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Hypoxia-related Gene Expressions Affecting Prognosis of High-grade Serous Adenocarcinoma of the Ovary

고등급 장액성 난소암의 예후에 영향을 미치는 저산소증 연관 유전자 발현 연구

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김 희 승의 박사학위논문을 인준함

2017년 7월

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부 위 원 장 박 노 현 (인)
위 원 배 덕 수 (인)
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Abstract

Hypoxia-related Gene Expressions Affecting Prognosis of High-grade Serous Adenocarcinoma of the Ovary

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Background: Ovarian cancer is the most lethal disease among malignancies of the female genital tract because of a higher rate of diagnosis at advanced-stage disease. Thus, maximal debulking surgery followed by adjuvant chemotherapy is essential to treat ovarian cancer. However, drug resistance of ovarian cancer is expected to be associated with microenvironment, which may hinder the improvement of prognosis and the development of optimal biomarkers for ovarian cancer. Among various types of tumor microenvironment, hypoxia-related genes are known to be associated with carcinogenesis, proliferation and invasion of tumor cells. However, they have been not investigated sufficiently for their roles affecting drug resistance and prognosis of ovarian cancer. Thus, we evaluated expressions of hypoxia-related genes in the most common histologic type, high-grade serous adenocarcinoma of the ovary (HGSO), and investigated the roles of hypoxia-related genes affecting platinum-resistance and survival of HGSO.

Methods: We determined IC_{50} of cisplatin, and then evaluated cell viability after treatment of cisplatin under hypoxia in OV90 cells. Basic *mRNA* levels of *CBP*, *P300*, *HIF-1α*, *HIF-1β*, *FIH* and *VHL* were evaluated in each five tissues of normal ovary and HGSO by using of quantitative RT-PCR. Then, we investigated protein levels

after treatment of cisplatin under hypoxic conditions (normoxia, 5%O_2, 3%O_2 and 1%O_2) in OV90 cells by using Western blot. Furthermore, we estimated cellular proliferation and reactive oxygen species (ROS) production in OV90 cells by N-acetylcysteine (NAC) to determine relationship between hypoxia and ROS generation. Finally, we performed immunohistochemistry (IHC) for the six genes in patients with HGSO (n=149), and investigated prognostic factors affecting platinum-resistance and progression-free survival (PFS) among clinico-pathologic factors and the six hypoxia-related genes.

Results: IC_{50} of cisplatin were 50 μmol/L in OV90 cells, and hypoxic cells were less inhibited than normoxic cells after treatment of IC_{50} of cisplatin. In HGSO, *mRNA* levels of *CBP*, *P300*, *HIF-1a* and *HIF-1β* were lower than those in normal tissues, whereas *mRNA* levels of *FIH* and *VHL* were higher than those in normal tissues. In Western blot, the protein level of *HIF-1β* was increased, whereas that of *VHL* was decreased under hypoxic conditions after treatment of cisplatin in OV90 cells. Furthermore, the protein level of *HIF-1β* was increased, whereas that of *VHL* was decreased when the concentration of NAC was increased with cisplatin in OV90 cells. On IHC, a low expression of *HIF-1β* and a high expression of *VHL* were favorable factors for reducing platinum-resistance in patients with HGSO (Adjusted ORs, 0.657 and 0.381; 95% CI, 0.443-0.975 and 0.165-0.878). In addition, a low expression of *HIF-1β* and a high expression of *VHL* were related with improved PFS in patients with HGSO (adjusted HRs, 0.657 and 0.381; 95% CIs, 0.443-0.975 and 0.165-0.878).

Conclusions: A high expression of *HIF-1 β* and a low expression of *VHL* may be related with platinum-resistance and poor prognosis in patients with HGSO.

Keywords: *HIF-1β*, *VHL*, platinum-resistance, progression-free

survival, high-grade serous adenocarcinoma, ovarian cancer.

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Contents

Introduction

Ovarian cancer is known to be the most lethal disease among malignancies of the female genital tract because of no effective screening method for early detection, and thereby a higher rate of diagnosis at advanced-stage disease (1, 2). To overcome these limitations, many studies have focused on the effect of new molecular targeted drugs to improve survival, and the role of biomarkers to predict early-stage disease. However, most of molecular targeted drugs have failed to improve overall survival (3, 4), and different biomarkers have not shown their effect for early detection of ovarian cancer (5, 6).

In particular, drug resistance of tumor is expected to be associated with tumor heterogeneity and microenvironment, which may hinder the improvement of prognosis and the development of optimal biomarkers for ovarian cancer (7, 8). Among different types of tumor microenvironment, hypoxia-related genes are known to be related with carcinogenesis, proliferation and invasion of tumor cells (9, 10), which may act as roles to predict drug resistance and consequent prognosis in various types of malignancies such as kidney and lung cancers (11, 12).

Although *hypoxia inducible factor-1a* (*HIF-1a*) and *vascular endothelial growth factor* (*VEGF*) have been shown to related with carcinogenesis and progression of ovarian cancer (13, 14), other hypoxia-related genes such as *HIF-1β*, *cAMP-response element-binding protein* (*CREB*)-binding protein (*CBP*), a*denovirus early region 1A* (*E1A*)-*binding protein P300* (*P300*), *factor-inhibiting HIF* (*FIH*) and *von Hippel-Lindau* (*VHL*) have not been investigated sufficiently for their roles affecting drug resistance and prognosis of ovarian cancer.

Thus, we evaluated expressions of hypoxia-related genes in the

most common histologic type, high-grade serous adenocarcinoma of the ovary (HGSO), and investigated the roles of hypoxia-related genes affecting platinum resistance and prognosis of HGSO.

Materials and methods

Reagents

Cisplatin and N-acetylcysteine (NAC) were obtained from Sigma-Aldrich (Missouri, USA). For immunohistochemistry (IHC) and Western blot, anti-*HIF-1α* was obtained from Abcam (Cambridge, UK). Anti-*CBP,* anti-*FIH,* anti-*HIF-1β* and anti-*VHL* antibodies were purchased from Cell Signaling (Danvers, USA). Moreover, anti-*P300* antibody was obtained from Santa Cruz (California, USA).

Cell lines and tissue samples

A human ovarian cancer cell line, OV90 (high-grade serous adenocarcinoma) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). We purchased each five frozen tissue samples with normal ovary and HGSO from Cancer Tissue Bank of Seoul National University.

Cell culture

We used a 1:1 mixture of MCDB 105 medium and Medium 199 (Sigma-Aldrich) and 15% fetal bovine serum for OV90 cell line with penicillin and streptomycin. It was cultured at 37℃ in an incubator with a humidified atmosphere of 5% of CO_2. For the experiment, monolayer cultures of OV90 cells were grown in culture medium to 70% confluence in 100-mm tissue culture dishes. Cells were serum starved for 24 hr, and then treated with cisplatin. This design was replicated in three independent experiments.

Culturing cells under hypoxic conditions

OV90 cells were grown in MCDB 105/Medium 199 1:1 mixture containing 15% FBS. For normoxic oxygen conditions (21% O_2), cells were incubated in a standard humidified incubator at 37℃ and 5%

CO_2. The hypoxia culturing was performed using a commercially available hypoxia incubator chamber (STEMCELL Technologies Inc., Canada). For hypoxic oxygen conditions, cells were incubated in hypoxia chamber containing 90% N_2;5% CO_2 (5% O_2), 92% N_2;5% CO_2 (3% O_2) or 94% N_2;5% CO_2 (1% O_2).

Cytotoxicity and cell viability assays

To investigate the cell viability in normoxic- or hypoxic culture conditions, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay was used. OV90 cells were seeded and exposed to different concentrations (0, 1, 10, 20 or 100 μmol/L) of cisplatin in normoxia or hypoxia. MTT solution (5 mg/ml) was then added to each well after 24h treatment and incubated during additional 4 h at 37°C. Subsequently, the medium was discarded and replaced by 100 μL of 0.04 N HCl in isopropanol to dissolve the formazan crystals followed by quantified at 595 nm and 655 nm as a background using a microplate reader (Bio-Rad, South Korea). The percentage of cell viability was calculated according to the following equation: cell viability percentage (%) = (absorbance of hypoxia or chemical compound-treated cells/absorbance of control cells)×100%.

Determination of cellular reactive oxygen species

Intracellular reactive oxygen species (ROS) production was estimated using 2',7'-dichlorofluorescin diacetate (DCFH-DA, Sigma) which is converted to fluorescent 2',7'-dichlorofluorescin (DCF) in the presence of peroxides. OV90 cells were detached with trypsin-EDTA, collected by centrifugation, and washed with PBS. The cells were treated with 10 μM DCFH-DA for 30 min at 37°C. Then, the cells were washed with PBS twice, and treated with NAC in a dose dependent manner (0, 1, 2, 5 and 10 mM) or a combination of cisplatin with NAC for

1h at 37°C in a CO_2 incubator. The treated cells were washed with PBS again. Fluorescent DCF intensity was analyzed using a flow cytometer (BD Biosceince, Franklin Lakes, NJ, USA).

RNA isolation

Total cellular RNAs of frozen tissue samples were obtained using Trizol reagent (Invitrogen, Carlsbad, CA) based on the manufacturer's guideline. The quality of total RNA were determined by spectrometry.

Quantitative RT-PCR analysis

Complementary DNA was synthesized using total RNA extracted from OV90 cell line and each of the tissuesby AccuPower RT PreMix (Bioneer, Daejeon, Republic of Korea). Gene expressions were estimated using SYBR Green (Sigma, St. Louis, MO, USA) and a StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). *GAPDH* gene was simultaneously analyzed as a control and used for normalization for variation in loading. We produced forward and reverse primers of the six hypoxia-related genes depicted in Table 1, and for *GAPDH* gene, the forward primer (5'-ACA CAG AAG ACG GTG GAT GG-3') and the reverse primer (5'-GGC AGG TCA GGT CAA CAA CA-3') were amplified as a 193 - bp product.

Thereafter, we determined expression levels of the six genes using the standard curve method and C_T values, and normalized them based on *GAPDH* expression. The PCR conditions were 95℃ for 3 minutes, followed by 40 cycles at 95℃ for 30 seconds, 60℃ for 30 seconds, and 72℃ for 30 seconds using a melting curve program, which could increase the temperature from 55℃ to 95℃ at a rate of 0.5℃ per 10 seconds, and continuous fluorescence measurement. We used ROX reference dye (Invitrogen, CA, USA) as a negative control for the fluorescence measurements. Sequence-specific products were identified

by producing a melting curve in which the C_T value represented the cycle number at which a fluorescent signal was statistically greater than background, and relative gene expressions were quantified using the $2^{-\Delta\Delta CT}$method (15).

Table 1. List of forward and reverse primers of the six hypoxia-related genes for high-grade serous adenocarcinoma of the ovary

Gene	Strand	5′→3′	RT size	qRT size
CBP	Forward	AGGACCTGACGTACCTGTGC	363	114
	Reverse	AATGACTCTGGGTCCTGTCG		
P300	Forward	ATATGCCACCATGGAGAAGC	481	147
	Reverse	ATGGACCAGAGACTGGATGC		
HIF-1α	Forward	TCAGCTATTTGCGTGTGAGG	475	178
	Reverse	AGCACCAAGCAGGTCATAGG		
HIF-1β	Forward	AGGAACAGATGCAGGAATGG	308	177
	Reverse	GGCTGGTAGCCAACAGTAGC		
FIH	Forward	CCCATACCCTGTTCATCACC	469	121
	Reverse	GTGCAGCGTGCAATACTAGC		
VHL	Forward	CCCAAATGTGCAGAAAGACC	486	109
	Reverse	AGGCAGACAAGTCACCAACC		

Abbreviation: *CBP*, cAMP-response element-binding protein (CREB)-binding protein; *P300*, adenovirus early region 1A (E1A)-binding protein p300; *FIH*, factor-inhibiting hypoxia inducible factor; *HIF-1a*, hypoxia inducible factor-1α; *HIF-1β*, hypoxia inducible factor-1β; *VHL*, von Hippel-Lindau.

Western blot analysis

Concentrations of proteins in whole-cell extracts were determined using the Bradford protein assay (Bio-Rad, CA, USA) with bovine serum albumin as the standard. Proteins were denatured, separated using SDS - PAGE and transferred to nitrocellulose. Blots were developed using enhanced chemiluminescence detection (SuperSignal West Pico, IL, USA) and quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a ChemiDoc EQ system and Quantity One software (Bio-Rad, CA,

USA). Immunoreactive proteins were detected using goat anti-rabbit polyclonal antibodies against phosphor-proteins and total-proteins at a 1:1000 dilution and 10% SDS/PAGE gel. As a loading control, total proteins were used to normalize the results from detection of proteins by Western blotting. We performed multiple exposures of each Western blot to ensure linearity of chemiluminescent signals.

Human study population

We collected clinico-pathologic data of patients with HGSO treated between July 2001 and November 2012. Approval of the Institutional Review Board was obtained for the current study (No. 1311-050-533). The inclusion criteria were as follows: patients with HGSO; those treated with cytoreductive surgery followed by adjuvant chemotherapy; those with Eastern Cooperative Oncology Group performance status of 0-2; those without underlying diseases affecting survival. We extracted data of International Federation of Gynecology and Obstetrics (FIGO), age, extent of cytoreduction, platinum-resistance, progression-free survival (PFS).

Optimal cytoreduction was defined as the maximal diameter of a residual tumor $\leq$1 cm, whereas suboptimal cytoreduction was defined as the maximal diameter of a residual tumor >1 cm. All patients received adjuvant chemotherapy using paclitaxel (175 mg/m^2) and carboplatin (AUC 5 or 6) after surgery, and the chemotherapy was repeated for 6 cycles every three weeks. Platinum-resistance was defined as poor response to the chemotherapy with treatment-free interval of less than six months. PFS was defined as the time elapsed from the date of surgery to the date of disease recurrence.

Immunohistochemistry

The six hypoxia-related proteins were evaluated by immunohistochemistry (IHC), and the substitution of the primary

antibody with purified non-immune mouse IgG was included at the same concentration for negative controls. For IHC of HGSO, we took representative core tissue sections (diameter of 2 mm) from paraffin blocks, and arranged them in new tissue microarray (TMA) blocks using trephine apparatus (Superbiochips Labratories, Seoul, Republic of Korea). After IHC, one pathologist unaware of clinico-pathologic characteristics interpreted expression levels for the six genes semi-quantitatively based on the product of staining intensity (I score; 1, weak; 2, moderate; 3, strong) and percentage of positive cells (P score, 1, <25%; 2, 25-50%; 3, 51-75%; 4, >75%). The final immunoreactive score defined as I score times P score ranged from 1 to 12, and the high expression of each gene was considered when the final immunoreactive score was six or more.

Statistical analysis

Data for cell viability assays were evaluated by analysis of variance (ANOVA) based on the general linear model (PROC-GLM) of SAS program (SAS Institute Inc., Cary, NC, USA), For investigating the role of hypoxia-related genes affecting platinum-resistance and PFS, we performed Chi-squared and Student's t-tests, Kaplan-Meier analysis with the log-rank test, Cox' proportional hazard and logistic regression analyses to determine hazard ratio (HR), odds ratio (OR) and 95% confidence interval (CI). Statistical analyses were performed using SPSS software version 19.0 (SPSS Inc., Chicago, IL, USA), and a P value <0.05 was defined to be statistically significant.

Results

Hypoxia increased cell viability with decreased cytotoxicity to cisplatin

To determine inhibitory concentration 50 (IC_{50}) of cisplatin, OV90 cells were treated with cisplatin (0, 10, 25, 50, and 100 μmol/L) for 24 hr under normoxic condition. As a result, IC_{50} of cisplatin was 50 μmol/L in OV90 cells shown in Figure 1A. When OV90 cells were treated with cisplatin of 50 μmol/L, hypoxic cells were less inhibited than normoxic cells, and the less O_2 concentration, the weaker cisplatin inhibited the cell growth (Figure 1B). These results suggested that hypoxia may lead to the increase of cell viability in comparison with normoxia, and it may induce drug resistance of ovarian cancer cells to cisplatin.

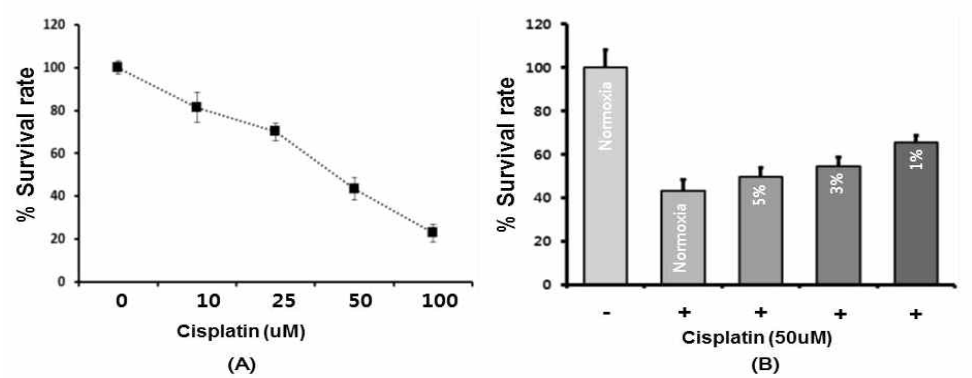

Figure 1. Effects of cisplatin on cell viability of OV90 cells in response to hypoxia.

(A) Cytotoxicity assay for determining the half maximal inhibitory concentration (IC_{50}) of cisplatin in OV90 (high-grade serous adenocarcinoma of the ovary) cells. MTT assay indicated effects of cisplatin in a dose-dependent manner (0, 10, 25, 50 and 100 μmol/L). Data was revealed as a percentage relative to non-treated control

cells (100%). (B) Cell viability assay under different hypoxic conditions (normoxia, 5% O_2, 3%O_2 and 1% O_2) after treatment of 50 μmol/L cisplatin in OV90 cells in response to IC_{50} of each chemicals. When OV90 cells were treated with cisplatin of 50 μmol/L, survival rate of hypoxic cells was less inhibited than normoxic cells.

Basic mRNA levels of hypoxia-related genes in high-grade serous adenocarcinoma of the ovary

Next, we compared *mRNA* levels of the six genes including *CBP*, *P300*, *HIF-1α*, *HIF-1β*, *FIH* and *VHL* between normal and cancer tissues of the ovary by using quantitative RT-PCR. In HGSO, *mRNA* levels of *CBP*, *P300*, *HIF-1α* and *HIF-1β* were lower than those in normal tissues, whereas *mRNA* levels of *FIH* and *VHL* were higher than those in normal tissues (Figure 2). It means that some specific genes such as *FIH* and *VHL* may be highly expressed in HGSO when compared with normal ovary, showing the basic tendency toward proteolytic degradation of hypoxia-related proteins such as *HIF-1α* and *HIF-1β.*

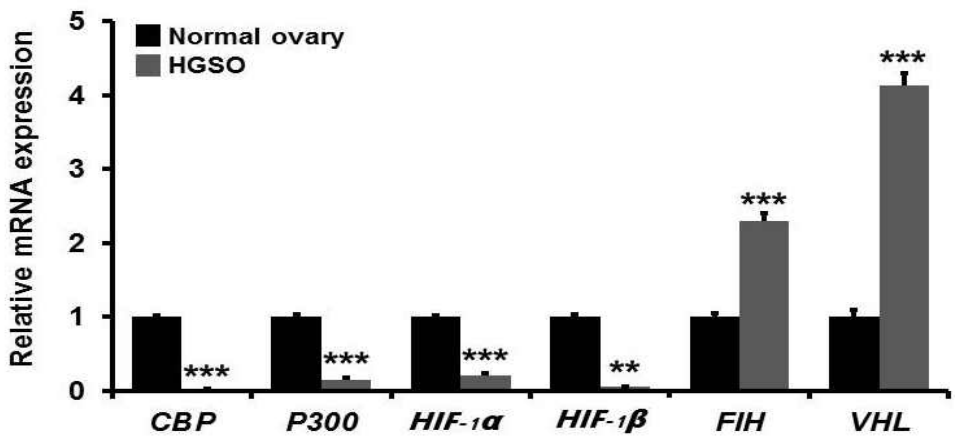

Figure 2. Quantitation of relative *mRNA* expression of hypoxia-related genes in high-grade serous carcinoma of the ovary

Relative *mRNA* levels of hypoxia-related genes in high-grade serous adenocarcinoma of the ovary were estimated by quantitative RT-PCR as compared with normal ovary. *mRNA* levels of *CBP*, *P300*, *HIF-1α* and *HIF-1β* were lower than those in normal ovary, whereas *mRNA* levels of *FIH* and *VHL* were higher than those in normal ovary ($*P < 0.05$).

Changes of protein levels of hypoxia-related genes under hypoxia in high-grade serous adenocarcinoma of the ovary

Next, we compared protein levels of the six genes under hypoxic conditions after treatment of cisplatin by using Western blot. As a result, the protein level of *HIF-1β* was increased, whereas that of *VHL* was decreased under hypoxic conditions after treatment of cisplatin (Figure 3). This result shows that changes of protein levels of *HIF-1β* and *VHL* may be associated with drug resistance in HGSO.

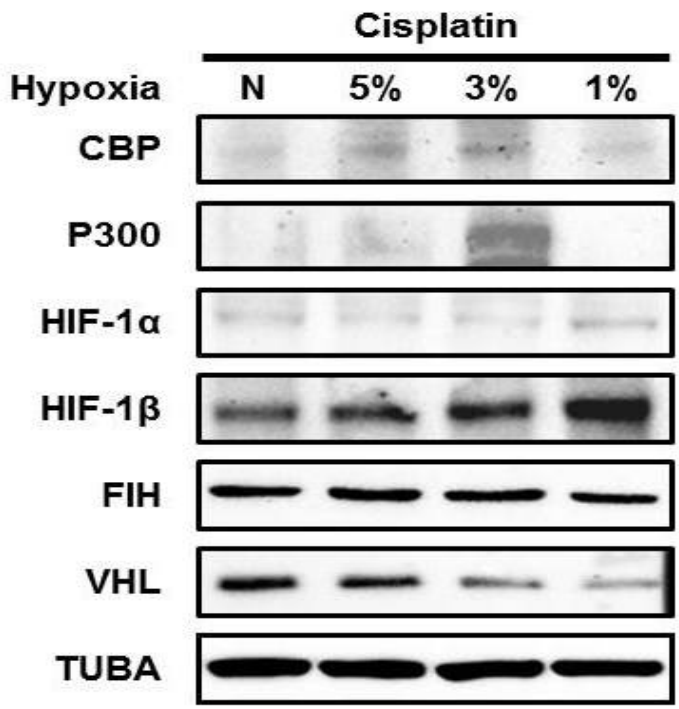

Figure 3. Comparison of protein levels after treatment of cisplatin under hypoxic conditions in OV90 cells by Western blot.

The protein level of *HIF-1β* was increased, whereas that of *VHL* was decreased under hypoxic conditions after treatment of cisplatin in OV90 cells.

As illustrated in Figure 4A, cell proliferation was not significantly changed in response to NAC (1 to 10 mM) in OV90 cells whereas it was dramatically decreased by 20 mM NAC. Moreover, ROS production was gradually decreased in a dose-dependent manner (0, 1, 2, 5 and 10 mM) of NAC in OV90 cells (Figure 4B). Then, we analyzed the production of ROS in response to NAC with cisplatin in OV90 cells. As a result, a combination of NAC with cisplatin did not reduce ROS generation in OV90 cells (Figure 4C). These results show that NAC may reduce ROS production in OV90 cells, whereas a combination of NAC with cisplatin affect ROS generation in OV90 cells.

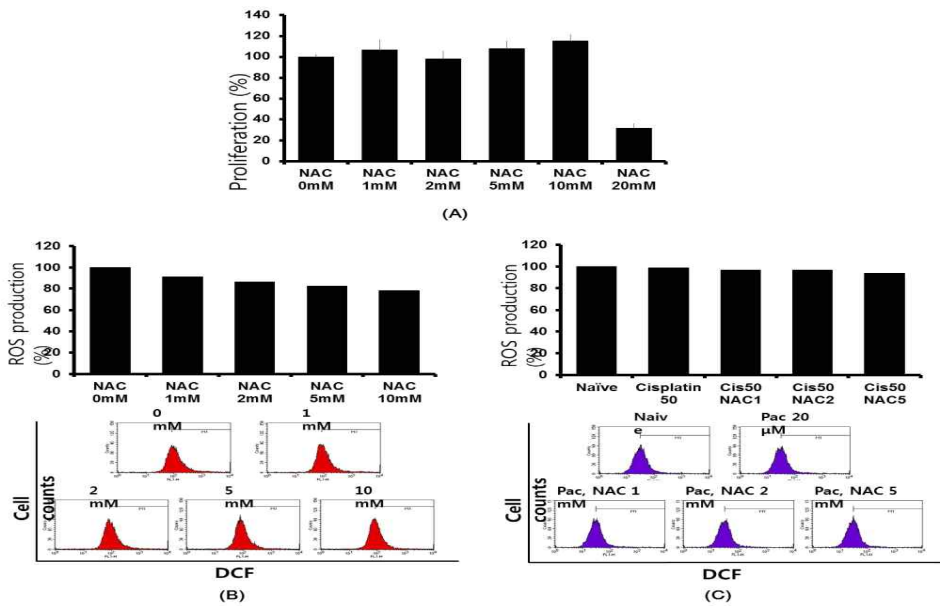

Figure 4. (A) Proliferation of OV90 cells after treatment of N-acetylcysteine (NAC) by proliferation assay; production of reactive oxygen species (ROS) in OV90 cells after treatment of (B) NAC and (C) NAC with cisplatin by flow cytometry

Cell proliferation was not significantly changed in response to NAC (1 to 10 mM), whereas it was dramatically decreased by 20 mM NAC in OV90 cells. ROS production was gradually decreased in a dose-dependent manner (0, 1, 2, 5 and 10 mM) of NAC. Moreover, cisplatin (50 μmol/L) did not reduce ROS production as compared to non-treated cells, and additional treatment of NAC (1, 2 or 5 mM) with cisplatin did not also show a decrease of ROS production as compared to cisplatin only treatment.

Effects of N-acetylcysteine with cisplatin on expression of hypoxia-related genes in high-grade serous adenocarcinoma of the ovary

To identify regulatory effects of the combined substrate including NAC with cisplatin, we performed Western blot analyses in OV90 cells. As a result, the protein level of *HIF-1β* was increased, whereas that of *VHL* was decreased when the concentration of NAC was increased with cisplatin in OV90 cells (Figure 5). This result indicates that changes of hypoxia-related protein levels may be involved in drug resistance under anti-oxidant condition in HGSO.

Roles of hypoxia-related genes as prognostic factors in high-grade serous adenocarcinoma of the ovary

A total of 149 patients with HGSO were included in the current study. Table 2 shows clinico-pathologic characteristics and expressions of the six genes of all patients. Among the six genes, *CBP* protein was expressed in the nucleus and cytoplasm, and *HIF-1β* protein was shown in the nucleus, whereas *P300, HIF-1a*,

FIH and *VHL* proteins demonstrated cytoplasmic immunostaining (Figure 6).

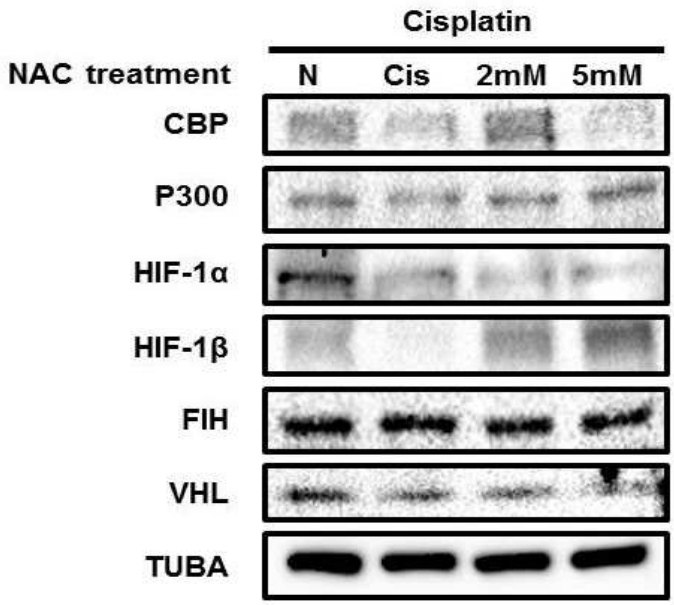

Figure 5. Protein levels after treatment of cisplatin and N-acetylcysteine (NAC) in OV90 cells by Western blot

HIF-1β expression was increased, whereas *VHL* expression was decreased when the concentration of NAC was increased with cisplatin in OV90 cells.

Table 2. Clinico-pathologic characteristics and expression of hypoxia-related genes by immunohistochemistry in patients with high-grade serous adenocarcinoma of the ovary (HGSO)

Characteristics or expreesions	HGSC (n=149, %)	CCC (n=61, %)
Age (median, range, y)	53 (28-80)	49 (30-77)
FIGO stage		
I	5 (3.4)	36 (59)
II	6 (4)	4 (6.6)
III	112 (75.1)	19 (31.1)

IV	26 (17.5)	2 (3.3)
Extent of cytoreduction		
Optimal	82 (55)	53 (86.9)
Suboptimal	67 (45)	8 (13.1)
Platinum resistance		
No	114 (76.5)	52 (85.2)
Yes	35 (23.5)	9 (14.8)
CBP		
Low	37 (24.8)	13 (21.3)
High	112 (75.2)	48 (78.7)
P300		
Low	41 (27.5)	20 (32.8)
High	108 (72.5)	41 (67.2)
HIF-1α		
Low	61 (40.9)	30 (49.2)
High	88 (59.1)	31 (50.8)
HIF-1β		
Low	88 (59.1)	36 (59)
High	61 (40.9)	25 (41)
FIH		
Low	40 (26.8)	22 (36.1)
High	109 (73.2)	39 (63.9)
VHL		
Low	128 (85.9)	54 (88.5)
High	21 (14.1)	7 (11.5)

Abbreviation: CBP, cAMP-response element-binding protein (CREB)-binding protein; FIGO, International Federation of Gynecology and Obstetrics; FIH, factor-inhibiting hypoxia inducible factor; HIF, hypoxia inducible factor; adenovirus early region 1A (E1A)-binding

protein p300, P300, VHL, von Hippel-Lindau.

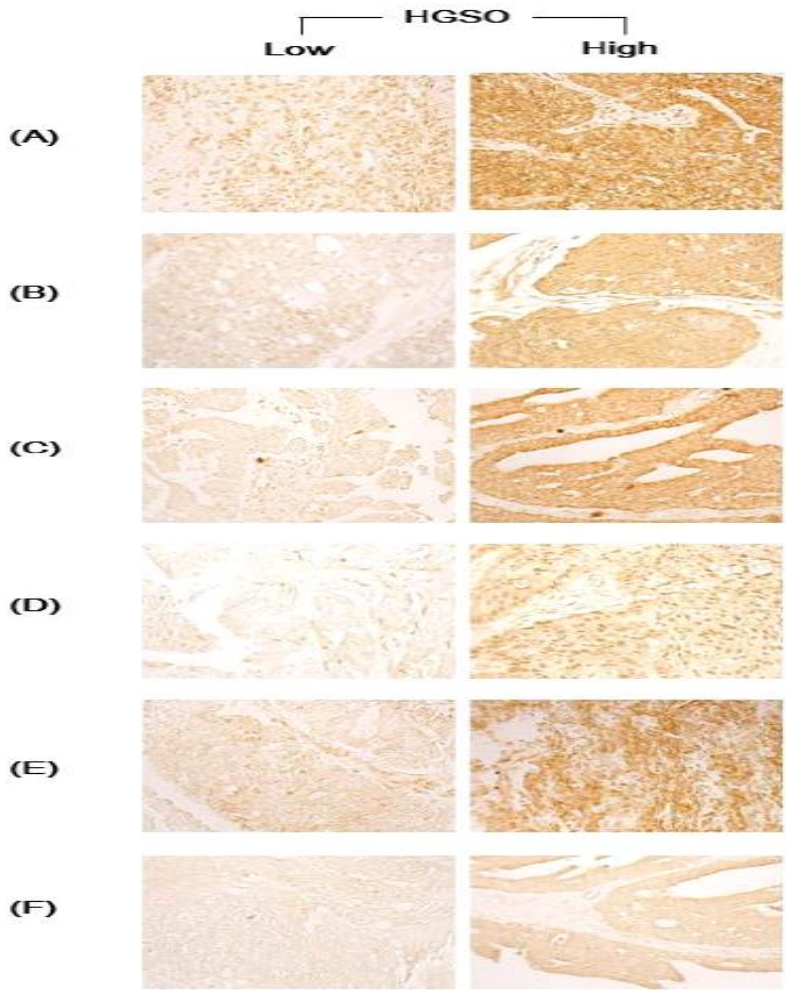

Figure 6. Immunohistochemistry for the six hypoxia-related genes: (A) *CBP*; (B) *P300*; (C) *HIF-1α*; (D) *HIF-1β*; (E) *FIH*; (F) *VHL*

Among the six genes, *CBP* protein was expressed in the nucleus and cytoplasm, and *HIF-1β* protein was shown in the nucleus, whereas *P300*, *HIF-1α*, *FIH* and *VHL* proteins demonstrated cytoplasmic immunostaining (original magnification ×400).

In terms of platinum resistance, early-stage (FIGO stage I or II) disease, optimal cytoreduction, a low expression of *HIF-1β* and a high expression of *VHL* were favorable factors for reducing platinum-resistance in patients with HGSO (Adjusted ORs, 0.273, 0.593, 0.657 and 0.381; 95% CI, 0.085-0.878, 0.397-0.884, 0.443-0.975 and 0.165-0.878; Table 3).

Table 3. Univariate and multivariate analyses for platinum-resistance in patients with high-grade serous adenocarcinoma of the ovary

Characteristics	Univariate			Multivariate		
	OR	95% CI	P value	Adjusted OR	95% CI	P value
Age< 54 years	0.837	0.575-1.219	0.353	-	-	-
Early-stage (I or II) disease	0.211	0.067-0.666	0.008	0.273	0.085-0.878	0.029
Optimal cytoreduction	0.476	0.325-0.697	<0.001	0.593	0.397-0.884	0.010
High expression of CBP	0.790	0.507-1.229	0.296	-	-	-
High expression of P300	0.777	0.511-1.182	0.238	-	-	-
Low expression of HIF-1 α	0.775	0.526-1.143	0.198	-	-	-
Low expression of HIF-1 β	0.631	0.428-0.932	0.021	0.657	0.443-0.975	0.037
High expression of FIH	0.678	0.444-1.034	0.071	-	-	-
High expression of VHL	0.309	0.135-0.707	0.005	0.381	0.165-0.878	0.023

Abbreviation: *CBP*, cAMP-response element-binding protein (CREB)-binding protein; CI, confidence interval; *FIH*, factor-inhibiting hypoxia inducible factor; *HIF*, hypoxia inducible factor; OR, odds ratio; *P300*, adenovirus early region 1A (E1A)-binding protein p300; *VHL*, von Hippel-Lindau.

In terms of survival, a low expression of *HIF-1β* and a high expressions of *VHL* were associated with improved PFS in patients with HGSO (Figure 7). In multivariate analyses, early-stage disease, optimal cytoreduction, a low expression of *HIF-1β* and a high expression of *VHL* were related with improved PFS in patients with

HGSO (adjusted HRs, 0.273, 0.593, 0.657 and 0.381; 95% CIs, 0.085-0.878, 0.397-0.884, 0.443-0.975 and 0.165-0.878; Table 4).

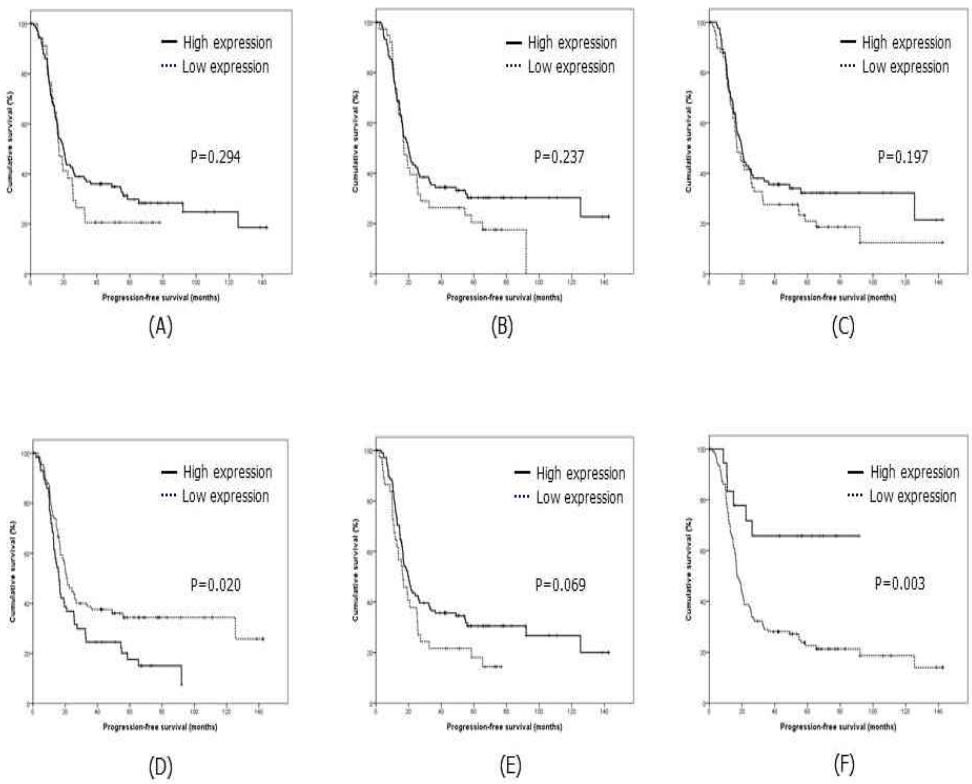

Figure 7. Comparison of progression-free survival based on expressions of the six hypoxia-related genes in patients with high-grade serous adenocarcinoma of the ovary: (A) *CBP*; (B) *P300*; (C) *HIF-1α*; (D) *HIF-1β*; (E) *FIH*; (F) *VHL*

A low expression of *HIF-1β* and a high expressions of *VHL* were associated with improved progression-free survival.

When we compared PFS based on the combination of *HIF-1β* and *VHL* expressions, a low expression of *HIF-1β* and a high expression of *VHL* showed better PFS, whereas a high expression of *HIF-1β* and a low expression of *VHL* was related with poor PFS (Figure 8).

Table 4. Univariate and multivariate analyses for progression-free survival in patients with high-grade serous adenocarcinoma of the ovary

Characteristics	Univariate			Multivariate		
	HR	95% CI	P value	Adjusted HR	95% CI	P value
Age< 54 years	1.202	0.821-1.760	0.345	-	-	-
Early-stage (I or II) disease	0.211	0.067-0.666	0.008	0.273	0.085-0.878	0.029
Optimal cytoreduction	0.476	0.325-0.697	<0.001	0.593	0.397-0.884	0.010
High expression of CBP	0.790	0.507-1.229	0.296	-	-	-
High expression of P300	0.777	0.511-1.182	0.238	-	-	-
Low expression of HIF-1α	1.290	0.875-1.092	0.198	-	-	-
Low expression of HIF-1β	0.631	0.428-0.932	0.021	0.657	0.443-0.975	0.037
High expression of FIH	1.476	0.967-2.252	0.071	-	-	-
High expression of VHL	0.309	0.153-0.707	0.005	0.381	0.165-0.878	0.023

Abbreviation: *CBP*, cAMP-response element-binding protein (CREB)-binding protein; CI, confidence interval; *FIH*, factor-inhibiting hypoxia inducible factor; *HIF*, hypoxia inducible factor; HR, hazard ratio; *P300*, adenovirus early region 1A (E1A)-binding protein p300; *VHL*, von Hippel-Lindau.

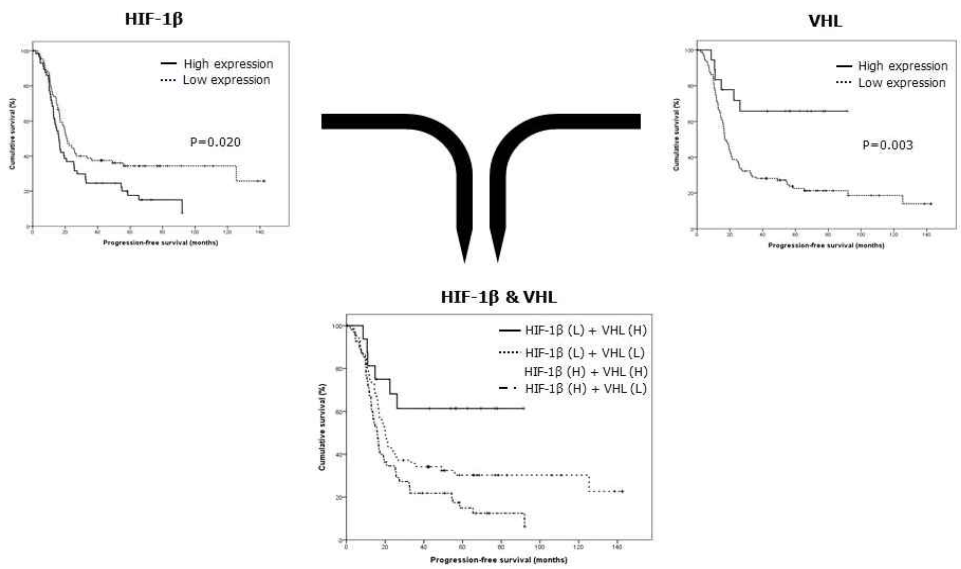

Figure 8. Comparison of progression-free survival according to the combined expressions of *HIF-1β* and *VHL* genes in patients with high-grade serous adenocarcinoma of the ovary

A low expression of *HIF-1β* and a high expression of *VHL* showed better prognosis, whereas a high expression of *HIF-1β* and a low expression of *VHL* was related with poor prognosis.

Discussion

The presence of hypoxic regions within tumors has been reported to adversely affect clinical outcomes, and to be associated with a worse prognosis. Although it seems to be important to select patients with more burden of hypoxic tumors in clinical setting, there is no consensus for the best method suited for routine assessment of hypoxia. The reasons are as follows. First, many studies included patients with various types of histology and tumor differentiation of the ovary. Second, fragmentary genes associated with hypoxia were investigated among all hypoxia-related genes in most of studies. In the current study, we included only the most common histologic type, HGSO, and thereby investigated the roles of most genes associated with hypoxia.

Up to now, representative hypoxia-related genes known to affect prognosis are *HIF-1a* and *VEGF* in ovarian cancer. Previous studies have indicated that *HIF-1a* acts as a positive key regulator of tumor growth in many malignant solid tumors. It plays an important role in the up-regulation of certain hypoxia-related proteins involved in angiogenesis, gluocse transport, glycolysis, erythropoiesis, and inhibition of apoptosis (16). Moreover, *HIF-1a* overexpression is involved in the development of malignancy in adenoma, borderline tumor to adenocarcinoma (17), and that hypoxic status differs according to histologic type and microenvironment (18).

Moreover, *VEGF*, a downtream gene of *HIF-1a*, is known to be expressed strongly in 36-80% of patients with ovarian cancer (19). Bevacizumab, monoclonal antibody for targeting *VEGF-A*, has been shown to improve survival of advanced-stage ovarian cancer (20, 21), which recently have change the protocol of drug therapy in the disease. However, we did not fail to find the role of *VEGF* in the current study because a high expression of *VEGF* was observed in

most of patients with HGSO (98%). Since a high expression of *VEGF* was shown in ovarian cancer cells or tissues in the current study, we can expect the role of *VEGF* for predicting prognosis in serum levels like previous studies (22, 23).

On the other hand, we found that a high expression of *HIF-1β* may be related with platinum-resistance and poor PFS. Although *HIF-1β* is known to be constitutively expressed in cells, loss of *HIF-1β* has been reported to result in reduced tumor growth, decreased angiogenesis, and resistance to chemotherapy (24). This hypothesis can be supported by a previous study where knockdown of *HIF-1β* reduced cisplatin resistance in cervical cancer cells (25). Moreover, activation of *EGFR* signaling may increase nuclear accumulation of *HIF-1β*, which interacts with transcriptional factor c-Jun and binds to the *CRE* site, and results in an increased of *COX-2* gene expression related with tumorigenesis (26).

However, we found that expressions of *CBP* and *P300* were not related with hypoxia, and relevant prognosis. *CBP* and *P300* are known as histone acetyltransferase (HAT) molecules, and considered to be important transcriptional coactivators that act to regulate relevant gene expression (27). In colon cancer, overexpression of *CBP* and *P300* has been reported to be
related with poor prognosis (28). Nevertheless, we did not find the relation between *CBP* or *P300*, and hypoxia or related prognosis in the current study. The limited role has been also reported in renal cancer (29). It means that their roles as a biomarker may different among various types of cancers, which requires more studies to evaluate their roles in ovarian cancer.

Moreover, *FIH* was not a biomaker associated with prognosis of HGSO. FIH inhibits *HIF-1a* in an oxygen-dependent manner, and it remains active unless severe hypoxia occurs. This suggests that *FIH* may have an important function as one of the final checks on *HIF-1*

a transcriptional activity (30). The presence of *FIH* has been investigated in various normal and neoplastic human tissues, in which the intensity and subcellular localization are very heterogeneous. Although in normal human tissues *FIH* is predominantly cytoplasmic, nuclear expression of *FIH* can be relatively strong in certain neoplasms (31).

Among solid tumors, *FIH* has provided variable prognostic values in several tumour types. In pancreatic endocrine tumors (PETs), cytoplasmic *FIH* levels were significantly higher in more malignant PETs, but were not associated with survival. Nuclear *FIH* did not correlate with any histopathologic variables in this study (32). In invasive breast cancer, both cytoplasmic *FIH* expression and absence of nuclear *FIH* were independent
prognostic factors for a shorter disease-free survival (33). In addition, renal clear cell carcinoma (CCC) showed that a low
expression of nuclear *FIH* is a significant independent predictor
for worse survival. In particular, *FIH* can be detected both in the nucleus and cytoplasm, and the specific subcellular localization varied between different renal CCC patients (34). The absence of nuclear *FIH* in more aggressive phenotypes could be explained by increasing gene mutations within renal CCC including *FIH* gene mutations.

A low expression of *VHL* may be also associated with platinum-resistance and PFS. Under normoxic conditions, *HIF-1a* is hyroxylated, and this hyroxylation promotes rapid degradation of it by the ubiquitin-proteosome pathway mediated by *VHL* (35). Under hypoxic condition, however, *HIF-1a* can be stabilized and accumulated by decreasing prolyl hydroxylases activity and hydroxylation of *HIF-1a*. Consequently, stabilized *HIF-1a* heterodimerizes with *HIF-1β* to form *HIF-1* and binds to target DNA at the hypoxic response element, which leads to tumorigenesis and angiogenesis (36).

However, the current study did not show the significant correlation between *HIF-1a* and *VHL*. This result has also been reported in a previous study where it was found in clear cell carcinoma of the ovary, suggesting that a more hypoxic or only a partially oxygenated microenvironment does not allows *VHL* to regulate *HIF-1a* (37). On the other hand, low expression of *VHL* can be related with a high expression of *HIF-1β* because a previous study reported that *HIF-1β*, not *HIF-1a*, may suppress the development of *VHL*-related tumor (38).

On the other hand, a high expression of *HIF-1β* and low expression of *VHL* may act as a role of biomakers for only HGSO. In contrast, these results did not be shown in CCC. This result can be explained by the hypothesis that expressions of hypoxia-related genes can be suppressed by some scavengers in CCC. A previous study supports the hypothesis where detoxification of oxidative stress by glutathione peroxidase 3 was the most active in CCC than in the other histologic types, suggesting the role as a tumor suppressor gene (39).

However, the current study has some limitations as follows. First, we did not show consistent changes of hypoxia-related gene expressions after treatment of paclitaxel according to hypoxia in spite of adjuvant chemotherapy using paclitaxel. In spite of relevant evidence, It means that paclitaxel can act as a minor role to change hypoxia-related genes during chemotherapy in ovarian cancer. Second, we failed to show consistent trend of change of *mRNA* levels after treatment of paclitaxel and cisplatin according to hypoxia. In particular, *mRNA* levels of hypoxia-related genes was reduced in the lowest hypoxia condition (1%O_2). Moreover, we found the similar result that cell viability was also decreased when the highest dose of NAC (20 mM) was administered. It means that the extreme hypoxic condition can suppress tumor proliferation inversely.

Conclusively, *HIF-1β* and *VHL* may be biomarkers for predicting

platinum-resistance and survival in patients with HGSO, and relevant targeted therapy can be considered for treating HGSO in the future.

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국문요약 (국문초록)

고등급 장액성 난소암의 예후에 영향을 미치는 저산소증 연관 유전자 발현 연구

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배경: 난소암은 조기 발견을 위한 적절한 선별검사가 없기 때문에 대부분의 경우 진행성 병기에서 진단되고, 여성생식기에서 발생하는 종양 중 가장 예후가 불량한 것으로 알려져 있다. 이러한 진행성 난소암의 일차 표준 치료로 최적종양감축술과 보조적 항암화학요법이 중요한 것으로 알려져 있으나, 진행성 난소암의 80%에서 일차 표준 치료 후 재발 소견을 보인다. 재발의 원인 중 약물 저항성은 치료 실패 및 예후의 불량의 가장 중요한 원인으로 알려져 있으며, 이러한 약물 저항성의 관련 인자 중 종양미세환경이 중요한 역학을 하는 것으로 알려져 있다. 특히, 종양미세환경 중 저산소증 관련 유전자들은 암성화, 종양세포의 증식 및 침습과 관련이 있는 것으로 알려져 있으나 난소암에서 약물 저항성 및 예후와 관련하여 저산소증 관련 유전자들에 대한 연구는 아직 충분하지 않은 상황이다. 따라서 본 연구에서는 난소암 중 가장 흔한 조직학적 유형인 고등급 장액성 난소암을 대상으로 저산소증 관련 유전자들의 발현은 확인하고, 이러한 유전자들의 발현이 약물 저항성 및 예후에 미치는 영향에 관하여 분석하고자 하였다.

방법: 먼저 고등급 장액성 난소암의 대표적인 세포주인 OV90 세포주를 이용하여 난소암의 치료 약물인 cisplatin의 IC_{50}을 확인하였고, cisplatin 처리 후 저산소증 조건에서 세포의 생존능력을 확인하였다. 또한, 정상 난소 및 고등급 장액성 난소암 조직에서 quantitative RT-PCR을 이용하여 저산조증 관련 유전자인 *CBP*, *P300*, *HIF-1α*, *HIF-1β*, *FIH*, *VHL*의 *mRNA* 기저 발현을 비교 평가하였고, OV90 세포주를 이용하여 저산조증 조건에서 cisplatin 처리 후 여섯 개의 저산소증 관련 유전자들 발현을 Western blot을 이용하여 비교 평가하였다. 또한, 저산소증과 활성산소 생성 간의 관계를 확인하기 위하여 N-acetylcysteine (NAC)에 의한 OV90 세포주의 증식 및 활성산소 생성을 측정하였다. 최종적으로 고등급 장액성 난소암 환자 149명을 대상으로 여섯 개 저산소증 관련 유전자들의 단백질 발현을 면역조직화학검사를 이용하여 평가하였고, 이 유전자들이 백금 저항성 및 무진행생존률에 미치는 영향을 분석하였다.

결과: OV90 세포주에서 cisplatin의 IC_{50}은 50 μmol/L이었고, cisplatin

처리 후 저산소증의 증가에 따라 OV90 세포주의 생존률이 증가함을 확인할 수 있었다. 조직에서 저산소증 관련 유전자들의 기저 *mRNA* 발현을 확인하였을 때, 정상 난소조직에 비하여 *CBP*, *P300*, *HIF-1a, HIF-1β* 의 *mRNA* 발현은 적었고, *FIH, VHL*의 *mRNA* 발현은 많았다. Western blot에서 cisplatin 처리 후 저산소증의 증가에 따라 *HIF-1β*의 발현은 증가하였고, *VHL*의 발현은 감소하였다. 이러한 결과는 OV90 세포주에 NAC와 cisplatin을 동시에 처리하였을 때 NAC의 증가에 따라 *HIF-1β*의 고발현 및 *VHL*의 저발현이 저산조증 조건에서와 유사하게 나타남을 확인할 수 있었다. 면역조직화학검사에서 *HIF-1β*의 저발현과 *VHL*의 고발현은 저분화 장액성 난소암에서 백금 저항성을 줄이고 (Adjusted ORs, 0.657 and 0.381; 95% CI, 0.443-0.975 and 0.165-0.878), 무진행생존률을 향상시키는 것과 연관이 있음을 확인할 수 있었다 (adjusted HRs, 0.657 and 0.381; 95% CIs, 0.443-0.975 and 0.165-0.878).

결론: 고등급 장액성 난소암에서 저산소증 관련 유전자들 중 *HIF-1β*의 발현증가 및 *VHL*의 발현 감소는 백금 저항성 및 예후 불량과 관련이 있음을 확인할 수 있었다.

주요어: *HIF-1β*, *VHL*, 항암제 저항성, 무진행생존률, 고등급 장액성암, 난소암.

학 번: 2011-31119

Supplements

Introduction

Drug resistance of tumor is expected to be associated with tumor heterogeneity and microenvironment, which may hinder the improvement of prognosis and the development of optimal biomarkers for ovarian cancer (7, 8). Among different types of tumor microenvironment, hypoxia-related genes are known to be related with carcinogenesis, proliferation and invasion of tumor cells (9, 10), which may act as roles to predict drug resistance and consequent prognosis in various types of malignancies such as kidney and lung cancers (11, 12).

In ovarian cancer, histologic types have been suggested to depend on exposure level and response to oxidative stress. Among all histologic types, clear cell carcinoma (CCC) is developed with persistent exposure to oxidative stress and inflammation, showing relatively slow growth and high drug resistance. On the other hand, HGSO is developed with at least oxidative stress, which demonstrate relatively fast growth and low drug resistance (24, 25). This hypothesis suggests the possibility of different roles of hypoxia-related genes affecting drug resistance and prognosis according to histologic types of ovarian cancer.

Thus, we compared expressions of hypoxia-related genes between two extreme histologic types based on oxidative stress by hypoxia, CCC and HGSO, and investigated the roles of hypoxia-related genes affecting platinum resistance and prognosis of each histologic type of ovarian cancer.

Materials and methods

Reagents

Paclitaxel was obtained from Sigma-Aldrich (Missouri, USA).

Cell lines and tissue samples

ES-2 (CCC) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). We purchased five frozen tissue samples with CCC from Cancer Tissue Bank of Seoul National University.

Cell culture

We used a 1:1 mixture of McCoy's 5A (modified) medium (ATCC, USA) and 10% fetal bovine serum for ES-2 cell line with penicillin and streptomycin.

Culturing cells under hypoxic conditions

ES-2 cells were grown in McCoy's 5A Medium Modified containing 10% FBS.

Cytotoxicity and cell viability assays

Human ovarian cancer cells were seeded and exposed to different concentrations (0, 1, 10, 20 or 100 μmol/L) of chemical compounds (paclitaxel or cisplatin) in normoxia or hypoxia.

Determination of cellular reactive oxygen species

ES-2 cells were detached with trypsin-EDTA, collected by centrifugation, and washed with PBS. NAC in a dose dependent manner (0, 1, 2, 5 and 10 mM) or a combination of paclitaxel with NAC for 1h at 37 °C in a CO_2 incubator.

RNA isolation

Total cellular RNAs of ES-2 cell line was obtained using Trizol reagent (Invitrogen, Carlsbad, CA) based on the manufacturer's

guideline.

Quantitative RT-PCR analysis

Complementary DNA was synthesized using total RNA extracted from ES-2 cells and AccuPower RT PreMix (Bioneer, Daejeon, Republic of Korea).

Human study population

The inclusion criteria were as follows: patients with CCC; those treated with cytoreductive surgery and adjuvant chemotherapy using paclitaxel and carboplatin; those with Eastern Cooperative Oncology Group performance status of 0-2; those without underlying diseases affecting survival.

Immunohistochemistry

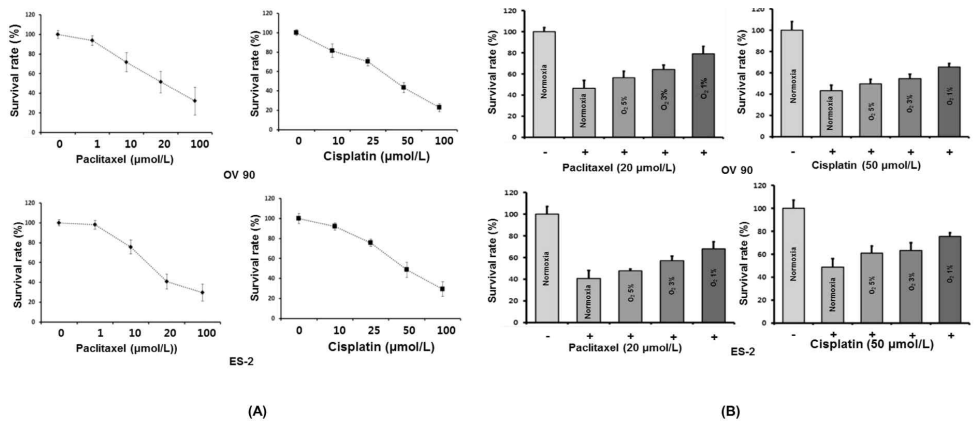
For IHC of CCC, we took representative core tissue sections (diameter of 2 mm) from paraffin blocks, and arranged them in new TMA blocks using trephine apparatus (Superbiochips Labratories, Seoul, Republic of Korea).

Results

Hypoxia increased cell viability with decreased cytotoxicity to paclitaxel and cisplatin

To determine IC_{50} of paclitaxel and cisplatin, OV90 and ES-2 cells were treated with paclitaxel (0, 1, 10, 20 and 100 μmol/L) or cisplatin (0, 10, 25, 50, and 100 μmol/L) for 24 hours under normoxic condition. As a result, IC_{50} of paclitaxel or cisplatin was 20 and 50 μmol/L in OV90 and ES-2 cells shown in Supplementary figure 1A. When OV90 and ES-2 cells were treated with paclitaxel of 20 μmol/L and cisplatin of 50 μmol/L, respectively, hypoxic cells were less inhibited

than normoxic cells, and the less O_2 concentration, the weaker paclitaxel and cisplatin inhibited the cell growth (Supplementary figure 1B).

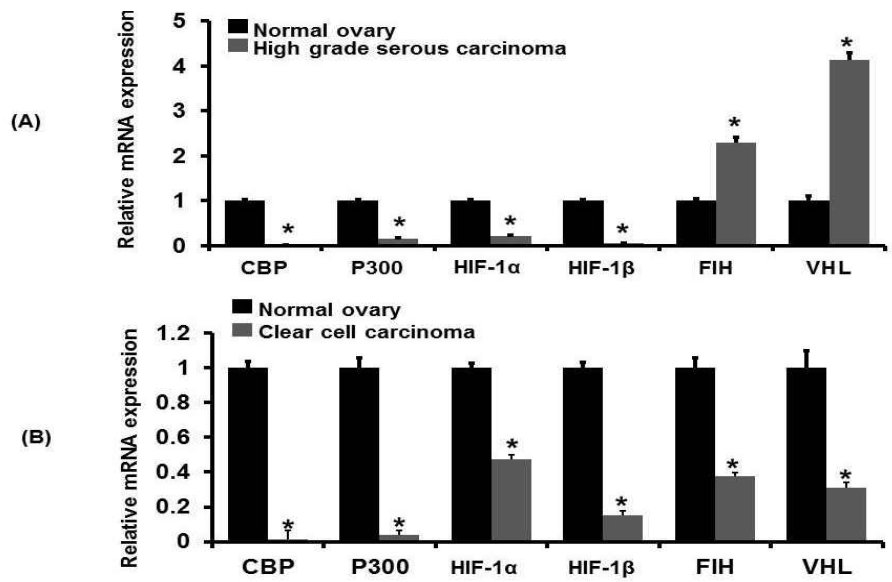


Supplementary figure 1. Effects of cisplatin and paclitaxel on cell viability of OV90 and ES-2 cells in response to hypoxia. (A) Cytotoxicity assay for determining IC_{50} of paclitaxel and cisplatin in OV90 (high-grade serous adenocarcinoma) and ES-2 (clear cell carcinoma) cells. MTT assay indicated effects of cisplatin and paclitaxel in a dose-dependent manner (0, 10, 25, 50 and 100 μmol/L). Data was revealed as a percentage relative to non-treated control cells (100%). (B) Cell viability assay under different hypoxic conditions (normoxia, 5% O_2, 3%O_2 and 1% O_2) after treatment of 20 μmol/L paclitaxel and 50 μmol/L cisplatin in OV90 and ES-2 cells in response to IC_{50} of each chemicals. When OV90 and ES-2 cells were treated with paclitaxel of 20 μmol/L and cisplatin of 50 μmol/L, survival rate of hypoxic cells was less inhibited than normoxic cells.

Basic mRNA levels of hypoxia-related genes were different between high-grade serous adenocarcinoma and clear cell carcinoma of the ovary

Next, we compared *mRNA* levels of the six genes including *CBP*,

P300, *HIF-1a*, *HIF-1β*, *FIH* and *VHL* between normal and cancer tissues of the ovary by using quantitative RT-PCR. In HGSO, *mRNA* levels of *CBP*, *P300*, *HIF-1a* and *HIF-1β* were lower than those in normal tissues, whereas *mRNA* levels of *FIH* and *VHL* were higher than those in normal tissues (Supplementary figure 2A). On the other hand, *mRNA* levels of all the six genes in CCC were lower than those in normal tissues (Supplementary figure 2B).

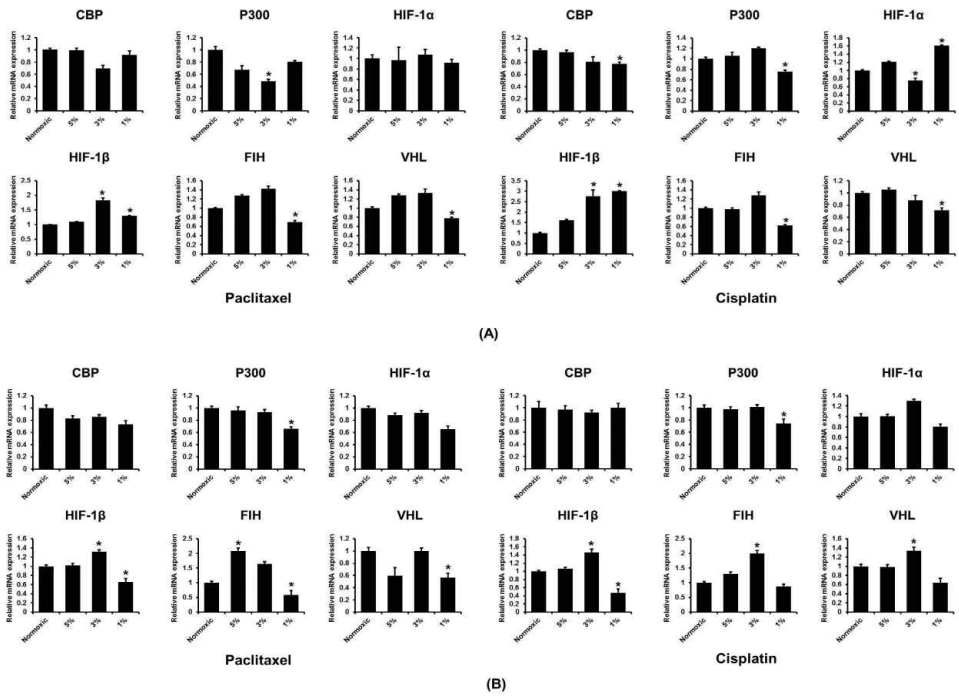


Supplementary figure 2. Quantitation of relative *mRNA* expression of hypoxia-related genes in high-grade serous adenocarcinoma (HGSO) and clear cell carcinoma (CCC).

Relative *mRNA* levels of hypoxia-related genes in (A) high-grade serous carcinoma (HGSO) and (B) clear cell carcinoma (CCC) were estimated by quantitative RT-PCR as compared with normal ovary. In HGSO, *mRNA* levels of *CBP*, *P300*, *HIF-1a* and *HIF-1β* were lower than those in normal ovary, whereas *mRNA* levels of *FIH* and *VHL* were higher than those in normal ovary. On the other hand, *mRNA* levels of all the six genes in CCC were lower than those in normal ovary ($*P < 0.05$).

Changes of mRNA levels of hypoxia-related genes under hypoxia were different between high-grade serous adenocarcinoma and clear cell carcinoma

We also compared *mRNA* levels of the six genes under hypoxic conditions after treatment of paclitaxel and cisplatin by using quantitative RT-PCR. As a result, the *mRNA* level of *HIF-1β* tended to be increased, whereas *mRNA* levels of *FIH* and *VHL* tended to be decreased under hypoxic conditions after treatment of paclitaxel in OV90 cells (P <0.05). When OV90 cells were treated with cisplatin, *mRNA* levels of *HIF-1α* and *HIF-1β* tended to be increased while those of *P300*, *FIH* and *VHL* tended to be decreased under hypoxic conditions (P <0.05; Supplementary figure 3A). In ES-2 cells, *mRNA* levels of *P300*, *HIF-1β*, *FIH* and *VHL* tended to be decreased after treatment of paclitaxel (P <0.05), whereas those of *P300* and *HIF-1β* tended to be reduced after treatment of cisplatin under hypoxic conditions (P <0.05; Supplementary figure 3B).



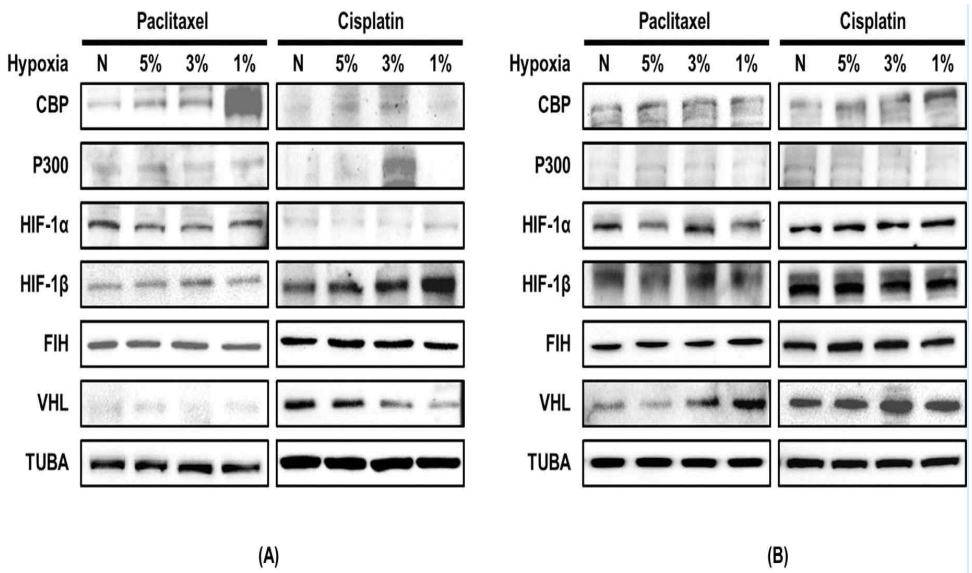
Supplementary figure 3. Comparison of *mRNA* levels after treatment of paclitaxel and cisplatin under hypoxic conditions.
Relative *mRNA* expression of hypoxia-related genes including *CBP*, *P300*, *HIF-1α*, *HIF-1β*, *FIH* and *VHL* was analyzed by quantitative RT-PCR in (A) OV90 and (B) ES-2 cells under hypoxic conditions in response to paclitaxel and cisplatin. The *mRNA* levels of *HIF-1β* was increased, whereas expressions of *FIH* and *VHL* were decreased under hypoxic conditions after treatment of paclitaxel in OV90 cells. When OV90 cells were treated with cisplatin, the *mRNA* levels of *HIF-1α* and *HIF-1β* were increased while those of *P300*, *FIH* and *VHL* were decreased under hypoxic conditions. In ES-2 cells, *mRNA* expressions of *P300*, *HIF-1β*, *FIH* and *VHL* were decreased after treatment of paclitaxel, whereas those of *P300* and *HIF-1β* were reduced after treatment of cisplatin under hypoxic conditions ($*P < 0.05$).

These results suggest that changes of *mRNA* levels of hypoxia-related genes may be different between HGSO and CCC under hypoxia. Specifically, chemotherapy under hypoxia may increase *HIF-1β* expression in HGSO and decrease *HIF-1β* expression in CCC, whereas it may decrease *P300*, *FIH* and *VHL* expressions in both HGSO and CCC. Afterwards, we compared protein expressions of the six genes based on hypoxia because these results did not show consistent changes of *mRNA* levels of the six gene expressions according to hypoxia.

Changes of protein levels of hypoxia-related genes under hypoxia were different between high-grade serous adenocarcinoma and clear cell carcinoma

Next, we compared protein levels of the six genes under hypoxic conditions after treatment of paclitaxel and cisplatin by

using Western blot. As a result, the protein level of *HIF-1β* was increased, whereas that of *VHL* was decreased under hypoxic conditions after treatment of cisplatin in spite of no changes of protein levels after treatment of paclitaxel in OV90 cells (Supplementary figure 4A). On the other hand, the protein level of *VHL* was increased under hypoxic conditions after treatment of paclitaxel despite no changes of the other genes in ES-2 cells (Supplementary figure 4B).



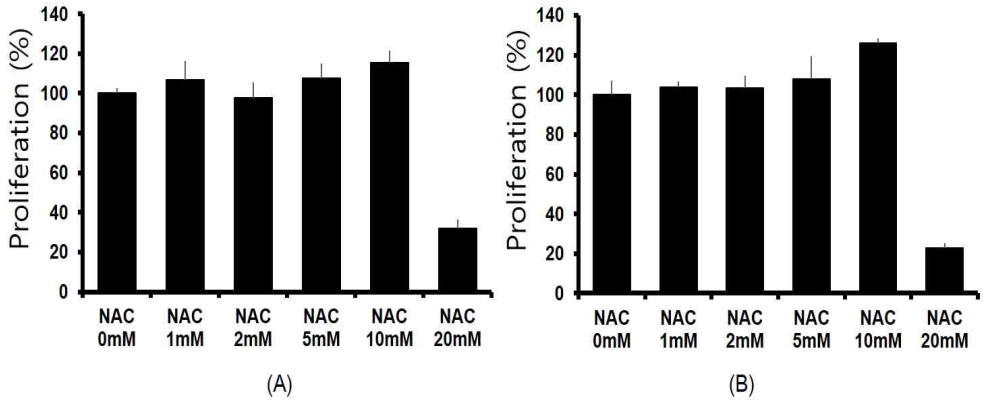
Supplementary figure 4. Comparison of protein levels after treatment of paclitaxel and cisplatin under hypoxic conditions in (A) OV90 and (B) ES-2 cells by Western blot.

The protein level of *HIF-1β* was increased, whereas that of *VHL* was decreased under hypoxic conditions after treatment of cisplatin in spite of no changes of protein levels after treatment of paclitaxel in OV90 cells. On the other hand, the protein level of *VHL* was increased under hypoxic conditions after treatment of paclitaxel despite no changes of the other genes in ES-2 cells.

Effects of N-acetylcysteine on cell proliferation and reactive oxygen species production in human ovarian cancer cells

To determine relationship between hypoxia and ROS generation, we estimated cellular proliferation and ROS production in OV90 and ES-2 cells by NAC which is a potent

antioxidant. As illustrated in Supplementary figure 5, cell proliferation was not significantly changed in response to NAC (1 to 10 mM) in both OV90 and ES-2 cells whereas it was dramatically decreased by 20 mM NAC. ROS production was gradually decreased in a dose-dependent manner (0, 1, 2, 5 and 10 mM) of NAC in OV90 and ES-2 cells (Supplementary figure 6).

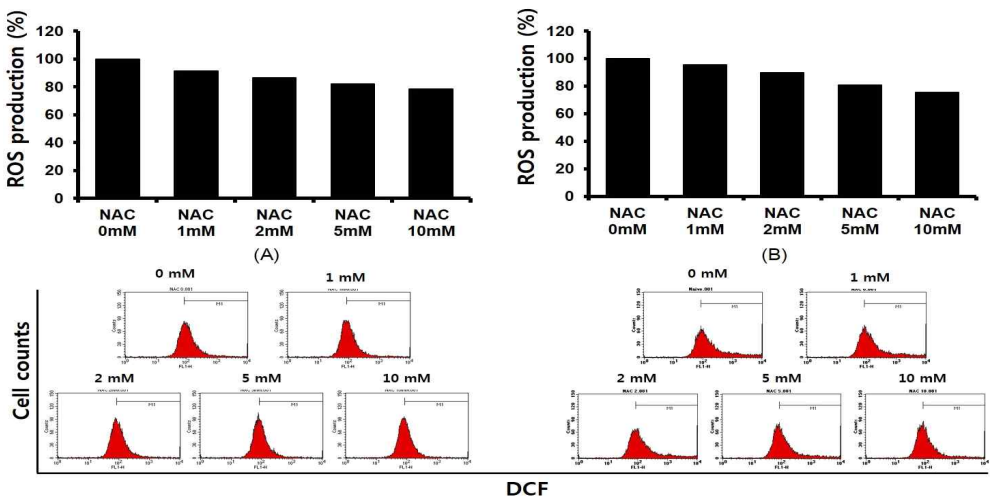


Supplementary figure 5. Proliferation of human ovarian cancer cells after treatment of N-acetylcysteine (NAC): (A) OV90 and (B) ES-2 cells by proliferation assay.

Cell proliferation was not significantly changed in response to NAC (1 to 10 mM) in both OV90 and ES-2 cells whereas it was dramatically decreased by 20 mM NAC.

Then, we analyzed the production of ROS in response to NAC with paclitaxel or cisplatin in human ovarian cancer cells. In OV90 cells, paclitaxel reduced ROS production as compared to non-treated cells, and additional treatment of NAC (1, 2 or 5 mM) with paclitaxel

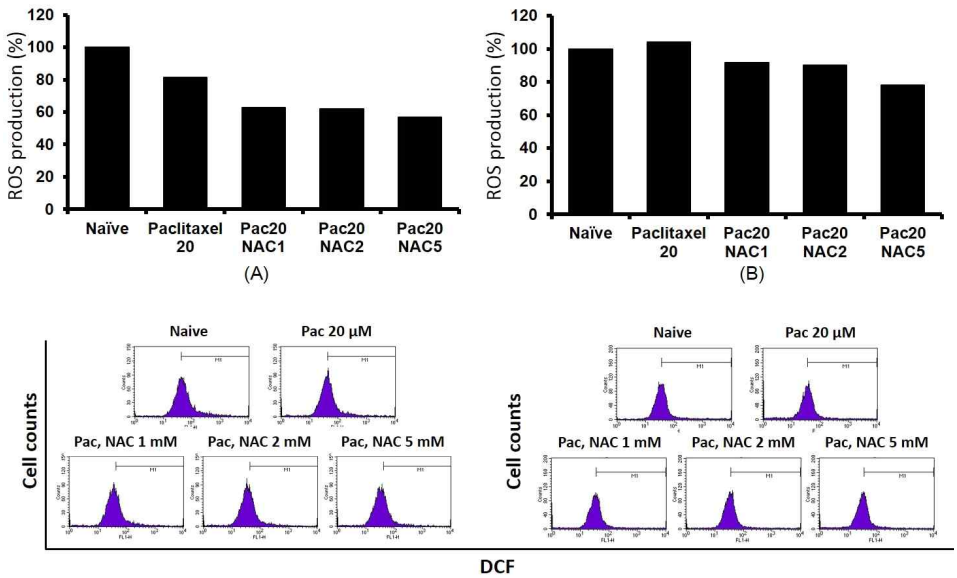
showed a decrease of ROS production as compared to paclitaxel only treatment (Supplementary figure 7A). On the other hand, ROS production was slightly increased by paclitaxel, and elevated ROS levels were decreased by additional treatment of NAC in a dose-dependent manner (1, 2 and 5 mM) with paclitaxel in ES-2 cells (Supplementary figure 7B).



Supplementary figure 6. Production of reactive oxygen species (ROS) in human ovarian cancer cells after treatment of N-acetylcysteine (NAC): (A) OV90 and (B) ES-2 cells by flow cytometry.

ROS production was gradually decreased in a dose-dependent manner (0, 1, 2, 5 and 10 mM) of NAC in OV90 and ES-2 cells.

Moreover, a combination of NAC with cisplatin did not reduce ROS generation in OV90 cells (Supplementary figure 8A), whereas it reduced faintly ROS production in ES-2 cells (Supplementary figure 8B). These results show that NAC reduces ROS production in both OV90 and ES-2 cells and a combination of NAC with cisplatin or paclitaxel affect ROS generation in OV90 and ES-2 cells.



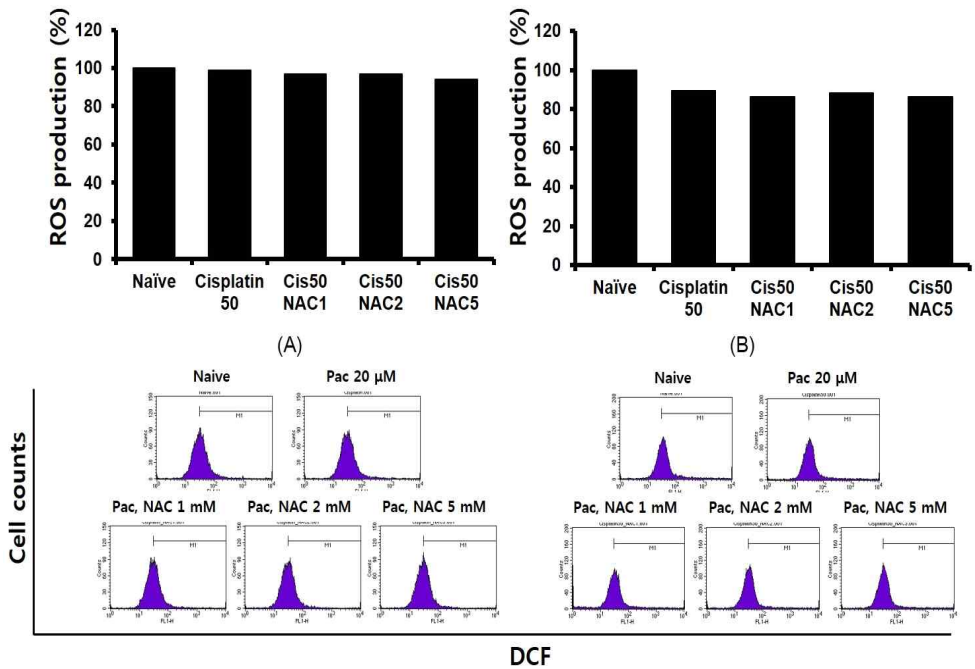
Supplementary figure 7. Production of reactive oxygen species (ROS) in human ovarian cancer cells after treatment of paclitaxel and N-acetylcysteine (NAC): (A) OV90 and (B) ES-2 cells by flow cytometry.

In OV90 cells, paclitaxel (20 μmol/L) reduced ROS production as compared to non-treated cells, and additional treatment of NAC (1, 2 or 5 mM) with paclitaxel showed a decrease of ROS production as compared to paclitaxel only treatment. On the other hand, ROS production was slightly increased by paclitaxel, and elevated ROS levels were decreased by additional treatment of NAC in a dose-dependent manner (1, 2 and 5 mM) with paclitaxel in ES-2 cells.

Effects of N-acetylcysteine with paclitaxel or cisplatin on expressions of hypoxia-related genes in high-grade serous adenocarcinoma and clear cell carcinoma

To identify regulatory effects of the combined substrate including NAC with paclitaxel or cisplatin, we performed Western blot analyses in OV90 and ES-2 cells. When we treated NAC with paclitaxel, *CBP*

and *FIH* expressions were decreased while *HIP-1a* expression was increased in OV90 cells. In ES-2 cells, *HIF-1a* and *FIH* expressions were reduced (Supplementary figure 9).

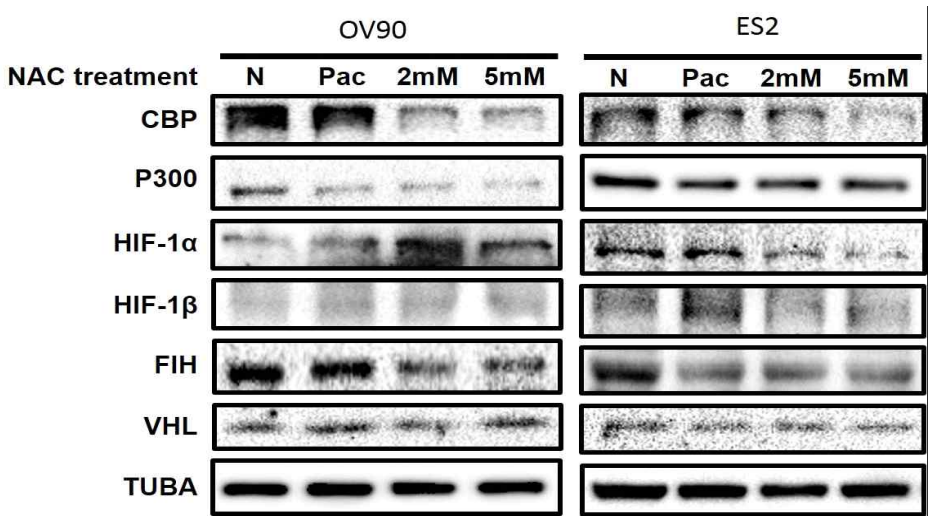


Supplementary figure 8. Production of reactive oxygen species (ROS) in human ovarian cancer cells after treatment of cisplatin and N-acetylcysteine (NAC): (A) OV90 and (B) ES-2 cells by flow cytometry.

In OV90 cells, cisplatin (50 μmol/L) did not reduce ROS production as compared to non-treated cells, and additional treatment of NAC (1, 2 or 5 mM) with cisplatin did not also show a decrease of ROS production as compared to cisplatin only treatment. On the other hand, ROS production was faintly decreased by cisplatin, and elevated ROS levels were also faintly decreased by additional treatment of NAC (1, 2 and 5 mM) with cisplatin in ES-2 cells.

Furthermore, the protein level of *HIF-1β* was increased, whereas that of *VHL* was decreased when the concentration of NAC was

increased with cisplatin in OV90 cells. In ES-2 cells, *HIF-1β* expression was also increased in response to additional treatment of NAC in cisplatin, whereas *VHL* expression was gradually decreased in response to increasing concentration of NAC levels in cisplatin as compared to cisplatin alone (Supplementary figure 10). These results indicate that changes of hypoxia-related protein levels may be involved in drug resistance under anti-oxidant condition and differently expressed between HGSO and CCC.

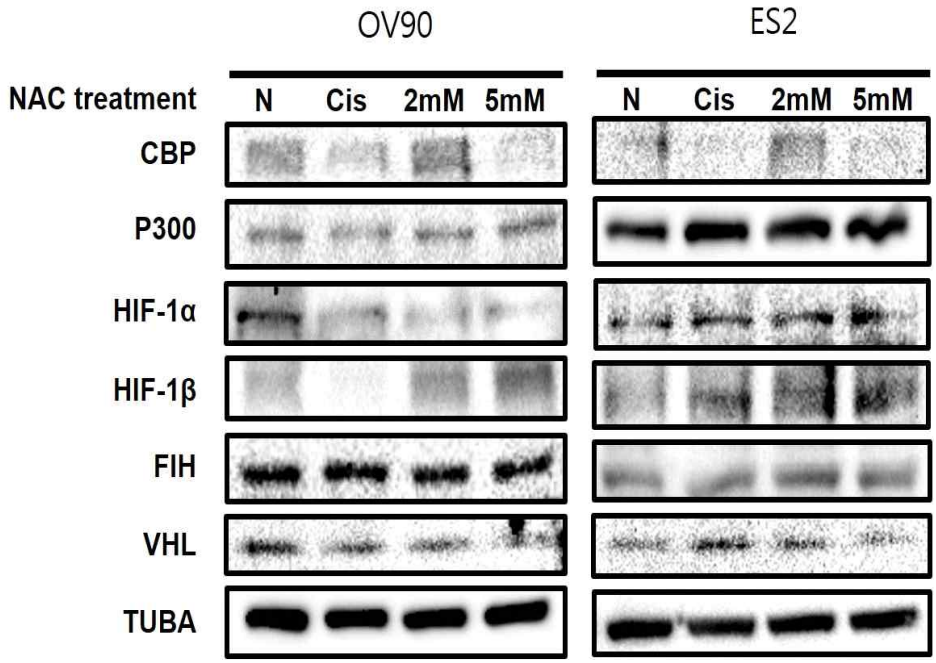


Suppelmentary figure 9. Change of protein levels after treatment of paclitaxel and N-acetylcysteine (NAC) in OV90 and ES-2 cells by Western blot

CBP and *FIH* expressions were decreased while *HIP-1a* expression was increased in OV90 cells, whereas *HIF-1a* and *FIH* expressions were reduced in ES-2 cells.

Roles of hypoxia-related genes as prognostic factors were different between high-grade serous adenocarcinoma and clear cell carcinoma

A total of 210 patients (n=149, HGSO; n=61, CCC) were included in



Supplementary figure 10. Change of protein levels after treatment of cisplatin and N-acetylcysteine (NAC) in OV90 and ES-2 cells by Western blot

HIF-1β expression was increased, whereas *VHL* expression was decreased when the concentration of NAC was increased with cisplatin in OV90 cells. In ES-2 cells, *HIF-1β* expression was also increased in response to additional treatment of NAC in cisplatin, whereas *VHL* expression was gradually decreased in response to increasing concentration of NAC levels in cisplatin as compared to cisplatin alone.

the current study. Supplementary table 1 shows clinic-pathologic characteristics and expression of the six genes. Among the six genes, *CBP* protein was expressed in the nucleus and cytoplasm, and *HIF-1β* protein was shown in the nucleus, whereas *P300, HIF-1a*, *FIH* and *VHL* proteins demonstrated cytoplasmic immunostaining

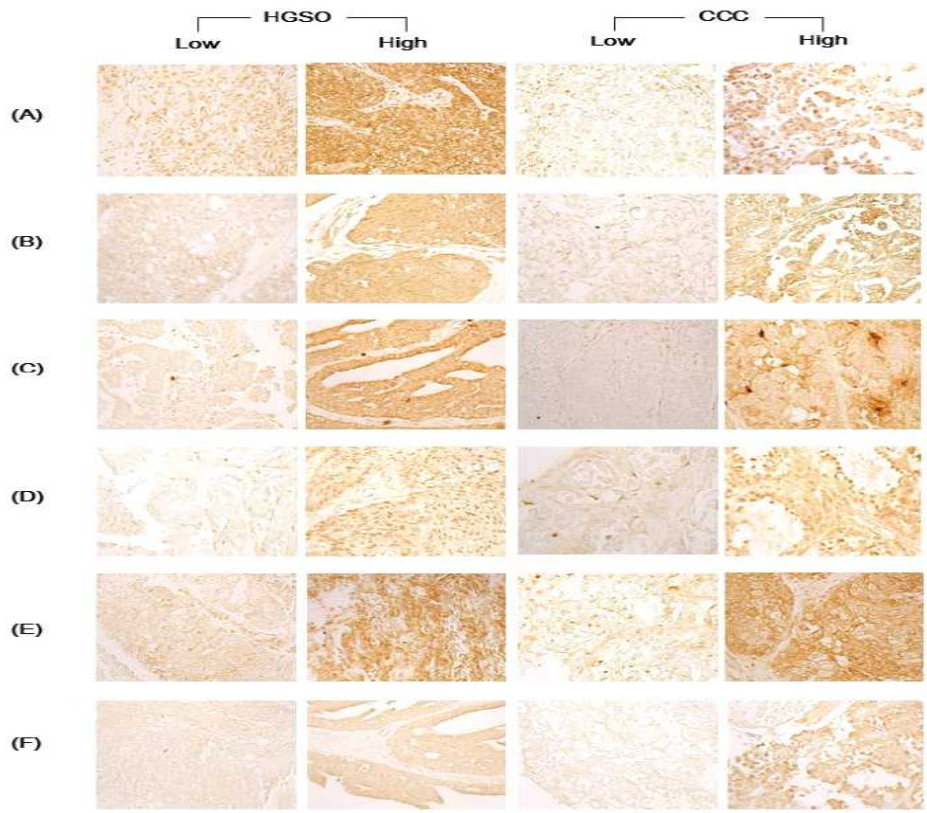
(Supplementary figure 11).

Supplementary table 1. Clinico-pathologic characteristics and expressions of hypoxia-related genes by immunohistochemistry in patients with high-grade serous adenocarcinoma (HGSO) or clear cell carcinoma of the ovary (CCC)

Characteristics or expressions	HGSO (n=149, %)	CCC (n=61, %)
Age (median, range, y)	53 (28-80)	49 (30-77)
FIGO stage		
I	5 (3.4)	36 (59)
II	6 (4)	4 (6.6)
III	112 (75.1)	19 (31.1)
IV	26 (17.5)	2 (3.3)
Extent of cytoreduction		
Optimal	82 (55)	53 (86.9)
Suboptimal	67 (45)	8 (13.1)
Platinum resistance		
No	114 (76.5)	52 (85.2)
Yes	35 (23.5)	9 (14.8)
CBP		
Low	37 (24.8)	13 (21.3)
High	112 (75.2)	48 (78.7)
P300		
Low	41 (27.5)	20 (32.8)
High	108 (72.5)	41 (67.2)
HIF-1α		
Low	61 (40.9)	30 (49.2)
High	88 (59.1)	31 (50.8)
HIF-1β		
Low	88 (59.1)	36 (59)
High	61 (40.9)	25 (41)
FIH		
Low	40 (26.8)	22 (36.1)
High	109 (73.2)	39 (63.9)
VHL		
Low	128 (85.9)	54 (88.5)
High	21 (14.1)	7 (11.5)

Abbreviation: *CBP,* cAMP-response element-binding protein (CREB)-binding protein; FIGO, International Federation of Gynecology and Obstetrics; *FIH*, factor-inhibiting hypoxia inducible factor; *HIF*,

hypoxia inducible factor; *P300,* adenovirus early region 1A (E1A)-binding protein p300; *VHL*, von Hippel-Lindau.



Supplementary figure 11. Immunohistochemistry for the six hypoxia-related genes: (A) *CBP*; (B) *P300*; (C) *HIF-1α*; (D) *HIF-1β*; (E) *FIH*; (F) *VHL*

Among the six genes, *CBP* protein was expressed in the nucleus and cytoplasm, and *HIF-1β* protein was shown in the nucleus, whereas *P300*, *HIF-1α*, *FIH* and *VHL* proteins demonstrated cytoplasmic immunostaining (original magnification ×400).

In terms of platinum resistance, early-stage (FIGO stage I or II) disease, optimal cytoreduction, a low expression of *HIF-1β* and a

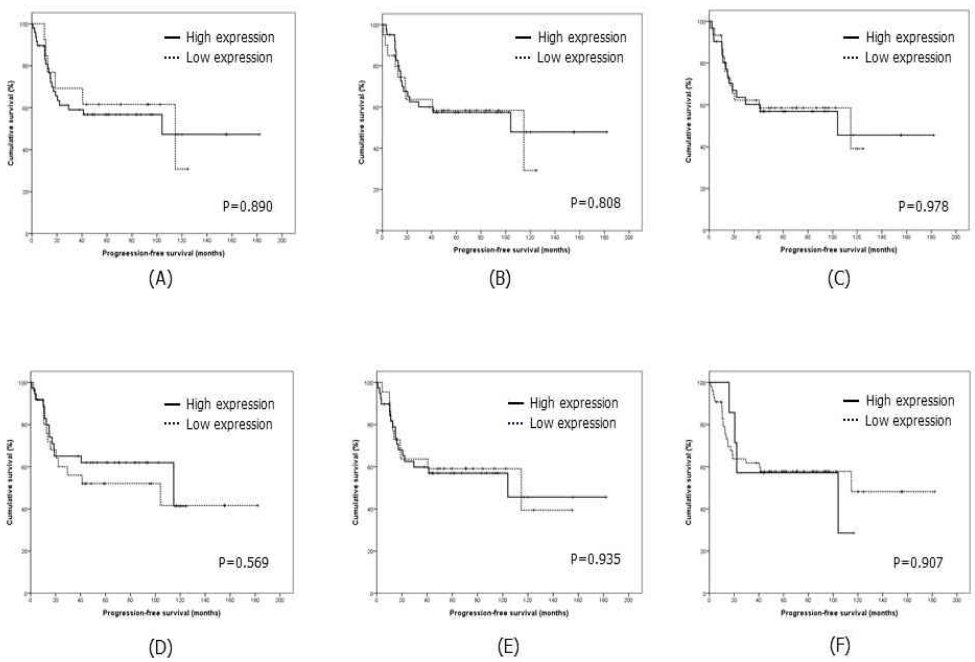
high expression of *VHL* were favorable factors for reducing platinum-resistance in patients with HGSO (Adjusted ORs, 0.273, 0.593, 0.657 and 0.381; 95% CI, 0.085-0.878, 0.397-0.884, 0.443-0.975 and 0.165-0.878), whereas only early-stage disease and optimal cytoreduction were favorable in those with CCC by multivariate logistic analysis (adjusted ORs, 0.088 and 0.210; 95% CIs, 0.030-0.263 and 0.073-0.603; Supplementary table 2).

Supplementary table 2. Univariate and multivariate analyses for platinum-resistance in patients with high-grade serous adenocarcinoma (HGSO) or clear cell carcinoma (CCC) of the ovary

Characteristics	Univariate			Multivariate		
	OR	95% CI	P value	Adjusted OR	95% CI	P value
HGSC						
Age< 54 years	0.837	0.575-1.219	0.353	-	-	-
Early-stage (I or II) disease	0.211	0.067-0.666	0.008	0.273	0.085-0.878	0.029
Optimal cytoreduction	0.476	0.325-0.697	<0.001	0.593	0.397-0.884	0.010
High expression of CBP	0.790	0.507-1.229	0.296	-	-	-
High expression of P300	0.777	0.511-1.182	0.238	-	-	-
Low expression of HIF-1 α	0.775	0.526-1.143	0.198	-	-	-
Low expression of HIF-1 β	0.631	0.428-0.932	0.021	0.657	0.443-0.975	0.037
High expression of FIH	0.678	0.444-1.034	0.071	-	-	-
High expression of VHL	0.309	0.135-0.707	0.005	0.381	0.165-0.878	0.023
CCC						
Age< 50 years	0.525	0.230-1.200	0.127	-	-	-
Early-stage (I or II) disease	0.065	0.025-0.172	<0.001	0.088	0.030-0.263	<0.001
Optimal cytoreduction	0.123	0.051-0.296	<0.001	0.210	0.073-0.603	0.004
High expression of CBP	0.938	0.378-2.326	0.890	-	-	-
High expression of P300	0.905	0.406-2.017	0.808	-	-	-
Low expression of HIF-1 α	0.989	0.465-2.017	0.978	-	-	-
Low expression of HIF-1 β	0.802	0.376-1.714	0.570	-	-	-
High expression of FIH	0.968	0.443-2.115	0.935	-	-	-
High expression of VHL	0.938	0.323-2.722	0.907	-	-	-

Abbreviation: *CBP,* cAMP-response element-binding protein (CREB)-binding protein; CI, confidence interval; *FIH*, factor-inhibiting hypoxia inducible factor; *HIF*, hypoxia inducible factor; OR, odds ratio; *P300*, adenovirus early region 1A (E1A)-binding protein p300; *VHL*, von Hippel-Lindau.

In terms of survival, there was no related genes affecting PFS in those with CCC (Supplementary figure 12). In multivariate analyses, early-stage disease, optimal cytoreduction, a low expression of *HIF-1β* and a high expression of *VHL* were related with improved PFS in patients with HGSO (adjusted HRs, 0.273, 0.593, 0.657 and 0.381; 95% CIs, 0.085-0.878, 0.397-0.884, 0.443-0.975 and 0.165-0.878), whereas only early-stage disease and optimal cytoreduction prolonged PFS without related gene expressions in those with CCC (adjusted HRs, 0.088 and 0.210; 95% CIs, 0.030-0.263 and 0.073-0.603; Supplementary table 3)



Supplementary figure 12. Comparison of progression-free survival based on the six hypoxia-related gene expressions in patients with clear cell carcinoma of the ovary: (A) *CBP*; (B) *P300*; (C) *HIF-1α*; (D) *HIF-1β*; (E) *FIH*; (F) *VHL*

There were no related genes affecting progression-free survival in patients with clear cell carcinoma of the ovary.

Supplementary table 3. Univariate and multivariate analyses for progression-free survival in patients with high-grade serous adenocarcinoma (HGSO) or clear cell carcinoma (CCC).

Characteristics	Univariate			Multivariate		
	HR	95% CI	P value	Adjusted HR	95% CI	P value
HGSC						
Age< 54 years	1.202	0.821-1.760	0.345	-	-	-
Early-stage (I or II) disease	0.211	0.067-0.666	0.008	0.273	0.085-0.878	0.029
Optimal cytoreduction	0.476	0.325-0.697	<0.001	0.593	0.397-0.884	0.010
High expression of CBP	0.790	0.507-1.229	0.296	-	-	-
High expression of P300	0.777	0.511-1.182	0.238	-	-	-
Low expression of HIF-1α	1.290	0.875-1.092	0.198	-	-	-
Low expression of HIF-1β	0.631	0.428-0.932	0.021	0.657	0.443-0.975	0.037
High expression of FIH	1.476	0.967-2.252	0.071	-	-	-
High expression of VHL	0.309	0.153-0.707	0.005	0.381	0.165-0.878	0.023
CCC						
Age< 50 years	0.354	0.134-0.935	0.036	-	-	-
Early-stage (I or II) disease	0.065	0.025-0.172	<0.001	0.088	0.030-0.263	<0.001
Optimal cytoreduction	0.123	0.051-0.296	<0.001	0.210	0.073-0.603	0.004
High expression of CBP	1.066	0.430-2.642	0.890	-	-	-
High expression of P300	0.905	0.406-2.017	0.808	-	-	-
Low expression of HIF-1α	0.989	0.465-2.107	0.978	-	-	-
Low expression of HIF-1β	1.246	0.584-2.661	0.570	-	-	-
High expression of FIH	0.968	0.443-2.115	0.935	-	-	-
High expression of VHL	1.066	0.367-3.091	0.907	-	-	-

Abbreviation: *CBP,* cAMP-response element-binding protein (CREB)-binding protein; CI, confidence interval; *FIH*, factor-inhibiting hypoxia inducible factor; *HIF*, hypoxia inducible factor; HR, hazard ratio; *P300*, adenovirus early region 1A (E1A)-binding protein p300; *VHL*, von Hippel-Lindau.

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