대흉외지 2007;40:529-535

비소세포 폐암조직에서 Apurinic/Apyrimidinic Endonuclease-1/Redox Factor-1의 발현변화

유대군* · 송윤정* · 조은정* · 강민웅** · 한종희**
나명훈** · 임승평** · 유재현** · 전병화* · 이 영**

Alteration of Apurinic/Apyrimidinic Endonuclease-1/Redox Factor-1 in Human Non-small Cell Lung Cancer

Dae Goon Yoo, M.D.*, Yun Jeong Song, M.D.*, Eun Jung Cho, M.D.*, Min-Woong Kang, M.D.**,
Jong Hee Han, M.D.**, Myung Hoon Na, M.D.**, Seung Pyung Lim, M.D.**,
Jae Hyeon Yu, M.D.**, Byeong Hwa Jeon, M.D., Ph.D.*, Young Lee, M.D.**

Background: An imbalance between oxidants and antioxidants leads to oxidative stress, and this has been proposed to play an important role in the pathogenesis of lung neoplasm. Apurinic/apyrimidinic endonuclease-1/redox factor-1 (APE/ref-1) is a multifunctional protein involved in DNA base excision repair and the redox regulation of many transcription factors. However, the alteration of the expressed levels of APE/ref-1 in non-small cell lung cancer is unknown. ***Material and Method:*** Forty-nine patients with surgically resected non-small cell lung cancer (NSCLC) were included in this study. Immunohistochemical staining with APE/ref-1 antibodies was performed, and their expressions were analyzed via Western blotting for specific antibodies. ***Result:*** APE/ref-1 was localized at the nucleus and mainly in the non-tumor region of the NSCLC tissue specimens; it was expressed in the cytoplasm and nucleus of the NSCLC. The nuclear and cytoplasmic expressions of APE/ref-1 in lung cancers were markedly up-regulated in the NSCLC, and this was correlated with the clinical stage. Catalase, as first-line antioxidant defense, was dramatically decreased in the NSCLC. ***Conclusion:*** Taken together, our results suggest that APE/ref-1, and especially cytoplasmic APE/ref-1, was upregulated in the lung cancer regions, and this may contribute to the compensatory defense system against oxidative stress. A low expression of catalase might have fundamental effects on the extracellular redox state of lung tumors, along with the potential consequences for the tumors.

(Korean J Thorac Cardiovasc Surg 2007;40:529-535)

Key words: 1. Lung neoplasms
2. Proteins

BACKGROUND

Lung cancer continues to be a one of the major cause of cancer death, and smoking tobacco which containings reactive oxygen species, is the primary cause of lung cancers. Lung cancer is a common pathology with high mortality due to late diagnosis. The lungs are directly exposed to higher oxygen concentrations than most other tissues. Lung tissue is pro-

*충남대학교 의과대학 생리학교실
Department of Physiology, College of Medicine, Chungnam National University
**충남대학교 의과대학 흉부외과학교실
Department of Thoracic and Cardiovascular Surgery, College of Medicine, Chungnam National University
†이 연구는 충남대학교 임상의학 연구소와 한국과학재단, 한국학술진흥재단 연구비에 의해 연구되었음.
논문접수일 : 2007년 4월 11일, 심사통과일 : 2007년 7월 2일
책임저자 : 이 영 (301-721) 대전시 중구 대사동 640, 충남대학교 의과대학 흉부외과학교실
(Tel) 042-280-7375, (Fax) 042-280-7373, E-mail: y_lee@cnu.ac.kr

tected against these oxidants by a variety of antioxidant mechanisms such as superoxide dismutases. An imbalance between of oxidants and antioxidants induced oxidative stress in lung tissues. Increased oxidative stress is a significant part of the pathogenesis of lung cancer as well as obstructive lung diseases and parenchymal lung diseases. In addition to oxidative stress, oxidative DNA damage and DNA repair may mediate several cellular processes, like replication and transcription, mutagenesis and apoptosis and thus may be important for the organism development as well as its pathogenesis, including cancer. Among the endogenous mechanisms that repair oxidative DNA damage is the ubiquitously expressed apurinic/apyrimidinic endonuclease/redox factor-1 (APE/ref-1). APE/ref-1 is a multifunctional protein that is responsible not only for DNA repair[1]. It is an essential endonuclease in the base excision repair pathway of oxidative damaged DNA, as well as having reducing properties that promote the binding of redox-sensitive transcription factors such as activator protein-1 to their cognate DNA sequences[2,3]. In particular APE/ref-1 also has an important function against oxidative stress. Reduction of APE activity by antisense RNA has been reported to sensitize cells to oxidative DNA damage[4,5]. Conversely, overexpression of APE repair function provokes an increase in resistance to some alkylating agents and oxidative stress[4,6]. Additionally, several mutation of APE/ref-1 were uncovered in lung cancer[7,8]. As the N-terminal of APE/ref-1 has putative nuclear localization signals, its mutation may affect the subcellular localization. As well as a nuclear role of APE/ref-1, an extra-nuclear role of APE/ref-1 in the regulation of endothelial oxidative stress has been discovered. APE/ref-1 suppresses oxidative stress through modulation of cytoplasmic rac1-regulated reactive oxygen species generation[9,10]. Although previous studies suggest that antioxidant activity is impaired in lung cancers[11-13], the net oxidative stress levels in lung cancer are unknown due to several kinds of antioxidant enzyme that could be up-regulated. Because of the critical role of APE/ref-1 has in DNA repair and oxidative stress, and its previously altered levels of expression and localization in the lung cancer[14,15], we have focused our attention on APE/ref-1 in lung cancer. We investigated the expression levels and cellular localization of APE/ref-1 in the human lung cancer specimens.

Table 1. Characteristics of patients subjected in lung cancer

Characteristics	Results
Sex (M : F)	35 : 14 (71.4% : 28.6%)
Mean age (year)	63.2±7.9 (47～77)
Histology	
Squamous cell carcinoma	23 (46.9%)
Adenocarcimona	25 (51.0%)
Other	1 (2.0%)
Pathologic stage	
I	29 (59.2%)
II	11 (22.4%)
III	8 (16.3%)
IV	1 (2.0%)

MATERIAL AND METHOD

1) Tissue specimens

Samples from normal lung and a consecutive series of tumor specimens from 49 patients with operable non-small cell lung cancer (NSCLC) were obtained from the archives of the Department of Pathology and Thoracic surgery at the Chungnam National Hospital between June 2004 and September 2006. Forty-nine samples of fresh frozen lung tumors, 23 squamous cell lung carcinomas, 25 adenocarcinoma, and 1 other tumor in liquid nitrogen were used for immunohistochemistry and Western blot analysis. No chemotherapy or radiotherapy was given before surgery. Distant healthy-looking adjacent tissue samples from the same patients were used as control specimens. The ages of the patients ranged from 47 to 77 years. The histopathologic and data for cases are summarized in Table 1.

2) Immunohistochemistry for APE/ref-1

Cellular localization of APE/ref-1 was determined using immunohistochemical staining. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections. Tissue specimens were cut into 4-um sections from representative tumor and non-tumor blocks. Monoclonal antibody for human

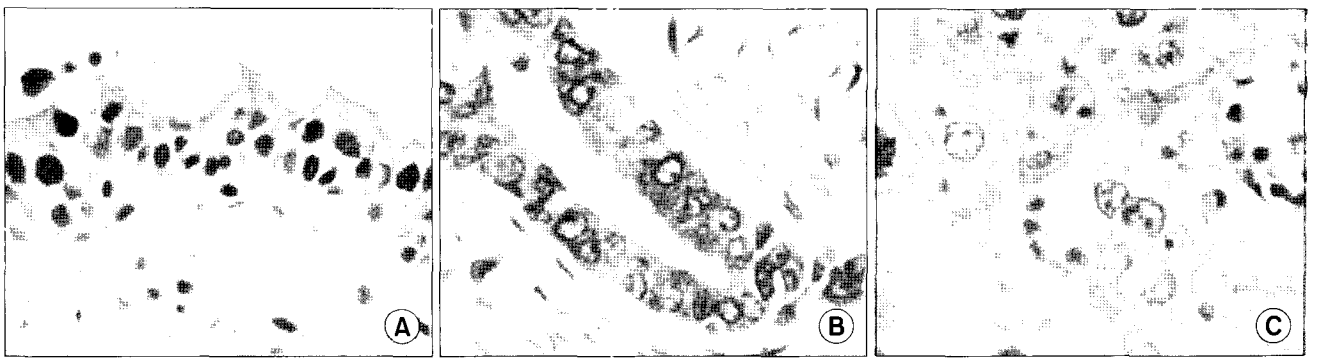

Fig. 1. Immunohistochemical stain for APE/ref-1 in lung tissues. (A) Normal bronchus, (B) Adenocarcinoma, (C) Squamous cell carcinoma.

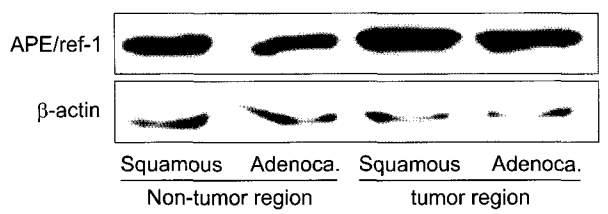

Fig. 2. Expression of APE/ref-1 in the non-tumor and tumor regions of squamous and adenocarcinoma. APE/ref-1 expression levels was assessed by the Western blot as described in Material and Method. β-actin used as loading control.

APE/ref-1 were used with dilutions of 1 : 600 for an anti-APE/ref-1 antibodies (1 : 600, Novus Biological). The color was developed with 3,3'diaminobenzidine (DAKO, USA). The sections were counterstained lightly with hematoxyline and mounted by using Immu-Mount (Thermo Shandon, USA).

3) Western blot

For the Western blot analysis, 50 g of protein was run under denaturating and reducing condition, transferred to nitrocellulose membranes, and treated with APE/ref-1, Catalase (dilution 1 : 1,000, 1 : 2,500, respectively)[16,17]. β-actin was used as a maker for protein loading. 30 mg of samples were first homogenized with a Dounce homogenizer. To obtain cytoplasmic fractions, cell pellets were suspended in cytoplasmic lysis buffer (20 mmol/L HEPES, pH 7.9, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1% Nonidet P-40, 10 mmol/L NaCl, 0.4 mmol/L PMSF). The nuclear soluble fraction was obtained by re-suspending pellets from these lysates in nuclear lysis buffer (20 mmol/ L HEPES, pH 7.9, 1 mmol/L EDTA, 1 mmol/L DTT, 0.1% Nonidet P-40, 25% glycerol, 420 mmol/L NaCl, 0.4 mmol/L phenylmethylsulfonyl fluoride). Immunoreactivity was detected with an enhanced chemiluminescence (ECL) kit (Amersham Pharmacia Biotech).

4) Statistical analysis

Values are expressed as the mean±SEM. Statistical evaluation was performed using Student's t-test, with $p<0.05$ considered significant.

RESULT

1) Expression of apurinic/apyrimidinic endonuclease (APE) in lung carcinoma

Immunohistochemical analysis for APE/ref-1 was performed in the tissue specimens with NSCLC. In the non-tumor regions, the predominant pattern of APE/ref-1 expression was nuclear, however, cytoplasmic and mixed nuclear/cytoplasmic expression was observed in the tumor region (Fig. 1). To evaluate the expression levels of APE/ref-1, Western blot was performed in the tissues of lung tumor and non-tumor regions of squamous and adenocarcinoma. APE/ref-1 expression was upregulated in the tumor region compared with non-tumor regions. No significant difference was observed in APE1/ref-1 pattern according to histotype (squamous vs adenocarcinoma) (Fig. 2).

To study the expression level of APE/ref-1, Western blot was performed in the nuclear and cytoplasmic fractions of the lung cancer tissue specimens. Nuclear APE/ref-1 expression in the tumor region was significantly higher than that in

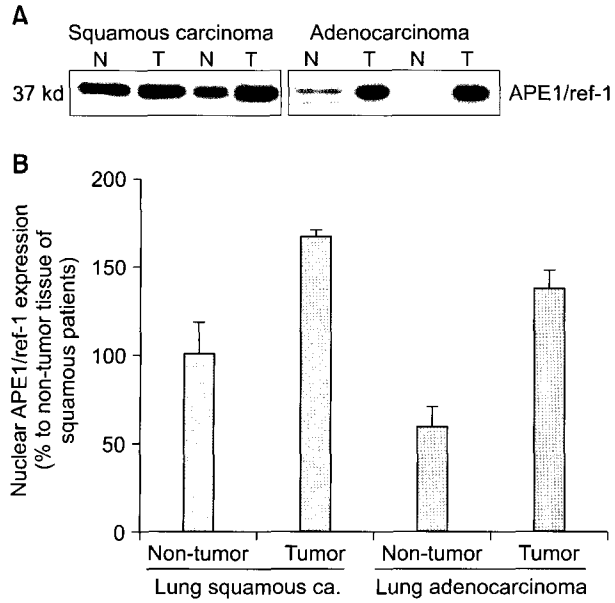

Fig. 3. Nuclear expression of APE/ref-1 in the non-tumor (N) and tumor (T) regions of squamous and adenocarcinoma. (A) Western blot analysis for APE/ref-1. (B) Summarized data of nuclear expression of APE/ref-1 (n=16). Expression levels were represented as % expression to non-tumor tissue of squamous carcinoma patients.

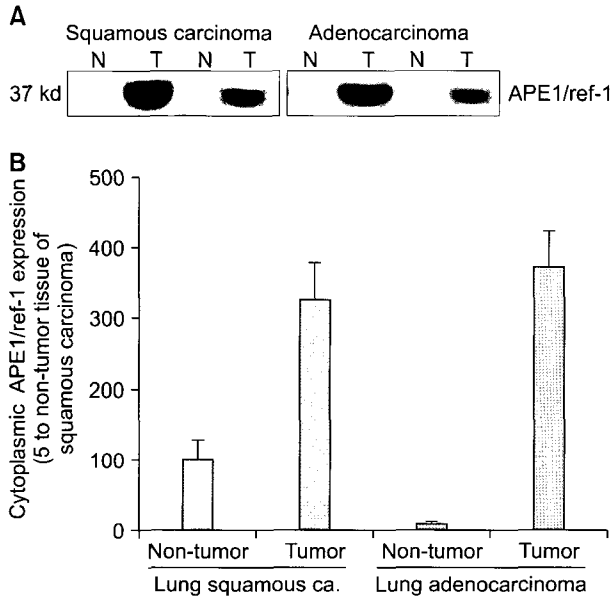

Fig. 4. Cytoplasmic expression of APE/ref-1 in the non-tumor (N) and tumor (T) regions of squamous and adenocarcinoma. (A) Western blot analysis for APE/ref-1. (B) Summarized data of nuclear expression of APE/ref-1 (n=16). Expression levels were represented as % expression to non-tumor tissue of squamous carcinoma.

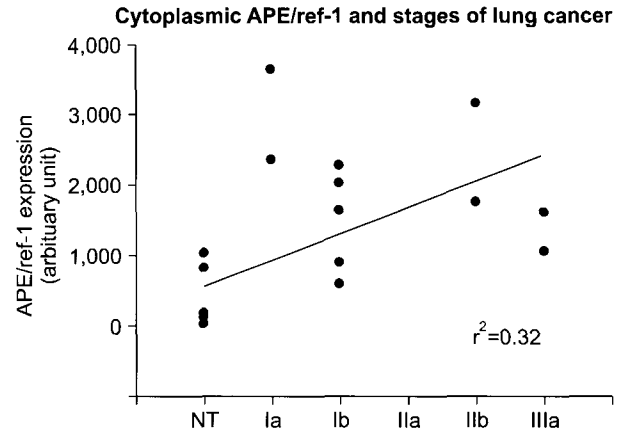

Fig. 5. Correlation of cytoplasmic APE/ref-1 and lung cancer stages. As a control, APE/ref-1 expression levels of non-tumor regions (NT) were used.

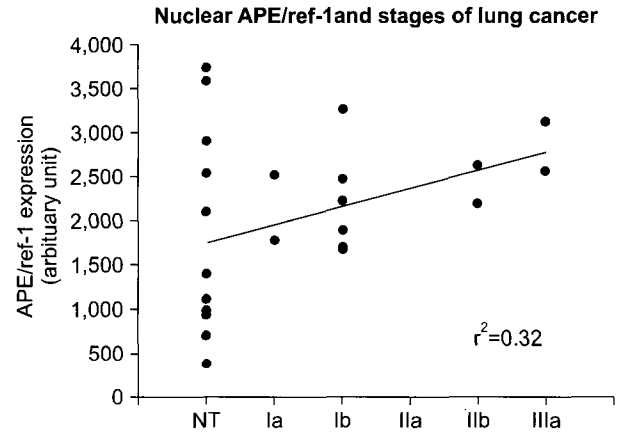

Fig. 6. Correlation of nuclear APE/ref-1 and lung cancer stages. As a control, APE/ref-1 expression levels of non-tumor regions (NT) were used.

non-tumor region in both lung cancer (Fig. 3). Cytoplasmic APE/ref-1 in the cytoplasmic fraction of the non-tumor region was minimal, however, it in tumor-region was markedly upregulated (Fig. 4).

2) Correlation of APE/ref-1 with stage of lung cancer

To see whether APE/ref-1 is associated with a particular stage of lung cancer, expression levels of cytoplamic or nuclear APE/ref-1 were analyzed with the stage of lung cancer. For this analysis, expression levels of non-tumor regions were evaluated as stage 0. Cytoplasmic APE/ref-1 expression was strongly correlated with stage of lung cancer (r^2=0.32)(Fig. 5),

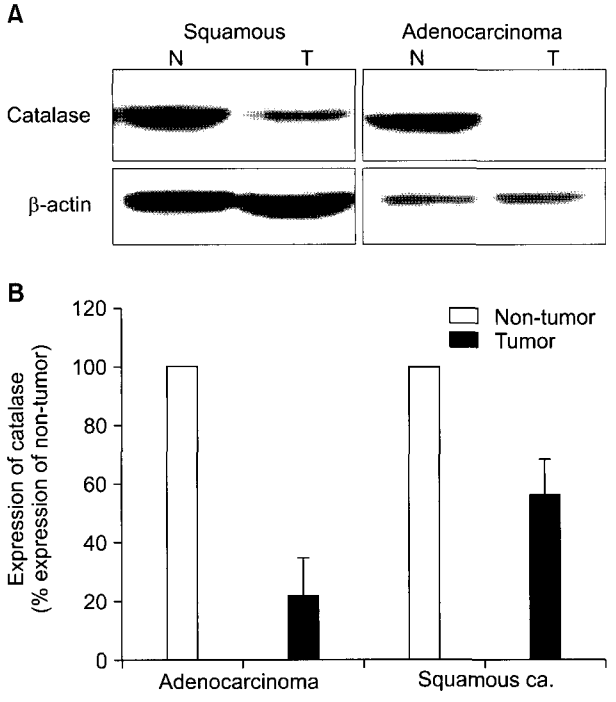

Fig. 7. Expression of catalase, antioxidant enzymes, in the non-tumor (N) and tumor (T) regions of squamous and adenocarcinoma. (A) Western blot analysis for catalse. (B) Summarized data of the expression levels of catalase (n=8). Expression levels were represented as % expression to each non-tumor tissue. β-actin used as loading control.

however nuclear APE/ref-1 expression was slightly correlated with stages of lung cancer(r^2=0.13)(Fig. 6).

3) Altered expression of catalase in the lung cancer

Catalase is known as an important antioxidant to remove hydrogen peroxide in the cellular system. Therefore, we investigated the expression level of catalase with Western blot in the fresh frozen lung cancer specimens. Catalase expression of lung cancer was significantly decreased in the tumor regions, compared with non-tumor regions (Fig. 7). No significant difference was observed in catalase expression according to histotype (squamous vs adenocarcinoma).

DISCUSSION

In this study, we demonstrated the altered expression of APE/ref-1 and catalase in the NSCLC. In addition, a cytoplasmic expression of the APE/ref-1 protein was strongly correlated with advanced stage, regardless of histotype. APE/ref-1 is a multifunctional protein that is not only decisively involved in base excision repair (BER), but also in the activation of several proliferation-associated transcription factors[3,18]. Therefore, tumor cells that overexpress APE/ref-1 might not only be protected against endogenous and exogenous DNA damaging agents inducing lesions repaired by BER but could also have a selection advantage, leading to faster and/or more aggressive proliferation. Whether different expression of APE/ref-1 in various tissues is related to the repair or redox function of APE/ref-1, and/or to cell proliferation and differentiation, is still unclear. Superoxide dismutases are known to be potential regulators of intracellular and extracellular redox status. It is highly likely that low expression of extracellular superoxide dismutase might have fundamental effects on the extracellular redox state of lung cancer with potential consequences on cancer behavior[19]. Also, lipid peroxidation in cancer was increased[20] which it may be due to the poor antioxidant system as observed in the previous studies[21]. In this study, APE/ref-1 expression in lung cancer was increased compared with non-tumor regions, which suggests that upregulated APE/ref-1 expression could be related to a compensatory mechanism against increased oxidative stress in the lung cancer tissue. An interesting observation was the inverse correlation between nuclear and cytoplasmic expression of APE/ref-1. APE/ref-1-positive cytoplasm was higher in tumor region than in non-tumor regions. However, lung tumors showed a weak nuclear staining, compared with cytoplasmic staining. In particular, these tumors showed a relatively strong cytoplasmatic staining. This suggests that some tumors may be deficient in nuclear translocation of APE, resulting in strong cytoplasmic and negative nuclear APE/ref-1 reactivity. Interestingly, intensity of cytoplasmic APE/ref-1 expression was associated with stage of lung cancer. In contrast, intensity of nuclear APE/ref-1 expression was neither associated with it. These data suggest that strong expression of APE/ref-1 in cytoplasm has been correlated with the progression or stage of lung cancer. The nuclear localization signal is potentially located in the 1~35 amino acid of APE/ref-1[22,23]. Therefore mutation of this nuclear localization signal of APE/ref-1 would be related to the cytoplasmic APE/ref-1 in the lung cancer. It was even re-

ported that mutation of APE/ref-1 (Ile64Val, Asp148Glu) was uncovered[7,8]. Further study needs to verify why cytoplasmic APE/ref-1 is upregulated in the lung cancer using nucleotide mutational analysis.

CONCLUSION

We have shown that high levels of cytoplasmic APE/ref-1 are associated with tumor stages in lung cancer. Furthermore a low expression of the catalase system may be an early event in the multi-step process of carcinogenesis of lung cancers.

REFERENCES

1. Xanthoudakis S, Miao GG, Curran T. *The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains.* Proc Natl Acad Sci USA 1994; 91:23-7.
2. Xanthoudakis S, Curran T. *Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity.* Embo J 1992;11:653-65.
3. Evans AR, Limp-Foster M, Kelley MR. *Going APE over ref-1.* Mutat Res 2000;461:83-108.
4. Grosch S, Fritz G, Kaina B. *Apurinic endonuclease (Ref-1) is induced in mammalian cells by oxidative stress and involved in clastogenic adaptation.* Cancer Res 1998;58: 4410-6.
5. Silber JR, Bobola MS, Blank A, et al. *The apurinic/apyrimidinic endonuclease activity of Ape1/Ref-1 contributes to human glioma cell resistance to alkylating agents and is elevated by oxidative stress.* Clin Cancer Res 2002;8:3008-18.
6. Tomicic M, Eschbach E, Kaina B. *Expression of yeast but not human apurinic/apyrimidinic endonuclease renders Chinese hamster cells more resistant to DNA damaging agents.* Mutat Res 1997;383:155-65.
7. Zienolddiny S, Campa D, Lind H, et al. *Polymorphisms of DNxA repair genes and risk of non-small cell lung cancer.* Carcinogenesis 2006;27:560-7.
8. Popanda O, Schattenberg T, Phong CT, et al. *Specific combinations of DNA repair gene variants and increased risk for non-small cell lung cancer.* Carcinogenesis 2004; 25:2433-41.
9. Angkeow P, Deshpande SS, Qi B, et al. *Redox factor-1: an extra-nuclear role in the regulation of endothelial oxidative stress and apoptosis.* Cell Death Differ 2002;9: 717-25.
10. Ozaki M, Suzuki S, Irani K. *Redox factor-1/APE suppresses oxidative stress by inhibiting the rac1 GTPase.* Faseb J 2002;16;889-90.
11. Jaruga P, Zastawny TH, Skokowski J, Dizdaroglu M, Olinski R. *Oxidative DNA base damage and antioxidant enzyme activities in human lung cancer.* FEBS Lett 1994;341:59-64.
12. Guner G, Islekel H, Oto O, Hazan E, Acikel U. *Evaluation of some antioxidant enzymes in lung carcinoma tissue.* Cancer Lett 1996;103:233-9.
13. Coursin DB, Cihla HP, Sempf J, Oberley TD, Oberley LW. *An immunohistochemical analysis of antioxidant and glutathione S-transferase enzyme levels in normal and neoplastic human lung.* Histol Histopathol 1996;11:851-60.
14. Puglisi F, Aprile G, Minisini AM, et al. *Prognostic significance of Ape1/ref-1 subcellular localization in non-small cell lung carcinomas.* Anticancer Res 2001;21: 4041-9.
15. Kakolyris S, Giatromanolaki A, Koukourakis M, et al. *Nuclear localization of human AP endonuclease 1 (HAP1/ Ref-1) associates with prognosis in early operable non-small cell lung cancer (NSCLC).* J Pathol 1999;189: 351-7.
16. Jeon BH, Gupta G, Park YC, et al. *Apurinic/apyrmidinic endonuclease 1 regulates endothelial NO production and vascular tone.* Circ Res 2004;95:902-10.
17. Kim CS, Son SJ, Kim EK. *Apurinic/apyrimidinic endonuclease1/redox factor-1 inhibits monocyte adhesion in endothelial cells.* Cardiovasc Res 2006;69:520-6.
18. Fritz G. *Human APE/Ref-1 protein.* Int J Biochem Cell Biol 2000;32:925-9.
19. Svensk AM, Soini Y, Paakko P, Hiravikoski P, Kinnula VL. *Differential expression of superoxide dismutases in lung cancer.* Am J Clin Pathol 2004;122:395-404.
20. Kaynar H, Meral M, Turhan H, Keles M, Celik G, Akcay F. *Glutathione peroxidase, glutathione-S-transferase, catalase, xanthine oxidase, Cu-Zn superoxide dismutase activities, total glutathione, nitric oxide, and malondialdehyde levels in erythrocytes of patients with small cell and non-small cell lung cancer.* Cancer Lett 2000;227:133-9.
21. Szatrowski TP, Nathan CF. *Production of large amounts of hydrogen peroxide by human tumor cells.* Cancer Res 1991;51:794-8.
22. Jeon BH, Gupta G, Park YC. *Apurinic/apyrimidinic endonuclease 1 regulates endothelial NO production and vascular tone.* Circ Res 2004;95:902-10.
23. Jackson EB, Theriot CA, Chattopadhyay R, Mitra S, Izumi T. *Analysis of nuclear transport signals in the human apurinic/apyrimidinic endonuclease (APE1/Ref1).* Nucleic Acids Res 2005;33:3303-12.

=국문 초록=

배경: 산화제와 항산화제 사이의 불균형은 폐암의 발생에 중요한 역할을 하는 것으로 생각되는 산화스트레스를 유발한다. APE/ref-1은 DNA의 복구와 많은 전사인자들의 산화환원 조절에 연관된 다 기능성 단백질이다. 그러나 폐암에서 APE/ref-1 발현수준의 변화는 알려져 있지 않다. **대상 및 방법**: 49명의 수술적으로 제거한 비소세포성 폐암 환자를 대상으로 하였다. APE/ref-1 항체에 대한 면역조직화학적 염색을 하였고, 특이 항체에 대한 Western blot을 시행해 발현 정도를 분석하였다. **결과**: APE/ref-1은 주로 폐암 조직의 비 암세포 부분은 핵에 국한되어 존재하였고, 암세포는 핵과 세포질 모두에 존재하였다. 비소세포성 폐암의 핵과 세포질의 APE/ref-1의 발현은 증가되어 있었고 이는 임상적인 병기와도 상관관계를 보였다. 대표적인 항산화 물질인 catalase는 비소세포성 폐암에서 현격하게 감소되어 있었다. **결론**: APE/ref-1, 특히 세포질 내의 APE/ref-1의 증가는 산화스트레스에 보상적으로 일어나는 것으로 생각하며 catalase의 감소는 폐암의 특성을 나타내는 세포 외부의 산화환원 과정에 근본적인 역할을 하는 것으로 생각한다.

중심 단어 : 1. 폐암
2. 단백질