Expression profiles of ID and E2A gene in ovarian cancer and suppression of ovarian cancer by E47

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Directed by Professor Sang Wun Kim

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ABSTRACT

Expression profiles of ID and E2A gene in ovarian cancer and suppression of ovarian cancer by E47

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Basic helix-loop-helix/inhibitor of DNA binding (bHLH/ID) networks have been associated with oncogenesis and shown to be dysregulated in many types of carcinomas. We investigated ID and E2A expression levels and assessed if ovarian cellular homeostasis can be reprogrammed by restoring the ID/E2A axis in ovarian serous cystadenocarcinoma. We analyzed ID1-4 and E2A copy number alterations, mutations, methylation, and mRNA expression status using The Cancer Genome Atlas (TCGA) data of 570 ovarian serous cystadenocarcinoma patients. We determined the effect of E47 induction on ovarian cancer cell growth arrest in vitro and in vivo using SKOV-3/Luc cells introduced with tamoxifen-inducible E47 proteins. We also analyzed ID and E2A expression levels and corresponding survival outcomes in ovarian cancer patients. We found that ID1 and ID4 showed copy number gain in most cases, while E2A showed copy number loss in most cases (88.2%). In total, 554/570 (97.2%) cases showed gain of ID1-4 genes or loss of E2A. Predominantly, ID1-4 were hypomethylated but E2A was hypermethylated. Only ID3 alteration was associated with worse overall survival ($p = 0.006$). E47 induced SKOV-3 cell death in vitro and inhibited tumor growth in SKOV-3 implanted mouse model. The expression level of ID3 and ID4 was

higher and that of E2A was lower in cancerous ovarian tissues than in normal tissues. Patients with high ID3 expression levels showed worse overall survival than those with low ID3 expression levels ($p = 0.042$). We suggest that the restoration of the balance between ID and E2A in ovarian cancer may be a promising approach to treat ovarian cancer.

Key words : basic helix-loop-helix protein, inhibitor of differentiation protein, E protein, transcription factor-3, The Cancer Genome Atlas, ovarian cancer

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I. INTRODUCTION

Basic helix-loop-helix (bHLH) proteins are transcription factors that bind the E-box sequence to induce the expression of specific genes, including those that promote the differentiation of stem cells or progenitor cells into specific cell types. They play a key role in maintaining a differentiated state and inhibiting cell proliferation. The bHLH family members assemble as either homodimer or heterodimer complexes with other bHLH proteins such as E47.[1]

The *E2A* gene, encoding protein E2A or transcription Factor-3 (TCF-3), is the founding member of the E-protein gene family; E2A encodes two distinct bHLH transcription factors E12 and E47 produced by alternative splicing; they are collectively known as E2A proteins. E2A proteins bind the E-box sequence by forming a homodimer or heterodimer and regulate the expression of genes that control cell growth and differentiation in a variety of cell lineages[2] and therefore, play a key role in differentiation and apoptosis. E2A proteins are negatively regulated by the inhibitor of DNA binding (ID) family of proteins (ID1-4 in humans). IDs contain an α-helical HLH dimerization motif but lack a DNA binding domain, resulting in bHLH/ID heterodimers that are dominant-negative inhibitors of bHLH.[3] Therefore, ID proteins control cell differentiation by interfering with the DNA binding activity of E2A proteins

and are the master regulators of cancer stem cells and tumor aggressiveness. ID proteins act primarily by sequestering the ubiquitously expressed E2A proteins. Recent studies have shown that *ID* genes may function as oncogenes and ID, as inhibitors of G1 cell cycle arrest and differentiation.[4,5] Overexpression of ID proteins has been shown in various cancers.[6,7] ID genes have been known to promote cell cycle progression and their overexpression, to induce apoptosis and oncogenesis.[8] In addition, the ID proteins are required for angiogenesis and vascularization of tumors.[9]

Ovarian cancer is a highly lethal gynecological malignancy in women.[10] Optimal cytoreductive surgery combined with platinum-based chemotherapy is the standard treatment for advanced-stage ovarian cancer.[11] However, even after complete remission after primary treatment, approximately 60-80% of patients with advanced-stage ovarian cancer experience relapse.[12] To improve survival outcomes, several new anti-cancer drugs have been developed, including targeted therapeutic agents, antiangiogenic agents, and immunologic agents.[13] ID or E2A proteins as possible targets for novel therapeutic purposes have long been speculated. Inhibition of ID proteins by a peptide aptamer (ID1/3-PA7) induces cell-cycle arrest and apoptosis in ovarian cancer.[14] The level of ID-1 expression correlates with the malignant potential of ovarian cancer, resulting in poor survival outcomes.[15] Overexpression of ID3 was observed in esophageal squamous cell carcinoma[16] and cervical cancer.[17] ID4 gene is amplified in ovarian cancer and that ID4-specific tumor-penetrating nanocomplex suppresses cancer growth and significantly improves the survival of tumour-bearing mice.[18] In breast cancer, high expression levels of ID proteins are associated with cancer proliferation and metastasis in breast cancers.[19,20] In addition, the expression level of E2A has been found to be lower in colorectal cancer tissue than in normal colon mucosa, and in more advanced colon cancer, the lower the expression level of E2A, the poorer is the prognosis. Furthermore, cancer cell proliferation is accelerated when E2A is

suppressed in colon cancer cells and is inhibited when E2A is overexpressed.[21] Expression of E2A was lower in colorectal cancer, low E2A expression was associated with advanced-stage and larger tumor size, and predicted poor prognosis.[22] However, a precise understanding of ovarian cancer with respect to the levels of ID and E2A expression is needed to develop more efficient therapeutic concepts.

High ID protein level in ovarian cancer suggests the dysregulation of bHLH networks and that restoration of the ID expression may promote ovarian cellular homeostasis. Therefore, we hypothesized that dysregulated bHLH activity may be integral to ovarian cancer pathogenesis, and increased E47 expression levels may promote E47 heterodimerization with ID, sequestering ID protein. The restoration of bHLH activity may induce ovarian cancer cell growth arrest or apoptosis.

In this study, we analyzed ovarian cancer samples from the TCGA database to investigate the degree of mutation or expression of *ID1-4* and *E2A* genes and the role of E47 as a growth suppresser in ovarian cancer cells. In addition, we examined ID and E2A expression in tumor tissues of ovarian cancer patients.

II. MATERIALS AND METHODS

1. TCGA data

We analyzed the data of 570 ovarian serous cystadenocarcinoma patients from The Cancer Genome Atlas (TCGA; cBioPortal for Cancer Genomics, UCSC cancer genomics browser) database. The copy number variations, methylation status, and mRNA expression levels of ID1-4 and E2A genes were analyzed at different stages of cancer. We analyzed the association between copy number alterations in the genes and corresponding mRNA expression levels, as well as the difference in mRNA expression levels and the degree of gene methylation.

2. Cell Culture

The human ovarian cancer cell line SKOV-3 was obtained from the American Type Culture Collection (Manassas, VA) and was cultured in RPMI1640 (Gibco) medium containing 10% fetal bovine serum in a humid atmosphere with 5% CO_2 at 37 °C. E47-inducible cells (SKOV-3/E47^{MER}) were generated by infecting SKOV-3 cells with a retroviral vector expressing E47 fused to a tamoxifen-inducible modified estrogen receptor (MER). This vector also contained the *IL2RA* gene encoding CD25; therefore, CD25 expressing cells were selected to isolate stable lines by fluorescence-activated cell sorting (FACS) as previously described.[23,24] E47 activity was induced by incubating cells with 4 μmol/L tamoxifen (Sigma) for 48 h unless otherwise noted.

3. Transfections and FACS

For flow cytometry, SKOV-3 cells were transfected (Lipofectamine 2000; Invitrogen) with the green fluorescent protein (SKOV-3/Luc) or inducible E47 plasmids (SKOV-3/Luc/E47^{MER}) and incubated with tamoxifen for 48-72 h. For live sorting, SKOV3 cells were immunostained with fluorescein isothiocyanate-conjugated mouse antihuman CD25 (1:100, BD Biosciences) as previously described.[25]

4. Microarray analysis

Cells were harvested from the group of cells incubated with tamoxifen for 48 h (SKOV-3/Luc/E47$^{MER)}$ and untreated cells (SKOV-3/Luc). For microarray analysis, RNA was labeled with biotin-16-UTP and hybridized to the HumanHT-12 v4 Expression BeadChip (Illumina, Inc). The BeadChip was then scanned and normalized using a BeadArray Reader. The resulting data were collected by a scanner software and preprocessed by GenomeStudio software (Illumina, Inc). Principal component analysis of differential gene expression was performed using the Partek Genomics Suite software (Partek,

Inc). Hierarchical clustering and other statistical analyses were performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/summary.jsp). The functions of E47 and the genes significantly associated with E47 alterations were predicted by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of differentially expressed genes in the DAVID.

5. Real-Time qPCR Analysis

ID and *E2A* expression levels were analyzed by real-time qPCR in ovarian tissues from patients with ovarian cancer (n = 98) and normal ovarian tissues (n = 20). Total RNA was extracted from ovarian tissue samples using the RNeasy Mini Kit (Qiagen) and reverse transcribed with qScript cDNA Supermix (Quanta). Real-time qPCR was performed using the LightCycler 480 II system with SYBR Green I (Roche), and gene expression level was normalized to 18S rRNA level.

6. Animal Studies

Fourteen Severe Combined Immunodeficiency (SCID)/beige mice were obtained from Orient Bio Co. Ltd (Seoul, Korea). For implantation, SKOV-3/Luc and the SKOV-3/Luc/E47^{MER} cells were treated with tamoxifen (4uM) and cell proliferation changes were observed. Subsequently, 3,000,000 μL and 300 μL of SKOV-3/Luc and SKOV-3/Luc/E47^{MER} cells, respectively, were subcutaneously implanted into SCID/beige mice. Three days after implantation, tamoxifen pellets (25 mg) were implanted subcutaneously at other sites to analyze the effect of E47 on tumorigenesis and the growth of SKOV-3/Luc cells. Tumor size was measured weekly using ultrasound. The Xenogen IVIS Imaging System was used for bioluminescence imaging, and after intraperitoneal injection of luciferin solution (15 mg/mL or 30 mg/kg, in PBS, dose of 150 mg/kg), every 2 min between 5 and 20 min, luminescence

was measured and the maximum value was used for analysis.

7. Immunohistochemistry (IHC)

Formalin fixed paraffin embedded (FFPE) tissues were stained using the Ventana BenchMark XT automated immunostainer BenchMark XT (Ventana Medical Systems, Tucson, AZ, USA). The slides were dried at 60 °C for 1 h and deparaffinized using EZ Prep (Ventana Medical Systems) at 75 °C for 4 min. Cell conditioning was performed using CC1 solution (Ventana Medical Systems) at 100 °C for 64 min. The slides were incubated with ID-1, ID-2, ID-3, ID-4 (rabbit polyclonal antibody, (Abcam, USA), and E2A (rabbit polyclonal antibody, (Santa Cruz Biotechnology) diluted to 1:50 at 37 °C for 32 min. The signals were detected using the OptiView DAB IHC Detection Kit (Ventana Medical Systems). Counterstaining was performed with Hematoxylin I (Ventana Medical Systems) for 4 min at room temperature.

8. IHC scoring

Nuclear and/or cytoplasmic reactivity was assessed using the H-score, which is the sum of the product of the percentage of cells and corresponding staining intensity (0, 1, 2, and 3). The ordinal values for staining intensity are as follows: 0 for no detectable staining, 1+ for weak reactivity mainly detectable at high magnification (20-40X objective), and 2+ or 3+ for more intense (moderate and strong signal, respectively) reactivity easily detectable at low magnification (4X objective). Positivity was defined as an H-score of 100 or more, the extent of 75% or more, or the presence of 2+ or 3+ intensity.

9. Statistical Analyses

Data are expressed as mean ± standard deviation (SD) or as mean ± standard error of the mean (SEM). Groups were compared using Student's t-test. Pearson coefficient was used when indicated. Progression-free survival (PFS)

was the time from the date of diagnosis to disease progression, and overall survival (OS) was the time from diagnosis until death due to any cause. PFS and OS curves were estimated using the Kaplan–Meier method.

III. RESULTS

1. Expression of *ID* gene and *E2A* gene in ovarian cancer

TCGA data analysis for ID and E2A are shown in Figure 1. TCGA pan-cancer (PANCAN) gene expression by RNA seq (Illumina HiSeq) pancan normalized (n = 7083) showed decreased ID1-3 and increased ID4 gene expression levels (Figure 1A). Cross-cancer alteration showed that ID1-4 genes were amplified and the E2A gene was deleted in ovarian cancer (Figure 1B,1C). GISTIC analysis to identify the most significant regions of copy-number alterations in ovarian serous cystadenocarcinoma (n = 570) revealed copy number gain in ID-1 in 58.1% samples and loss in 3.5% samples; in ID-2, gain in 38.1% and loss in 18.9% samples; in ID-3, gain in 22.8% and loss in 43.3% samples; and in ID-4, gain in 50.7% and loss in 14.4% samples. E2A gene showed copy number loss in 88.2% but gain only in 3.5% samples. The ratio of gain in ID-1-4 genes to loss in the E2A gene was 97.2% (554/570), and most ovarian cancers showed ID gain or E2A loss (Figure 1D, Table 1). Analysis of the relationship between the copy number alterations in the ID gene and mRNA expression revealed that ID-1 mRNA expression level decreased in the case of ID-1 gene loss. However, in the case of ID-1 gain or amplification, ID-1 mRNA expression level was slightly higher than that in the case of ID-1 present in the diploid condition. In most cases, ID-2 mRNA expression level increased slightly while ID-3 mRNA expression level remained constant. However, ID-4 mRNA expression level was increased in most cases regardless of the change in copy number (Figure 1E). We also found that the change in the copy number of in ID and E2A was not related to the stage of ovarian cancer. However, the evaluation of ID and E2A mRNA expression level

changes in early ovarian cancer (stage 1) and advanced-stage ovarian cancer (stage 3C, 4) revealed that the E2A mRNA expression level increased in early ovarian cancer.

Table 1. Putative copy-number alterations from GISTIC in Ovarian Serous Cystadenocarcinoma, (TCGA, n=570).

Copy number.*	Gene (No, %)				
	ID1	ID2	ID3	ID4	E2A
-2	0 (0.0)	2 (0.4)	1 (0.2)	0 (0.0)	26 (4.6)
-1	20 (3.5)	106 (18.6)	246 (43.2)	82 (14.4)	477 (83.7)
0	219 (38.4)	245 (43.0)	193 (33.9)	199 (34.9)	47 (8.2)
1	289 (50.7)	195 (34.2)	118 (20.7)	223 (39.1)	18 (3.2)
2	42 (7.4)	22 (3.9)	12 (2.1)	66 (11.6)	2 (0.4)

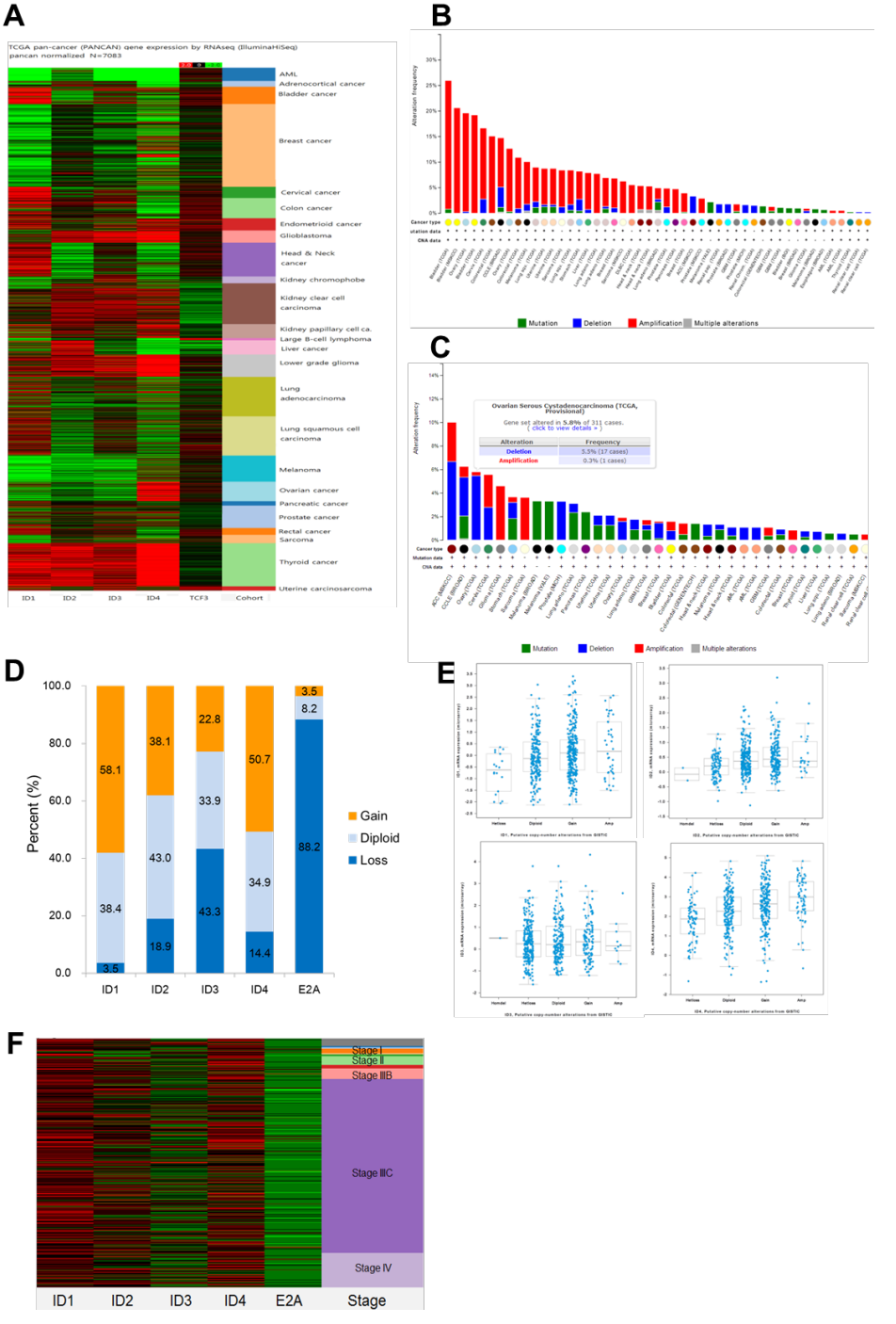

Figure 1. TCGA data analysis of gene alteration for ID and E2A. A: TCGA pan-cancer (PANCAN) gene expression by RNAseq (IlluminaHiSeq) pancan normalized (n = 7083), B: Cross-cancer alteration summary for ID1-4, C: Cross-cancer alteration summary for E2A, D: Putative copy-number alterations from GISTIC in ovarian serous cystadenocarcinoma, (TCGA, n = 570), E: Analysis of the relationship between copy number of ID1-4 gene and mRNA expression in ovarian serous cystadenocarcinoma using cBioportal, F: Copy-number alterations from GISTIC according to FIGO stage in ovarian serous cystadenocarcinoma (TCGA, n = 570).

2. Methylation level of *ID* gene and *E2A* gene in different stages of ovarian cancer

We found that the ID gene promoter was hypomethylated and the E2A gene promoter was hypermethylated in ovarian cancer, regardless of cancer stage (Figure 2A). Analysis of the relationship between ID methylation and mRNA expression revealed that although ID1-4 were hypomethylated, only ID-2 and ID-4 showed increased mRNA expression levels while ID-1 and ID-3 did not (Figure 2B). E2A was hypermethylated in most cases and mRNA expression level also decreased (Figure 2C). Furthermore, E2A mRNA expression level further decreased with increased E2A copy number loss. Besides, the expression level of E2A mRNA in ovarian cancer was low throughout regardless of the mRNA expression level of the ID gene (Figure 2C).

3. ID and E2A alterations and overall survival

OncoPrint analysis of ID1-4 genes revealed that gene alteration in ID1-4 in 28% (161/575) of ovarian cancers (Figure 2D). Most of these alterations included an increased number of copies of a gene and a subsequent increase in mRNA expression level. Further, the evaluation of the association between ID and E2A alteration and overall survival revealed that patients with ID-3 gene

alterations showed poor overall survival (p = 0.005992) (Figure 2E).

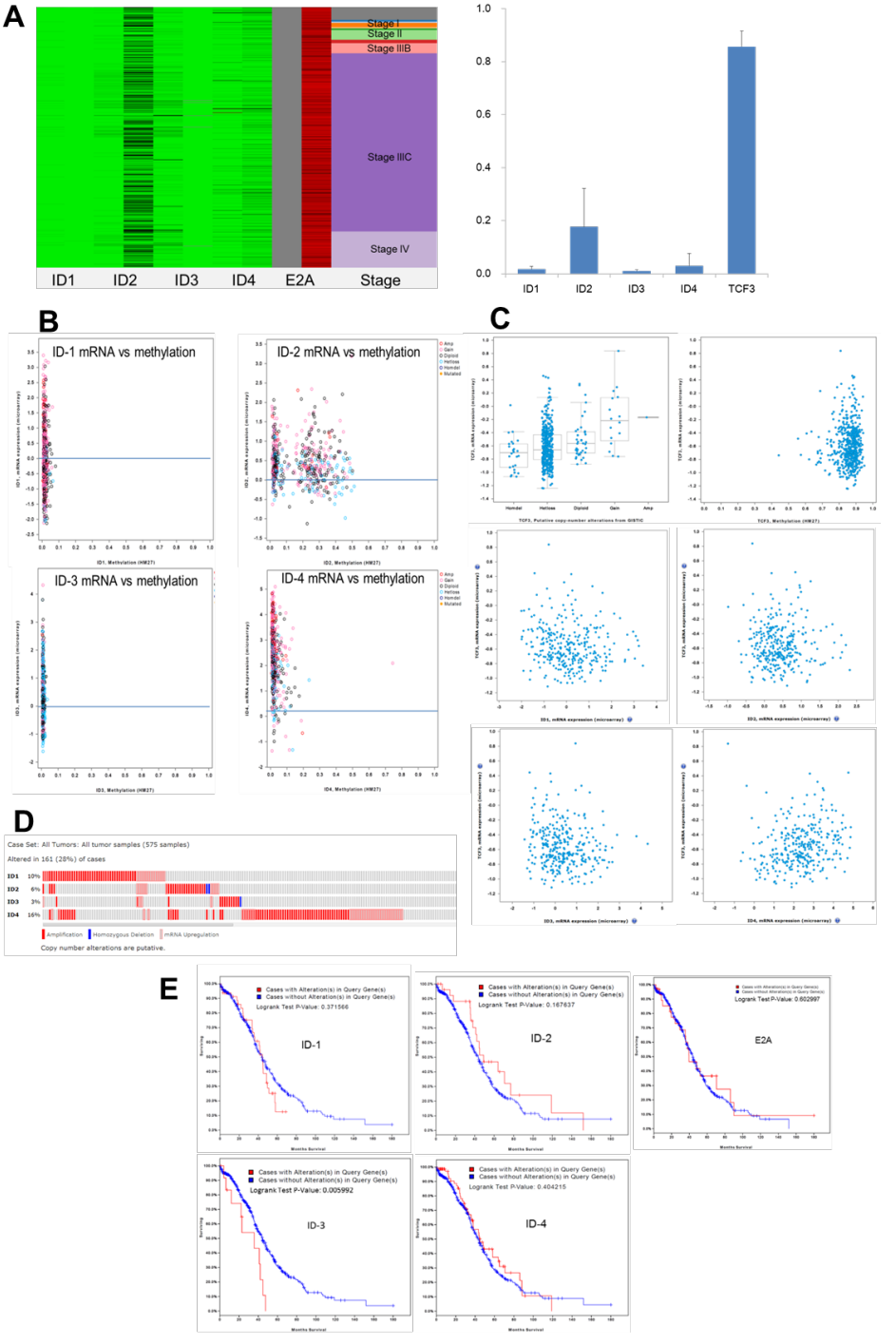

Figure 2. TCGA data analysis of methylation for ID and E2A. A: DNA Methylation (HM27) in Ovarian Serous Cystadenocarcinoma, (TCGA, n=616), B: The relationship between methylation of ID gene and mRNA expression in ovarian serous cystadenocarcinoma using cBioportal, C: Analysis of the relationship between copy number of E2A gene, ID1-4 gene and mRNA expression in ovarian serous cystadenocarcinoma using cBioportal, D: Analysis of ID-1~4, E2A gene alterations by OncoPrint, E: Overall survival in ovarian serous cystadenocarcinoma according to ID and E2A protein.

4. Induction of E47 cause cell growth arrest in ovarian cancer cells

To increase bHLH activity in aggressively growing human ovarian cancer cells, we stably transduced human ovarian cancer cell lines with a tamoxifen-inducible form of E47 fused to a MER, generating the SKOV-3/Luc/E47MER cell line. SKOV-3/Luc/E47MER cell lines express high levels of E47 and show nuclear localization of E47 induced by tamoxifen treatment.

As shown in figure 3A, E47MER induced SKOV-3 cells underwent growth arrest or death when incubated with tamoxifen for 3 days. Tamoxifen treatment of SKOV-3 cells lacking ectopic E47 did not affect cell growth arrest, indicating that cell cycle arrest was induced by E47 activity and not tamoxifen. Previous studies showed that upon the induction of E47 in pancreatic ductal adenocarcinoma cells, Ki67 expression levels were diminished (24, 26). We assessed if this response was universal among cancer cell lines. We found that the induction of E47 activity lead to a rapid loss of Ki67 expression level in ovarian cancer lines (Figure 3B). The number of tamoxifen-induced SKOV-3/Luc/E47MER cells declined, compared with uninduced SKOV-3/Luc cells (Figure 3C). In previous studies of PDA cells, E47 was found to be involved in G0/G1 arrest or cell death (24). Therefore, we assessed whether

E47 arrested ovarian cancer cells in G0/G1, S, or G2/M by assessing DNA content using flow cytometry and found that E47 expression indeed lead to G0/G1 arrest and apoptosis (Figure 3D). E47 was induced with tamoxifen for 2 days, followed by culture for an additional 2 days without tamoxifen. Remarkably, extent of apoptosis was significantly increased in tamoxifen-treated cells than in tamoxifen untreated cells (Figure 3E).

5. E47 induces global changes in the expression levels of genes associated with the cell cycle, cancer, and tissue differentiation

To evaluate the changes in gene expression levels promoted by E47, we performed qRT-PCR in tamoxifen-treated and tamoxifen-untreated SKOV-3 cells (Figure 3F). E47 induced considerable changes in gene expression levels in ovarian cancer cells. The genes most profoundly downregulated by E47 included the ovarian cancer-associated cell cycle activators for cyclin A2 (CCNA2) and aurora kinase A (AURKA), revealing conserved effects of E47 in ovarian cancer cells. The functions of E47 and the genes significantly associated with E47 alteration were predicted by GO and KEGG pathway enrichment analysis of differentially expressed genes in DAVID (httpsavid://d.ncifcrf.gov/summary.jsp). GO enrichment analysis predicted the functional roles of target host genes based on three aspects, namely, biological processes, molecular function, and Functional Annotation Tool. We found that defense response, platelet-derived growth factor binding, extracellular matrix was associated with E47 in ovarian cancer. KEGG analysis revealed 10 pathways related to E47 functions in ovarian cancer including cell cycle, DNA replication, pathways in cancer, p53 signaling pathway, among others (Figure 3H).

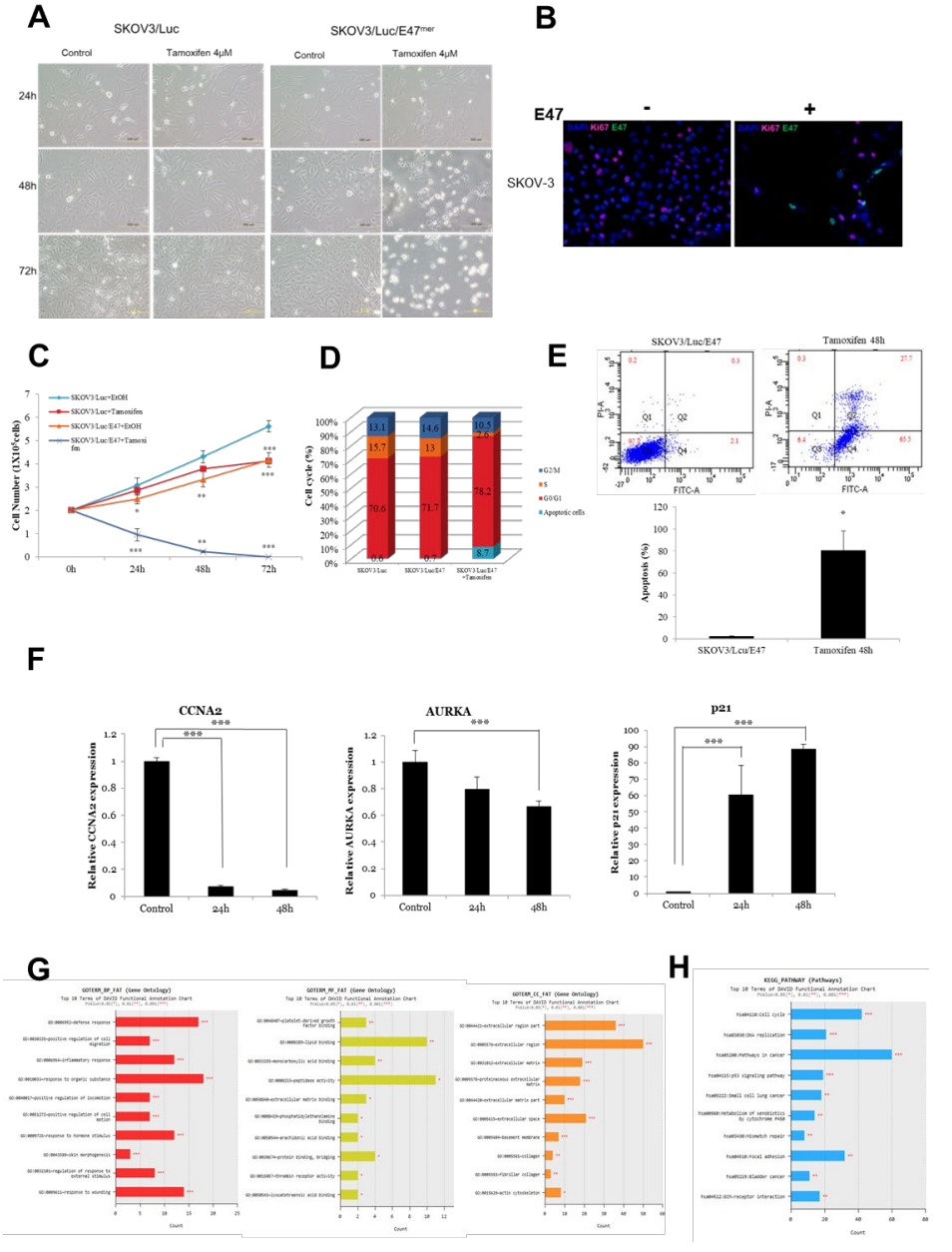

Figure 3. E47 induces cell growth or cell death in SKOV-3 cells. A: E47^{mer} induced SKOV-3 cells growth arrest when cells were incubated with tamoxifen. B. Immunostaining for the replication marker Ki67 (red) and DAPI (blue), x200. C: Growth curves (log scale) for SKOV-3/Luc and SKOV-3/Luc/E47^{mer} cells D:

Percentage of cells in individual cell cycle phases determined by flow cytometry. E: Analysis of apoptosis following tamoxifen treatment using flow cytometry in SKOV-3/Luc/E47^{mer} cells. F: qRT-PCR for CCNA2, AURKA, P21, TOP2A transcripts in all SKOV3/E47 lines. G: Gene Ontology (GO) biological process (BP), molecular function (MF) Functional Annotation Tool (FAT) categories. H: The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway were analyzed using DAVID software.

6. E47 inhibits ovarian cancer formation in vivo

Induced E47 activity has been found to be sufficient to inhibit tumor growth in ovarian cancer cells in vitro; therefore, we hypothesized that altering the bHLH transcription networks might also inhibit tumor growth in ovarian cancer cells in vivo. Therefore, to evaluate the effects of E47 on tumor growth dynamics, control SKOV-3/Luc cells and Skov-3/Luc/E47MER cells were treated with tamoxifen pellet from day 3 to day 45 after implantation into SCID/beige mice. As shown in Figure 4A, during the course of the 77 days, control SKOV-3/Luc cells treated with tamoxifen produced large tumors in mice, whereas tumor sizes in mice implanted with tamoxifen-induced SKOV-3/Luc/E47MER cells were significantly reduced ($P = 0.00012$) (Figure 4A, B).

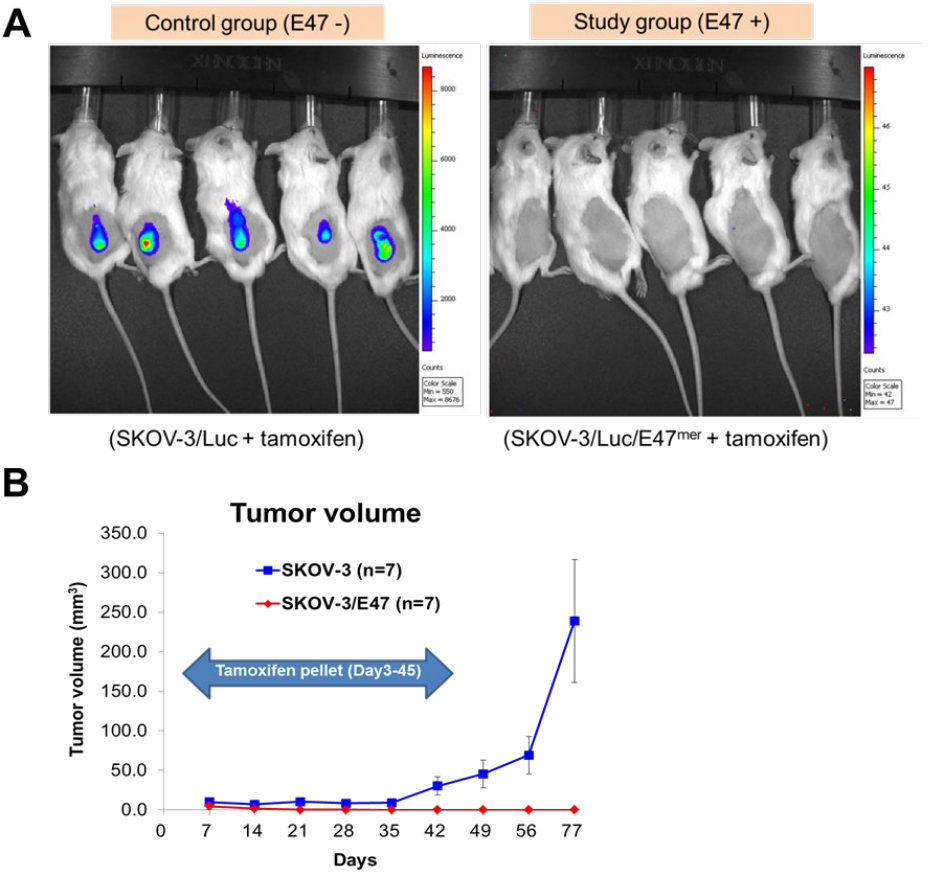

Figure 4. E47 inhibits ovarian cancer cell tumorigenesis in vivo. A: Bioluminescence imaging on Day 77 using Xenogen IVIS Imaging System. B: E47 inhibited tumor growth in SKOV-3 cells transplanted NSG mouse.

7. Clinical validity of ID and E2A in ovarian cancer patients.

To evaluate the clinical validity of ID and E2A, we performed the qRT-PCR and IHC in ovarian cancer patients. qRT-PCR analysis revealed the upregulation of ID and downregulation of E2A in the ovarian tissues of patients with ovarian cancer than in those without (Figure 5A, B).

IHC of cancerous ovarian tissues (n = 23) and normal ovarian tissues (n = 10) showed higher expression level of ID-3, ID-4 and lower expression level of

E2A in cancerous ovarian tissues than in normal ovarian tissues. There was no expression of ID-1 and ID-2 in cancerous ovarian tissue (Figure 5C). The Kaplan–Meier curve for PFS and OS according to ID and E2A expression are shown in figure 5D. Patients with low ID-3 expression levels showed better OS than those with high ID-3 expression levels. The expression levels of ID-1, ID-2, ID-4, and E2A did not show a significant effect on survival outcomes.

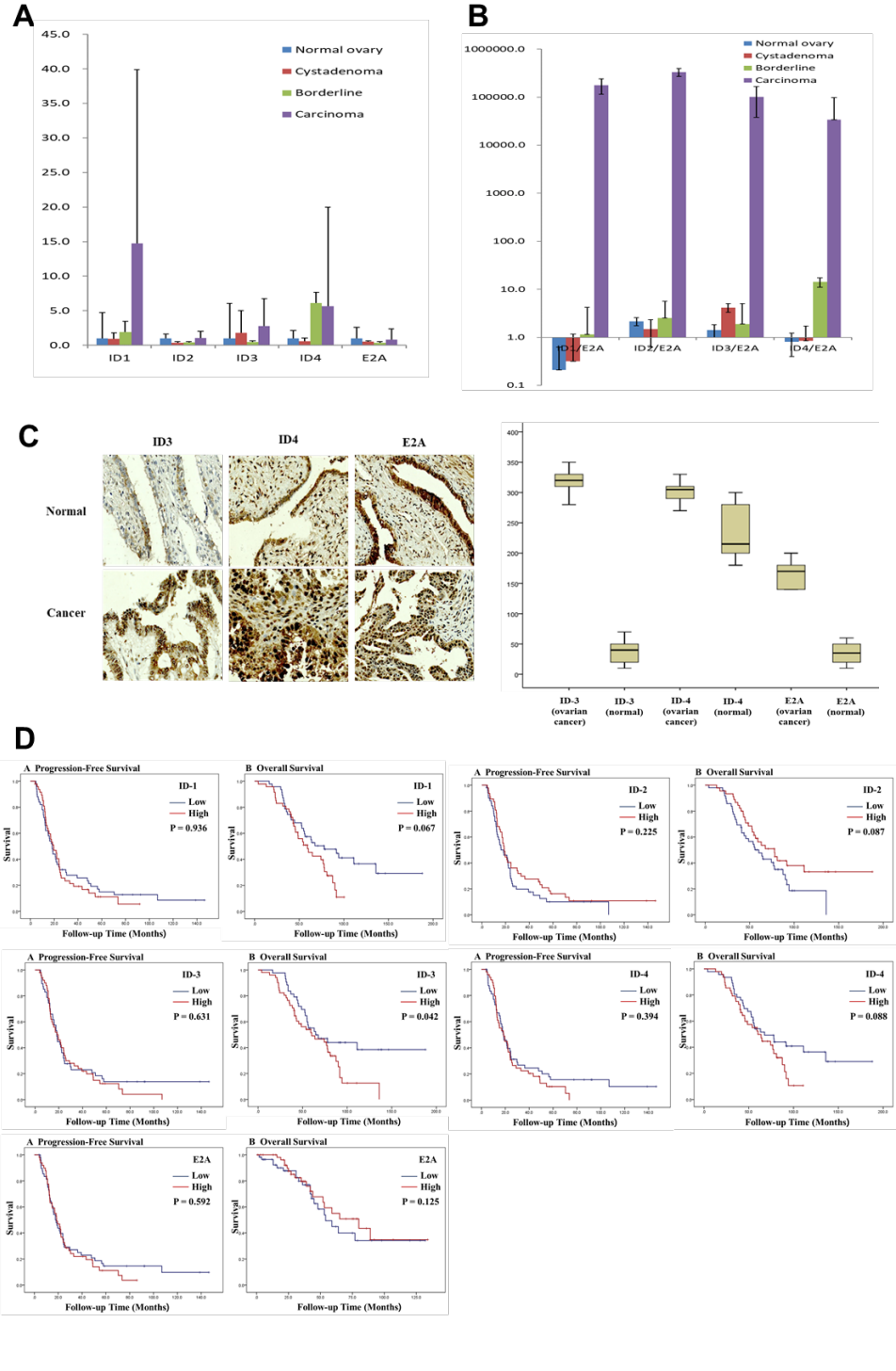

Figure 5. ID, E2A expression and survival outcome in patients with ovarian cancer. A: E47 induces expression of ID1, ID3, ID4 and E2A target genes in ovarian cancer tissue and benign ovarian tissue. B Expression ratio of ID1, ID3, ID4 and E2A target genes in ovarian cancer tissue and benign ovarian tissue. C Protein expression status of ID and E2A and immunohistochemistry score in ovarian cancer. D: Kaplan-Meier curves of progression-free survival and overall survival according to ID-1~4 and E2A gene expression in advanced-stage ovarian cancer patients.

IV. DISCUSSION

In our study, TCGA data analysis revealed copy number gain in *ID-1* and *ID-4* in most cases but *E2A* showed copy number loss in 89.3% in serous ovarian cancer. Besides, *ID-1-4* genes were hypomethylated but the *E2A* gene was hypermethylated. The *ID-4* mRNA expression level increased and *E2A* mRNA expression level decreased in ovarian cancer and the decrease was more prominent in advanced-stage disease. Furthermore, E47 was found to induce SKOV-3 cell death in vitro and inhibited tumor growth in the SKOV-3 mouse model. In addition, ID expression level increased and E2A expression level decreased in tumor tissues of high-grade serous ovarian cancer (HGSOC) patients. Low expression level of ID-3 in patients was consistent with better OS.

Ovarian cancer is the deadliest gynecological cancer in Korea[26]. The standard treatment of ovarian cancer is surgical cytoreduction followed by taxane-platinum combination chemotherapy. However, approximately 60-80% of patients with advanced-stage ovarian cancer experience relapse after primary treatment. To improve survival outcomes, several new anti-cancer drugs have been proposed, including targeted therapeutic agents, antiangiogenic agents, and immunologic agents. However, currently available

targeted therapeutic agents have limited efficacy in ovarian cancer. Therefore, we analyze *ID* and *E2A* as potential candidates for targeted therapy in ovarian cancer patients.

E2A is a ubiquitously expressed transcription regulator and encodes two bHLH transcription factors, E12 and E47[27] that are characterized by a broad expression pattern and the ability to bind DNA.[28,29] E47 plays a critical role in promoting B-cell lymphopoiesis, T-cell development, myogenesis, and cell proliferation.[30-33] In addition, E47 can function as a growth suppresser in adenocarcinoma.[32,33] However, the role of E47 in ovarian cancer is unclear. ID proteins are transcriptional regulators, which play critical roles in normal cell growth and differentiation.[34] The primary function of ID1-4 proteins is to bind and inhibit the activity of bHLH transcription factors.[35] The bHLH proteins activate transcription by forming heterodimers that bind to regulatory enhancer box sequences in target genes. ID proteins lack a basic DNA-binding domain and function as a dominant-negative regulator of bHLH proteins through the formation of ID/bHLH heterodimers. They have roles in a variety of biologic processes regulating tumorigeneses, such as G1-S cell cycle transition,[36] activation of potential proto-oncogenes,[37] and exogenous tumor cell growth and metastasis.[38]

In this study, we showed that ID3 is highly expressed in HGSOC and negatively associated with OS. This can be a result of the following reason. ID3 promotes the Wnt/β-catenin signaling and Wnt/β-catenin pathway activation plays a significant role in HGSOC chemotherapy resistance. Huang et al.[39] showed that ID3 promote the stemness of intrahepatic cholangiocarcinoma and which leads to activation of Wnt/β-catenin signaling. β-catenin signaling is hyperactivated in numerous cancers, and is associated with cancer initiation, progression, therapeutic resistance and recurrence.[40,41] Nagaraj et al.[42] reported that Wnt/β-catenin pathway maintains the stemness of HGSOC and platinum resistance. Wnt/β-catenin signaling is associated with intrinsic and acquired

platinum chemo-resistance. Furthermore, the Wnt/β-catenin pathway is associated with suppressing the anticancer immune response within the tumor microenvironment,[43,44] and enhancing tumor angiogenesis.[45] Because of these factors, we believe that high expression of ID3 may impact survival outcomes in patients with HGSOC.

We hypothesized that dysregulated ID protein activity could be integral to ovarian cancer pathogenesis and that modulating the bHLH/ID balance could be a promising approach to treat ovarian cancer. We found that the imbalance of ID and E2A in ovarian cancer cells and tissues of ovarian cancer patients and the change in bHLH may affect survival outcome. In addition, temporary induction of E47 in vitro and in vivo produced G0/G1 arrest and apoptosis. We showed that the restoration of bHLH expression in ovarian cancer can be achieved by the induction of E47 and this strategy could be used for ovarian cancer treatment.

V. CONCLUSION

In conclusion, ID and E2A play key roles in not only the proliferation homeostasis of cancer cells, but also in the suppression of cancer cells. This study revealed the imbalance between ID and E2A expression in ovarian cancer cell lines and tumor tissues and the correlation between ID3 expression and poor overall survival. Therefore, E47 overexpression may induce ovarian cancer cell growth arrest by restoring the bHLH/ID balance.

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ABSTRACT(IN KOREAN)

난소암에서의 ID와 E2A 유전자의 발현 양상과 E47에 의한 난소암의 억제

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이 용 재

bHLH/ID 네트워크는 종양 발생과 관련이 있으며 많은 유형의 암종에서 조절되지 않는다. 이 연구의 목적은 ID 및 E2A 발현 수준을 조사하고 난소암에서 ID/E2A 축을 복원하여 난소 세포 항상성을 재프로그래밍할 수 있는지 여부를 조사하는 것이다. 본 연구에서는 장액성 난소암에서 ID-1~4 및 E2A 유전자 돌연변이 및 발현 수준을 확인하고자 하였다. 570명의 장액성 난소암 환자의 The Cancer Genome Atlas 데이터를 사용하여 ID-1~4 및 E2A 유전자 변이, 유전자 복제수 변이, 메틸화 및 mRNA 발현을 분석하였다. In vitro 와 in vivo에서 타목시펜 유도성 E47 단백질이 도입된 SKOV-3/Luc 세포를 이용하여 E47 단백질에 의해 난소암 세포들의 성장 억제 및 사멸을 유도할 수 있는지 분석하였다. 실제 난소암 환자의 검체에서 ID 와 E2A 유전자 및 단백질의 발현 분석 및 발현양에 따른 생존율 차이를 비교하였다. The Cancer Genome Atlas 데이터에서 ID-1 와 ID-4는 대부분의 경우 유전자 복제 수 증가 (gain) 를 보였지만, E2A는 89.3%에서 유전사 복제 수 손실 (loss) 이 확인되었다. ID 유전자 복제 수 증가 또는 E2A의 유전자 손실이 있는 경우는 554/570 (97.2%)로 확인되었다. ID 유전자는 저메틸화 양상, E2A는 과메틸화 양상을 나타냈다. ID3 유전자 변이가 있는 경우 전체생존기간이 감소하였다. ($p = 0.006$) E47은 in vitro에서 SKOV-3 세포 사멸을 유도하고 SKOV-3 이종이식 마우스

모델에서 종양 성장을 억제하는 것을 확인하였다. 난소암 환자의 검체에서 ID-3과 ID-4는 정상 난소에 비해 난소암에서 높은 단백질 발현 수준, E2A는 낮은 단백질 발현 수준으로 나타났다. 또한 난소암 환자의 검체에서 ID 유전자는 과발현되어 있었고, E2A 유전자는 발현양이 감소되어 있었다. ID-3 유전자의 발현이 높은 환자군은 ID-3 유전자의 발현이 낮은 환자군보다 전제생존기간이 감소하였다. ($p = 0.042$) 본 연구에서는 ID-1, ID-3, ID-4는 발현이 증가되어 있었고, E2A는 발현이 감소되어 있는 것을 확인하였다. 결론적으로 이번 연구에서는 난소암에서 E47의 과발현을 유도하면 난소암 세포의 성장 억제 및 세포 사멸이 유도됨을 확인하였다. 즉 E2A 발현을 증가시켜서 ID와 E2A의 균형을 회복해 줌으로써 난소암을 치료할 수 있고 이러한 방법은 난소암 치료의 새로운 접근법이 될 수 있음을 확인하였다.

핵심되는 말 : basic helix-loop-helix 단백질, ID 단백질, E 단백질, transcription factor-3, The Cancer Genome Atlas, 난소암