Autophagy contributes to the chemoresistance of non-small cell lung cancer

Jin Gu Lee

Department of Medicine

The Graduate School, Yonsei University

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Directed by Professor Kyung Young Chung

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Jin Gu Lee

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This certifies that the Doctoral Dissertation of Jin Gu Lee is approved.

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Thesis Supervisor: Kyung Young Chung

.....

Thesis Committee Member: Young Sam Kim

.....

Thesis Committee Member: In Kyu Park

.....

Thesis Committee Member: Jae-woo Kim

.....

Thesis Committee Member: Chul Hoon Kim

The Graduate School

Yonsei University

December 2012

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Autophagy contributes to the chemoresistance of non-small cell lung cancer

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Lung cancer is the leading cause of cancer-related death in the world. Chemotherapy plays a major role in treating patients with non-small cell lung cancer (NSCLC). However, even the best regimens available today are only capable of providing overall response rate of 30–50%, with the mechanisms involved in chemotherapy resistance remaining unknown. Although recent studies have suggested that autophagy, a catabolic digestion process of cellular organelles, plays a role in chemoresistance in hypoxic conditions, the underlying mechanisms remain elusive. In the present study, the role of hypoxia-induced autophagy in acquiring chemoresistance was evaluated in NSCLC. To

confirm the existence of hypoxia in human NSCLC specimens, we evaluated the expression of hypoxia induced factor-1α (HIF-1α) in both solid tumor and normal lung tissues of patients who underwent therapeutic pulmonary resection for NSCLC. *In vitro* human lung cancer cells (A549) were grown in standard culture media. Cells were exposed to 1% oxygen for 2, 4, 6 and 8 hours and control cells were cultured in normoxia. We analyzed expressions of HIF-1α and autophagy related proteins by Western blot analysis. We also analyzed autophagosomes by transmission electron microscopy (TEM) and GFP-LC3 puncta staining. To confirm chemoresistance, cells were exposed to cisplatin at different concentrations for 8 hours under normoxia or hypoxia, and the percentage of viable cells was determined by MTT colorimetric assay. Finally, we compared the expression of autophagy-related proteins between the tumor tissues after neoadjuvant treatment and the tumor tissues with treatment-naïve, both of which underwent pulmonary resection.

HIF-1α proteins were predominantly expressed in tumor tissue compared with normal tissue, as shown by immunohistochemical stainings and Western blot. *In vitro* hypoxia caused a time-dependent increase in the conversion of LC3B-I to LC3B-II as well as HIF-1α

expression, and a decrease in p62/sequestosome1(SQSTM1) expression in A549 cells as determined by Western blots. Hypoxia also induced GFP-LC3 puncta staining and a marked accumulation of autophagosomes as determined by TEM. Compared with normoxia, cisplatin-induced cell death under hypoxia was significantly decreased. Finally, the tumor tissues after neoadjuvant treatment showed increased LC3B-I to LC3B-II conversion and decreased levels of p62/SQSTM1 compared with the treatment-naïve tumor tissue. The present study suggests that autophagy may play an important role in acquiring chemoresistance in NSCLC induced by hypoxia in solid tumors.

Keywords; NSCLC, drug resistance, hypoxia, autophagy

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I. Introduction

Lung cancer is the leading cause of cancer-related death in the world[1]. In Korea, lung cancer accounts for the fourth highest cancer incidence and the highest cancer-related mortality[2]. Non-small cell lung cancer (NSCLC) constitutes more than 80% of lung cancers. Surgical resection is the only potentially curative therapy for NSCLC, but only 20 to 30% of patients are candidates for curative surgical resection at diagnosis. Even after surgery, 50 to 60% of patients develop local or distant recurrence. Consequently, chemotherapy plays a major role in the treatment of patients who experience recurrence or present with advanced stage cancer at diagnosis. Since its introduction, platinum-based chemotherapy has been widely used for the treatment of NSCLC. Although chemotherapeutic regimens with greater

efficacy continue to be developed, even the best regimens produce an overall response rate of only 30–50%. Indeed, lack of response to chemotherapy in some individuals is due to the development of resistance. Though there have been many efforts to elucidate the mechanism of drug resistance, the mechanisms involved in chemotherapy resistance remain unknown.

Recent studies show that autophagy, a catabolic digestion process of cellular organelles[3], plays an important role in protecting cells against adverse conditions[4-8], including hypoxia[9-12]. Autophagy is an evolutionarily conserved catabolic process for the degradation and recycling of cytosolic, or aggregated proteins, as well as excess or defective organelles. The process of autophagy has been implicated in both cellular survival and cell death[13,14]. Autophagy is a response to the stress of irradiation[15], chemotherapeutic agents[16], serum starvation, or viral infection[17]. During autophagy, cytoplasmic components are engulfed by double membrane structures and delivered to lysosomes/vacuoles for degradation. The formation of double-membrane vesicles is a complex process involving autophagy-related proteins[13]. LC3, a mammalian homolog of yeast Apg8, is one such autophagy-related protein that is associated with autophagosome membranes. Specifically, the cytoplasmic form (LC3-I) is processed and recruited to autophagosomes, where LC3-II is generated by site-specific proteolysis and lipidation near the C-terminus. The mechanisms

required to target cytoplasmic cargo to the autophagosome are now beginning to be characterized. The cellular protein p62/sequestosome1(SQSTM1) can function as an adaptor to target ubiquinated protein aggregates to the autophagosome by binding both ubiquitin and LC3[18]. Following binding of p62 to LC3-II, the autophagosome completes and fuses with lysosomes, resulting in the degradation of both p62 and LC3-II. Thus p62 degradation can be used as another marker of autophagy flux[19].

Autophagy has been considered to have dual functions in cancer, as both a tumor suppressor and a protector of cancer cell survival[20,21]. Before the development of a tumor, autophagy is considered to act as a tumor suppressor; however, after tumor develops, it supports the growth of the tumor. Specifically, autophagy-mediated support of tumor cell survival may play a critical role in cancer progression at later stages, such as dissemination and metastasis. In favor of this hypothesis, starvation-induced autophagy is accompanied by suppression of protein synthesis, cell division and motility in an energy conservation effort that sustains cells in a dormant state with the capacity to resume cell growth and proliferation upon restoration of regular growth condition[7,21-23]. Many studies have documented that, in cancer cells, autophagy is up-regulated in response to metabolic stress caused by chemotherapy or radiation as a cell survival mechanism by, not only

contributing to treatment resistance, but also providing a novel therapeutic target in cancer[16,20,21,24,25]. Cancer cells are often under higher metabolic stress than normal cells, which in turn increases tumor dependence on autophagy for survival, implying a therapeutic window for cancer cell targeting by pharmacologic autophagy modulation.

Hypoxia is considered to play an important role in chemoresistance and is also known to induce autophagy. Hypoxia is present in 90% of solid tumors, because the vascular system cannot supply the growing tumor mass with adequate amounts of oxygen[26-29]. Numerous studies have shown that patients with hypoxic tumors exhibit a worse prognosis than patients with better-oxygenated tumors. This association is partially due to the physical and chemical properties within the hypoxic microenvironment that prevent effective delivery of chemotherapeutic agents and reduce the DNA damaging ability. In addition to the microenvironments, hypoxia influences several cancer related pathways relevant to cancer, including angiogenic signaling, energy metabolism, cell migration, DNA repair, and cell growth. Recent studies have shown relations between multiple hypoxic signaling events and the activation of autophagy. These include transcriptional and translational responses initiated by oxygen-dependent stabilization of the HIF-1 transcription factor, activation of the unfolded protein response (UPR) and

inhibition of the mammalian target of rapamycin (mTOR) kinase signaling pathway[30]. These processes facilitate survival during metabolic stress and may also be an important mechanism for the removal of potentially toxic damaged proteins and organelles[30]. HIF-1 is considered the main regulator of transcription during hypoxia, including a diverse transcriptional response that involves more than 60 genes. Over-expression of HIF-1α is frequently observed in tumors, and numerous studies have demonstrated its importance in mediating tumor development and growth. HIF is thought to contribute to tumor cell survival and growth principally through its ability to regulate both angiogenesis and glycolysis.

However, the role of autophagy in cancer cells under hypoxia is not fully understood. We hypothesized that hypoxia-induced autophagy may contribute to the resistance of NSCLC cells to chemotherapeutic agents by enhancing their survival and decreasing their apoptotic potential. To test this hypothesis, we investigated the effect of hypoxia on cancer cells exposed to chemotherapeutic agents and analyzed the involvement of autophagy. Moreover, in order to gather data supporting our hypothesis, we measured HIF-1α expression in tumor tissue as well as autophagy markers in tumor tissues obtained from NSCLC patients before and after neo-adjuvant treatment.

II. Materials and Methods

1. Cell lines and culture

The human lung cancer cell line, A549, was obtained from the Korean Cell Line Bank (KCLB). A549 cells were maintained as monolayers in RPMI 1640 medium (GIBCOBRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum, 100 U/ml of penicillin (Thermo Scientific, Rockford, IL, USA) and 0.1 mg/ml of streptomycin (Thermo Scientific,) at 37.0^{o}C under 5% CO_2.

2. Reagents

The chemotherapeutic agent, cisplatin (Sigma, St. Louis, MO, USA), was dissolved as a stock solution (1 mM) and added directly into media at the final concentrations indicated.

3. Hypoxia treatment

For induction of hypoxic conditions, cells were cultured in a CO_2 incubator (Forma Scientific, Marietta, OH, USA) maintained at 94% N_2, 5% CO_2 and 1% O_2. Cells (1 x 10^4 cells/well) were seeded in 96-well flat-bottomed plates overnight, then exposed to the chemotherapeutic agent, cisplatin, at the indicated concentrations for 8 hours under normoxia (20% O_2) or hypoxia (1% O_2).

4. MTT colorimetric assay

To measure the effects of hypoxia on chemosensitivity of human adenocarcinoma cells, A549 cells were seeded in 96-well plates at a density of 1 x 10^4 cells/well and cultured in 1% or 20% O_2 in the medium containing various concentrations of cisplatin as indicated for 8 hours. The percentage of cells killed in each well was examined with a 3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Sigma). The spectrophotometric absorbance of the sample was measured using a plate reader at 570 nm. All experiments were carried out in triplicate.

5. Transient transfection and identification of autophagy

A549 cells were seeded (6.25 x 10^5 cells/well) in 6-well plates overnight, and a GFP-LC3 expressing plasmid was transiently transfected into the cells using Lipofectamine LTX Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After 24 hours and after, verifying expression of GFP-LC3, cells were subjected to hypoxia (1% O_2) for 8 hours. At the end of the treatment, autophagy was detected by counting the percentage of cells with GFP-LC3-positive dots under a confocal laser scanning microscope, LSM700 (Carl Zeiss, Jena, Germany).

6. Electron microscopy

After thermal stresses for 12 hours, each sample was fixed with 2% glutaraldehyde - paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 2 hours and washed three times for 30 min in 0.1 M PB. Samples were then postfixed with 1% OsO4 dissolved in 0.1 M PB for 2 hours and dehydrated in an ascending gradual series (50 ~ 100%) of ethanol and infiltrated with propylene oxide. Specimens were embedded using a Poly/Bed 812 kit. After pure fresh resin embedment and polymerization at 60℃ in an electron microscope oven (TD-700, DOSAKA, Kyoto, Japan) for 24 hours 350 ㎚ thick sections were cut and stained with toluidine blue for light microscopy. Next, 70 ㎚ thin sections were double stained with 7% uranyl acetate and lead citrate for contrast staining. The sections were cut by a LEICA Ultracut UCT Ultra-microtome (Leica Microsystems, Wetzlar, Germany). All of the thin sections were observed by transmission electron microscopy (JEM-1011, JEOL, Tokyo, Japan) at an acceleration voltage of 80 ㎸.

7. Western blot

Expression of autophagy-related genes was analyzed by Western blotting of proteins extracted from cells. At the end of the designated treatments, A549 cells were lysed in RIPA lysis buffer (Biosesang, Seongnam, Gyeonggi, KOREA) with 1 mM PMSF. Equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore Corporation, Billerica, MA, USA). After blocking with 5% nonfat milk, membranes were probed with ether anti-HIF-1α (BD Biosciences, San Jose, California, USA), anti-LC3 (Sigma), anti-p62C-terminus/SQSTM1(Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-β-actin antibody (Sigma) and then developed with the ECL (Thermo Scientific, Rockford, IL, USA) reagent.

8. NSCLC patients and samples

To prove over-expression of autophagy related factors after chemotherapy, we selected five NSCLC patients who had not yet been treated and five NSCLC patients who received neoadjuvant treatment following operation as our study group. All patients underwent complete tumor resection and mediastinal lymph node dissection at the Department of Thoracic and Cardiovascular Surgery of Severance Hospital of Yonsei Medical University. Diagnosis and histological type of lung cancer is according to the World

Health Organization as revised. Pathologic stage was evaluated and determined according to the revised tumor-node-metastasis (TNM) classification. The research has been approved by the Institutional Review Board of Yonsei University College of Medicine, and all the patients had signed an informed consent. Tumor specimens and the surrounding normal lung tissue (separated from tumor by 5 cm) were obtained at surgery, or selected by a pathologist, and stored in liquid nitrogen. The specimens were divided into three groups: one group was for protein preparation, the second was for EM and the last one was for immunohistochemisty.

9. Immunohistochemisty for HIF-1α expression

Surgical specimens were immediately transferred from the operating room to the laboratory, fixed in 10% formaldehyde for four days, and then embedded in paraffin. Formalin-fixed and paraffin-embedded tissues were sectioned at a thickness of 4 um and stained with an antibody against HIF-1α (ab2185, 1:50, Abcam, Cambridge, UK) using a Ventana automated immunostainer (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's protocol. Signals were detected using a DAB Map detection kit (Ventana Medical Systems) based on the labeled streptavidin-biotin method. The frequency of nuclear staining was evaluated without any

knowledge of either the clinical or pathologic status data. Five fields (x200) were analyzed to determine the frequency of HIF-1α stained nuclei. At least 500 tumor cells in the five fields were counted. HIF-1α staining was evaluated utilizing a semi-quantitative grading system based on the number of tumor cells showing completely dark stained nuclei within the tumor tissue (0:none, 1:less than 10% positivity, 2:10% or greater positivity). Any cytoplasmic staining which was occasionally observed, was ignored because active HIF-1α is located only in the nucleus[31]. Stained sections were independently graded by one pathologist.

10. Protein extraction and Western blotting of tumor tissues

Tumor tissues and adjacent tissues were homogenized in 1 ml RIPA buffer (50 mM Tris-HCl, pH 7.5, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100) and protease inhibitor cocktail. The homogenate was incubated for 20 minutes on ice and then centrifuged at 14000 rpm for 15 minutes at 4℃. The supernatant was collected and the same volume of 5X SDS buffer was added to the supernatant. The mixture was then boiled for 5 minutes and stored at -80 ^{o}C. Proteins were separated by sodium dodecy1 sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 8-16% polyacrylamide gels. After electrophoresis, proteins were transferred to PVDF

membranes (Millipore Corporation, Billerica, MA, USA), Membranes were blocked in 5% nonfat milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature for 1 hour. The membranes were then incubated with a rabbit monoclonal antibody against LC3 (1:2000 diluted in TBST, Novus, USA) overnight at 4℃ and washed five times with TBST for 10 minutes at room temperature. Membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000 dilution in TBST, Santa Cruz) at 37℃ for 1 hour and were washed five times with TBST for 10 minutes each at room temperature. Membranes were treated with ECL (Thermo Scientific, Rockford, IL,USA) reagent and exposed to photographic film. Each band was quantified using densitometry, and the results were shown as relative expression of each protein from different samples compared to that of control sample.

11. Statistical analysis

MTT assays were analyzed using SoftMax Pro5.4 (Molecular Devices Corporation, Sunnyvale, CA, USA). All the experiments were repeated at least three times. The data were expressed as mean ± SEM. Statistical analysis was performed using Student's t-test or ANOVA (two-tailed). The criterion for statistical significance was $p < 0.05$.

III. Results

1. Tumors of NSCLC patients experiences hypoxia

To confirm the role of hypoxia in solid tumors, as we hypothesized, we first investigated whether hypoxia appeared in tumors of NSCLC patients. Expression of HIF-1α was assessed by both immunohistochemical stains and Western blot and compared with normal lung tissue.

1.1 Immunohistochemical stains

HIF-1α proteins were detected and localized in ten paraffin embedded human lung tissue sections using IHC. The mean tumor size was 58.3±39.38mm. The pattern of HIF-1α expression was predominantly diffuse nuclear staining. Tumors showed higher expression of HIF-1 α compared with normal lung tissue on IHC (1.4 vs. 0.2, $p<0.05$) (Figure 1).

1.2. Western blot analysis

Specimens from six NSCLC patients (neoadjuvant:3, chemo-naïve:3) were analyzed by Western blot analysis. As shown in Figure 2, HIF-1α protein was more highly expressed in tumors compared with normal lung tissue.

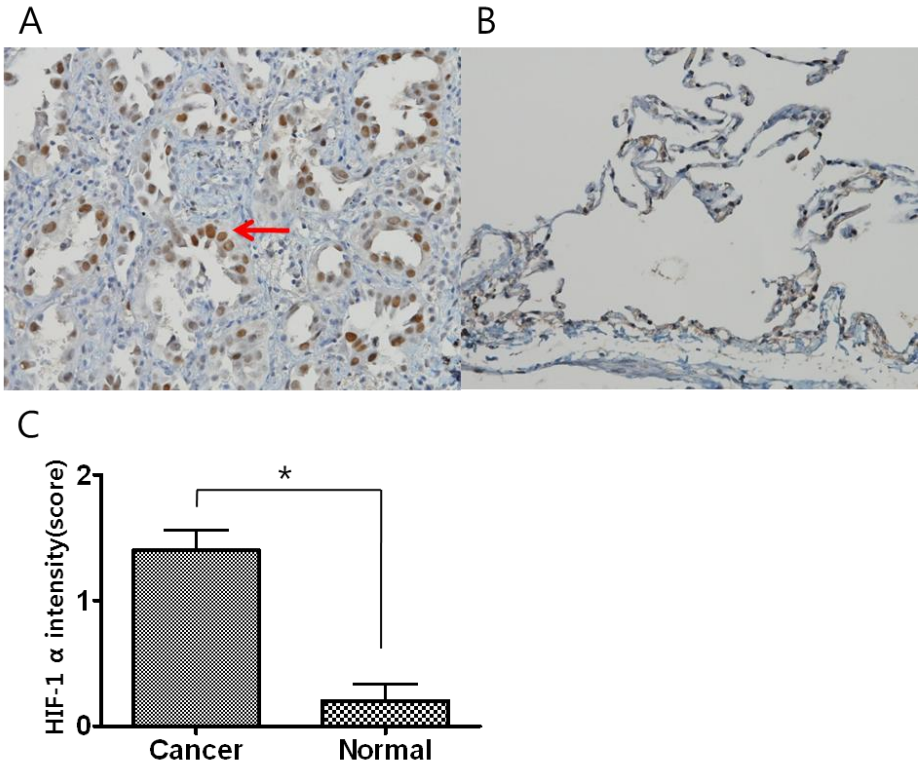

Figure 1. Immunohistochemical analysis of tumor tissues of non-small cell lung cancer (NSCLC) shows higher expression of HIF-1α compared with normal lung tissue. The red arrow shows HIF-1α expression in the nucleus of a tumor cell. A: NSCLC. B: normal lung (magnification x200). C: HIF-1α staining was scored utilizing a semi-quantitative grading system based on tumor cells showing completely darkly stained nuclei (0:none, 1:less than 10% positivity, 2:10% or greater positivity). Tissues from ten patients were used for analysis. The data are mean±SEM, ∗($p<0.05$).

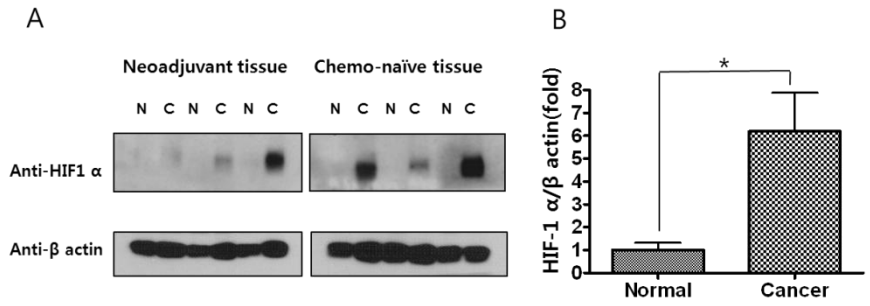

Figure 2. Western blot analysis shows higher expression of HIF-1α in tumor tissues of non-small cell lung cancer (NCSLC). A: Tumor tissues from both patients after treatment and patients with chemo-naïve tumors show higher expression of HIF-1α compared with normal lung tissue. N, Normal; C, Cancer. B: Expression intensity of Western blot was measured using the Image J. Tissues from six patients were used for analysis. The data are mean ±SEM, *($p<0.05$).

2. *In vitro* hypoxia induces expression of HIF-1α in a cancer cell line

To mimic the hypoxia experienced in a solid tumor, A549 cells were exposed to 1% hypoxia for 2, 4, 6 and 8 hours and compared with control cells cultured under normoxia. To confirm the existence of hypoxia, HIF-1α expression was measured after 2, 4, 6 and 8 hours by Western blot analysis.

The hypoxic induction of HIF-1α proteins was dependent on the duration of exposure to hypoxia. HIF-1α protein started to increase rapidly within 2 hours of exposure to 1% oxygen and increased further with a longer exposure to hypoxia (Figure 3).

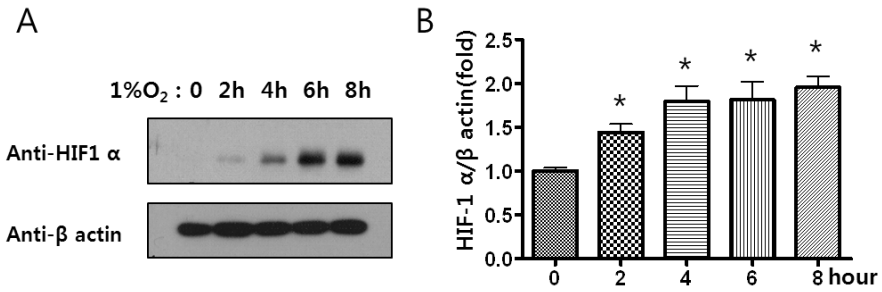

Figure 3. A549 cells exposed to 1% hypoxia exhibit a time-dependent increase in expression of HIF-1α. A: Cells were incubated under 1% hypoxia for 0, 2, 4, 6 and 8 hours. Western blot analysis shows that expression of HIF-1α increased with a longer duration of hypoxia. B: The expression intensity of Western blot data was quantified by Image J. The data are mean±SEM, *($p<0.05$). The graphs are representative of seven independent experiments.

3. Hypoxia induces autophagy in A549 cells

We next analyzed the occurrence of autophagy under hypoxia with an expression vector encoding GFP-LC3 which is concentrated in autophagic vacuoles, resulting in punctate fluorescence within cells undergoing autophagy. A549 cells were transiently transfected with GFP-LC3 plasmids, and 24 hours post transfection, cells were incubated under normoxia or hypoxia. After 6 hours exposure to hypoxia, cells were observed under a confocal microscope and the cells with diffused or punctate GFP were counted. Under normoxia, GFP‑LC3 was diffusely fluorescent whereas GFP‑LC3 became punctate under hypoxia, indicating formation of autophagosomes (Figure 4).

To confirm the involvement of autophagy by additional independent assays, we analyzed the conversion of LC3B-I to LC3B-II protein processing, which is a hallmark of autophagy, using Western blotting. Levels of endogenous LC3B-I to LC3B-II conversion were markedly increased in A549 cells incubated at 1% hypoxia as compared with normoxia. At the same time, we determined the levels of p62/SQSTM1, the degradation of which is used as another marker of autophagy flux. Levels of endogenous p62/SQSTM1 were markedly decreased in A 549 cells incubated at 1% hypoxia as compared with normoxia treated cells (Figure 5).

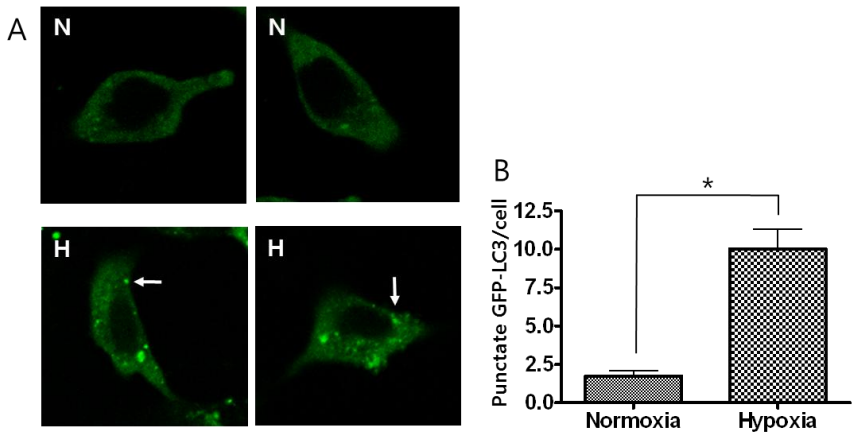

Figure 4. Hypoxia induces autophagy in cancer cells in confocal microscopy. A: LC3-GFP distribution. A549 cells were incubated in normoxia or hypoxia for 6 hours. GFP-LC3 displayed diffuse intracellular localization under normoxic conditions, while membrane translocation (punctate localization) indicative of autophagy was observed in hypoxic cells (magnification x40). N, Normoxia; H, Hypoxia. White arrows indicate punctate GFP-LC3. B: Quantification of GFP‑LC3 translocation in transiently‑transfected normoxic and hypoxic cells was performed as described. The number of punctate GFP-LC3 in each cell was counted and 79 cells were included. Only well preserved spindle-shaped A549 cells were included in the analysis. The data are mean±SEM, *($p<0.05$).

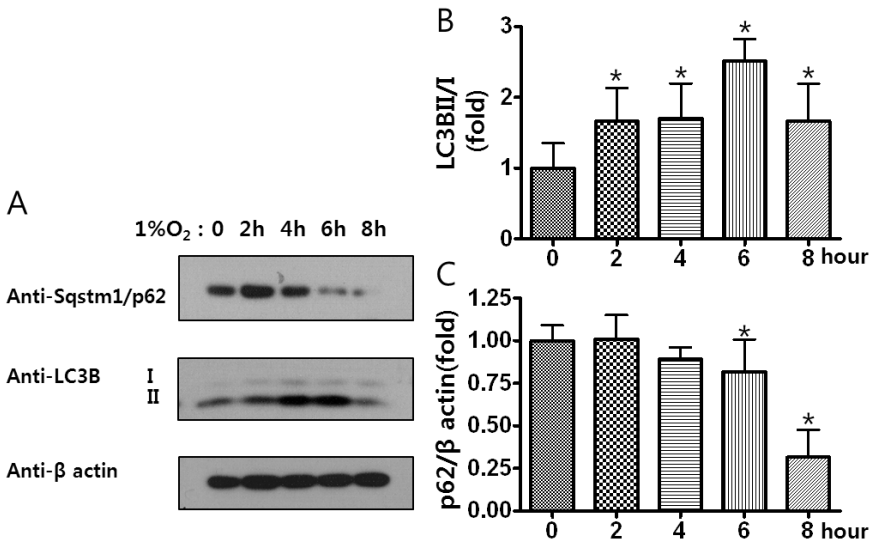

Figure 5. Hypoxia induces autophagy in cancer cells according to Western blotting. A: A549 cells were incubated under normoxia or hypoxia for 2, 4, 6 and 8 hour. Western blot analysis shows increased LC3B-I to LC3B-II conversion in A549 cells incubated in 1% hypoxia as compared with normoxia. At the same time, the expressions of p62, another marker of autophagy flux, decrease in A549 cells incubated at 1% hypoxia as compared with nomoxia. B,C: Expression intensity of Western blotting was measured by Image J. The data are mean±SEM, *($p<0.05$). The graphs are representative of six independent experiments, *($p<0.05$).

As another independent assay of autophagy, cells were processed by transmission electron microscopy after hypoxic treatment. Cells under hypoxia revealed a marked accumulation of autophagosomes, where cytoplasmic material and/or membrane vesicles were encapsulated in vacuoles (Figure 6, black arrows), suggesting that real stimulus of autophagy is hypoxia. Thus, autophagy was activated significantly in response to hypoxia.

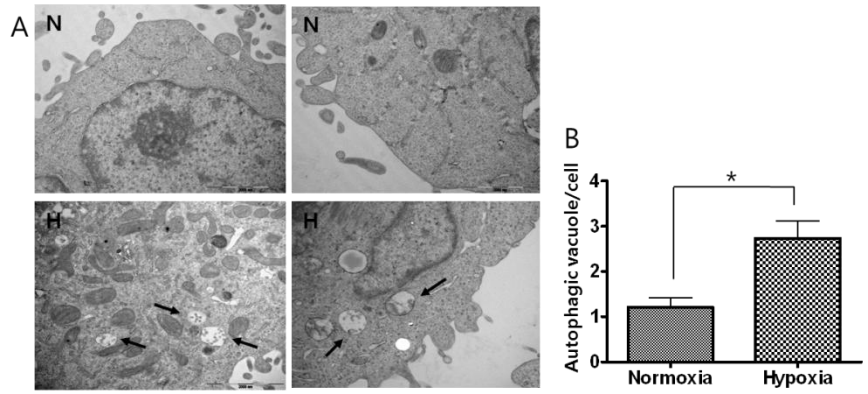

Figure 6. Hypoxia induces autophagy in cancer cells as determined by electron microscopy. A: Electron micrographs showing the ultrastructure of A549 cells in normoxia or hypoxia for 6 hours. Arrows indicate double-membraned autophagic vacuoles (magnification x20,000), N, Normoxia; H, Hypoxia. B: Quantification of autophagic vacuoles in normoxic and hypoxic cells was performed as described. The number of autophagic vacuoles in each cell was counted and 76 cells were included. The data are mean±SEM, *($p<0.05$).

4. Hypoxia enhances chemoresistance of a lung cancer cell line

To investigate the effect of hypoxia on chemoresistance, A549 cells were cultured in complete medium at 20% or 1% O_2 for 6 hours in the presence or absence of the chemotherapeutic agent, cisplatin. Cell death determined by MTT assay, which showed that 32.0% of cells under normoxia were killed by cisplatin (512 μM), compared with 11.8% under hypoxia (Figure 7). These data demonstrated that A549 cells exposed to hypoxia exhibited enhanced resistance to a chemotherapeutic agent. Together, these results suggest that hypoxia protects cells from chemotherapeutics-induced cell death.

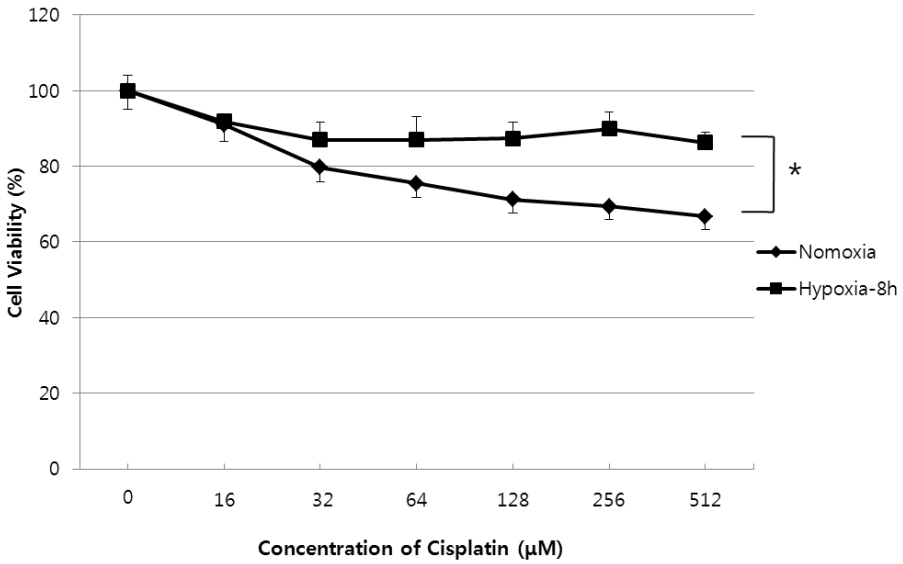

Figure 7. Hypoxia enhances the chemoresistance of cancer cells. A549 cells were treated at the indicated concentration of cisplatin with 20% or 1% O_2 for 8 hours. Cell death was measured by MTT assay. The percentage of cell death is shown as the mean±SEM of at least three independent determinations. Statistically significant differences are marked, *($p<0.05$).

5. Chemotherapy enhanced autophagy induction in patients with NSCLC

To confirm the induction of autophagy in patients with NSCLC after chemotherapy, we analyzed the conversion of LC3B-I to LC3B-II protein and the protein levels of p62/SQSTM1 in both patients with NSCLC who underwent therapeutic pulmonary resection after neoadjuvant chemotherapy and patients with NSCLC (chemotherapy naïve) who underwent only therapeutic pulmonary resection. Patients who received chemotherapy before pulmonary resection showed increased LC3B-I to LC3B-II conversion and decreased level of p62/SQSTM1 compared with patients who were chemotherapy-naïve (Figure 8). These results indicate that autophagy is induced by chemotherapy in the tumor tissue of NSCLC patients.

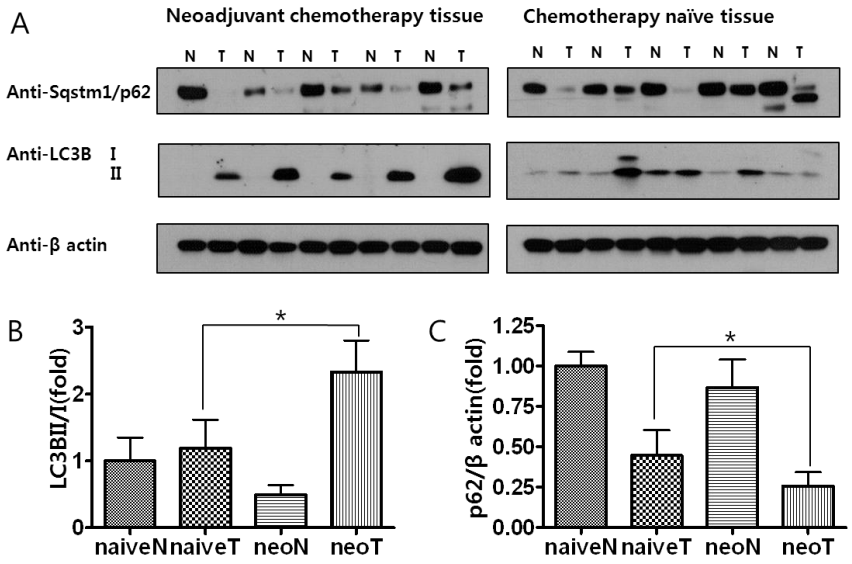

Figure 8 Chemotherapy enhances autophagy in tumor tissues of non-small cell lung cancer (NSCLC). A: Western blot analysis for conversion of LC3B-I to LC3B-II protein and p62/SQSTM1 in tumors of NSCLC. The conversions of LC3B-I to LC3B-II were higher in tumor tissue after treatment than in non-treated tumor tissues. Conversely, p62 expressions of tumor tissue after treatment were lower than in chemotherapy-naïve. B,C: Expression intensity of Western blot was measured by image J program. The data are mean±SEM, *($p<0.05$). N, Normal; T, Tumor; neo, neoadjuvant.

IV. Discussion

In this study, we showed that autophagy induced by hypoxia results in enhanced cell survival upon chemotherapy. At the same time we confirmed the existence of hypoxia and enhanced autophagy in response to chemotherapy in human solid tumor tissue.

In spite of recent advances in the fields of chemotherapeutic agents, cancer cells have shown drug resistance, which can manifest clinically as tumor progression or recurrence. Although there have been many efforts to elucidate the mechanism of drug resistance, the mechanisms associated with chemotherapy resistance remain unknown.

Recently, autophagy has been introduced to be an explanation for the mechanism of drug resistance. Autophagy, a catabolic digestion process of cellular organelles[3], plays an important role in maintaining cellular homeostasis and facilitating cell survival against adverse conditions[4-8], including hypoxia[9-12]. The role of autophagy in tumor is controversial, as it can function as both a tumor suppressor and a protector of cancer cell[20,21]. Specifically, after a tumor develops, autophagy can support the persistence of the tumor. According to many studies, autophagy activity has been documented in several cancer cells. Autophagy is up-regulated in response to

metabolic stress caused by chemotherapy and radiation as a cell survival mechanism, likely contributing to treatment resistance, but also providing a novel therapeutic target in cancer[16,20,21,24,25]. In the present study, we also showed that autophagy contributes to chemo-resistance in a lung cancer cell line. Moreover, we could observe that there is enhanced autophagy activity in post treatment lung cancer tissue (neoadjuvant treatment) obtained from NSCLC patients compared with tumor tissue obtained from treatment-naïve NSCLC patients. Most of the advances in the understanding the role of autophagy in cancer have come from cancer cell lines studies expressing autophagy markers. There is little information regarding autophagy in clinical tumor samples, especially the extent to which it varies in tumor tissues. As a step to support our hypothesis, we measured autophagy activity in both pre-treatment tumors and post-treatment tumors (neo-adjuvant treatment) obtained from NSCLC patients. To the best of our knowledge, this is the first report regarding enhanced autophagy activity in human lung cancer tissue after treatment.

Among several conditions that induce autophagy, hypoxia is one of the commonly faced microenvironments in solid tumors. Hypoxia is often found in solid tumors including lung cancer and also important because it triggers several crucial pathways in cancer cells. Cells are thought to adapt to hypoxia

through various pathways, such as angiogenesis, metastasis, glycolytic metabolism or autophagy for survival.[32]

Hypoxia is reported to correlate with poor prognosis and high patient mortality[28], due in part to resistance to chemotherapy[26,27,33,34]. Recent studies have demonstrated that hypoxia can induce autophagy[10-12], and that hypoxia-induced autophagy may protect cancer cells from apoptosis caused by chemotherapeutic agents. Hypoxia inducible factor 1α (HIF-1α) is the main transcription factor leading to autophagy. In the present study we showed high expression of HIF-1α in tumor tissue obtained from NSCLC patients indicating that hypoxia in solid tumors seems to have a role in the tumor microenvironment. In addition, lung cancer cells exposed to 1% O_2 hypoxia showed resistance to the chemotherapeutic agent, cisplatin (Figure 7).

With respect to its molecular mechanism, the HIF-1 transcription factor is regarded to play a crucial role in hypoxia[35-39]. Indeed, the resistance of cancer cells to treatment modalities such as radiation or chemotherapy may be partially due to the induction of HIF-1α and activation of HIF-1[40-42]. HIF-1 controls autophagy under hypoxia by regulating the expression of the downstream target BNIP3 and BNIP3L. Recent studies have shown that BNIP3 induces autophagy by disrupting the interaction of Beclin 1 with Bcl-2 and Bcl-XL[43-45]. On the contrary, there is a report that oxygen deprivation-

induced autophagy does not require HIF-1 activity, or expression of the HIF-1 target gene BNIP3 or BNIP3L, but rather the activity of 5'-AMP-activated protein kinase (AMPK)[4]. Therefore, further studies concerning the molecular mechanism of chemoresistance in hypoxia-mediated autophagy induction are important. However, as hypoxia and autophagy play vital roles in tumor progression and the effectiveness of cancer treatments, it is reasonable to predict that drugs that alter the hypoxia-autophagy pathway may be beneficial to increase the responsiveness of chemotherapy in NSCLC patients.

V. Conclusion

1. Tumor tissues obtained from NSCLC patients showed higher expression of HIF-1 α compared with normal lung tissue according to IHC and Western blot analysis.
2. Induction of HIF-1 α expression by 1% hypoxia in A549 cells was verified by Western blot analysis.
3. Hypoxia induces autophagy in cancer cells: A549 cells were incubated in normoxia or hypoxia for 6 hours, and GFP-LC3 displayed diffuse intracellular localization under normoxic conditions, while membrane translocation (punctate localization) indicative of autophagy was observed in hypoxic cells.
4. Hypoxia induces autophagy in cancer cells: The conversions of LC3B-I to LC3B-II were markedly increased in A549 cells incubated at 1% hypoxia as compared with normoxia. Levels of endogenous p62/SQSTM1 were markedly decreased in A549 cells incubated at 1% hypoxia as compared with normoxia.
5. Hypoxia induces autophagy in cancer cells: Transmission electron microscopy revealed a marked accumulation of autophagosomes under 1% hypoxia, where cytoplasmic material and/or membrane vesicles were encapsulated in vacuoles suggesting that the real

stimuli of autophagy are hypoxia.

6. Hypoxia induces chemoresistance in cancer cells: MTT assay demonstrated that A549 cells exposed to hypoxia showed enhanced resistance to chemotherapeutic agents.
7. Chemotherapy enhances autophagy in NSCLC tumor tissue : Western blot analysis for the tumors of NSCLC showed higher LC3B-I to LC3B-II conversion and lower level of p62/SQSTM1after chemotherapy.

The present study demonstrated that autophagy plays a pivotal role in acquiring drug resistance in NSCLC and especially hypoxic condition as a micro-environment is dominantly involved in these process.

VI. References

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Abstract (in Korean)

비소세포폐암에서 자가포식작용 증대에 의한 항암제 내성기전 획득

<지도교수 정 경 영>

연세대학교 대학원 의학과

이 진 구

폐암은 서구뿐 아니라 전세계적으로 암 관련 사망 주요 원인 중 하나이다. 항암요법은 비소세포폐암의 치료에 있어 중요한 역할을 하고 있지만, 최신의 요법에도 반응율은 30-50% 정도로 불량하며 이러한 불량한 반응의 기전으로 생각되는 항암제 저항에 대한 기전은 아직 명확하지 않다. 최근의 연구에서 세포 소기관의 소화작용에 관여하는 자가포식작용(autophagy)이 항암제 내성에 관여하는 것으로 보고 되고 있으나 그 기전은 알려져 있지 않다. 이에 본 연구에서는 비소세포폐암과 같은 고형암에서 종양 내 흔히 존재하는 저산소증에 의해 유도된 자가포식작용의 항암제 내성 획득에서의 역할을 규명하고자 하였다. 우선 종양 내에서 저산소증의 존재를 확인하기 위해 비소세포폐암으로 수술을 받은 환자의 종양 조직에서 저산소증 인자인 HIF-1a 증가 여부를 확인하고, 폐암 세포주인

A549 세포를 배양하여 1% 저산소증 또는 정상 산소 상태에서 2, 4, 6, 8시간 노출하여 HIF-1α의 발현을 확인하고 동시에 저산소증 하에서 자가포식작용에 관여하는 단백질의 발현을 Western blot을 통해 분석하고 자가포식소체(autophagosome)의 형성을 투과 전자 현미경 사진 및 GEF-LC3 puncta 염색을 통해 확인하였다. 저산소증 상태에서 유도된 자가포식에 의한 항암제 내성을 확인하기 위해 폐암 세포주를 여러 농도의 cisplatin하에서 8시간의 저산소증 또는 정상 산소상태에서 노출한 후 MTT assay를 통해 세포의 사멸 정도를 확인하였다. 마지막으로 치료 목적의 폐절제를 통해 얻은 비소세포폐암 조직을 이용하여 항암 치료를 받았던 종양과 이전 치료병력이 없는 종양에서 자가 포식작용 발현 정도를 비교하였다. 폐암 조직에서 면역염색 및 Western blot에서 HIF-1α의 발현이 정상 조직에 비해 증가함을 확인할 수 있었다. 폐암 세포주에서 1% 저산소증 하에서 시간의 증가에 따라 HIF-1α이 증가하였고 동시에 자가포식 단백질인 LC3BI에 대한 LC3BII의 비율이 역시 증가하고 p62/sequestosome1은 반대로 시간에 따라 감소하였다. 또한 저산소증 하에서 GEP-LC3 puncta의 증가와 TEM을 통해 세포질내 autophagosome의 축적을 확인하였다. 여러 농도의 cisplatine에 처리한 후 1% 저산소증에 노출시 A549 세포주는 정상 산소 상태에 비해 항암제에 저항을 보였다. 수술 전 항암 치료를 받았던 종양 조직에서 항암 치료의 경험이 없는 조직에 비해 자가 포식 작

용의 증가를 확인할 수 있었다. 본 연구를 통해 비소세포폐암에서 저산소증에 유도된 자가포식작용이 항암제 저항에 중요한 역할을 하는 것을 확인할 수 있었다.

핵심되는 말: 비소세포폐암, 약제 내성, 자가포식 작용