농학석사 학위논문

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2020년 8월

서울대학교 대학원

농생명공학부 바이오모듈레이션 전공

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지도교수 송 용 상
이 논문을 농학석사 학위논문으로 제출함

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2020년 6월

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Abstract

Autophagy contributes to the insensitivity to gefitinib in ovarian cancer cells with wild-type EGFR

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The epidermal growth factor receptor (EGFR) has been a therapeutic target for many epithelial cancers due to its frequent overexpression and hyperactivation. Gefitinib (ZD1839, Iressa), which is a tyrosine kinase inhibitor (TKI), is a small molecule that competes with the binding of ATP to the intracellular tyrosine kinase domain of EGFR. Clinical studies have suggested that treatment with gefitinib monotherapy did not provide survival benefits to patients with wild-type EGFR cancers including ovarian cancer. However, several cases have been reported that non-small-cell lung cancer (NSCLC) patients with wild-type EGFR responded to gefitinib. This study was conducted to elucidate underlying mechanisms contributing to the insensitivity to gefitinib in ovarian cancer cells. Recently, several studies have demonstrated that kinase-independent EGFR has a role in autophagy initiation and this was involved in the resistance to EGFR-targeted therapy. This study investigated the pro-survival role of autophagy induced in response to gefitinib in ovarian cancer cells. To evaluate the role of autophagy in gefitinib-insensitive ovarian cancer cells, ovarian cancer cells (SKOV3) were treated with gefitinib and autophagic flux was examined by expression of autophagy markers and acidic vesicle measurement. Treatment with gefitinib increased conversion to LC3-II and acidic vesicle formation in ovarian cancer cells with wild-type EGFR. To evaluate whether autophagy contributes to the

insensitivity to gefitinib, wortmannin, chloroquine (autophagy inhibitor) and rapamycin (autophagy inducer) were used. Treatment with autophagy inhibitors significantly improved sensitivity to gefitinib in ovarian cancer cells suppressing cell proliferation. Using FDA-approved anti-malarial drug, chloroquine (CQ), we investigated the anti-cancer effect of the combination of gefitinib with CQ and its molecular mechanisms in ovarian cancer cells (SKOV3). We found that combining CQ improved the tumor-suppressive effect of gefitinib by inducing apoptosis. Taken together, our results suggest that induction of autophagy could be critical for the insensitivity to gefitinib in ovarian cancer cells. Our results suggest that inhibition of autophagy can be a novel therapeutic strategy potentiating gefitinib sensitivity of ovarian cancer. Therefore, CQ could be a potential therapeutic agent enhancing the anti-cancer effect of gefitinib in ovarian cancer cells.

Keywords: EGFR-targeted therapy, gefitinib, EGFR wild-type, kinase-independent EGFR, autophagy, chloroquine (CQ), ovarian cancer

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List of Abbreviations

EOC: Epithelial ovarian cancer

EGFR: epidermal growth factor receptor

TKI: Tyrosine kinase inhibitor

AVO: Acidic vesicular organelles

LC3: MAP1LC3B: Microtubule-associated proteins 1A/1B light chain 3B

NBR1: Neighbor of BRCA1 gene 1 protein

CQ: Chloroquine

BAK: Bcl-2 homologous antagonist/killer

BAX: Bcl-2-associated X protein

MOMP: mitochondrial outer membrane permeabilization

PUMA: p53 upregulated modulator of apoptosis

TCGA: The Cancer Genome Atlas

GDC: Genomic Data Commons

CNV: Copy-number variation

CNA: Copy-number alteration

Introduction

Ovarian cancer is the fifth highest cancer death rate among women, and it has the highest mortality rate among any of the cancers in the female reproductive system. According to the American Cancer Society (ACS), 21,750 women are predicted to be diagnosed and 13,940 women will die from ovarian cancer in 2020. The 5-year relative survival rate for ovarian cancer is 48% in all races [1]. Only about 25% of patients with ovarian cancer are diagnosed at an early stage because of unrecognizable symptoms and lack of powerful screening strategies [2]. Since 1980s, standard treatment strategy has been conducted and this includes primary debulking surgery (PDS) followed by chemotherapy for the ovarian cancer at advanced stage disease [3, 4]. Although majority of patients receiving chemotherapy are responsive, 70-85% of patients have a relapse and show the more aggressive disease progression [5]. Therefore, it is an urgent need to exploit strategies for the treatment of advanced and recurrent ovarian cancer.

The epidermal growth factor receptor (EGFR, also known as HER1 and ErbB1) is receptor tyrosine kinases (RTK) which is a member of the ErbB family. Binding of its specific ligands including epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), and epigen (EPG) triggers conformational change to form EGFR dimerization and induces autophosphorylation of several tyrosine residues [6]. As a consequence, EGFR activates its downstream signaling cascades including the phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) pathway, Ras/mitogen-activating protein (MAP) kinase/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway. EGFR is involved in fundamental functions to normal physiology and cancer including cell

proliferation, survival, cell-cycle progression, angiogenesis, and motility [7]. In epithelial ovarian cancer (EOC), some genetic defects in EGFR has been reported that amplification (4-22%) and mutation (<4%) are found on the gene. On the other hand, rate of EGFR overexpression varies with the occasion (9-62%), depending on the experimental conditions and cutoff criteria [8]. Increased EGFR expression has been associated with poor prognosis of patients with ovarian cancer [9, 10], which has led to conducting clinical trials of EGFR-targeted therapy including gefitinib [11-15].

Gefitinib (Iressa, ZD1839) is one of the first-generation EGFR tyrosine kinase inhibitors that reversibly binds to the tyrosine kinase domain of EGFR and inhibits ATP binding suppressing phosphorylation of downstream molecules. However, clinical studies in patients with ovarian cancer could not show a survival advantage for monotherapy using the gefitinib [13] and other agents including erlotinib [11, 12] and cetuximab which is a chimeric monoclonal antibody to target EGFR [15]. Especially, gefitinib and erlotinib are more effective in cancers with activating EGFR mutations (exon 19 deletions and the L858R substitution mutation) than in those with wild-type EGFR in non-small-cell lung cancer (NSCLC) [16]. However, some cases of EGFR wild-type NSCLC responded to these agents in clinical studies [17]. Recently, several studies have been conducted to elucidate mechanisms of insensitivity in NSCLC with wild-type EGFR [18-20]. Thus, identifying mechanisms involved in the insensitivity and developing combination strategies for sensitizing to EGFR-targeted therapy is essential to ovarian cancer patients with overexpression of wild-type EGFR.

There are several studies on the kinase-independent functions of EGFR which have a possibility to contribute to the resistance to EGFR-targeted therapy to patients with

wild-type EGFR through increasing cancer survival [21-25]. One of them is the induction of autophagy [26]. Autophagy (or autophagolysosome) is a conserved process to maintain homeostatic and catabolic balance against cellular stress. Autophagy is crucial for the degradation or recycling of cytoplasmic components, proteins, and organelles [27]. In oncology, recent evidence suggests that dysregulation of autophagy is associated with tumorigenesis, chemoresistance, metastasis, maintenance of stemness in cancer stem cells, and poor prognosis [27-29]. Several studies revealed that EGFR-overexpressed tumors were dependent on autophagy for survival, growth, and drug resistance [30, 31]. Also, several studies found that EGFR TKIs induced autophagic flux and it would play role in increasing the cell viability [32-36]. Xiaojun Tan et al. demonstrated that treatment of gefitinib or erlotinib stimulates the endocytic trafficking of EGFR localized in endosomes and the inactive EGFR facilitates autophagy initiation via Sec5-dependent subcomplex [25]. However, whether autophagy is associated with the insensitivity to EGFR-targeted therapy in ovarian cancer with wild-type EGFR is unclear.

Chloroquine (CQ) and hydroxychloroquine (HCQ) are 4-aminoquinolines which are derivatives of quinoline. These are an FDA-approved drugs for the prevention and treatment of malaria [37]. Recently, CQ and HCQ have been studied for drug repurposing to new indications such as treatment of viral infectious diseases [38, 39] and cancer [40]. In oncology, CQ and HCQ prevent the endosomal acidification which is related to lysosomal degradation. Several clinical studies have shown radio- and chemo-sensitizing effect of CQ and HCQ in glioblastoma, breast cancer, prostate cancer, myeloma and pancreatic cancer increasing possibility of drug-repurposing [40].

In the present study, we investigated the mechanism of insensitivity to gefitinib

involved in EGFR kinase-independent function in ovarian cancer cells with wild-type EGFR. We found that treatment of gefitinib induced cytoprotective autophagy. Then, we examined whether repurposed drug, CQ could support tumor-suppressive effect of gefitinib in ovarian cancer cells with wild-type EGFR. Our findings would imply the potential new strategy for the treatment of patients with ovarian cancer with wild-type EGFR.

Materials and Methods

1. Cell culture and cell line

Human ovarian cancer cell line, SKOV3 was purchased from American Type Culture Collection (Rockville, MD). SKOV3 cells were grown in RPMI-1640 medium (WELGENE, Seoul, Korea). Medium was supplemented with 10% fetal bovine serum (Gibco), 1 % P/S (100 Units/ml Penicillin and 100 μg/ml Streptomycin, Invitrogen, Carlsbad, CA). The cells were cultivated at 37°C in a humidified incubator with an atmosphere of 5% CO2. For assays, SKOV3 cells were incubated with agents in 1% serum-containing medium.

2. Reagents and antibodies

Gefitinib (S1025) was purchased from Selleckchem (Houston, TX). Chloroquine diphosphate salt (C6628), wortmannin (W1628), rapamycin (R8781) and acridine orange hydrochloride solution (A8097) were purchased from Sigma Aldrich (St Louis, MO). Primary antibodies used for Immunoblotting analysis were as follows: EGFR (1:1000; sc-373746) and caspase-3 (1:000; sc-7148) from SantaCruz Biotechnology, p-EGFR Y1068 (1:1000; #2234), LC3B (1:1000; #3868), NBR1 (1:1000; #9891), cleaved caspase-3 (1:1000; #9661), Bax (1:1000; #2774), Bak (1:1000; #3792) and β-actin (1:5000, #4970) from Cell Signaling Technology, α-tubulin (1:5000; T5168) from Sigma-Aldrich.

3. Detection of acidic vesicular organelles (AVOs)

Cells were seeded in 6-well plate and treated with 2 μM of gefitinib for indicating

time. For quantification of AVOs, cells were washed and stained with a final concentration of 1 μg/ml acridine orange in phenol red free RPMI 1640 (Gibco) for 15 min at 37°C in a humidified incubator with an atmosphere of 5% CO2. Using trypsin/EDTA, the cells were harvested and resuspended with PBS. AVOs were analyzed by BD FACS Canto II flow cytometer (BD Bioscience).

4. Immunoblotting analysis

Cells were lysed with 2X RIPA buffer (50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1 mM EGTA, 280 mM NaCl, 0.2% SDS), 1% Triton X-100, 0.1% sodium deoxycholate, EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), 1 mM PMSF, and 1 mM Na3VO4 for 30 min. The concentration of protein was measured using BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). 10-20 μg of proteins were loaded onto 9-12% SDS-PAGE gels for separation and transferred onto nitrocellulose membrane (GE Healthcare Life Sciences). Membranes were blocked with 5% dry skim milk (w/v) or 5% BSA (w/v) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature and incubated overnight at 4 °C with specific primary antibody. Membranes were then washed with TBS-T and incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) diluted in 5% dry skim milk or 5% BSA for 2 h at room temperature. Signals were visualized with the enhanced chemiluminescence detection kits, WESTSAVE up (AbFrontier, Seoul, Korea) and ECL Select Western Blotting Detection Reagent (GE Healthcare Life Sciences) using ChemiDoc™ Touch Imaging System (BIO-RAD). Signal intensities of bands were quantified by densitometry (BIO-RAD's Image Lab software 5.2).

5. Cell viability assay

Cell viability was measured by MTT assay. Cells were plated onto 96-well plates at a density of 2000-3000 cells per well. The cells were treated with various concentration of gefitinib alone or co-treated with chloroquine diphosphate salt for 72 h. DMSO was used as solvent control. At the end of treatment, cells were incubated with 50 µl of MTT solution (2 mg/ml, dissolved in phosphate-buffered saline) onto 96-well plates for 3 h at 37 ℃ in humidified conditions with 5% CO2. The culture media containing agents and MTT solution were removed. Finally, DMSO was added to solubilize formazan formed for 30 min at room temperature. Optical density values were measured at 540 nm using a spectrophotometer (LabSystem, Helsinki, Finland). To make data easily comparable, the values were normalized to DMSO-treated control using GraphPad Prism 5 software.

6. Apoptosis analysis

Cells were collected by trypsinization, centrifuged and washed in 1X PBS buffer. The apoptotic cells were analyzed using the Annexin V apoptosis assay kit (Annexin V-FITC apoptosis detection kit I, BD Bioscience, San Jose, CA). The cells were resuspended in 1X binding buffer at a concentration of 1X10^6 cells/ml. Then 1X10^5 cells were transferred to a 5 ml culture tube. The 3 µl of Annexin V-FITC (556419, BD Bioscience) and 3 µl of PI (propidium iodide, 51-66211E, BD Bioscience) were added to the cell suspension in the dark and incubated for 15 min at room temperature followed by gently vortexing cells. The apoptotic cells were then analyzed by BD FACS Canto II flow cytometer (BD Bioscience) under a fluorescence microscope using a dual filter set for FITC and PE (Phycoerythrin). The following controls are used to set up compensation and quadrants; Unstained cells, cells stained with Annexin V-FITC (no PI) and cells stained with PI (no Annexin V-

FITC). The apoptosis index was calculated as percent apoptotic cells in the total number of cells counted using a hemocytometer.

7. Statistical analysis

All data were generated using GraphPad Prism 5 software and expressed as mean ± SEM of three independent experiments. The statistical significance of differences was determined using Student's t-test and One-way ANOVA with Bonferroni or Tukey's post hoc test. All statistical analyses were performed using IBM SPSS Statistics 23 software (SPSS Inc., Chicago, IL). For all analyses, differences with p-value < 0.05 were considered statistically significant.

Results

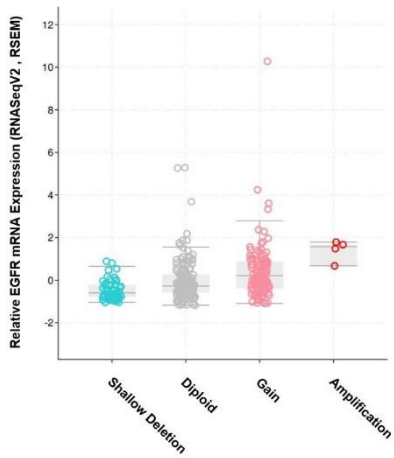
The potency of EGFR as a therapeutic target in ovarian cancer.

To explore the clinical significance of EGFR in ovarian cancer, we obtained copy-number variations (CNVs) data set from NIH GDC data portal (Figure 1A). Amount 20 different cancer types, ovarian cancer shows significantly elevated EGFR copy-numbers. Also, we investigated copy-number alterations (CNAs) data in ovarian cancer from cBioportal (Figure 1B). Tumor samples from 572 patients with ovarian cancer were analyzed. EGFR copy-number alterations were categorized into gene amplifications, gains (low-level amplifications), diploid and shallow deletions (heterozygous loss). This result indicated that copy-number gain of EGFR gene accounted for a large proportion of ovarian cancers. Furthermore, we confirmed the Kaplan-Meier curve for overall survival in patients according to EGFR expression within TCGA (Figure 1C). High EGFR expression was significantly associated with poor patient survival in TCGA of ovarian cancer patients. These data suggest EGFR as a potential target of ovarian cancer. Thus, a strategic approach is required for adopting EGFR-targeted therapy would be necessary in clinical settings.

A

Rank	Cancer type	CNV Gains
1	Glioblastoma	46.31% (276/596)
2	Esophageal Carcinoma	15.22% (28/184)
3	Lung Squamous Cell Carcinoma	12.15% (61/502)
4	**Ovarian Cancer**	**11.97% (70/585)**
5	Head and Neck Squamous Cell Carcinoma	11.90% (62/521)
6	Lung Adenocarcinoma	10.53% (54/513)
7	Bladder Urothelial Carcinoma	9.31% (38/408)
8	Low Grade Glioma	8.05% (40/497)
9	Stomach Adenocarcinoma	7.64% (33/432)
10	Sarcoma	6.54% (17/260)
11	Breast Invasive Carcinoma	4.10% (44/1072)
12	Cervical Squamous Cell Carcinoma and Endocervical Adenocarcinoma	3.74% (11/294)
13	Uterine Corpus Endometrial Carcinoma	3.73% (19/510)
14	Colon Adenocarcinoma	3.35% (15/448)
15	Adrenocortical Carcinoma	3.33% (3/90)
16	Cholangiocarcinoma	2.78% (1/36)
17	Liver Hepatocellular Carcinoma	2.70% (10/371)
18	Testicular Germ Cell Tumors	2.67% (4/150)
19	Kidney Renal Papillary Cell Carcinoma	1.86% (5/269)
20	Rectum Adenocarcinoma	1.83% (3/164)

B



C

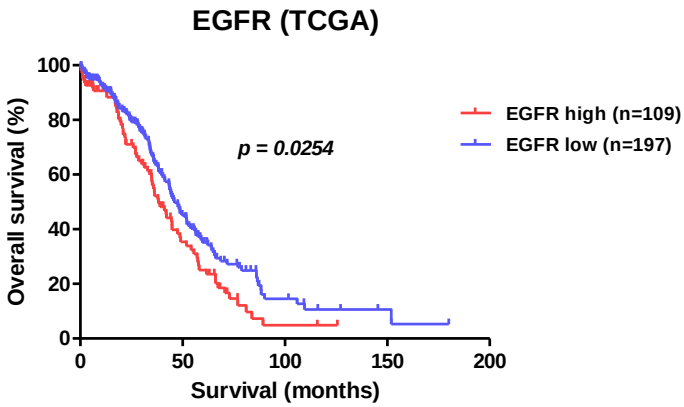


Figure 1. EGFR is closely related to progression of epithelial ovarian cancer.

(A) The distribution of EGFR copy-number variations (CNVs) in various cancer types within TCGA. 1347 cases affected by 966 CNV events across 27 projects. The CNV data set was obtained from NIH GDC data portal (https://portal.gdc.cancer.gov).

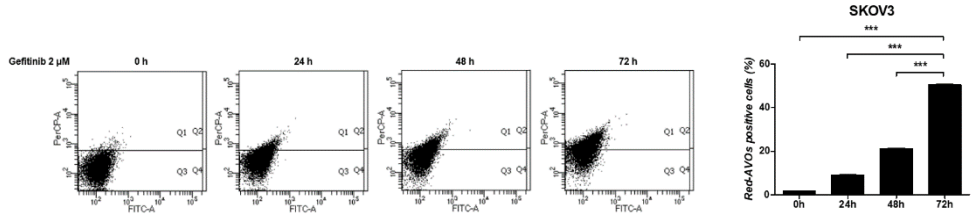
(B) EGFR copy-number alterations (CNAs) in ovarian cancer. The CNA data set was obtained from cBioportal (http://www.cbioportal.org).

(C) Survival analysis of EGFR in TCGA of ovarian cancer patients (mRNA expression of EGFR). Kaplan-Meier curves showing the overall survival analysis in patients with high and low expression of EGFR. (TCGA ovary data set; n = 109 for EGFR high, n = 197 for EGFR low; P = 0.0254 with log-rank analysis) High and low expression groups were separated by mean expression of EGFR.

Gefitinib induces autophagy in ovarian cancer cells with wild-type EGFR.

To examine whether autophagy is stimulated by treatment of gefitinib in ovarian cancer cells with overexpression of wild-type EGFR, cancer cells were exposed to gefitinib for different times (24, 48 and 72 h) and the amount of acidic vesicular organelles (AVO) was measured by FACS analysis through the acridine orange staining (Figure 2A). To confirm whether treatment of gefitinib activates autophagic flux, we assessed the LC3 turnover and NBR1 degradation pattern by immunoblotting analysis (Figure 2B). Gefitinib treatment increased level of AVO in time-dependent manner. Upon treatment with gefitinib, LC3B-II ratio and autophagic cargo receptor, NBR1 degradation were increased indicating the activation of autophagic flux. These results suggest that gefitinib induces autophagy in ovarian cancer cells with wild-type EGFR.

A


Gefitinib 2 μM
0 h
24 h
48 h
72 h
PerCP-A
FITC-A
Q1
Q2
Q3
Q4
SKOV3
***
***
***
Red-AVOs positive cells (%)
0
20
40
60
0h
24h
48h
72h

B

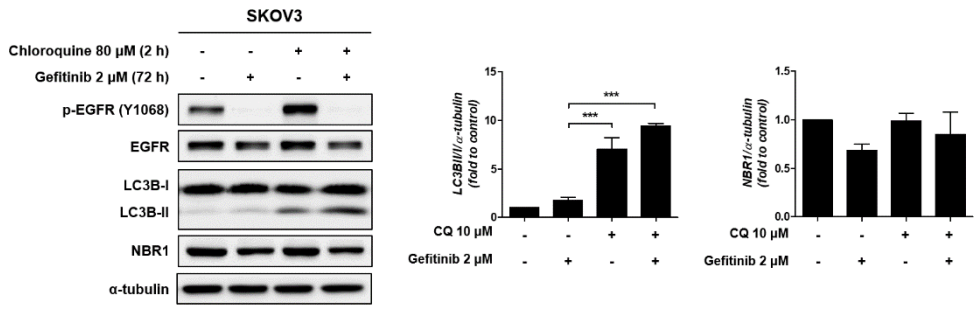

SKOV3
Chloroquine 80 μM (2 h) - - + +
Gefitinib 2 μM (72 h) - + - +
p-EGFR (Y1068)
EGFR
LC3B-I
LC3B-II
NBR1
α-tubulin
LC3BII/α-tubulin (fold to control)
***
***
0
5
10
15
CQ 10 μM - - + +
Gefitinib 2 μM - + - +
NBR1/α-tubulin (fold to control)
0.0
0.5
1.0
1.5
CQ 10 μM - - + +
Gefitinib 2 μM - + - +

Figure 2. Gefitinib stimulates the autophagic flux in ovarian cancer cells with wild-type EGFR.

(A) Quantification of acidic vesicular organelles (AVO). After treatment with gefitinib 2 μM for indicated time, cells were stained with 1 μg/ml acridine orange (AO) for 15 min. AO fluorescent cells were analyzed by flow cytometry using green fluorescence (FITC; 495-519 nm) in nonacidic compartments, and red fluorescence (PerCP; 675 nm) in acidic vacuoles.

(B) SKOV3 cells were treated with 2 μM gefitinib for 72 h. At the end of incubation, 80 μM chloroquine (CQ) was added at 2 h prior to examination of autophagy flux. The expression of LC3B-II/I and NBR1 was determined by western blotting. Signal intensities of bands were quantified by densitometry (Bio-Rad's Image Lab software 5.2) and normalized against the α-tubulin signal. Data are presented as the mean±SEM (n=3). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

Gefitinib-induced autophagy has a role in the cytoprotective response in ovarian cancer cells with wild-type EGFR.

Autophagy can play either a tumor-suppressing role involved in inducing autophagic cell death or a tumor-promoting role in response to stressful conditions. To identify whether gefitinib-induced autophagy contributed to the insensitivity in ovarian cancer cells as cytoprotective functions, cells were treated with gefitinib and autophagy inhibitors (wortmannin and CQ) or inducer (rapamycin) and cell viability was checked by MTT assay (Figure 3A). Combining gefitinib and autophagy inhibitors (wortmannin, or CQ) significantly suppressed the cell proliferation in a dose-dependent manner. On the other hand, pre-treatment of autophagy inducer, rapamycin, significantly increased the insensitivity to gefitinib increasing the cell viability in a dose-dependent manner. These results suggest that autophagy is crucial for the insensitivity to gefitinib in ovarian cancer cells with wild-type EGFR.

A

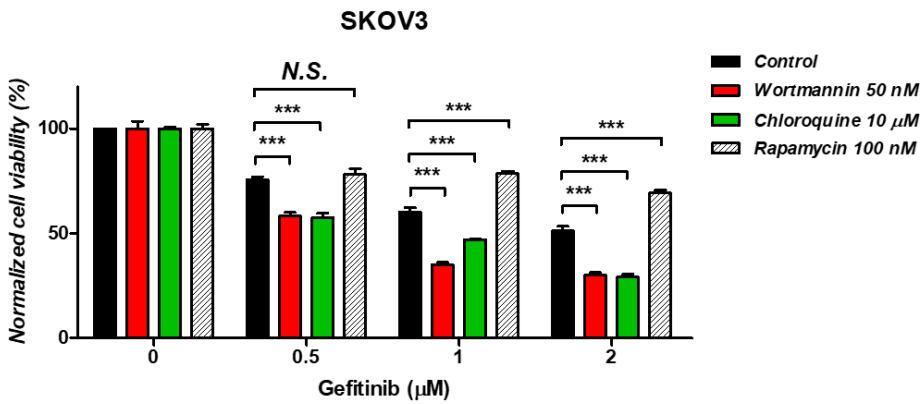

SKOV3
N.S.
***
***
***
***
***
***
***
Normalized cell viability (%)
100
50
0
0
0.5
1
2
Gefitinib (μM)
Control
Wortmannin 50 nM
Chloroquine 10 μM
Rapamycin 100 nM

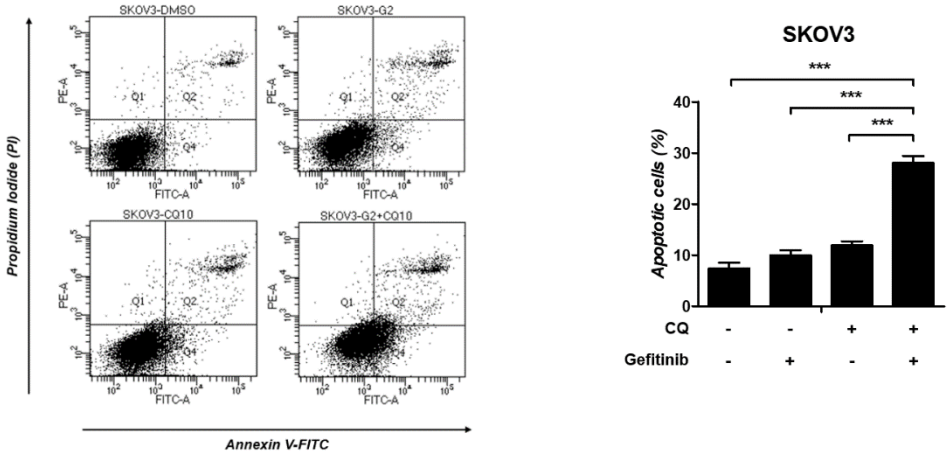
Figure 3. Inhibition of autophagy improves response to gefitinib by suppressing cell proliferation.

(A) SKOV3 cells were treated with 0, 0.5, 1, 2 μM of gefitinib in combination with autophagy inhibitors or inducer. Wortmannin (50 nM) or CQ (10 μM) was co-treated with gefitinib for 72 h. Rapamycin was pre-treated for 2 h. After washing with PBS, cells were treated with gefitinib for 72 h. Then, cell viability assay was performed. To access comparable data, cell viability was normalized to standardize on DMSO-treated control using GraphPad Prism 5 software. Data are presented as the mean±SEM (n=3). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

Disruption of autophagy increases the tumor-suppressive effect of gefitinib by inducing apoptosis in ovarian cancer cells with wild-type EGFR.

Then, we analyzed the cell apoptosis by double staining of Annexin V and PI to determine whether the combined treatment with gefitinib and CQ induced apoptotic cell death (Figure 4A). To analyze the apoptotic cells, flow cytometry was conducted. Co-treatment with gefitinib and CQ enhanced Annexin V and PI positive cell population indicating apoptosis was induced. To identify its mechanisms, we confirmed the protein contents involved in apoptosis by immunoblotting (Figure 4B). We found that combination of gefitinib and CQ significantly increased contents of BAX which acts as a pro-apoptotic molecule damaging the membrane potency of mitochondria. Also, we confirmed that BAK and cleaved caspase-3 were also increased in the co-treatment group compared to the gefitinib-alone treatment. These results indicate that combination of gefitinib and CQ has anti-proliferative effect by inducing apoptosis.

A


SK0V3-DMSO
SK0V3-G2
SK0V3-CQ10
SK0V3-G2+CQ10
Q1
Q2
Q4
FE-A
FITC-A
Propidium Iodide (PI)
Annexin V-FITC
SKOV3
Apoptotic cells (%)
***
CQ
Gefitinib

B

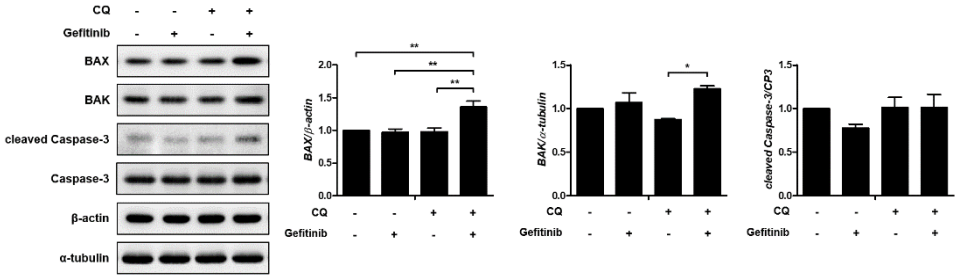

CQ
Gefitinib
BAX
BAK
cleaved Caspase-3
Caspase-3
β-actin
α-tubulin
BAX/β-actin
BAK/α-tubulin
cleaved Caspase-3/CP3
**
*

Figure 4. Combination of CQ and gefitinib increases apoptotic cell death in ovarian cancer cells with wild-type EGFR.

(A) The cell proportion of apoptosis after co-treatment with gefitinib and CQ for 72 h. The apoptosis was assessed through the Annexin V-FITC/PI staining. The early apoptotic cells were counted from the lower right quadrant, and the late apoptotic cells were counted from the upper right quadrant. Data are presented as the mean±SEM (n=3). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

(B) The effect on protein contents of apoptosis markers (BAX, BAK and cleaved Caspase-3/Caspase-3) through the combination of gefitinib and CQ in SKOV3 cells. Signal intensities of bands were quantified by densitometry (Bio-Rad's Image Lab software 5.2) and normalized against the β-actin or α-tubulin signal. Data are presented as the mean±SEM (n=3). *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

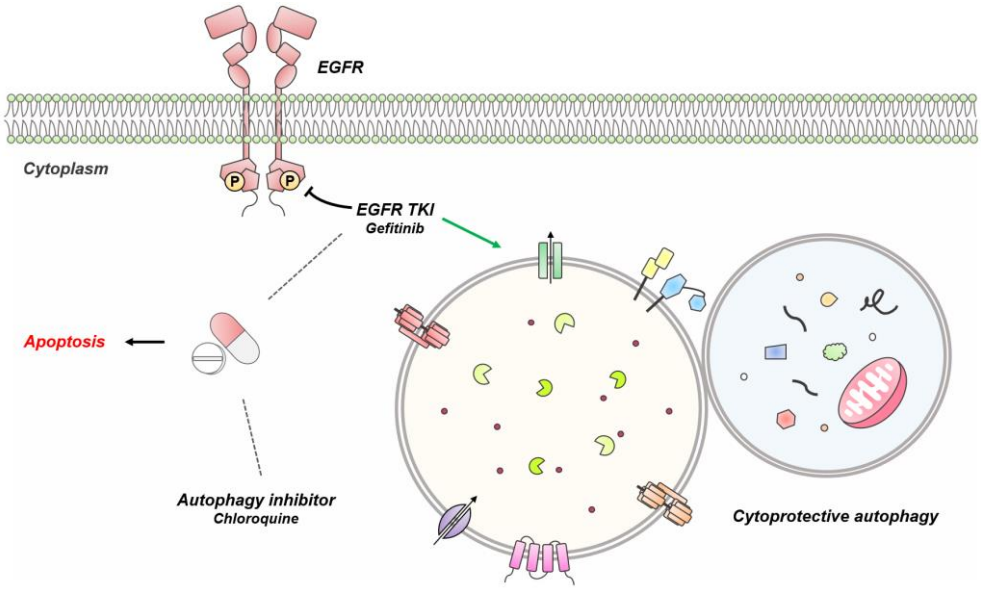

EGFR
Cytoplasm
P
P
EGFR TKI
Gefitinib
Apoptosis
Autophagy inhibitor
Chloroquine
Cytoprotective autophagy

Figure 5. Gefitinib-induced autophagy is associated with the insensitivity of gefitinib in ovarian cancer cells with wild-type EGFR.

Gefitinib induces cytoprotective autophagy, which contributes to the insensitivity in ovarian cancer cells with wild-type EGFR. Inhibitor of lysosomal acidification, CQ could enhance the tumor-suppressive effects of gefitinib in ovarian cancer cells through suppressing cell proliferation and increasing apoptotic cell death. The CQ, FDA-approved drug for the treatment of malaria could be repositioned to sensitizer for gefitinib in ovarian cancer cells with wild-type EGFR.

Discussion

In this study, using both autophagy inhibitors and inducer, we found that inhibition of autophagy can sensitize the gefitinib to ovarian cancer cells with wild-type EGFR through suppressing cell proliferation and increasing apoptotic cell death.

There are several studies involved in clinical significance of EGFR in epithelial ovarian cancer. Brustmann et al. examined the expression of EGFR in ovarian serous carcinoma involved in outcome and clinicopathologic parameters. They found that the expression of EGFR located in the cytoplasm and membrane was 64% in ovarian serous carcinomas and it was associated with high grade ($p=0.0005$) and poor disease-free survival ($p=0.0137$) [41]. Jingying Zheng et al. revealed that the EGFR was more expressed in ovarian cancer than normal ovary ($p=0.003$) and positively correlated with tumor grade ($p=0.004$), FIGO stage ($p=0.005$) and lymph node metastasis ($p=0.015$) [42]. In term of tumor microenvironment which is recognized as a major factor to contribute to the disease progression, Ke Wang et al. investigated the clinical significance and biological function of EGFR expressed in tumor stroma of ovarian cancer. They found that overexpression of EGFR in tumor stroma was correlated with stage ($p=0.008$), metastasis ($p<0.001$) and survival ($p=0.002$). Also, they confirmed that tumor stromal EGFR could predict the patient survival ($p=0.019$) in the multivariate analysis [43]. Using TCGA data, we analyzed copy-number gain, copy-number alterations, and survival rate involved in EGFR expression. Our results support previous findings that EGFR is a significant factor for survival and has a potency to therapeutic targets in ovarian cancer (Figure 1).

Likewise, wild-type EGFR is more commonly overexpressed than its mutants and co-related with poor prognosis in many solid cancers including prostate cancer [44],

pancreatic ductal carcinoma [45], lung cancer [46, 47], hepatocellular carcinoma [48], clear cell renal cell carcinoma [49], head and neck cancer [50], and glioblastoma [51]. However, various cancers including EOC with wild-type EGFR positive have faced the problem of having limited clinical benefits on the current EGFR-targeted therapy. For that reason, EGFR-targeted therapy has been used only three cases; NSCLC with activating EGFR mutations for TKIs [52-54], metastatic colorectal cancers (CRCs) for anti-EGFR mAbs [55, 56], and head and neck cancers (HNCs) for combination of mAbs combined with radiotherapy [57, 58].

Especially the first-generation of EGFR TKIs, gefitinib and erlotinib are greatly effective in cancers harboring specific EGFR mutations (exon 19 deletions and the L858R point mutation), whether these agents are not suitable for patients with wild-type EGFR is controversial for several reasons; first, these TKIs could suppress the growth of cancer cells with wild-type EGFR [59-62]. Even if EGFR TKIs work properly, other RTKs could activate downstream signaling molecules by forming bypass pathway [63]. Several studies have been conducted to identify mechanisms involved in the insensitivity of EGFR-targeted therapy regarding downstream signal cascades in ovarian cancer. PI3K/AKT/mTOR pathway is dysregulated approximately 70% in ovarian cancer due to mutations or amplification of PIK3CA, loss of PTEN [64, 65]. On the basis of genetic alteration, Glaysher et al. found that combining with ZSTK474, a PI3K inhibitor, increased anti-cancer effect of gefitinib or erlotinib in primary cells from ovarian tumor [66]. In addition, Muranen et al. found that combination of gefitinib and BEZ235, a PI3K/mTOR inhibitor, had a synergistic effect increasing cell death remarkably in ovarian cancer cells [67]. In aspects on other downstream pathways of EGFR, Wei Wen et al. revealed that combined blockade of JAK/STAT3 and EGFR was shown to enhance the tumor-suppressive effect in ovarian cancer cells and xenograft mouse model [68]. Benoît

Thibault et al. confirmed that combining with dasatinib, c-Src inhibitor, improved gefitinib response through inhibiting cell growth and enhancing anti-metastatic potency in ovarian cancer cells [69]. Lastly, kinase-independent EGFR has a role in various aspects. The possibility that kinase-independent EGFR are crucial for cell survival has been proposed for years. Coker, K. J. et al. found that D813A, kinase-dead EGFR mutant, is able to stimulate DNA synthesis [21]. Shiaw-Yih Lin et al. found that nuclear EGFR was capable of a transcription factor to promote cell proliferation. They demonstrated that nuclear EGFR bound to the promoter of cyclin D1 in vivo [22]. Zhang Weihua et al. reported that kinase-inactive EGFR was interacted with the sodium/glucose cotransporter 1 (SGLT1) at plasma membrane. Inactive EGFR could stabilize SGLT1 and maintain intracellular glucose level. As consequence, it contributed to increasing cell survival through prevention of autophagic cell death [23]. Indeed, Yasuko Hanabata et al. identified that clinicopathologically, co-expression of SGLT1 and EGFR was inversely associated with tumor differentiation ($p=0.004$) in oral squamous cell carcinoma (OSCC) [24]. In connection with autophagy, Xiaojun Tan et al. found that kinase-independent EGFR was accumulated in the endosome and interacted with LAPTM4B in response to starvation. As a consequence, endosomal inactive EGFR could initiate autophagy [25]. In fact, Rama Krishna Kancha et al. found that 4 of 30 EGFR mutations which are clinically observed in NSCLC did not have a kinase activity showing independence of ligand stimulation [62]. These previous findings suggest that we should consider the EGFR biology to treat cancers with overexpression of wild-type EGFR. However, no evidence involved in the resistance of EGFR-targeted therapy associated with the non-canonical functions of EGFR has been revealed in ovarian cancer with wild-type EGFR.

Thus, we investigated whether kinase-independent EGFR has a role involved in

insensitivity to gefitinib in ovarian cancer cells with wild-type EGFR. It is known that cancers with EGFR overexpression have a dependency on autophagy to promote cell growth and survival [30, 31]. Several studies observed that agents of EGFR-targeted therapy including gefitinib induce autophagy in various cancer cells [25, 32-36]. As underlying mechanism, erlotinib and gefitinib promote autophagy via endosomal inactive EGFR in Sec5-dependent manner. The inactive EGFR binds to Rubicon, which releases Beclin-1 stimulating autophagy initiation [25]. Based on these evidences, to identify whether gefitinib induces autophagy, we detected percentage of acidic vesicular organelles and measured the contents of LC3B-II/I and NBR1 which is autophagic cargo receptor. Our result revealed that gefitinib stimulated autophagic flux in ovarian cancer cells (Figure 2). Then, we investigated whether gefitinib-induced autophagy is responsible for cell survival. We co-treated gefitinib with either autophagy inhibitors (CQ and wortmannin) or inducer (rapamycin) and then, cell viability was measured. We found that autophagy inhibition enhanced the tumor-suppressive effect of gefitinib in ovarian cancer cells with wild-type EGFR. This result suggests that autophagy could be involved in the insensitivity to gefitinib in ovarian cancer cells with wild-type EGFR (Figure 3). Next, we identified whether dual treatment of gefitinib and autophagy inhibitor reduces cell viability by enhancing apoptosis. Our results show that inhibitor of lysosomal acidification, chloroquine could improve the anti-cancer effects of gefitinib in ovarian cancer cells through increasing apoptotic cell death (Figure 4A). Regarding how inhibiting autophagy increases apoptosis, sensitizing to other anti-cancer drugs, Fitzwalter et al. revealed that interruption of autophagy prevents lysosomal degradation of FOXO3a. As consequence, PUMA expression is elevated which causes apoptotic cell death [70]. PUMA, one of the activator BH3s, directly binds to BAK and BAX, which promotes the reorganization of BAK and BAX involved in mitochondrial outer membrane permeabilization (MOMP) [71]. On that

base, we confirmed whether combination of gefitinib and CQ induces the intrinsic apoptosis pathway. We found that contents of BAK, BAX, and cleaved caspase-3 was elevated in co-treatment group (Figure 4B). Through the results, we would speculate on mechanisms that gefitinib-induced autophagy may be related with regulation of FOXO3a degradation in ovarian cancer cells with wild-type EGFR.

In conclusion, we proposed the possibility of chloroquine, FDA-approved drug for treatment of malaria to repurpose to anti-cancer drug combining with gefitinib in ovarian cancer cells with wild-type EGFR. Our results suggest that CQ is a potential therapeutic agent for sensitizing to gefitinib in ovarian cancer cells with wild-type EGFR overexpression. Further studies would be conducted to estimate the effectiveness of combining gefitinib with CQ in ovarian cancer cells as in vivo models or patient-derived tumor organoids for preclinical studies.

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국문 초록

야생형 EGFR을 가진 난소암 세포에서 Gefitinib에 의해 유도된 오토파지와 불감증의 연관성 연구

표피 성장인자 수용체는 (EGFR) 잦은 과발현과 과활성화로 인해 많은 상피암의 치료적 타겟이다. Gefitinib (ZD1839, Iressa)은 EGFR의 세포 내 티로신 키나아제 영역에 ATP와 경쟁적으로 결합하여 티로신 키나아제 기능을 억제하는 약제이다. Gefitinib의 단독 투여는 난소암을 포함한 야생형 EGFR을 가진 암 환자에게 생존 혜택을 제공하지 못했다. 그러나 야생형 EGFR을 가진 비소세포성 폐암 환자 일부에서 Gefitinib에 대한 항암 효과를 보인 임상 사례가 보고된 바 있다. 현재 EGFR 표적 치료와 관련한 연구는 선천적인 내성이나 후천적인 내성을 유발하는 기전을 규명하고, 병용 요법의 효과를 확인하는 것으로 활발히 진행되고 있다. 최근 여러 연구에서 EGFR이 키나아제 기능과 독립적으로, 오토파지를 (autophagy, 자가포식작용) 유도하여 EGFR 표적 치료법에 대한 내성에 관여한다는 것이 입증된 바 있다. 본 연구에서는 Gefitinib에 대한 불감증을 가진 난소암 세포에서 오토파지의 역할을 분석하였다. Gefitinib에 대해 불감증을 보이는 난소암 세포에서 오토파지의 역할을 평가하기 위해 난소암 세포주인 SKOV3에 Gefitinib을 처리하고 오토파지의 유동과 산성 소낭 세포소기관의 양을 측정하였다. Gefitinib 처리 시, 야생형 EGFR을 가진 난소암 세포에서 LC3-II로의 변환과 산성 소낭 세포소기관의 양이 증가하였다. 오토파지가 Gefitinib에 대한 불감증에 기여하는지 검증하기 위해 Wortmannin, Chloroquine (CQ) (오토파지 억제제), Rapamycin (오토파지 유도제)을 사용하여 세포 증식 양상을 확인하였다. 야생형 EGFR을 가진 난소암 세포에서 오토파지 억제제와 병용 처리는 세포 증식을 억제하여 Gefitinib에 대한 민감도를

유의적으로 향상시켰다. 또한 말라리아 치료제인 클로로킨을 (CQ) 사용하여 Gefitinib과 병용 처리 시, 세포 사멸을 유도하여 Gefitinib의 종양 억제 효과가 향상됨을 확인하였다. 결론적으로, 본 연구를 통해 Gefitinib에 의해 유도된 오토파지는 야생형 EGFR을 가진 난소암 세포에서 Gefitinib에 대한 불감증에 관여함을 확인하였다. 이러한 연구 결과는 대부분 야생형 EGFR을 가진 난소암 세포에서 오토파지 억제가 Gefitinib에 대한 반응성을 향상시키는 새로운 치료 전략이 될 수 있고, 말라리아 치료제인 클로로킨은 Gefitinib의 항암 효과를 높이는 잠재적 치료제가 될 수 있음을 제시한다.

주요어: EGFR 표적 치료, Gefitinib, 야생형 EGFR, 키나아제 독립적 EGFR, 오토파지 (autophagy, 자가포식작용), 클로로킨 (Chloroquine, CQ), 난소암

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