Nrf2-Dependent Overexpression of Sulfiredoxin and Peroxiredoxin III in Human Lung Cancer, Especially Squamous Cell Carcinoma

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This certifies that the dissertation

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− ABSTRACT −

Nrf2-Dependent Overexpression of Sulfiredoxin and Peroxiredoxin III in Human Lung Cancer, Especially Squamous Cell Carcinoma

Oxidative stress results in protein oxidation and is implicated in the pathogenesis of human carcinogenesis. Sulfiredoxin (Srx) has been identified as a responsible protein for the enzymatic reversal of the inactivated Peroxiredoxins (Prxs). The nuclear factor E2 related factor 2 (Nrf2) is a well known master transcription factor controlling transcription of many anti-oxidant responsive element (ARE) proteins, which up-regulates Srx expression during oxidative stress. We attempted to elucidate the biological functions of Srx and explore the potential association between Nrf2-dependent ARE-Protein up-regulation in human carcinogenesis.

In order to study the contribution of Srx and Prx III in human lung cancer, we examined the expression of these ARE-proteins, consisting of Nrf2, Prxs, Trx and Srx proteins from 40 surgically resected human lung cancer tissue compared to their normal counterparts using immunoblot and immunohistochemical analysis. For in *vitro* study, transforming growth factor (TGF-β_1), TNF-α, and Camptothecin treatment were used to examine Prx III inactivation in Mv1Lu mink lung epithelial cells and A549 lung cancer cells lines.

There was a marked overexpression of Prx I and Prx III proteins in lung cancer. Significant increase in oxidized form of catalytic site Cys-SO_2H of Prxs was found in lung

cancer tissue compared to their paired normal lung counterparts. In addition, there was a significant overexpression of sulfiredoxin in 12/20 (> 60 %) squamous cell carcinoma tissues by densito-metric analysis of the immunoblot, whereas only 8/20 (< 20%) of adenocarcinoma tissues overexpressed sulfiredoxin. Furthermore, Nrf2 was found to be present in the nuclear compartment of the cancer tissue.

In conclusion, Srx protein was markedly overexpressed in human squamous cell carcinoma along with Prx III, suggesting these proteins may play a protective role against oxidative injury and provide compensation for high mitochondrial metabolism occurring in human lung cancer.

Key words: Nuclear factor E2 related factor 2 (Nrf2), Peroxiredoxin (Prx), Sulfiredoxin (Srx), Human squamous cell carcinoma.

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LIST OF ABBREVIATION

Prx, peroxiredoxin

Srx, sulfiredoxin

Trx, thioredoxin

ROS, reactive oxygen species

Nrf2, nuclear factor E2-related factor 2

Keap1, Kelch like ECH-associating protein 1

ARE, antioxidant response element

TNF-α, tumor necrosis factor-α

TGF-β1, transforming growth factor-β1

HM, heavy membrane

siRNA, small interfering RNA

FITC, fluorescein isothiocanate

COPD, chronic obstructive pulmonary disease

SOD, superoxide dismutase

MnSOD, Mn^{2+}-dependent superoxide dismutase

NADPH, nicotinamide adenine dinucleotide phosphate

DAPI, 4', 6-diamidino-2-phenylindole

I. INTRODUCTION

Various oxidative stresses and many growth factor stimulation cause mutagenic changes to DNA, proteins, and lipids. Thus ROS signaling especially hydrogen peroxide as the second messenger plays a central role in a variety of cellular events such as cellular growth, proliferation, and human carcinogenesis (Rhee et al., 2006). Lung is very unique and complex major organ of the human body. It is exposed to constant oxidative stress and xenobiotic challenges during gas exchange through the alveolar capillary membrane. Alveolar and bronchial epithelium are in contact with environmental pollutions, toxic fumes, and cigarette smokes. They are also under the influence of cytokines released from macrophages, neutrophils, and lymphocytes. Lung is also the battle ground where the counteractions between protease and anti-protease take place. In order to compensate for heavy loading of oxidative stress, pulmonary epithelial cells are armed heavily with many specialized and dedicated proteins which serve to function as anti-oxidants. These anti-oxidant related element (ARE) group include glutathione, catalase, SOD, MNSOD, NADPH oxidases, peroxiredoxins, etc (Chowdhury et al., 2009).

Peroxiredoxins (Prxs) are a relatively new ubiquitous family of peroxidases which functions to protect human cells from oxidative stress by removing hydrogen peroxides produced as a result of normal cellular metabolism, growth, differentiation, inflammation, and proliferation (Kang et al., 1998; Kang et al., 2005). The conserved cysteine residue Sulfydril (-SH) at the active catalytic sites of (Prx) I, II and III are reversibly hyperoxidized to cysteine sulfinic acid ($-SO_2H$), with concomitant loss of their peroxidase activity (Chang

et al., 2004).

Prx III which is exclusively present in mitochondria plays a pivotal role in initiating cellular apoptosis, proliferation, carcinogenesis, and degenerative disorders, etc (Chang et al., 2004). Isoforms of Prxs and Hydrogen Peroxide signal transduction by Prxs are reviewed elsewhere (Bozonet et al., 2005; Rhee et al., 2005).

Sulphiredoxin (Srx) has been identified as a responsible protein for the enzymatic reversal of the inactivated Prxs in ATP, Mg2+, and Ca2+ dependent manner (Chang et al., 2004; Jonsson et al., 2008; Lei et al., 2008; Rhee et al., 2007) (Fig. 1). In the subsequent reports, crystal structure of pair embrace between Prx and Srx had been identified (Johnson et al., 2008). Srx is a family of low molecular weight sulfur containing proteins functioning to maintain cellular redox balance. Srx has been suggested to be implicated in oncogenic transformation of cell lines which overexpress Srx (Lei et al., 2008). In addition there has been a very recent report using nrf2 knock out mice clearly demonstrating that hyperoxia induced expression of Srx in mouse lung is dependent on nrf2 signaling pathway (Bae et al., 2008). We have previously reported the increased expression of Prx I, III, and thioredoxin (Trx) in human lung cancers (Park et al., 2006).

The nuclear factor E2 related factor 2 (nrf2) is well known master transcription factor controlling transcription of many Anti-oxidant related element (ARE) proteins (Kwak et al., 2003; Lau et al., 2008) (Fig. 2). Keap1-Nrf2-ARE plays critical role in protecting cells from both endogenous and exogenous stresses (Cho et al., 2006; Kensler et al., 2007). Nrf2 knockout mice are more susceptible to the hepatic pulmonary (Cho et al., 2002) and major organ damages caused by exposures to environmental agents and drugs, inflammatory

stresses, and many carcinogens (Kensler et al., 2007).

However, during carcinogenesis, cancer cells use the protective machinery of ARE to their survival advantage against oxidative stresses of growth (Colburn and Kensler, 2008; Lau et al., 2008) chemotherapy, and radiotherapy, endowing them with thecapacity to endure the toxic effects of the therapy (Kwak et al., 2001; Kwak et al., 2002; Okawa et al., 2006; Kensler et al., 2007; Osburn and Kensler, 2008). Recently it was reported that cigarette smoking induced Keap1 mutation causes release of Nrf2 and activation promoting transcription of ARE proteins contribute to the development of squamous carcinoma in the head and neck (Park et al., 2009; Stacy et al., 2006). However, the pathophysiologic role of Nrf2 and association between Nrf2, Prxs, Srx protein has not been clearly defined in human lung disease.

In order to elucidate the biological function of Srx and nrf2-dependent ARE-proteins in human carcinogenesis, we examined the expression of Srx protein and nrf2-dependent ARE-proteins in human lung cancer and its paired normal lung.

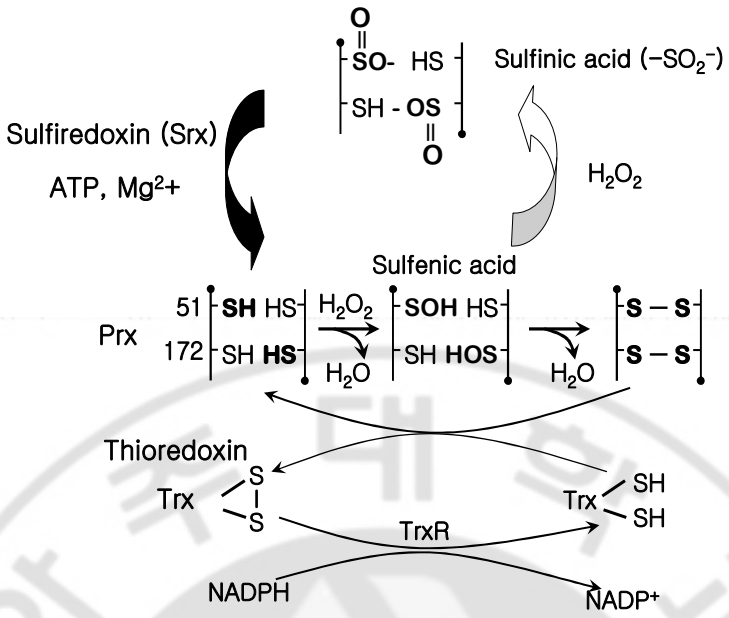

Fig. 1. Catalytic cycle of 2-Cys Prxs, their inactivation due to hyperoxidation by H_2O_2, and their reactivation via an ATP-dependent reduction catalyzed by Srx. The peroxidatic cysteine, Cp-SH (Cys^{51} in mammalian Prx 1) is selectively oxidized by H_2O_2 to Cp-SOH, which then reacts with the resolving cysteine, C_R–SH (Cys^{172} in Prx 1) of the other subunit in the homodimer to form an intermolecular disulfide. The disulfide is subsequently and specifically reduced by Trx, which in turn receives reducing equivalents from nicotinamide adinine dinucleotide phosphate via Trx reductase (TrxR). The Cp-SOH generated as an intermediate during catalysis occasionally undergoes further oxidation to Cp-SO_2H, leading to inactivation of peroxidase activity. Reactivation of the enzyme is achieved by reduction of the Cp-SO_2H moiety in reaction that requires ATP hydrolysis and is catalyzed by Srx, with reducing equivalents being provided by physiological thiols (RSH) such as glutathione and Trx (Rhee et al.,2007).

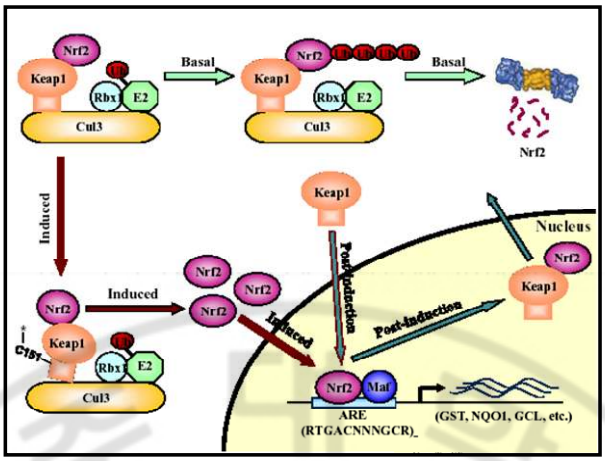

Fig. 2. Schematic model of Nrf2 regulation by Keap 1. Keap 1 is a key regulator of the Nrf2 signaling pathway and serves as a molecular switch to turn on and off the Nrf2-mediated antioxidant response**.** The switch is in off position: under basal conditions, keap1, functioning as an E3 ubiquitin ligase, constantly targets Nrf2 for ubiquitination and degradation. As a consequence, there are minimal levels of Nrf2. The switch is turned on: oxidative stress or chemopreventive compounds inhibit activity of the Keap1-Cul3-Rbx1 E3 ubiquitin ligase, resulting in increased levels of Nrf2 and activation of its downstream target genes. The switch is turned off again: upon recovery of cellular redox homeostasis, Keap1 travels into the nucleus to remove Nrf2 from the ARE. The Nrf2-Keap1 complex is then transported out of the nucleus by the NES in Keap1. In the cytosol, the Nrf2-Keap1 complex associates with the Cul2-Rbx1 core ubiquitin machinery, leading to degradation of Nrf2. For clarity, the constitutive cytoplasmic-nuclear shuttling of Nrf2, Keap1, and the complex is omitted (Lau et al., 2008).

II. MATERIALS AND MATHODS

A. Materials

RPMI1640 medium, fetal bovine serum, trypsin-EDTA, and penicillin-streptomycin were purchased from Gibco BRL (MD, U.S.A.). Alexa 488 conjugated goat antibodies to mouse IgG, Alexa 488 conjugated goat antibodies to rabbit IgG, Alexa 594 conjugated goat antibodies to rabbit IgG, Mitotracker Red CMXRos and DAPI were purchased purchased from Molecular probes (Eugene, OR, U.S.A.). Antibodies specific for Prx isoforms and Trx were purchased from Young in Frontier (Seoul, Korea). Nrf2, anti-HA and α-tubulin were purchased from Santacruz Biotechnology (CA, U.S.A.). An antibodies specific for Srx, pCGN vector and pCGN-Srx were kindly provided by Dr. HA WOO (Ewha Womans University, seodeamoon-gu, Korea). Camptothecin and protease inhibitor were purchased from Sigma-Aldrich (St. Louis, U.S.A.).

B. Subjects

A total of 40 patients who underwent surgery due to lung cancer Ajou University Medical center were enrolled in this study. Tumor tissue and paired sample of normal lung from each individual were collected after pathological evaluation. Informed consent was obtained and the study protocol was approved by Internal Review Board of at Ajou University Medical center

C. Cell culture and viability.

A549 cell line (Human Lung Carcinoma) was obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). Cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37ºC in a humidified atmosphere containing 5% CO2. 1 x 10^5 cells were seeded into 6-well plates, cultured in RPMI containing 10% FBS for 12h, and treated with H_2O_2 (200μM) and TGF-β1(2 or 10μg/ml) and TNF-α (10μg/ml) and Camptothecin (10μM/ml) for the indicated periods. The cells were continuously cultured by refreshing every day with the media containing the same growth factors, if necessary. At the indicated times, cells were harvested by trypsinization and counted with a haemacytometer after staining with final 0.2% (w/v) trypan blue (Gibco BRL) to exclude dead cells. To evaluate cellular viability, the population of trypan blue-stained cells were counted under the inverted microscope (Olympus, Tokyo, Japan).

D. Transfection and depletion of siRNA

Cells were transfected with pCGN vector and Srx-pCGN-HA using the FuGENE HD transfection reagent (Roche Diagnostics, GmbH, Mannheim, Germany). A small interfering RNA (siRNA) of human sulfiredoxin mRNA (Dharmacon, Chicago, U.S.A., SMART pool M-015263-00-0005) as well as siCONTROL® non-targeting siRNA were obtained from Dharmacon Research (Dharmacon). A day before transfection, cells were seeded into 6 well plates to allow them to reach 60% confluence. Then, the next day it was transfected with 20nM of Srx-siRNA or control siRNA using Lipofectamine RNAiMax reagents (Invitrogen).

E. Immunocytochemistry

Cells were grown on glass bottomed culture dishes, fixed with 4% para-formaldehyde and permeabilized for 5 min with 0.2% Triton X-100. Cells were blocked in 1xPBS containing 1%BSA for 1h and probed with primary antibody for overnight at 4ºC. After washing, the immune complex was probed with secondary antibodies. Immuno-stainined cells were washed with 1 x PBS and mounted with prolong GOLD (Molecular probes). HA-tagged Srx was detected with HA monoclonal antibodies (10μg/ml) and Alexa-488 conjugated goat antibodies raised against mouse IgG (5μg/ml). Prx III was detected with polyclonal antibodies (10μg/ml) and Alexa-488 conjugated goat antibodies raised against rabbit IgG (5μg/ml) or Alexa-594 conjugated goat antibodies raised against rabbit IgG (5μg/ml). Mitochondria were stained with 0.1uM MitoTracker Red CMXRos. Confocal fluorescence images were obtained using an LSM510 microscope (Carl Zeiss).

F. Co-Immunoprecipitation and Immunoblot analysis

For immunoblot analysis, lung tissues were homogenized in lysis buffer that contained 50mM Tris-HCl (pH7.5), 150mM NaCl, 1mM EDTA, 1% Triton X, Protease inhibitor cocktail (Sigma-Aldrich, St. Louis, U.S.A.). Cell lysates were centrifuged at 12000g for 10 min at 4ºC, and boiled for 5min in 2x sample buffer. For immunoprecipitation, A549 cells were lysed in lysis buffer and centrifuged at 12000g for 10 min at 4ºC. A549 cell lysates were precleared with Protein A agarose beads (Upstate, Charlottesville, U.S.A.). 2μl of anti-HA antibodies are added to the cell lysates and rotated at 4ºC for 2h. 20μl of Protein A agarose beads were then added, and samples in tubes were rotated for additional 2h. Samples

were washed four times and then boiled for 5min in 1x sample buffer. The samples were separated by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes (Scheicher & Schuell Bio Science Gmbh, Dassel, Germany). After blocking, each membrane was incubated with primary antibodies and sheep anti-mouse IgG or donkey anti-rabbit antibody (Amersham biosciences, England) was secondarily treated, immune complexs were analyzed with enhanced chemiluminescence reagent (ECL, Amersham biosciences, England).

G. Two-dimensional electrophoresis

A549 cells lysed in lysis (8M urea, 4%CHAPS, 40mM Tris, 20M DTT, 0.5% IPG buffer pH3-10 non-linear) containing protease inhibitor and then sonicated by three times 20s pulse. After removal of insoluble materials by centrifugation at 12,000g for 15min, cell lysates were mixed with rehydration buffer (8M urea, 2%CHAPS, 20mM DTT, 0.5% IPG buffer pH3-10 non-linear, bromophenol blue) and loaded onto immobilized pH gradient strips (pH3-10, non-linear, Amersham biosciences, Piscataway, NJ) for 16h at room temperature. Isoelectric focusing unit (Amersham biosciences) and preparation of the immobilized pH gradient strips for second-dimensional SDS-PAGE were carried out according to the procedures recommended by manufacturer. SDS-PAGE was conducted on 12% gel using an Amersham Bioscience SE600 13cm vertical unit and transferred to nitrocellulose membranes for immunoblot analysis.

H. Subcellular Fractionation

Cytosolic and mitochondrial-enriched fractions were prepared from A549 cells with the use of a Subcellular Proteome Extraction kit (Calbiochem, Darmstadt, Germany). Cells were washed two times in ice cold wash buffer at 4 ºC for 5 min and then incubated with ice cold extraction buffer I in the presence of protease inhibitor cocktail for 10min at 4 ºC under gentle agitation. The supernatant was collected as the cytosolic fraction. Then cells were incubated with ice cold extraction buffer II in the presence of protease inhibitor cocktail for 30min at 4 ºC under gentle agitation. This supernatant was collected as the mitochondria-enriched heavy membrane fraction.

I. Immunohistochemistry

Section of paraffin wax-embedded human tissue was used for immunohistochemistry. After deparaffinization, non-specific binding was blocked with 10% normal goat serum (Amersham biosciences) and then probed with sulfiredoxin specific polyclonal antibody for overnight at 4ºC. Tissue sections were rinsed three times in phosphate buffered saline before being incubated with streptavidin-biotin-peroxidase reagents (LSAB kit, Dako, Copenhagen, Denmark). The sections were developed with diaminobenzidine and were counterstained with haematoxylin and eosin.

J. Statistical analysis

Data were analyzed at least three times, and all values are represented as means ± SD of triplicates. Student's t test was used to compare continuous data. Values of $p<0.05$ were considered statistically significant. All data were analyzed by SPSS 11.0 version.

III. RESULTS

A. Localization of Prx III and Srx in A549 cells

To demonstrate subcellular localization of Prx III and Srx proteins in A549 cells, immunocytochemical analysis was performed with confocal microscopy. The Prx III in A549 cells specifically co-localized with Mitotracker dye in the mitochondrial fraction (Fig. 3A). When intracellular localization of Srx in A549 cells was investigated by immunocytochemistry, Srx protein under basal conditions existed in the cytosol (Fig. 3B).

To test the localization of Srx and Prx III in the different cellular compartments, the presence of Srx and Prx III protein was examined using immunoblot analysis of both proteins in the soluble cytosolic or heavy membrane (HM) fractions. Srx protein was also found predominantly in cytosol as in immunocytochemistry, whereas Prx III was detected in mitochondrial fraction (Fig. 3).

B. Oxidation of Prx III in mink lung epithelial cells

Mink lung epithelial cells (Mv1Lu) were exposed to TGF-β 1 (10μg/ml) for 72 hours. After 72 hours only oxidized form of Prx III was noticed on 2D electrophoresis with immunoblot analysis (Fig. 4).

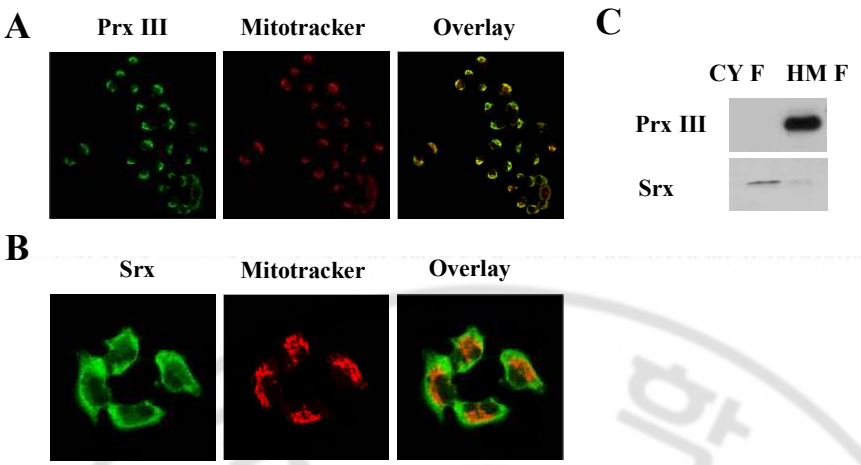

Fig. 3. Expression and cellular localization of peroxiredoxin III and sulfiredoxin. A549 cells were stained for the Prx III (FITC), Srx (FITC), and mitochondrial fraction (Mitotracker) and then examined by confocal microscopy (A, B). A549 cells were subjected to subcellular fractionation to yield mitochondria enriched (HM) and cytosolic fractions. Cell lysates were prepared and subjected to immunoblot analysis with antibodies to either Prx III or Srx (C) (CY F, cytosolic fraction; HM F, heavy membrane fraction).

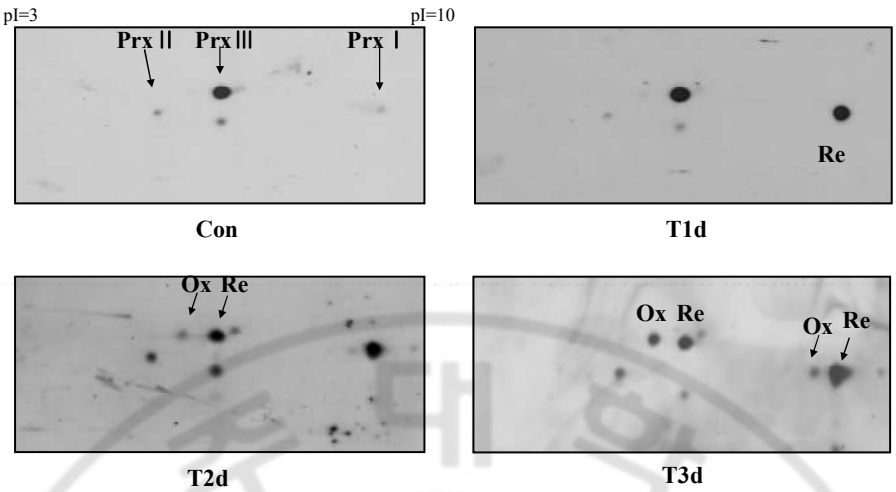

Fig. 4. Oxidation of Prx I, II, and III in Mv1Lu by TGF-β1. Mv1Lu cells were cultured in DMEM containing 10% FBS and were treated with 2ng/ml TGF-β1 for the periods indicated. Cell lysates were analyzed with 2D gel electrophoresis, followed by immunoblot analysis with indicated antibodies (Ox, oxidation form; Re, reduced form; Con, control; T1d, day 1; T2d, day 2; T3d, day3).

C. Srx dependent regulation of Prx III oxidation in A549 cells

The amount of Srx was reduced by 90% in A549 cells transfected with a small interfering RNA (siRNA) specific for Srx mRNA (20nM) (Fig.5A). A549 cells were treated by TGF-β1 (10μg/ml) for 72h and then cell lysates were subjected to two dimensional electrophoresis with subsequent immunoblot analysis. The sulfinic forms of Prx III were found only in A549 cells with Srx depletion. Oxidated forms of Prx III were also observed when A549 cells were treated with either TNF-α (10μg/ml) or Camptothecin (10μM) for 24h and then cell lysates were subjected to two dimensional electrophoresis with immunoblot anaylsis. (Fig. 6A, B).

D. Srx is translocated into the mitochondria under oxidative stress

The intracellular localization of Srx was investigated after oxidative stress using immunostaining and Immunoprecipitation (Fig. 7A, B). HA-tagged Srx was transfected in A549 cells to overexpress Srx. These cells were exposed to 200μM H_2O_2 for 10min to induce protein sulfinylation and then incubated for 4h in H_2O_2 free medium to reduce protein as reported previously (Noh et al., 2009). Srx was not found in the mitochondria under basal condition, but a significant increase of HA-tagged Srx was found in the mitochondria only 4and 8h after H_2O_2 treatment in the immunostaining. These results were confirmed by immunoprecipitation. The binding of Prx III and Srx through co-immnoprecipitation was verified at 4h after H_2O_2 treatment (Fig. 7B).

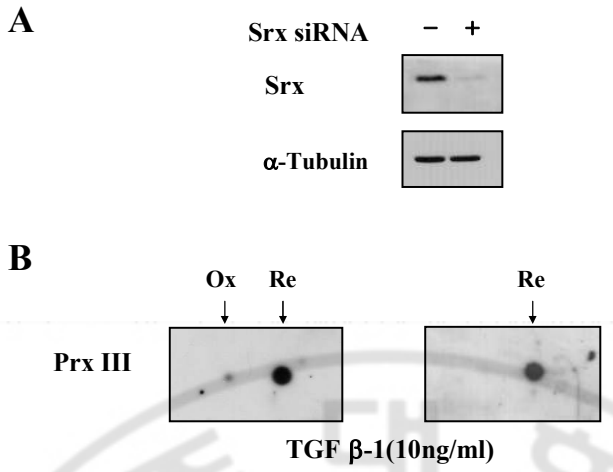

Fig. 5. Srx controls Prx III oxidation in A549 by TGF-β1. Cells were transfected with either a control siRNA or an siRNA specific for human Srx mRNA, after which cell lysates were prepared and subjected to immunoblot analysis with antibodies to Srx or α-tubulin (A). 48 h after cells were transfected as in (A), cells were exposed to 10ng/ml TGF-β1 for 3 days and cell lysates were analyzed with 2D gel electrophoresis, followed by immunoblot analysis with Prx III antibody (B).

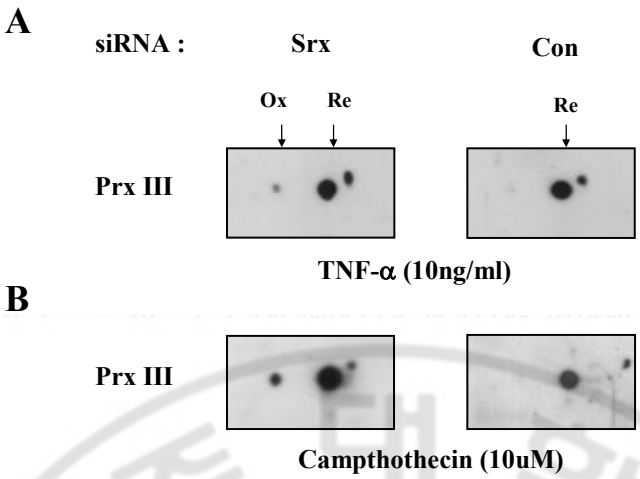

Fig. 6. Srx controls Prx III oxidation in A549 by TNF-α and Camptothecin. A549 Cells were transfected with either a control siRNA or a siRNA specific for human Srx mRNA. 48 h after cells were transfected, cells were exposed to 10ng/ml TNF-α for 24h (A) or 10μM camptothecin for 24h (B) and cell lysates were analyzed with 2D gel electrophoresis, followed by immunoblot analysis with Prx III antibody.

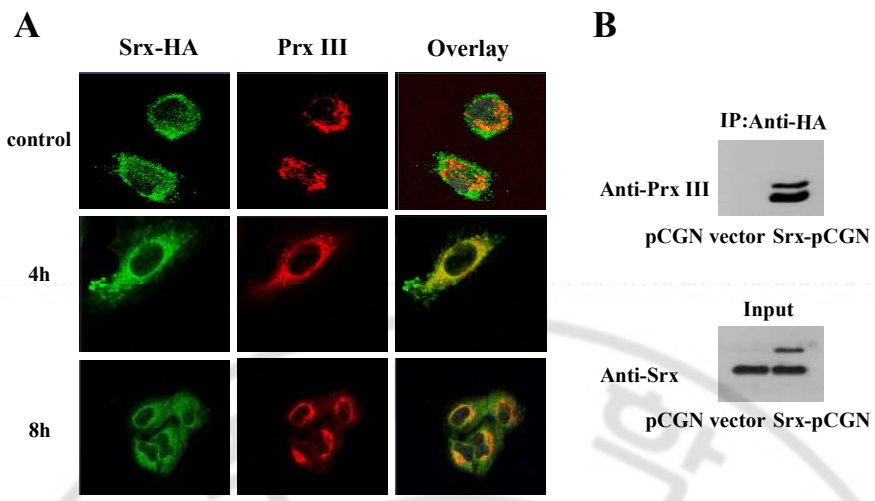

Fig. 7. Mitochondrial translocation of Srx under Oxidative conditions. A549 cells transfected with an expression vector for HA-tagged Srx were exposed to 200μM for 10min and then allowed to recover from oxidative stress. A549 cells were stained for HA epitope (FITC) and Prx III (Red) and then examined by confocal microscopy (A). After 4h, cells were lysed and then immunoprecipitated with HA antibody and immunoblotted with the indicated antibodies (B).

E. Overexpression of Prx I and III isoforms in human lung cancer

Increased amount of oxidized forms of Prx I and III was observed through 2D electrophoresis and immunoblot analysis in cancer tissue compared to the paired normal lung, as we have reported previously (Park et al., 2006) (Fig. 8).

F. Nrf2 dependent expression of ARE-proteins in human lung cancer

The expression of ARE-proteins, consisting of Nrf2, Prx, Trx, Srx was examined by a simultaneous immunoblot analysis of these proteins. The expression levels of Nrf2, Prxs, especially Prx I and III, Trx, and Srx were increased in cancer tissue compared to the paired normal lung (Fig. 9). Also significant overexpression of Nrf2 was observed in the nucleic fraction of the same lung cancer tissue.

G. Overexpression of Srx in human lung cancer

In 40 resected lung cancers with stage I and stage II of various cell types, we examined expression of Srx using immunoblot anaylsis. A markedly increased Srx expression was observed in 60% of squamous cell carcinoma compared to the paired normal lung tissue ($P<$ 0.001, n=20) (Fig. 10A), whereas Srx expression was overexpressed in only 20% of adenocarcinoma ($P<$ 0.001, n=20) (Fig. 10B). Expression of Srx in both lung cancer and paired normal tissue were measured by densitometry (Fig. 11A and Fig. 12A), Srx protein was overexpressed significantly in both squamous cell carcinoma and adenocarcinoma cell types (Fig. 11B and Fig. 12B). Srx protein was markedly overexpressed in all subtypes of lung cancer *($P<0.001$)* (Fig. 13A).

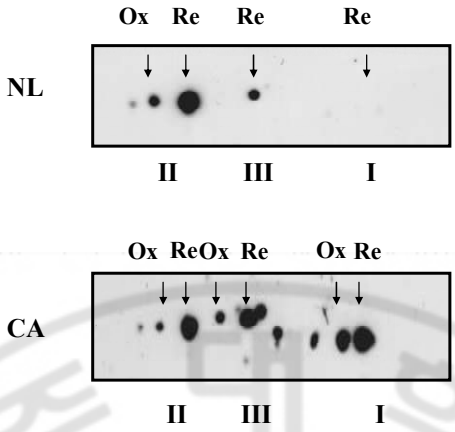

Fig. 8. Expression of Prx I and Prx III in lung cancer tissue and the paired normal lung tissue. 50μg of protein extracts from both lung cancer and the normal lung tissue were analyzed with 2D gel electrophoresis, followed by immunoblot analysis with indicated antibodies. Isoelectric focusing was done with gel strip (pH 3-10, non-linear) and second dimension was separation by the molecular weight on 12% SDS-PAGE (Ox, oxidation form; Re, reduced form; NL, normal lung tissue; CA, lung cancer tissue) (Park et al., 2006).

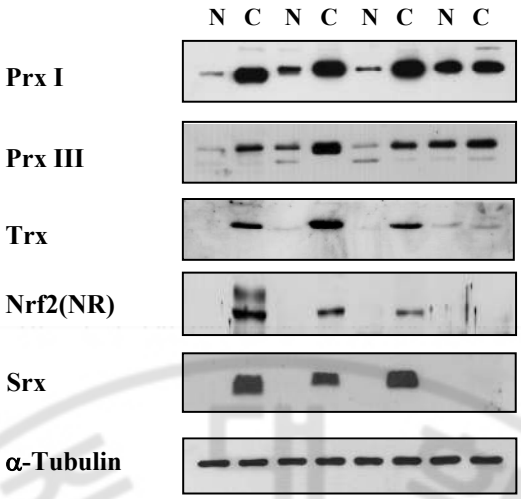

Fig. 9. Increased expression of ARE-proteins in Human lung cancer tissue (squamous cell carcinoma). Expression of Prx I, II, III, Trx and Srx in both lung cancer (C) and paired normal tissue (N) was subjected to immunoblot analysis with Prx I, Prx II, Prx III, Trx and Srx antibodies. Expression of Nrf2 in both lung cancer (C) and paired normal tissue (N) were subjected to subcellular fractionation and nucleic fraction was subjected to immunoblot analysis with Nrf2 antibody (NR, nucleic fraction).

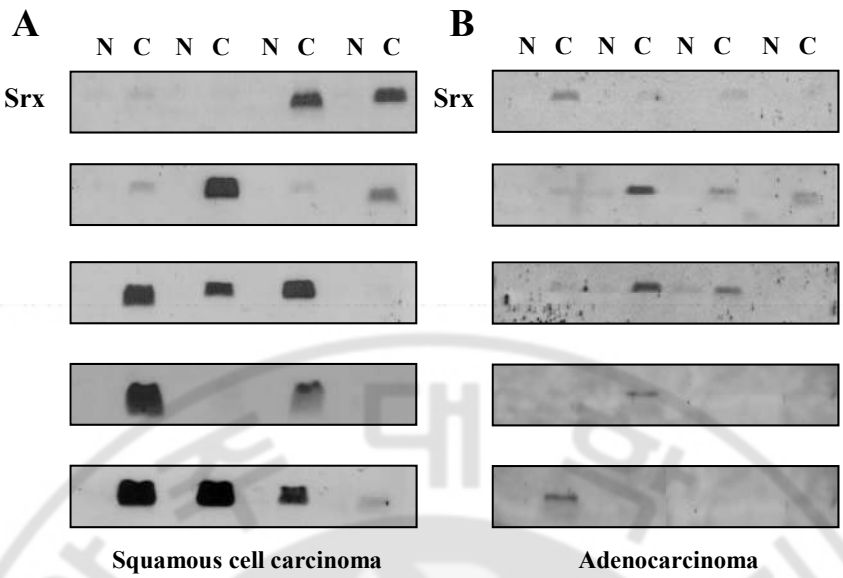

Fig. 10. Increased expression of Srx in Human lung cancer tissue - squamous cell carcinoma (A) and adenocarcinoma (B). Expression of Srx in both lung cancer (C) and paired normal tissue (N) were subjected to immunoblot analysis with Srx antibody.

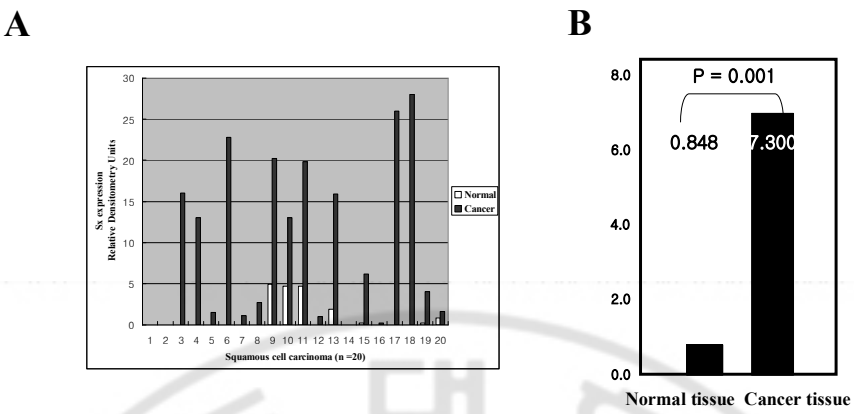

Fig. 11. Expression of Srx in human squamous cell carcinoma. Individual data were quantified as densitometry units (A) and expressed as relative to the corresponding value for expression of Srx in lung cancer and paired normal tissue (B).

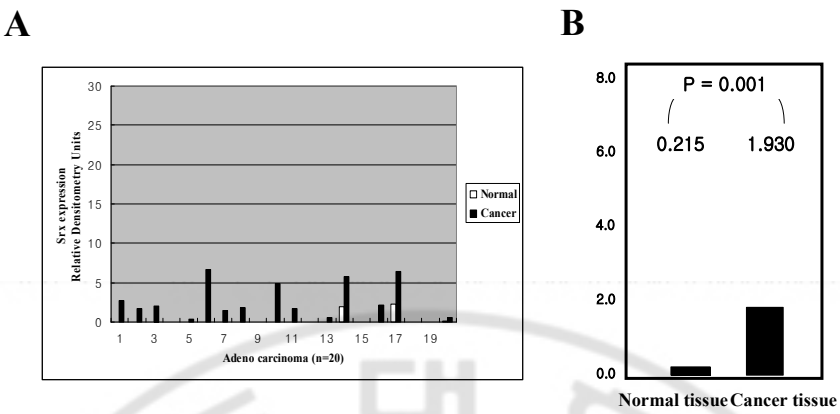

Fig. 12. Expression of Srx in human adenocarcinoma. Individual data were quantified as densitometry units (A) and expressed as relative to the corresponding value for expression of Srx in lung cancer and paired normal tissue (B).

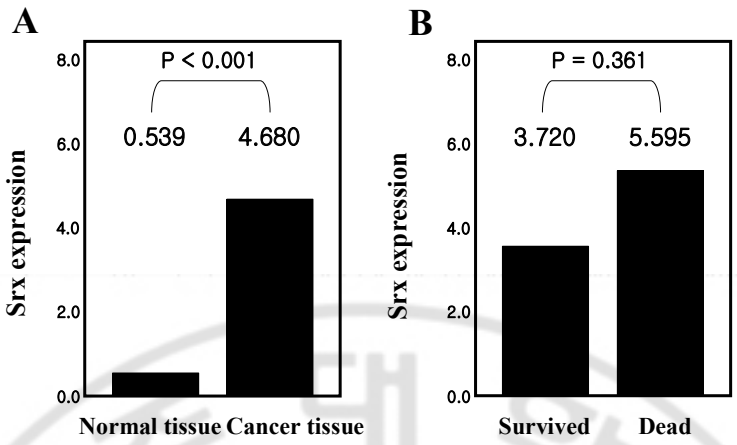

Fig. 13. Expression of Srx and survival in all cell types of lung cancer tissue. Data were expressed as relative to the corresponding value for expression of Srx in all cell types of lung cancer and paired normal tissue (A). Data were expressed as relative to the corresponding value for expression and survival of Srx in all cell types of lung cancer and paired normal tissue (B).

H. Survival of patients with lung cancer based on Srx expression

When the level of Srx expression was correlated with patient survival data a decreasing trend for survival was noticed. However, it was not statistically significant (Fig. 13B).

I. Expression of Srx in lung cancer tissues

In order to prove that the increase in Srx protein came from the lung cancer tissue, immnohistchemical staining of paraffin fixed slide was performed using monoclonal Srx antibody. The immunohistchemistry showed that the tumor tissue is much more heavily stained than the normal lung parenchyme. In addition, Srx is located mainly in the cytoplasmic part of the cancer cells. However, Srx was also found in the nuclei and nucleoli (Fig. 14).

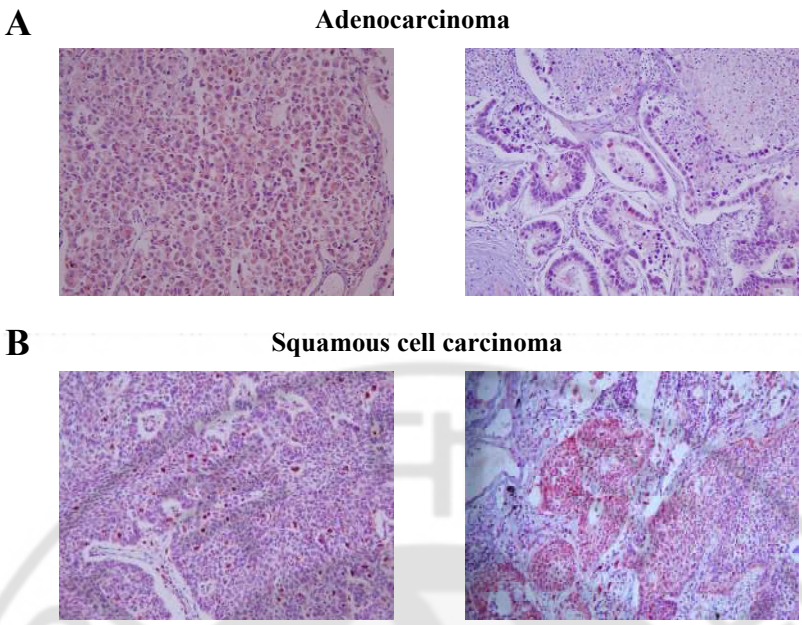

Fig. 14. Expression of Srx in human lung cancer tissue. Paraffin-fixed 5μM slide of lung cancer tissue was deparaffinized, incubated with Srx antibody and visualized with DAB as chromogen. The slide was counter-stained with haematoxylin and eosin (X400). Immunohstochemistry of adenocarcinoma (A) and squamous cell carcinoma (B) are shown.

IV. DISCUSSION

Principal finding of this study is that Srx protein was markedly overexpressed in human lung squamous cell carcinoma, suggesting Srx protein in co-operation with increased levels of Prxs, especially Prx I, III, and Trx may protect against oxidative injury to pulmonary epithelial cells from cigarette smoking. Another main finding from this study is that significantly increased Nrf2 was found in the nucleic fraction of lung cancer tissue. Which may act to control and regulate the expression levels of these ARE-proteins.

We have previously reported that Prxs are abundantly expressed in bronchial and pulmonary epithelial cells (Park et al., 2006). However, we could not elucidate detailed connections between these elevated proteins and underlying mechanism of overexpression. Our present study showed that the expression levels of Nrf2, Prxs, especially Prx I and III, Trx, and Srx were increased in cancer tissue compared to the paired normal lung. The presence of Nrf2 was also demonstrated in the same lung cancer tissue in which Srx was found to be overexpressed. Previous study revealed that increase of Srx which regenerates inactive 2-Cys Prx (Findlay et al., 2005; Jeong et al., 2006; Noh et al., 2009; Park et al., 2009; Woo HA et al., 2005) is dependent on Nrf2-dependent signaling pathway (Bae et al., 2008; Soriano et al., 2009). Therefore, we speculated that Nrf2 activation may be the master control switch which up-regulates the above mentioned ARE-proteins. Further experiment is concerning Keap 1 mutation may be necessary to confirm our current findings. Our result is

consistent with a recent report that oxidative stress induces expression of Prx6 and Srx in A549 cell lines through Nrf2 (Chowdhury et al., 2009).

Nrf2 activation resulting from both acute and chronic oxidative stresses of environmental carcinogens from smoking, acute and chronic inflammation may induce subsequent over-expression of ARE proteins which initially protect pulmonary epithelial cell injuries (Rahman et al., 2008; Hubner et al., 2009; Singh et al., 2009). In addition, it was reported that Keap1 mutation with increased transcription of ARE proteins was found in many patients head and neck squamous cell carcinomas (Park et al., 2009; Stacy et al., 2006; Shibata et al., 2008). We speculate that overexpression of Srx in squamous carcinoma is due to sulfinic forms of Prx III in mitochondria will increase in response to oxidative stress of carcinogens such as smoking, pollution, high growth and metabolic rate of tumor. Therefore, excess Srx was produced to regenerate sulfinic forms of Prx III to meet the increased anti-oxidant demand of tumor cell survival (Jeong et al., 2006). It is thought that increased expression of Nrf2 which regulates ARE-protein can be explained in the same way. In adenocarcinoma overexpression of Srx was less frequently found. This can be attributed to the fact that in adenocarcinoma patients tend to be at younger age, female, non smoker, previous chronic inflammation, and other genetic factors may be responsible for the development of cancer. Further study is needed to elucidate the role of Srx protein in human lung carcinogenesis and Srx inhibition with subsequent Prx III inactivation could be a potential therapeutic target for future lung cancer treatment.

V. CONCLUSION

In conclusion, Srx protein was markedly overexpressed in Nrf2 dependent manner in human squamous cell carcinoma along with Prx III, suggesting these proteins may play a protective role against oxidative injury and provide compensation for high mitochondrial metabolism occurring in lung cancer cells.

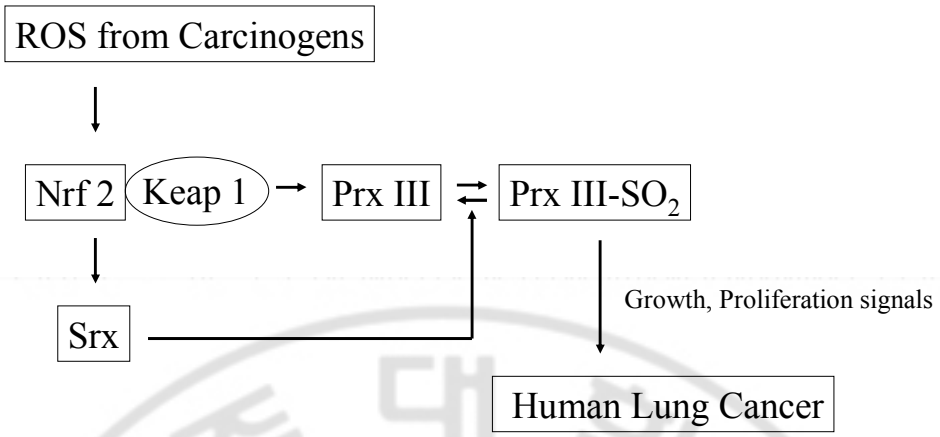

Fig. 15. **Proposed model for ROS leading to Srx and ARE-protein overexpression causing human lung cancer.** Oxidative stress from carcinogens increases the intracellular level of ROS which triggers Nrf2-dependent induction of Srx expression. The reduction of peroxides by Prx III results its hyperoxidation to the sulfinic form (Prx III-SO_2), the reversal of which is catalyzed by Srx. In order to meet the increased demand overexpression of ARE-proteins such as Srx, Prx I, III, Trx, and Nrf2 are overexpressed to support tumor cells growth.

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– 국문요약 –

인체 편평상피 세포암 조직에서 Nrf2 에 의존한 Sulfiredoxin 과 Peroxiredoxin III 의 과 발현 양상과 임상적 의의

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산화 스트레스는 체내 단백질의 산화와 인체 폐암의 발병을 초래할 수 있다. Sulfiredoxin(Srx)는 불활성 된 Peroxiredoxins (Prxs)의 효소적 활성을 되찾아 주는 단백으로 알려져 있다. The nuclear factor E2 related factor 2(Nrf2)는 anti-oxidant related element (ARE) 단백질들의 전사 인자로서 잘 알려져 있으며, 또한 산화 스트레스 아래에서 Srx 의 발현을 증가시키고 조절하게 된다. 이에 본 연구는 인체의 폐암 환자에서 Srx 의 생물학적 기능과 Nrf2 에 의존한 ARE 단백질들의 상승작용이 갖는 잠재적인 의미를 알아보고자 하였다.

인체의 폐암 조직에서 Srx 와 Prx III 의 역할을 알아보기 위하여 아주대학교 의과대학 부속병원에서 폐암으로 진단되어 수술 받은 40 명(선암 20 례, 편평상피 세포암 20 례)의 환자 폐암 조직과 동일 환자의 정상 부위 폐 조직에서, 1 차원 전기영동 방법과 면역 조직 화학 염색 기법을 이용하여 Nrf2,

Prxs, Thioredoxin (Trx), Srx 를 포함한 ARE 단백질들의 발현을 확인하였다. 또한 Mv1Lu 정상 폐 상피 세포주와 A549 폐암 세포주에 TGF-β1(10μg/ml), TNF-α(10μg/ml), 그리고 Camptothecin (10μM/ml)을 처리하여 불활성화 된 Prx III 에 대한 Srx 의 역할을 확인하였다.

Srx 의 발현은 인체 편평상피 세포암에서 동일 정상부위 폐 조직과 비교하였을 때 폐암 환자 조직에서 두드러지게 증가하였다($p < 0.001$). 또한 Prx I, Prx III, Trx 와 2-Cys Prxs 의 활성을 되살리는 Srx 의 발현 또한 높았으며, 핵 내에서 Nrf2 의 발현 또한 높은 것으로 확인되었다.

결론적으로 인체 편평상피 세포암에서 ARE 단백질들 Prx I, Prx III 그리고 Trx 의 발현과 더불어 2-Cys Prxs 의 활성을 되살리는 Srx 의 과발현 현상은 인체 편평상피 세포암에서 산화 스트레스에 대항하여 세포를 보호하고 기능을 유지하여 생명력을 주는 것으로 사료되어진다.

핵심어: Nuclear factor E2 related factor 2 (Nrf2), Peroxiredoxin (Prx), Sulfiredoxin (Srx), 인체 편평상피 세포암.