **(B & C)** p53
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38 529 reconstitution sensitized HKII depleted chemoresistant A2780cp cells to CDDP-induced
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40 530 apoptosis. A2780cp cells were transfected with HKII siRNA (100 nM, 16 h) infected with
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42 531 Adenoviral (Adv)-p53 (multiplicity of infection, MOI = 0 - 1.0, 12 h), treated with CDDP (10
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44 532 µM, 24 h) and apoptosis was assessed. Successful interrogation of HKII, P-p53 (Ser15), p53 and
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46 533 actin were confirmed by WB. **(D)** Glycolysis [resting extracellular acidification rate (ECAR)]
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48 534 was measured using sea horse analyzer with A2780cp cells transfected with 0-100 nM of HKII
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50 535 siRNA and subjected to CDDP treatment (10 µM, 24 h) on 96 wells. **(E)** Glucose consumption
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536 was also measured using glucose calorimetric assay with same cells treated as above but seeded
537 on to 12-well plate followed by CDDP treatment (10 μM, 24 h) (***P* < 0.01, ****P* < 0.001, CTL
538 vs. CDDP). Error bars denote ± SEM (n = 4).

539

540 **Fig 5. p53 transcriptionally regulates HKII via promoter binding.**

541 **(A)** A2780s cells were transfected with either scrambled or p53 siRNA (100 nM, 16 h), treated
542 with CDDP (10 μM, 24 h) followed by qPCR **(B)** WB of HKII, p53, and actin (loading control),
543 and **(C)** apoptosis assessment. **(D)** Schematic map of the p53 binding to the human HKII
544 promoter region (black color) showing potential p53 consensus binding site (gray color) and
545 primers used for ChIP (Arrow). p53 consensus binding sequence was located at the promoter
546 region from -1 to -1,000 upstream, and the amplicon targeting p53 bound to HKII was designed
547 in the region of (-265 to -137 bp). **(E)** CDDP-activated P-p53 (Ser 15), but not P-p53 (Ser 20)
548 regulates HKII via promoter binding in chemosensitive, but not in chemoresistant OVCA cells.
549 A2780s and A2780cp cells were treated with CDDP (0,10 μM, 24 h), and ChIP assays were
550 conducted with p53, P-p53 (Ser 15), and P-p53 (Ser 20) antibodies followed by qPCR. Glucose
551 consumption was measured using the spent medium of cells seeded on 12-well plate, and treated
552 with CDDP (10μM, 24 h). (**P* < 0.05, ***P* < 0.01, and ****P* < 0.001, CTL vs. CDDP; #*P* < 0.05,
553 ##*P* < 0.01, Scrambled vs. p53 siRNA). Error bars denote ± SEM (n = 4).

554

555 **Fig 6. PDK1 correlates with HKII and affects chemoresponsiveness to metformin in OVCA**
556 **cells in p53 dependent manner.**

557 **(A)** Correlation was analyzed between PDK1 and HK2 in OVCA patients in TGCA database
558 cohorts using the GEPIA tool. **(B)** Chemoresistant Hey (HGS, p53-wt) and OV-90 cells (HGS,

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3 559 p53-wt) were pre-treated with Metformin (0 – 2 mM, 6 h; Upper panel) and treated with CDDP
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5 560 (10 μM, 24h) followed by assessment of apoptosis with Hoechst nuclear staining. Nuclear
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7 561 morphology (broken/fragmented) was taken by immunofluorescence microscopy using Leica
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9 562 2400 system. **(C)** WB was simultaneously conducted using the same cell lysate as treated in (B)
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11 563 using antibody of HKII, P-PDK1 (Thr308), and actin (loading control). **(D)** Glucose
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13 564 consumption assay was conducted. Cells were seeded on 12-well plate, pre-treated with
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15 565 metformin (2 mM, 6 h) and treated with CDDP (10 μM, 24h) followed by collection of spent
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17 566 medium as cell viability was measured with Trypan blue for normalizing counted cell number.
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19 567 **(E)** A2780s cells were transfected with either scrambled or p53 siRNA (100 nM, 16 h), treated
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21 568 with CDDP (10 μM, 24 h) followed by WB of HKII, p53, P-p53 (Ser15), PDK1, P-PDK1 (Thr
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23 569 308), and actin (loading control). (*$P < 0.05$ and ***$P < 0.001$, CTL vs. CDDP). Error bars
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25 570 denote ± SEM (n = 3).
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29 572 **Fig 7. Hypothetical model illustrating the regulatory loop of PDK1-HKII-p53 and**
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31 573 **controlling role in cellular metabolism and chemosensitivity in OVCA.**
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33 574 **(A) <u>In chemosensitive OVCA cells:</u>** (1) CDDP/Metformin treatment activates p53 at the site of
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35 575 Ser15 and ; (2) this impairs activation of PDK1-Akt and transcriptionally suppresses its
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37 576 downstream molecule, HKII; (3) HKII mediated glycolysis is suppressed and apoptosis as a
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39 577 result is elicited in response to CDDP.
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41 578 (B) **<u>In chemoresistant OVCA cells</u>:** (1) CDDP failed to activate p53 due to mutant p53; (2)
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43 579 Signaling cascae of PDK1-Akt-HKII is activated and glycolysis is maintained, contributing to
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45 580 cell survival. Solid line indicates activated molecular action whereas dashed line indicates
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47 581 suppressed action. Arrows indicates direction whereas blocked line indicates inhibition.
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