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Chronic stress promotes Lung cancer development via IGF-IR pathway
만성적인 스트레스가 폐암 발생에 미치는 영향 연구

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ABSTRACT

Chronic stress promotes Lung cancer development via IGF-IR pathway

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Lung cancer is a leading cause of cancer-related death worldwide. Although smoking is a main cause, approximately 25% of lung cancer cases are not attributable to tobacco use. Although various risk factors, including Radon, ETS(Environmental Tobacco Smoke), genetic and viral factors, have been implicated in the pathogenesis of lung cancer in never smokers, this study suggests that chronic stress is also one of the risk factors. Recent mechanistic studies demon-

strate that chronic stress is associated with the progression of various cancer types. But the underlying mechanisms for the association between chronic stress and lung cancer development are poorly understood.

Stress hormone, norepinephrine (NE)-induced activation of beta-adrenergic receptor(ADBR) pathway is involved in various cellular process including cancer onset, cancer progression, inflammation, angiogenesis and immune system. We observed that a continuous exposure of NE induced transformation of human lung bronchial epithelial cells and activated type-1 insulin-like growth factor receptor (IGF-IR) signaling pathway. To examine the effect of chronic stress on lung cancer formation in vivo, we established CUS(Chronic unpredictable stress) mouse model. We found that chronic stress resulted in high levels of NE in mouse serum, activation of IGF-IR in lung tissue and promoted lung tumor formation. Moreover, we confirmed that inhibition of the IGF-IR pathway suppressed NE-induced cell transformation in vitro. To investigate the mecha-

nism underlying the NE-induced activation of IGF-IR pathway, we first analyzed expression level of IGF-IR axis in human lung bronchial epithelial cell lines. We confirmed that NE-induced IGF-IR activation was occurred in cell line with high-expression of IGF-II. Treatment of NE increased intracellular calcium level thought beta adrenergic receptor pathway and induced calcium-mediated secretion of IGF-II protein. Also, we showed that IGF-BP3 which regulates function of IGF-II protein is downregulated by treating NE. In conclusion, NE increased availability of IGF-II and then activated IGF-IR pathway. Our results indicate that stress hormone, NE may promote lung cancer development via the IGF-IR pathway, suggesting that blockade of IGF-IR is important for controlling chronic stress-promoted lung cancer development

Key words : Chronic Stress, Norepinephrine, Beta-adrenergic signaling, Lung cancer in never smokers(LCINS), IGF-IR pathway

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I. INTRODUCTION

Lung cancer is one of the most common cancers worldwide with over 1million deaths each year [1, 2]. Although the great majority of lung cancer cases are strongly associated with tobacco use, approximately 10 to 25% of all cases are not attributable to tobacco use. A common definition of a ‘never smoker’ is individual who has smoked less than 100 cigarettes per lifetime. If we consider lung cancer in never smokers (LCINS) as a separate category of cancers, it ranks the seventh most cause of cancer death worldwide [1-3]. Because many studies have focused on the pathogenesis of tobacco-mediated lung carcinogenesis, the molecular mechanisms of neoplastic transformation in never-smokers have been poorly understood [4]. Moreover, because a better understanding of mechanisms of lung carcinogenesis by smoking is likely to provide new insights on cancer prevention, the proportion of never smokers with lung cancer is expected to increase.

Therefore, the more study of mechanism underlying LCINS is needed. The mere existence of LCINS suggests that risk factors other than smoking must be present. Although various potential factors, including Radon, ETS(Environmental Tobacco Smoke), genetic and viral factors, have been implicated in the pathogenesis of LCINS, this study suggests that chronic stress is also one of the potential factors.

Epidemiological and clinical studies indicate that psychological factors including chronic stress, depression, and social support might influence cancer onset and progression. Recent mechanistic studies showed that stress hormone-mediated biological signaling pathways could contribute to such effects [4, 5]. In response to environmental stress, sympathetic nervous system (SNS) neural fibers and adrenal glands release the catecholamine neurotransmitter norepinephrine (NE) into target tissues to homeostasis. NE receptors, adrenergic receptors are present in many organs including brain, lung, liver, kidney, breast and ovary. By activating adrenergic receptor pathway,

NE initiates intracellular second messenger processes that could lead to carcinogenesis, with various times of onset. Many animal studies demonstrated that high level of NE in various organs increases tumorigenesis, whereas low level on NE decreases tumorigenesis [5-7]. Also, in humans, the use of tricyclic antidepressant, which may increase the level of NE throughout the body, may be related to increased rates of cancer [7-9]. Moreover, a cohort study of cardiovascular patients showed that the beta-blocker use relative to never-using appeared to decrease the cancer risk significantly [10-12]. These finding suggest that psychological stressors are associated with increased release of NE and exposure to stress is potentially a cancer risk factor.

The adrenergic receptors (or adrenoceptors) are cell surface receptors and a class of G protein-coupled receptors (GPCR), also known as seven-transmembrane domain receptors, 7TM receptors that have high affinity of the catecholamines, especially NE and EPI (epinephrine). There are two main groups of

adrenergic receptors, α and β, with several subtypes. It is well known that the beta-adrenergic receptor (ADRB) is the most involved in the carcinogenic processes. There are the three subtypes of β-adrenergic receptor, $\beta 1$, $\beta 2$, and $\beta 3$ in many sites of tumor growth and metastasis.

Ligation of their receptors by NE and EPI activates the Gas guanine nucleotide-binding protein which stimulates adenylyl cyclase, catalyzing a synthesis of cyclic adenosine monophosphate (cAMP). The resulting transient cAMP accumulation can regulate a diverse array of cellular processes including Protein kinase A (PKA) and Epac. These receptors are also directly associated with one of its ultimate effectors, the class C L-type calcium channel (LTCC). They can induce conformation change of LTCC and increase calcium influx. [5, 13-14]

Beta-adrenergic signaling has been found to regulate multiple cellular processes including inflammation, angiogenesis, cell motility, cellular immune response and epithelial-mesenchymal transition (EMT). Also, many studies demonstrated that ADRB

signaling pathway contributes to the initiation and progression of cancer. But the underlying mechanisms for the association between chronic stress and lung cancer development are poorly understood.

In the present study, I investigated the effect of chronic stress on lung tumorigenesis and mechanism underlying tumor promotional effect of stress hormone-NE. NE increased intra-cellular calcium level and bioavailability of IGF-II by increas-ing secretion of IGF-II and downregulating of IGF-BP3 which result in activation of IGF-IR pathway. The activation of this IGF-IR pathway is key role of the promotional effect of chronic stress on lung tumorigenesis.

II. METARIALS AND METHODS

Chemicals and reagent

Norepinephrine, Epinephrine, Cortisol and DMSO was purchased from sigma (Sigam-Aldrich, st.Louis, MO, USA). We obtained MTT[3-(4, 6-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] from MP Biomedicals LLC(Illkirch,. France). Atenolol, ICI 118,551 and propranolol were purchased from Tocris (Tocris Bio-science, MS, USA).

Cell lines, cell culture

HBEC, HBEC/Ras and HBEC/p53I were provided by Dr. J. Minna (University of Texas Southwestern Medical Center, Dallas, TX) . HBE cell lines and BEAS-2B cells were immortalized with a hybrid adenovirus/simian virus 40(provided form Dr. A. Klein-Szanto, Fox Chases Cancer Center, Philadelphia, PA). The human bronchial epithelial cell lines were grown at 37 in a humidified incubator under 5% CO2 in supplemented K-SFM medium (Invitrogen)

MTT assay

Increase of cell viability in the absence of growth factors by norepinephrine (Sigma-aldrich) was used to assess by 3-(4, 6-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro-mide(MTT) (MP Biomedical, LLC) assay. Briefly, Human bronchial epithelial cells seeded onto 96-sell plates at a den-sity of 1000-2000 cells per well. 24h after seeding, cells were treated with different concentration of NE and same concen-tration of NE with inhibitors in the absence of growth factors. After 5 days. 0.5 mg/mL of MTT solution was added to each sell and incubated for 4h at 37 in a humidified 5% Co2 air at-mosphere. When each cells stained clearly, remove medium. DMSO was added to each well for dissolve the crystals and the plates were read at 570nm using a microplate reader.

Anchorage-dependent / independent growth assay

For anchorage-dependent growth assay, BEAS-2B cells were seeded into six-well plate at low density (200 cells per

well). Every 3 days, cells were treated NE with different inhibitors. After 8~12days, in a humidified atmosphere with 37 and 5% Co2, colonies were fixed in 99% Methanol and stained with 0.05% crystal violet solution. We then counted the number of colonies.

For the anchorage-independent growth assay, as a bottom agar, 1 mL of medium containing 1% SeaPlaqueGTG agarose (Lonza Rockland, ME) was added to each well of a 24-well cell culture plate and left to solidify at room temperature. 5000 cells, resuspended in 0.5mL of medium containing 0.04% agar, were plated as triplicate. The plate was kept at room temperature for 2-3h until the top agar solidified. Every 3-4 days, medium containing NE with different inhibitors was changed. After 10-15days, colonies were stained with 500ug/mL MTT for 2h at 36. Images of colonies were taken by Fuji LAS-3000 imaging system, and counted the number of colones.

Foci formation assay

Transformed cells grow without contact inhibition. Normal cells divided until they reach a confluent monolayer. However, because transformed cells lose ability of contact inhibition, they can grow in dense and make the multilayer, foci. Briefly, HBE cell lines were seeded into 24-well cell culture plated at a density of 10000 cells per well. 24h after seeding, cells were treated NE with different inhibitors in the absence of growth factors. Every 3-4days medium on same conditions was changed. After 10-15days, foci were taken by microscopy and counted the number of foci.

Flow cytometry assay

BEAS-2B cells were plated at a concentration of $1.2X10^6$ cells/100 mm plates. The next day, cells were stained with fluo-4 am for 30min, and then treated with either PBS as a control or Norepinephrine (10uM) for various times. All cells were harvested, and subjected to flow cytometric analysis to

determine the percentage of intracellular calcium level in cells. Flow cytometric analysis was performed using a BD FACS Calibur.

siRNAs and transfection

BEAS-2B and HBEC/p53i cells were transfected with 20nmol/L si RNA against IGF-II at 80% confluence in growth factor free K-SFM media for 24 h using lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) according to the manu-facturer's instructions. At 24h after siRNA transfection, cells were treated NE or PBS. IGF-II and control siRNA oligonu-cleotieds were purchased from Dharmacon.

Western blotting analysis

To prepare whole cell lysate, cells were lysed with iced-cold lysis buffer (1% NP-40, 0.25% sodium deoxycholate, 1mM EDTA, 150mM NaCl, 50mM Tris-HCL[pH 7,4], 1% Triton X-100, 10% Glycerol) including a complete protease inhibitor

cocktail table (Roche diagnostics, Mannheim, Germany) and phosphatase inhibitors (Na3VO4 1mM, NaF 100mM, NaPP 10mM) or concentrated conditioned media. Concentration of protein samples was determined by using a biochinoconinic acid(BCA) assay kit (Pierce Biotechnology, Rockford, IL, USA). Equivalent amounts of protein were loaded using sodium dodecyl sulfate (SDS)-polyacrylamide gels (6-15%). After transferred to a polyvinyl difluoride (PVDF) membrane, mem-brane was rocked with 3% BSA in Tris-buffered saline 0.1% Tween20 (TBST) for 1h. Membrane was incubated for 1h at room temperature or 12h at 4℃. The membrane was then washed 3 times per 1h with Tris-buffered saline (TBS) con-taining 0.1% Tween20 (TBST). The membrane was incubated with appropriate secondary antibody for 1h at room tempera-ture and washed again in same way. And the protein-antibody complex was visualized by ECL solution (Femto) (Thermosci-entific INC, Bremen, Germany)

Animal experiment for chronic stress

For 1 month, we first induced stress through CUS mouse model. Chronic unpredictable mouse model were explained in table 1. And then we treated urethane (1g/kg) to induce initiation of lung tumor. After 3 months (mice were exposed to stress for 4month), mice were sacrificed.

Micro osmotic pumps (Alzet model 1002, 1004 and 2006. Durect, Cupertino, CA) were implanted subcutaneously on the back of the mice. The pumps were filled with physiological saline containing 30mM (1002) 68.1 mM (1004) and 50.1mM (2006) norepinephrine (which equals a daily dose of 1 lmol/100 g bodyweight) [33-34]. After 2-6 weeks, the pumps were renewed. We exposed NE to mice for 12 weeks.

Amlodipine (10mg/kg) administration was done oral gavage daily. Amlodipine was dissolved at 45% poly(ethylene glycol)(Sigma Aldrich) and 45% D.W and 10% DMSO. Amlodipine was purchased from Enzo.

MMP florescence combining CT imaging

After CUS experiment for 4month, 150 ul of MMPsence680 (PerkinElmer) was injected into teil vein. After 24 hours, flo-rescence combining CT image was gained using FLIT (Fluo-rescent Imaging topography) mode on IVIS SpectrumCT l In Vivo Imaging System.

Immunostaining assay

Formalin fixed paraffin embedded tissue specimens were pre-pared for immunofluorescence staining. First, deparaffinize sections in xylene, 5min 2 times then, Hydrate with 100% Ethanol 5min 2 times and hydrate with 90% ethanol, 5min 2times and rinse in distilled water, 5min. Next, blocking was assessed with BSA solution (Dako, carpinteria, CA). After blocking slides are stained with specific primary antibodies over-night in cold chamber. In next day slides are wash by PBST 5 times then, slides are stained with secondary antibody in room temperature. After 2h, wash slides 5 times again then,

gently drop the DAPI solution on the slides and evaluate under the fluorescence microscopy.

Tumor multiplicity and volume

Consecutive sections were made for every tissue block of the lungs and stained with H&E. We counted tumor number in 4 slide section, and tumor growth was monitored by the tumor volume: Volume (mm3) = width2 (mm2) × length (mm) /2.

III. Result

1. Effect of Stress hormone, norepinephrine (NE) on human lung bronchial epithelial cells

We first examine the effect of NE on the transformation properties of immortalized, non-tumorigenic human lung bronchial epithelial cell lines, BEAS-2B and HBEC/p53i. We observed that continuous exposure to NE increased cell viability (Figure 1A), anchorage dependent or independent colony formation (Figure 1B/ and C) in the absence of growth factors in BEAS-2B cells and foci formation in HBEC/p53i cells (Figure 1D).

2. Chronic stress promotes lung tumorigenesis in mice.

To investigate whether chronic stress influences lung tumorigenesis, we established CUS (chronic unpredictable stress) mouse model (Table 1). Briefly, After 4 month with CUS, we first detect the tumor by using the IVIS spectrum CT imaging system (Figure 2A). We observed that CUS had no effect on lung tumor initiation. Tumorigenesis is a multistep process. We

next investigated the effect of NE on tumor promotion stage. To induce lung tumor initiation, we used urethane (g/kg) which is a carcinogen to produce neoplasms of the lungs. We showed that CUS significantly increased urethane-induced lung tumor multiplicity (Figure 2B). KRAS mutations represent the most common genetic change in lung cancer. In the mouse, K-ras mutations are found in >90% of spontaneous and chemically induced lung tumors (Malkinson 1998). We also checked the effect of CUS on lung tumorigenesis in Kras-lung cancer mouse model. The signal was (Figure 2C) In this model, CUS mice showed significantly greater lung tumor growth than con-trol mice (Figure 2D). Taken together, These data suggest that CUS promotes lung tumorigenesis but not initiation.

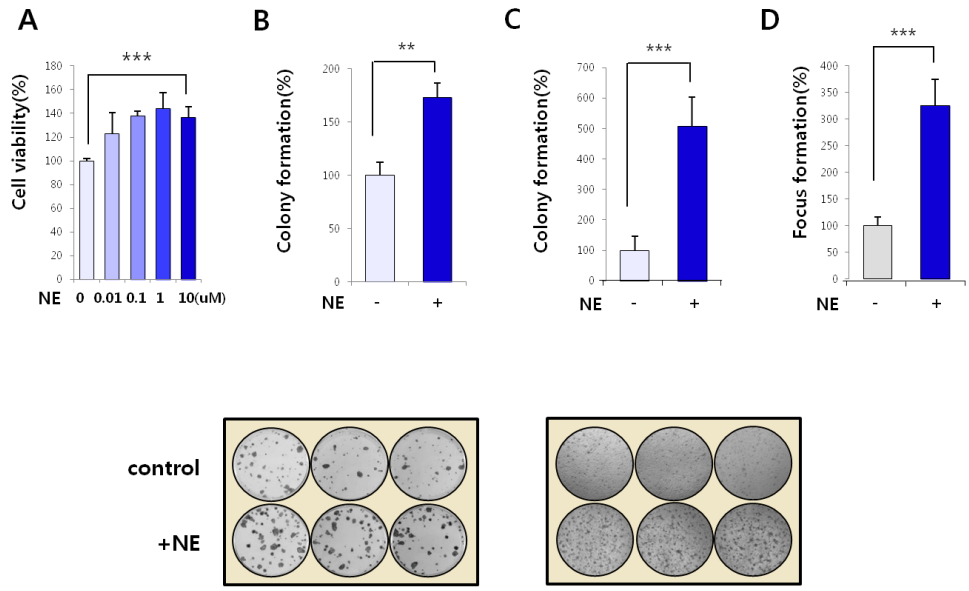

Figure 1. Norepinephrine induced transformation of human bronchial epithelial cells.

NE-induced cell transformation was confirmed by MTT assay (A), anchorage dependent assay (B), soft agar assay (C) in BEAS-2B cells and foci formation assay (D) in HBEC/p53i cells. Results are expressed as relative cell viability and the ability of colony formation compared with PBS as a control. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$

Table 1. CUS (Chronic Unpredictable Stress) Schedule.

Day	Chronic unpredictable stress
Day 1	Shaking (120 rpm, 30min) and light on overnight
Day 2	Free
Day 3	Restraint stress for 2hr and light on overnight
Day 4	Free
Day 5	Forced swim for 5~10min (warm or cold water)
Day 6	Light off for 2hr (pm 1:00 ~3:00)
Day 7	Free

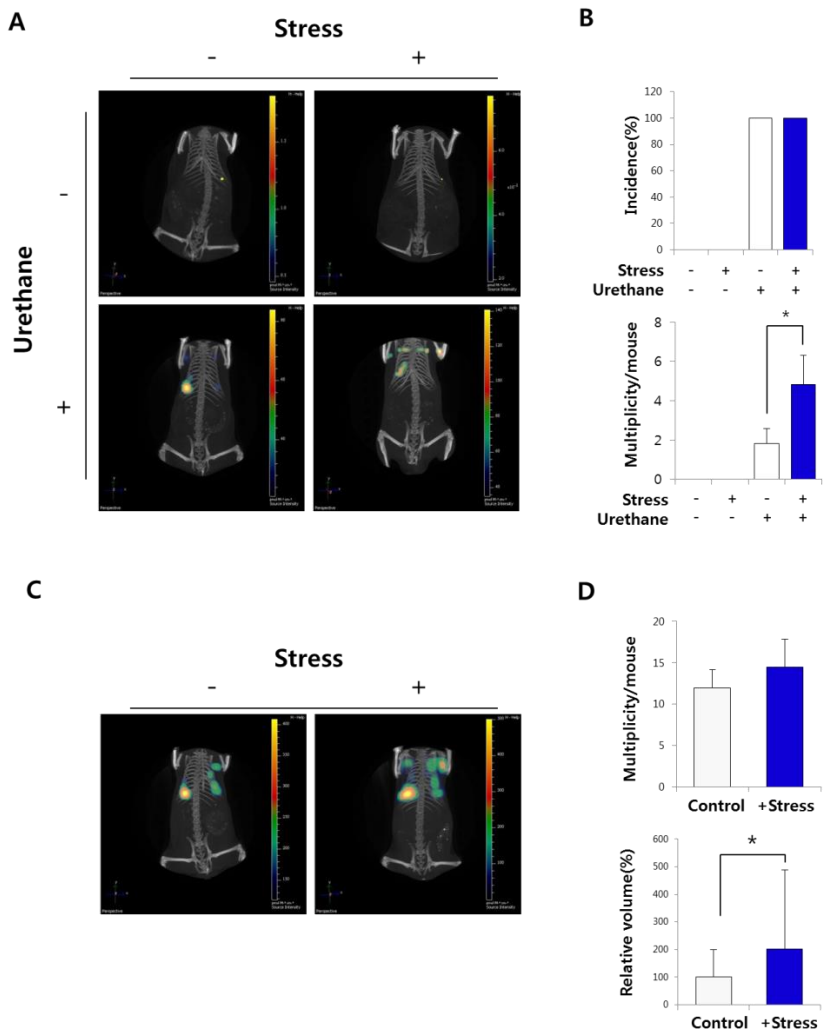

Figure 2. Chronic stress promoted lung tumorigenesis in mice.

Tumor in mouse lung was quantitatively measured by IVIS spectrum CT imaging system. Twenty-four hours after 2 nmol MMPSense680 probe was injected, mice were scanned (A, C). After sacrificing the mice, we counted the number of tumor (B, D) and measure the volume (D).

3. Stress hormone, norepinephrine (NE) promotes lung tumorigenesis in mice. NE is one of the main stress hormones. We first confirmed CUS could increase NE in mouse serum by using the ELISA kit (Figure 3A). We showed that the NE level in mice group with CUS was about five times higher than the group without CUS. To confirm whether the promotional effect of CUS on lung tumoigenesis is due to NE, we exposed mice to NE by using the micro osmotic pump which can deliver NE at controlled and continuous rate. After implantation of micro osmotic pump subcutaneously on the back of mice, we injected urethane for initiation of lung tumor. As shown in figure 3B, when exposed to NE, lung tumor multiplicity was increased. These result strongly suggest that stress-induced hormone, NE has a promotional effect on lung tumorigenesis.

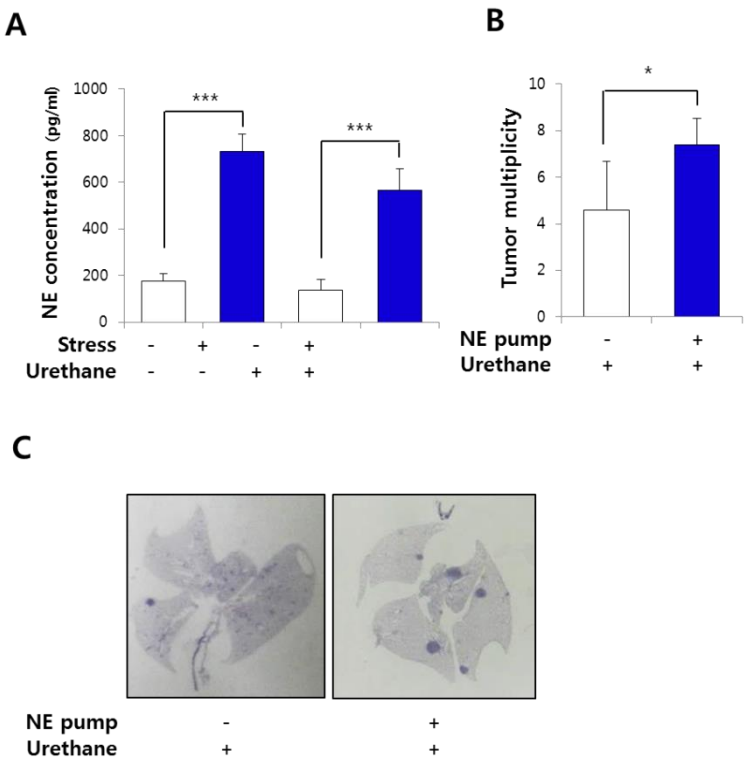

Figure 3. The effect of NE on lung tumorigenesis.

NE level in mouse serum was measured by using ELISA kit (MyBioSource, MBS2600834)(A). After micro osmotic pump containing NE was implanted on the back of mouse, we showed that the effect of NE on urethane-induced lung tumorigenesis. The tumor multiplicity in the mice group with NE was more increased than in the mice group without NE (B). Representative picture of H&E staining (C).

4. NE activates IGF-IR pathway in HBE cells and CUS activates IGF-IR in mouse lung tissue.

To investigate changes that are associated with NE, we used receptor tyrosine kinase signaling antibody array kit. As shown in Figure 4A, IGF-IR was up-regulated by stimulation of NE in BEAS-2B cell. We carried out the western blot and confirmed that NE activated IGF-IR pathway in dose and time dependent manners (figure 4B). We also identified that the level of phospho-IGF-IR in mouse lung tissue was higher in CUS group than control group (Figure 4C).

5. IGF-IR signal is strongly associated with cell transformation and the promotional effect of CUS on lung tumorigenesis.

The above findings suggested that NE-mediated IGF-IR signaling may have an important role in cell transformation and lung tumorigenesis in mice. Hence, we evaluated the effects of suppressing IGF-IR signaling on cell transformation. Anchor-

age dependent (Figure 5A) and Anchorage independent (Figure 5B)-colony formation was significantly decreased by inhibition of IGF-IR signaling. To determine the role of IGF-IR signaling in CUS-promotional effect of lung tumorigenesis in vivo, we analyzed mice (FVB/N background) carrying lung-specific human IGF-IR transgene (Tg). When we detect the tumor by using the IVIS spectrum CT imaging system, we showed that the signal was detected in urethane and urethane with CUS group in both IGF-IR Tg and FVB mice. But in comparison, the signal in IGF-IR Tg was higher than in FVB (Figure 5C). Also, CUS-promoted lung tumor formation was greater in IGF-IR Tg than in FVB mice (Figure 5D). We showed similar effect with micro osmotic pump containing NE (data not shown). Altogether, these results strongly indicate that IGF-IR synergizes with NE to accelerate lung tumorigenesis.

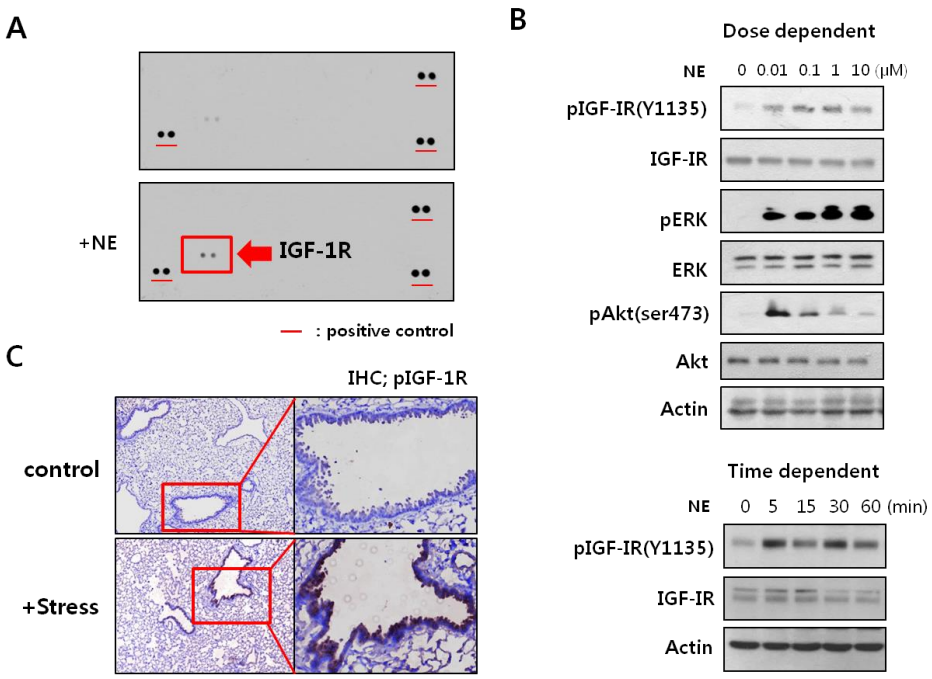

Figure 4. Stimulation of NE activated IGF-IR pathway in BEAS-2B cell and chronic stress also activated IGF-1R in mice lung tissue.

BEAS-2B cell ws treated with PBS or NE for 5min. NE increased phosphorylation of IGF-IR as detected by RTK signaling antibody array kit (R&D system, ARY001B)(A). NE-induced activation of IGF-IR pathway was detected by western blot analysis (B). IHC staining of phospho-IGF-IR (C). CUS increased phosphorylation of IGF-IR in mouse lung.

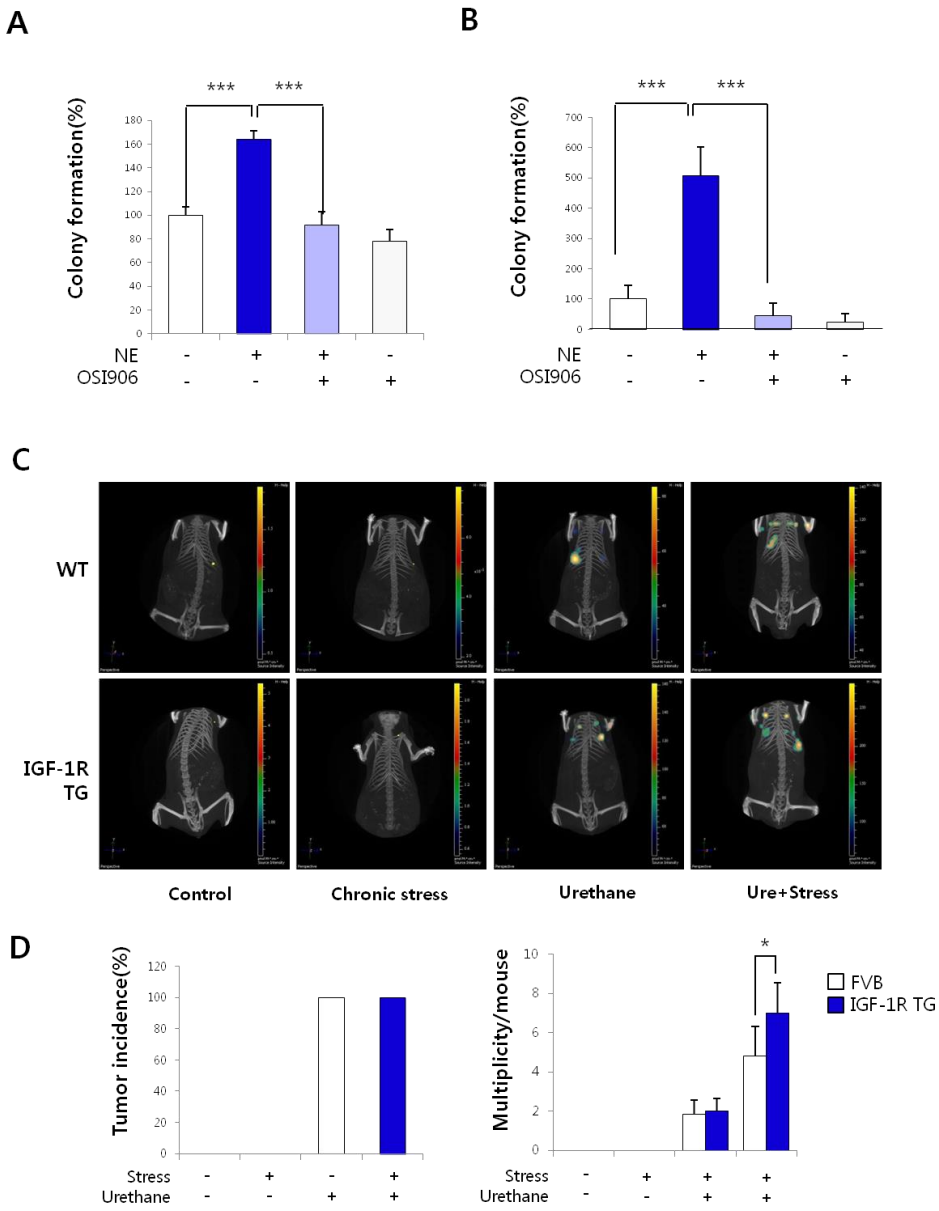

Figure 5. Effects of IGF−IR signal blocking on the NE−induced cell transformation and the promotional effect of NE on lung tu−morigenesis in IGF−IR Tg mice.

Effects of IGF-IR signaling blocking on the NE-induced cell transformation was confirmed by anchorage dependant assay (A) and soft agar assay (B). Tumor in mouse lung was quantitatively measured by IVIS spectrum CT imaging system (C). Tumor incidence and multiplicity (D). IGF-IR Tg showed greater CUS promotional effect than FVB. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$

6. NE induced cell transformation of HBE cell lines and activated IGF-1R in IGF-2 ligand- dependent manner.

To demonstrate the mechanism of NE-promoted lung tumorigenesis, we used CDK4/hTERT-immortalized normal human bronchial epithelial cell (HBEC) lines which are used to study lung cancer pathogenesis by introducing combinations of common lung cancer oncogenic changes(KRAS, p53). We first investigated the effect of NE on HBE cells. The stimulation of NE increased cell viability (Figure 6A) and foci formation (Figure 6B) in HBEC/p53i and HBEC/Ras cells but not in HBEC. NE also activated IGF-IR in HBEC/p53i and HBEC/Ras cells but not in HBEC (Figure 6C).

7. Dependence of HBE cells on autocrine IGF-II for cell transformation.

Because of the difference among HBE cell lines, we measured the expression level of IGF-IR axis by using real-time PCR. We observed that IGF-II expression was more highly ex-

pressed in HBEC/Ras and HBEC/p53i cells than in HBEC (Figure 7A). IGF-II is a secretory protein. To confirm whether NE increases secretion of IGF-II protein, we analyzed conditioned media after stimulating NE. We also showed that amount of IGF-II protein in conditioned media was increased by stimulation of NE in time-dependent manner. In addition, IGF-BP3 which regulates IGF ligands was also showed time-dependent decrease (Figure 7B). To assess whether bioavailability of IGF-II is a key role in NE-mediated IGF-IR activation, we performed the western blot analysis after stimulating NE with neutralizing IGF-II antibody. NE-induced IGF-IR activation was abrogated by neutralization of IGF-II (Figure 7C). We also showed that knock down of IGF-II by targeted siRNA in BEAS-2B and HBEC/p53i cells was similar to the effects with the neutralizing IGF-II antibody (Figure 7D). Moreover, NE-induced IGF-1R activation was enhanced by knock down of IGF-BP3 (Figure 7E). To directly test the role of IGF-II, we evaluated the effect of knockdown of IGF-II in BEAS-2B cells

on colony formation capability. As shown in Figure 7F, NE could not increase colony formative rates in BEAS-2B cells knocked down IGF-II. Taken together, these results suggest that the bioavailability of IGF-II is important role of NE-induced IGF-IR activation and the effect of NE on transformation of lung bronchial epithelial cells.

8. NE induced IGF-1R activation through Gas pathway of β-adrenergic receptor.

To determine the route of NE-induced IGF-IR activation, we treated NE with various inhibitors which are related to β-adrenergic receptor pathway. By western blot analysis, we found that NE activated IGF-IR through Gas pathway of β-adrenergic receptor (Figure 5A, B). Also, when we blocked PLC or IP3 receptor which are related to intracellular calcium, NE-induced IGF-IR activation was suppressed. These data indicated that NE-induced IGF-IR activation may be associated with intracellular calcium level.

9. The NE -induced IGF-IR activation is Ca-dependent process

To investigate whether NE-induced IGF-IR activation is associated with calcium, we first measured intracellular calcium level by FACS analysis (Figure 9A). We showed that NE could increase intracellular calcium level in BEAS-2B cell in short time. We next analyzed expression level of IGF-IR protein by stimulation of NE with calcium chelator, EGTA or BAPTA-AM. NE-induced IGF-IR activation was abrogated by calcium chelator. ADRB pathway is related to L-type calcium channel. Activation of ADRB signaling increased calcium influx by inducing conformational change of LTCC. To determine whether LTCC is related to increase intracellular calcium, and then occurs activation of IGF-IR pathway, we performed western blot analysis with LTCC blockers, Amlodipine and Nifedipine. LTCC blockers suppressed NE-induced activation of IGF-IR (Figure 9C) as well as secretion of IGF-II (Figure 9D). We already showed that IGF-BP3 also downregulated by stimulation of NE

in time dependent manner. It is known that calcium activate MMP family which can degrade IGF-BP3. We examined the association with MMPs on NE-induced IGF-IR activation through western analysis. As shown Figure 9E, inhibition of MMPs activity could suppress NE-induced IGF-IR activation. Taken together, these result suggest that increase of intracel-lular calcium through ADRB signaling pathway is related to IGF-II bioavailability, and it is important role of NE-induced IGF-IR activation.

10. Blockade of β-adrenergic receptor and L-type calci-um channel suppresses cell transformation and lung tu-morigenesis in vivo.

Because of above finding, we expected that blockade of ADRB and LTCC can suppressed NE-induced cell transformation. Hence, we evaluated the effects of suppressing ADRB or LTCC on cell transformation. We showed that NE-induced increase of cell viability (Figure 10A, D), anchorage dependent (Figure

5B) and anchorage independent (Figure 5C)－colony formation with inhibitor of ADRB or LTCC were significantly decreased. We also confirmed that blockade of LTCC could suppressed the promotional effect of CUS in lung tumorigenesis in vivo (Figure 11).

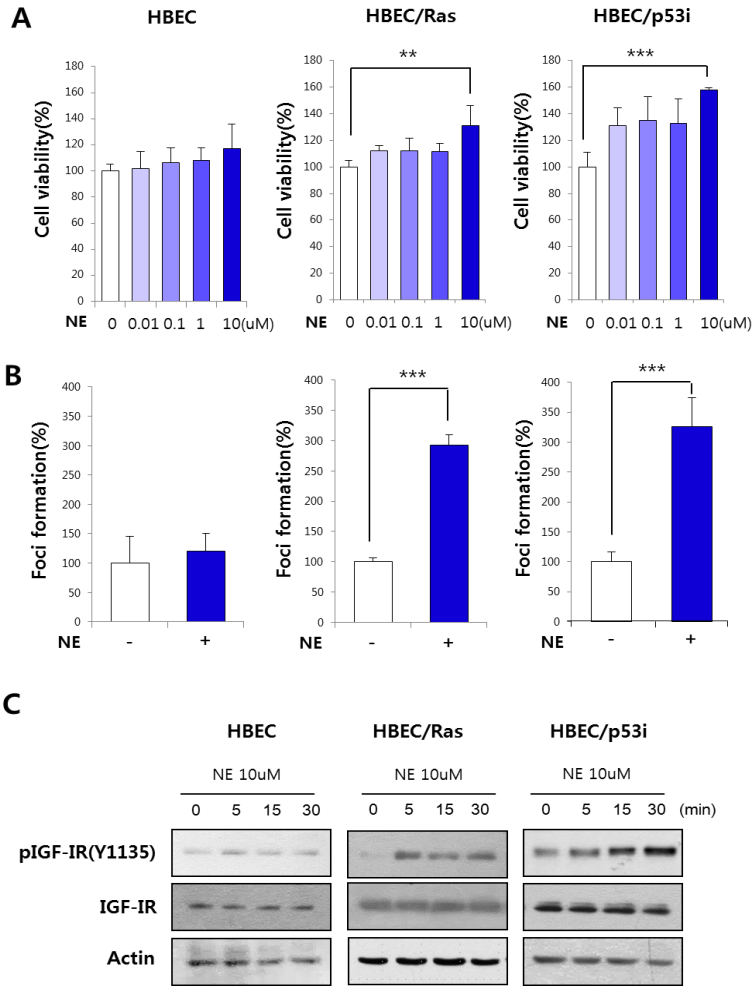

Figure 6. The effect of NE on HBE cell lines.

The effect of NE was confirmed by MTT assay (A) and foci formation assay (B) in HBE cell lines. Results are expressed as relative cell viability and the ability of foci formation com-pared with PBS as a control. HBE cells were treated with PBS or NE 10uM for indicated time. The expression of protein level was measured by western blot analysis (C).

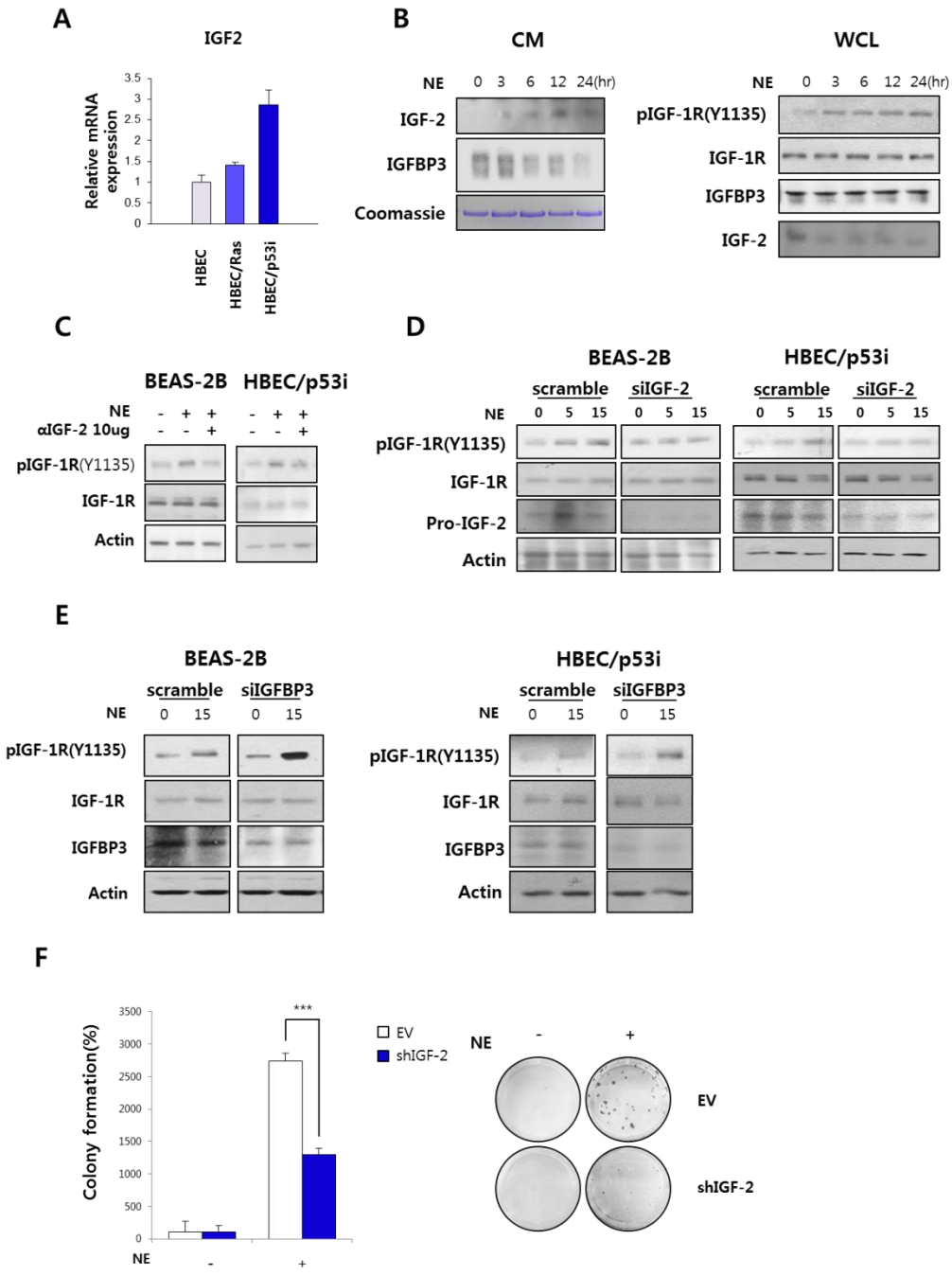

Figure 7. NE – induced IGF−1R activation is dependent on IGF−2 production

The expression level of IGF-II in HBE cell lines was measure by real-time PCR (A). After stimulation of NE for indicated time, amount of IGF-II in conditioned media was analyzed by western blot assay (B). After pretreatment of IGF-II neutralizing antibody for 1hr, BEAS-2B and HBEC/p53i was treated with NE (C). BEAS-2B and HBEC/p53i transfected with 20nM si scramble, 20nM siIGF-II (D) and si IGF-BP3 (E) was treated with PBS or NE. Whole cell lysates were analyzed by western blot with the indicated antibodies.

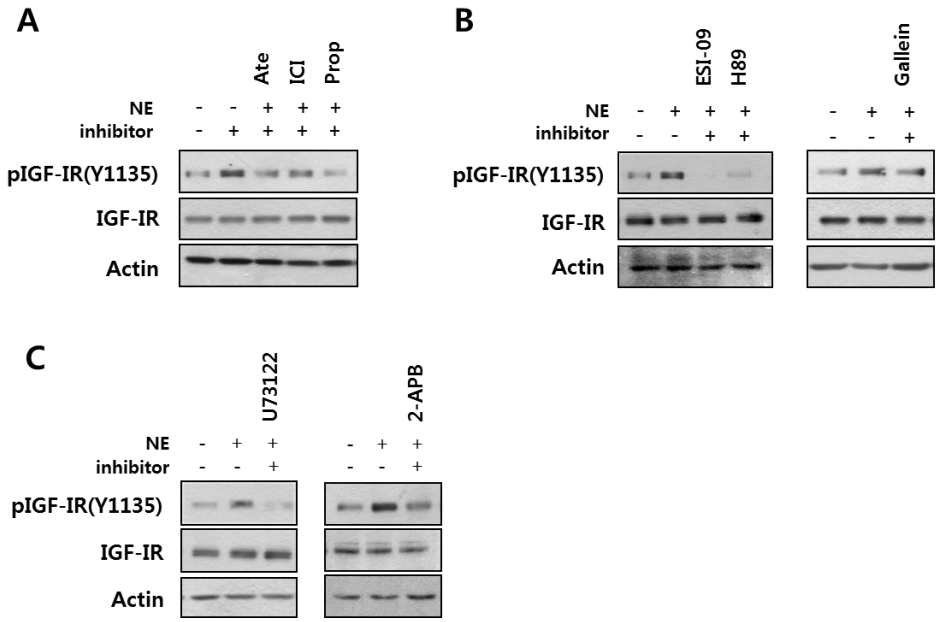

Figure 8. NE induced IGF-1R activation through Gas pathway of β-adrenergic receptor.

After pretreatment of various inhibitors related to GPCR path-way for 3hr, BEAS-2B were treat with NE. Whole cell lysates are analyzed by western blot assay. (Atenolol, β1-adrenergic receptor antagonist; ICI 118,551, β2-adrenergic receptor antagonist; Propranolol, non-selective β-adrenergic receptor antagonist; 10uM. ESI-09, Epac inhibitor; H89, PKA inhibitor; Gallein, G$\beta\gamma$ inhibitor; U73122, PLC inhibitor; 2-APB; IP3R inhibitor)

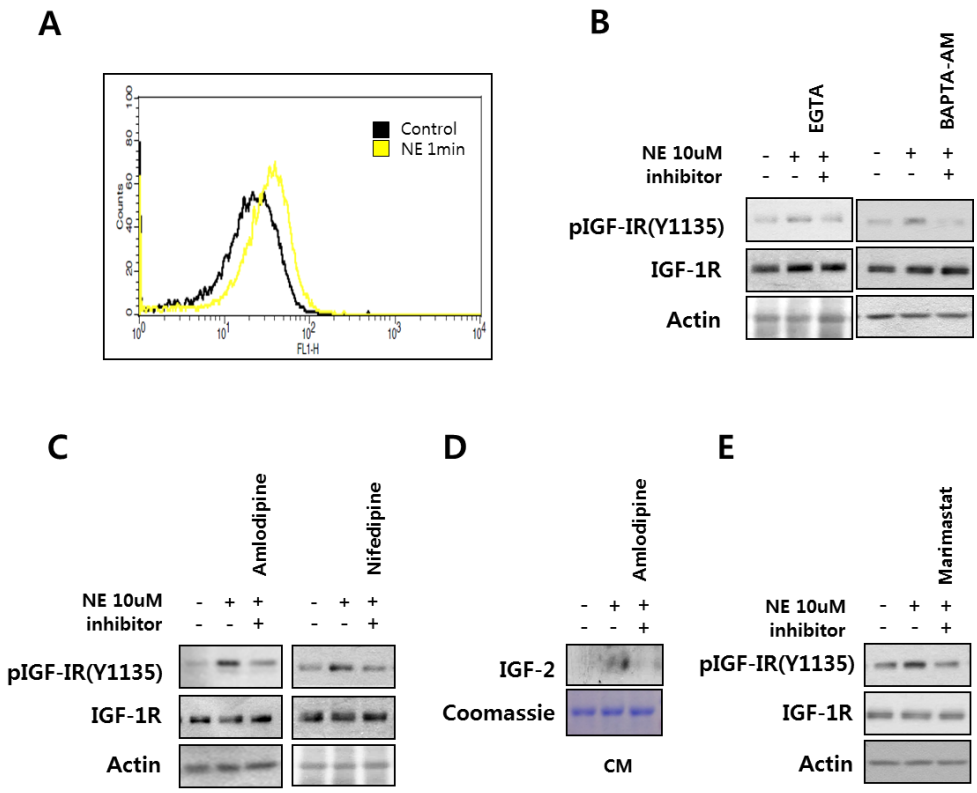

Figure 9. The NE – induced IGF-1R activation is Ca-dependent process

Intracellular calcium level was measure by FACS analysis (A). After pretreatment of calcium chelator (EGTA, BAPTA-AM, L-type calcium channel bloker (Amlodipine and Nifedipine) and pan-MMP inhibitor (Marimastat) for 3hr, BEAS2-2B were treated with NE. Expression of protein was analyzed by west-ern blot assay (B-E).

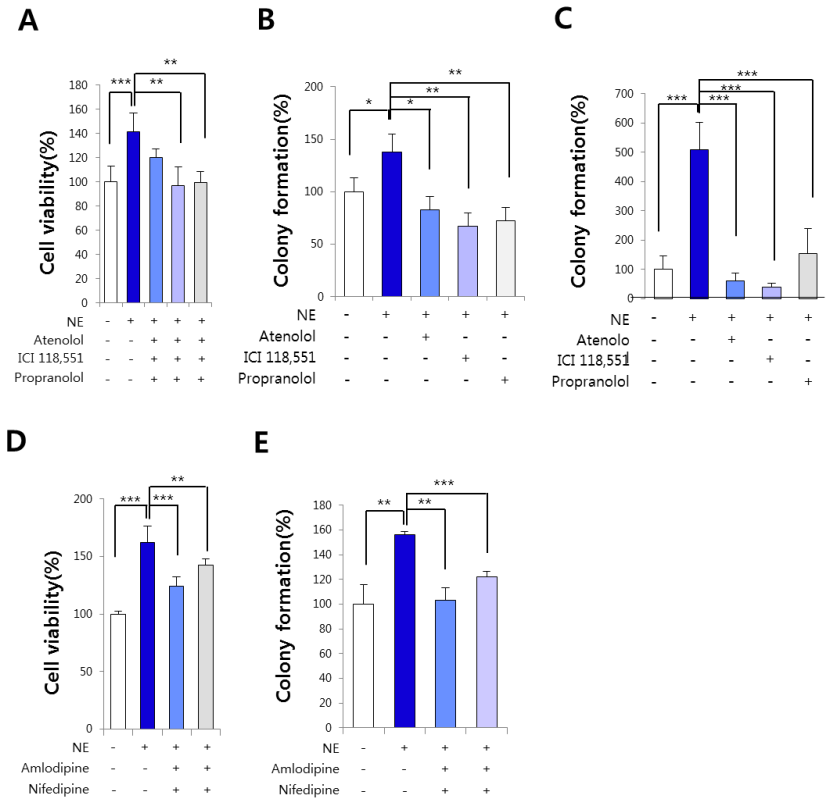

Figure 10. Blockade of β-adrenergic receptor and L-type calcium channel suppresses cell transformation

Effects of blockade of β-adrenergic receptor and L-type calcium channel on NE-induced cell transformation was confirmed by MTT assay (A, D), anchorage dependent assay (B), soft agar assay (C) in BEAS-2B cells. Results are expressed as relative cell viability and the ability of colony formation compared with PBS as a control. * P<0.05, ** P<0.01 and *** P<0.001

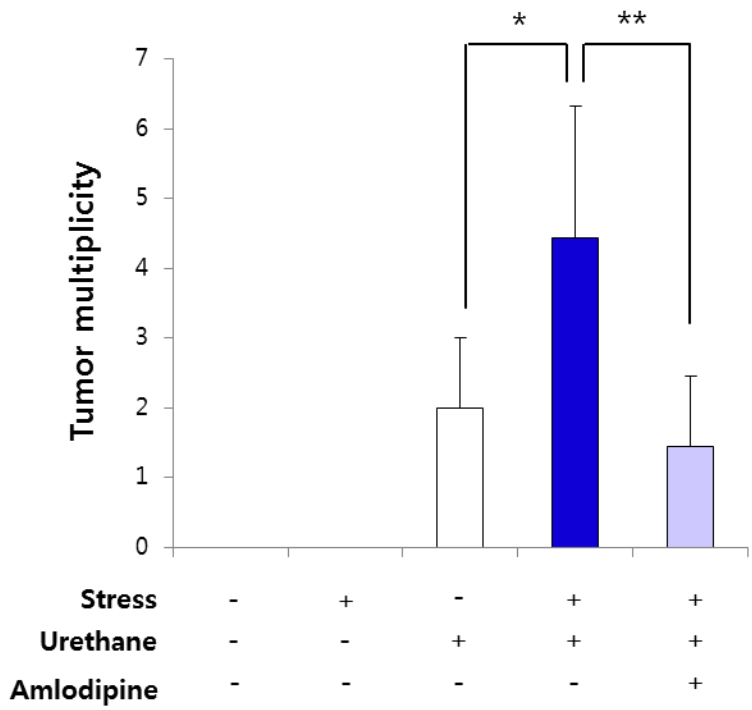

Figure 11. Blockade of L-type calcium channel suppresses lung tumorigenesis

In CUS model, mice were orally administrated amlodipine (10mg/kg) daily. Lung tumor was counted after H&E staining.

IV. Discussion

Chronic stress is a risk factor for the development of many psychopathological conditions in humans, including major depression and anxiety disorders. Recent mechanistic studies suggest that stress hormone-induced biological signaling contributes to various cellular process including inflammation, immune system, cancer onset and progression. Especially, it has been well established that NE-induced adrenergic receptor signaling is associated with these effects. [15-22].

In this study, I also focused on ADRB signaling pathway. It was identified that treatment of the β-adrenergic antagonist prior to each NNK (Nicotine-derived nitrosamine ketone) injection which is present in tobacco and induces lung cancer significantly suppressed the development of PACs (Pulmonary adenocarcinoma). Because of this, I expected that agonist of ADRB, NE also can influence on lung tumorigenesis via ADRB signaling pathway [23].

To investigate association between chronic stress and lung cancer in vivo, I established CUS (Chronic unpredictable stress) mouse model which is a widely used animal model of stress [24-26]. I showed that chronic stress has promotional effect on lung tumorigenesis but it is not enough to cause lung tumor alone. I showed that CUS increased NE level in mouse serum about 5-times more than control mouse. Notably, I identified that the promotional effect of CUS on lung tumorigenesis was due to stress hormone, NE (norepinephrine) by using the mi-cro osmotic pump system. Next, I discovered NE could activate IGF-IR pathway in human bronchial epithelial cells and CUS could also induced activation of IGF-IR in mouse lung tissue. Many studies have demonstrated that IGF-IR signaling is as-sociated with cell survival, proliferation and cancer progres-sion [26, 27]. In addition, our previous study demonstrated that IGF-IR pathway has a critical role of lung cancer devel-opment [29]. In this study, I verified IGF-IR is important role in NE-induced cell transformation and promotional effect of

CUS on lung tumorigenesis. I founded that NE-induced IGF-IR activation is dependent on their ligand, IGF-II. NE increased a bioavailability of IGF-II through (1) secretion of IGF-II and (2) downregulation of IGF-BP3. Moreover, I identified that these event are calcium-dependent process. It is already known that ADRB is associated with L-type calcium channel. ADRB signaling pathway induces conformational change of LTCC by phosphorylation and increase calcium influx. Calcium as second messenger, play an important role in signal transduction pathways. An increase in intracellular calcium directly triggers regulated exocytosis which is the movement of materials out of a cell via membranous vesicles [30]. I showed that blockade of LTCC with amlodipine and nifedipine decreased NE-induced IGF-II secretion. In addition, these inhibitors could suppress NE-induced cell transformation in vitro as well as CUS-promoted tumor formation in vivo.

Because IGF-IR has long been recognized for its role in tumorigenesis and growth, this pathway is regarded as a thera-

peutic target. But attempts to use drug targeted IGF-IR have shown serious side effects such as hyperglycemia, fatigue, and thrombocytopenia [31, 32]. Because of this reason, Blockade of IGF-IR is not appropriate for chemoprevention. This study shows that inhibition of ADRB and LTCC suppress NE-induced IGF-IR activation. And beta-blocker and LTCC blocker (amlodipine and nifedipine) are a medication used to regulation of blood pressure. So, I think that ADRB and LTCC are more appropriate than IGF-IR pathway as target for chemoprevention.

This study showed only NE effect on lung tumorigenesis. But, there are three main stress hormones, norepinephrine, epinephrine and cortisol. Epinephrine is NE's sister molecule and they also through adrenergic receptors. I showed that epinephrine has similar effects of NE on cell transformation and activation of IGF-IR signaling pathway. To confirmed whether cortisol induce transformation of human lung bronchial epithelial cells, I performed MTT assay and anchorage dependent colony formation assay with cortisol. Cortisol could not induce

cell transformation (data not shown). Also, stimulation of cor-tisol did not activate IGF-IR signaling pathway (data not shown). These data suggest that NE may be main hormone of CUS-promotional effect on lung tumorigenesis.

The significances of this study are (1) chronic stress could promote lung tumorigenesis without exposure tobacco, (2) stress hormone, NE-induced ADRB signaling pathway con-tributes to this effects (3) NE -induced IGF-1R activation is a key role in CUS-promotional effects of lung tumorigenesis and (4) this effects is calcium-dependent process.

This study suggests chronic stress may be one of the poten-tial causes in LCINS and we demonstrated the mechanisms of chronic stress-promoted lung cancer development. NE-induced IGF-IR pathway lead to lung tumorigenesis and this event is induced by increase of intracellular calcium through ADRB LTCC. These results indicate that ADRB and LTCC is an effective target for the chemoprevention of stress-induced lung cancer.

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국문초록

폐암은 전 세계적으로 암으로 인한 주요 사망 원인 중 하나이다. 폐암은 주로 흡연에 의해 유발되지만, 비흡연자에서의 폐암 발생이 전체 폐암의 약 25%정도 차지한다. 비흡연자의 폐암 발생에 관여하는 원인으로 라돈, ETS(간접흡연을 하게하는 연기), 유전적 요인, 바이러스 감염 등 여러 인자들이 보고되어 있으나 본 논문에서는 만성적인 스트레스를 주요 원인 중 하나로 제시하고 있다. 최근 연구들을 통해 만성적인 스트레스는 생물학적 신호전달체계를 통해 다양한 암의 유발 및 진행에 관여한다고 보고되어 있다. 그러나 비흡연자의 폐암 발생에서 만성적인 스트레스가 미치는 영향 및 그 조절기전에 관해서는 거의 알려져 있지 않다.

스트레스 호르몬인 노르에피네프린 (NE) 에 의해 활성화 되는 beta-Adrenergic 신호전달체계는 여러 가지 암의 진행뿐만 아니라 염증, 혈관 형성 및 면역반응을 포함한 다양한 cellular process에 관여한다고 보고되어 있다. NE의 지속적인 처리는 폐 기관지 상피세포주인 BEAS-2B 및 HBEC/p53i 세포주의 transformation을 증가시켰으며, 폐암 형성에서 중요한 역할을 하는 것으로 알려져 있는 IGF-IR 신호전달체계를 활성화 시키는 것이 관찰되었다. 본 연구를 위해 확립한 CUS(Chronic unpredictable stress) 마우스 모델에서 지속적인 스트레스의 유발은 마우스의 혈청 내 NE의

양을 증가시키고 폐 조직에서 IGF-IR 신호전달체계를 활성화 시켰으며, 또한 Urethane에 의해 유도되는 폐암 발생을 촉진하는 것이 관찰되었다. 게다가, IGF-IR 저해제인 OSI-906의 처리가 NE에 의한 폐 기관지 상피세포주의 transformation을 억제하는 것을 통해 IGF-IR 신호전달체계가 중요한 역할을 한다는 것을 알 수 있었다. NE에 의한 IGF-IR 신호전달체계의 활성 기전을 규명하고자 먼저 여러 세포주의 비교분석을 통해 IGF-II가 과발현 되어있는 세포에서 NE에 의한 IGF-IR 신호전달체계의 활성화가 일어나는 것을 확인하였다. NE의 지속적인 처리는 세포에서 IGF-II의 분비를 증가시켰고, IGF-II의 분비에는 칼슘이 중요하게 작용하는 것을 알 수 있었다. 또한 NE은 단백질 분해효소인 MMP의 활성을 증가시킴으로써 IGF-II의 역할을 저해할 수 있는 IGFBP3의 분해를 유도하는 것으로 보여진다.

종합적으로 본 논문에서는 만성적인 스트레스가 IGF-IR 신호전달체계를 활성화 시키므로 암의 유발을 촉진할 수 있다는 것을 처음으로 제시하고 있으며, 따라서 IGF-IR 신호전달체계의 저해를 통해 화학적 예방이 가능할 것으로 기대한다.

주요어 : 스트레스, 스트레스 호르몬, 노르에피네프린

폐암, 비흡연 폐암, IGF-IR