ANTICANCER RESEARCH *37*: 2381-2386 (2017)
doi:10.21873/anticanres.11576

# Knockdown of Importin 7 Inhibits Lung Tumorigenesis in K-ras$^{LA1}$ Lung Cancer Mice

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**Abstract.** *Background/Aim: Lung cancer shows the highest estimated deaths in both males and females in the Unites States. Importin 7 is overexpressed in lung adenocarcinoma tissues. In this study, we aimed to demonstrate the anticancer effect of importin 7 down-regulation, especially in lung cancer. Materials and Methods: Glycerol propoxylate triacrylate spermine (GPT-SPE) is a biocompatible carrier used for aerosol gene delivery. Repeated aerosol delivery of GPT-SPE/shImportin 7 complexes was performed to 10-week-old male K-ras$^{LA1}$ mice (a murine lung cancer model) twice a week for 4 weeks (8 times) in a nose-only exposure chamber. Results: Aerosol delivery of GPT-SPE/shImportin 7 inhibits lung cancer in K-ras$^{LA1}$ mice compared to control and scramble control groups. Moreover, importin 7-down-regulated stable cell-line demonstrates suppression of proliferation through Akt inhibition and apoptosis. Conclusion: Down-regulation of importin 7 significantly suppresses lung cancer in vitro and in vivo.*

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*Key Words:* Lung cancer, aerosol delivery, gene therapy, importin 7, GPT-SPE.

According to cancer statistics of American Cancer Society in 2016, lung and bronchus type cancer represent the leading cause of estimated deaths among cancer patients in both men and women (1). Lung cancer shows high mortality in patients; however, current lung cancer treatments have low therapeutic efficacy. Gene therapy is a promising treatment for genetically-based diseases, including cancer. Cancer gene therapy studies have been performed *in vitro* and *in vivo* (2).

Aerosol delivery is a non-invasive and effective approach for the expression of transgenes in the lungs. Fewer side-effects, uniform distribution and exposure to a large alveolar epithelial surface are advantages of aerosol delivery (3). Aerosol administration of plasmid and carrier complexes facilitates the efficiency of gene delivery *in vivo* (4). Various studies have reported that aerosol gene delivery using cationic polymers or viral vectors show therapeutic effects in animal lung cancer models (5).

Cationic carriers have advantages, including ease of modification and low toxicity, for aerosol gene delivery. Polymers containing natural materials, such as polyamines, are more biocompatible compared to polyethylenimine (PEI)-containing carriers (6). Glycerol propoxylate triacrylate spermine (GPT-SPE) has high transfection efficiency and biocompatibility *in vivo* (7). Moreover, therapeutic efficacy of aerosol gene delivery using GPT-SPE has been confirmed in *K-ras$^{LA1}$* lung cancer model mice (8, 9).

Importin 7, also called RanBP7, can cross the nuclear envelope in both directions and bind with GTPase Ran inside the nucleus (10). Importin 7 and importin β play an important role in nuclear import receptor of the histone H1 protein (11).

The heterodimeric complex of importin 7 and importin β also plays a chaperone-like function for H1 histones (12).

Extracellular signal-regulated kinase (ERK)-regulated proliferation requires serial phosphorylations by MEK and casein kinase 2 (13-15). Upon stimulation, the phosphorylation of kinase insert domain allows ERKs to bind with importin 7 that escorts ERKs into the nucleus *via* nuclear pores (16). Moreover, the nuclear penetration of ERKs by importin 7 could be a target for cancer treatment (17).

Here we show the therapeutic effect of importin 7 down-regulation in LA-4 mouse lung cancer cell-line and *K-ras$^{LA1}$* murine lung cancer model mice.

## Materials and Methods

*Materials*. Bax, Bcl-xL, GAPDH, actin and alpha-tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Akt, PARP and p53 antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Importin 7 antibody was purchased from Abcam (Beverly, MA, USA).

*Plasmids and shRNA target sequence*. Importin 7 shRNA in pRFP-C-RS vector was purchased from OriGene Technologies Inc. (Rockville, MD, USA). Sequence of shRNA against importin 7 is 'TTCAACACTGCTCCAGATTACTATGTCAG'.

*Cell culture and generation of importin 7-down-regulated stable cell line*. LA-4 mouse lung cancer cell-line was purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). LA-4 cell line was maintained in RPMI medium (HyClone, Logan, UT, USA) with 15 % fetal bovine serum (FBS) 1 % penicillin/streptomycin (GibcoBRL, Grand Island, NU, USA). For the generation of an importin 7-down-regulated stable cell line, shRNA of importin 7 plasmid was transfected using TransITR-LT1 (Mirus Bio Corp., Madison, WI, USA). Transfected clones were selected by culturing the cells in media containing puromycin (1 μl/ml; InvivoGen, San Diego, CA, USA) for a 2-week period.

*Western blot analysis*. Human lung and tumor tissues were obtained from the Korea Lung Tissue Bank (KLTB, Seoul, Korea). Experiments using human tissues were authorized by the KLTB (KU Guro Gene Bank 2012-004) and Seoul National University Institutional Review Board (SNUIRB-E1201/001-001). LA-4 cells were lysed using 1× Cell Culture Lysis Buffer (Promega, Madison, WI, USA), while human tissues and mouse lung tissues were homogenized using 2.5× Passive Lysis Buffer (Promega). The protein concentrations of lysates were measured using a Bradford kit (Bio-Rad, Hercules, CA, USA). Immunoblotting was performed by blocking in 5% skim milk in Tris-buffered saline + Tween 20 (TBS-T) for 1 h and incubating overnight with the primary antibody at 4˚C and with secondary antibody conjugated to horseradish peroxidase (Invitrogen, Carlsbad, CA, USA) for 3 h at room temperature. Bands were detected using an image detector Ez-Capture MG (ATTO, Tokyo, Japan).

*Aerosol delivery of GPT-SPE/shImportin 7 complexes*. All animal experiments in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University (SNU-121012-1). *K-ras$^{LA1}$* lung cancer model mice were obtained from Human Cancer Consortium-National Cancer Institute (Frederick, MD, USA) and cared in the laboratory animal facility with a 12-hour light/dark cycle. Humidity and temperature were controlled at 50±10 % and 23±2˚C, respectively. For aerosol delivery, ten-week-old male *K-ras$^{LA1}$* mice were randomly divided into the following 3 groups (4 mice per group, total 12 mice); control, small hairpin scramble vector control (shScr) and small hairpin of importin 7-delivered (shIPO7) groups. Aerosol-containing complexes of 8 mg GPT-SPE and 0.8 mg shRNA (small hairpin scramble or small hairpin importin7) were delivered to *K-ras$^{LA1}$* mice twice a week for 4 weeks. Aerosol was generated by a nebulizer (Korean patent #20304964) and delivered to mice in a nose-only exposure chamber. Tumors on the lung surface were carefully counted and measured using a digital caliper.

*Real-time cell proliferation analysis*. Cell proliferation was analyzed using xCELLigence RTCA DP system (Roche, Indianapolis, IN, USA) through measuring the electrical impedance of microelectrodes integrated in the bottom of a 16-well E-plate. For analysis assay, $1\times10^3$ of LA-4 cells (Control, shScr and shIPO7 stable cell-lines) were seeded in E-plates and incubated for 72 h.

*Statistical analysis*. Statistical analyses were performed using Microcal Origin Student's *t*-test two populations (Microcal Software, Northampton, MA, USA). The significances were set in terms of probability values ($*p<0.05$, $**p<0.01$ and $***p<0.001$) compared to corresponding values.

## Results

*Confirmation of importin 7 expression in human lung adenocarcinoma tissues and K-ras$^{LA1}$ mouse lung tissues*. Expression of importin 7 was confirmed in human normal lung and lung adenocarcinoma (Grade I, II and III) tissues of male smokers. Twelve human tissue samples (three samples per group) were analyzed by western blot. Importin 7 is over-expressed in human lung adenocarcinoma compared to normal lung tissues (Figure 1A). The up-regulation of importin 7 expression is also confirmed in lung tissues of *K-ras$^{LA1}$* mice compared to wild-type C57BL/6 mice ($*p<0.05$) (Figure 1B).

*Aerosol gene delivery of shImportin 7 suppresses lung tumorigenesis in K-ras$^{LA1}$ mice*. The complexes of GPT-SPE/shRNA were delivered to mice twice a week for 4 weeks (8 times) in nose-only exposure chamber. Repeated aerosol delivery of GPT-SPE/shImportin 7 complexes significantly decreased the total tumor number compared to control and shScr-delivered groups. The anti-tumorigenic effect of the GPT-SPE/shImportin 7 complexes is also shown as in terms of larger than 1 mm tumors and smaller than 1 mm tumors (n=4). The statistical significances of differences are presented as follows: $***p<0.001$ compared to control; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared to shScr (Figure 2).

Effects of importin 7 knockdown on cell proliferation and apoptosis. The effect of importin 7-knockdown on cellular

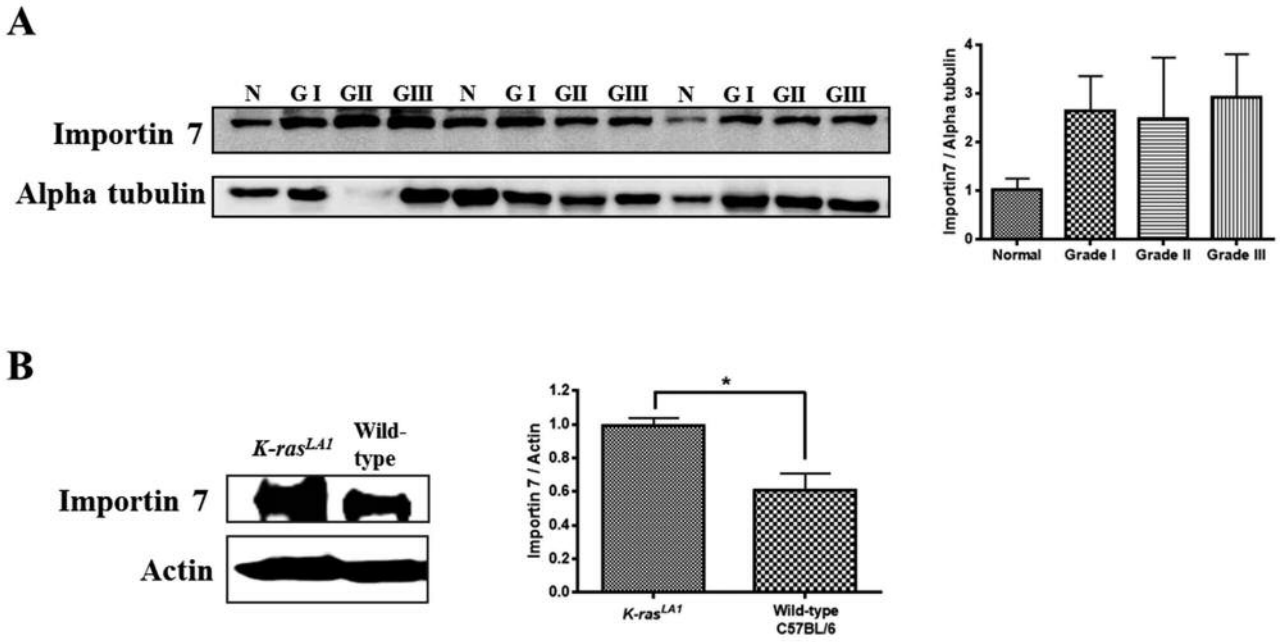


Figure 1. *Western blot and densitometric analysis of Importin 7 expression in human lung adenocarcinoma and K-ras$^{LA1}$ mouse lung tissues. (A) Confirmation of Importin 7 protein expression in human normal and adenocarcinoma lung tissues. GI, grade I adenocarcinoma tissues; GII, grade II adenocarcinoma tissues; GIII, grade III adenocarcinoma tissues (n=3). (B) Importin 7 expression using Western blot in K-ras$^{LA1}$ mouse and Wild-type C57BL/6 mouse lung tissues (n=4, *p<0.05, compared to wild-type C57BL/6 group). Each bar represents the mean±standard error of mean (SEM).*

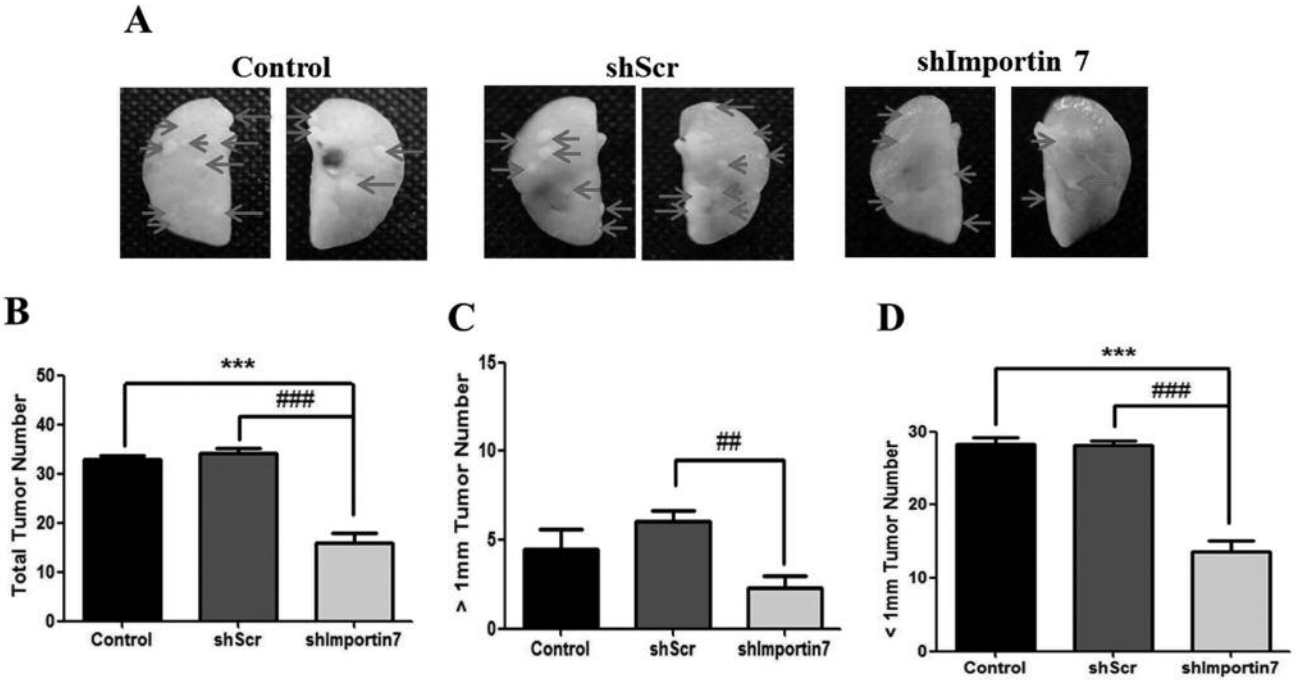


Figure 2. *Suppression of lung tumorigenesis in GPT-SPE/shImportin 7 complexes delivered K-ras$^{LA1}$ lung cancer mice. Aerosol delivery of GPT-SPE/shImportin 7 complexes to K-ras$^{LA1}$ mice twice a week for 4 weeks (8 times). (A) Figures of lung tumor lesions in control, shScr and shImportin 7 (shIPO7)-delivered K-ras$^{LA1}$ mice. (B) Total tumor number (n=4, ***p<0.001 compared to control; $^{###}$p<0.001 compared to shScr). (C) Tumor number of larger than 1 mm (n=4, $^{##}$p<0.01 compared to shScr). (D) Tumor number of smaller than 1 mm (n=4, ***p<0.001 compared to control; $^{###}$p<0.001 compared to shScr).*

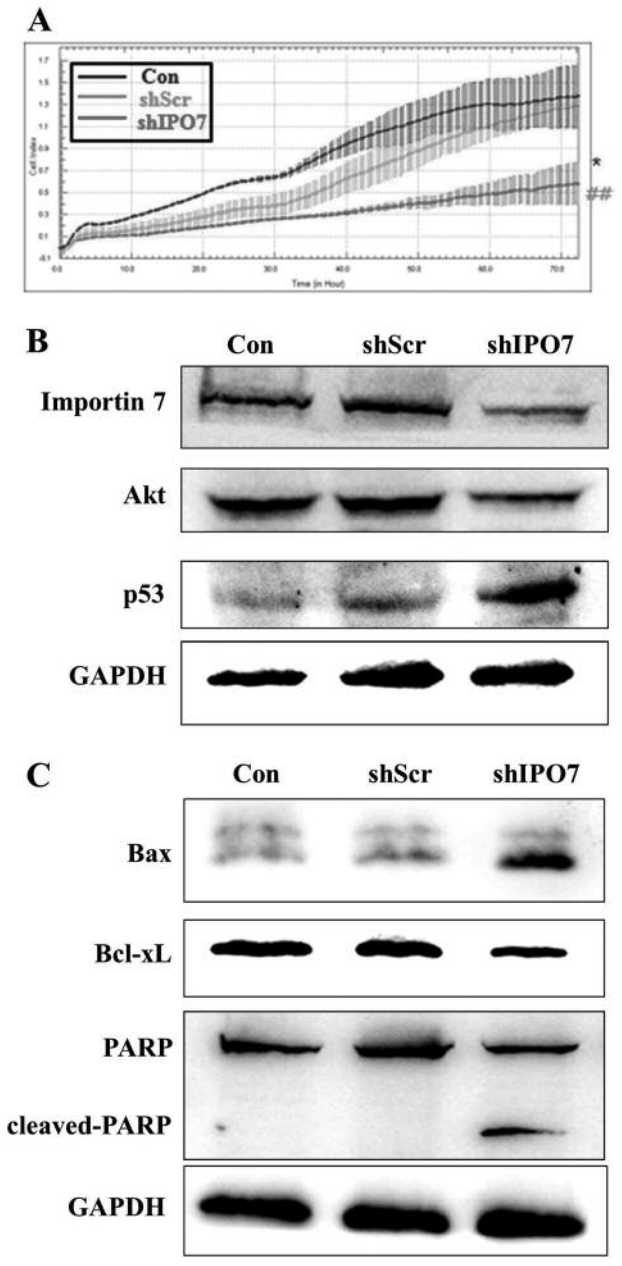


Figure 3. *Effect of Importin 7-down-regulated cell-line on cellular proliferation and apoptosis. (A) Cell proliferation analysis using xCELLigence RTCA DP system; $1\times10^3$ cells of control LA-4, shScr and shImportin 7 (shIPO7) stable cell-line were seeded in E-plates and analyzed for 72 h (n=3, *p<0.05 compared to control; ##p<0.01 compared to shScr). (B) Western blot of Importin 7, Akt and p53 (C) Bax, Bcl-xL and PARP in LA-4 control, shScr and shImportin 7 cell-line groups. Cells were incubated for 48 h and collected for Western blot.*

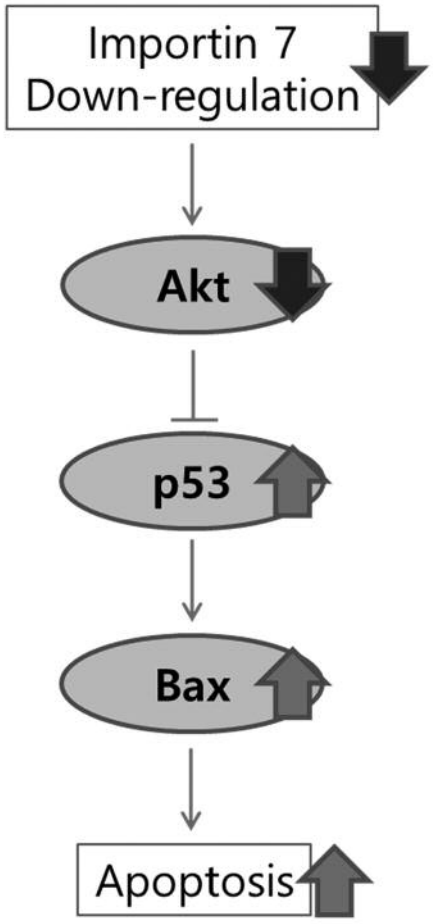


Figure 4. *A scheme describing the mechanism of Importin 7 down-regulation in cancer cells.*

proliferation is confirmed using the xCELLigence real-time proliferation detection system. Importin 7-down-regulated stable cell-line shows significant suppression of cell proliferation compared to control and shScr (*$p<0.05$ compared to control; $^{\#\#}p<0.01$ compared to shScr) (Figure 3A). The expression of Akt and apoptosis-related proteins were assessed using Western blot. Importin 7-down-regulated stable cell-line shows suppression of Akt expression, whereas p53 expression level is increased. To examine whether knockdown of importin 7 induces apoptosis, cleaved PARP, the expression of Bax (a proapoptotic protein) and Bcl-xL (an anti-apoptotic protein) was confirmed. Cleaved PARP and Bax are increased, whereas Bcl-xL is decreased in importin 7-down-regulated stable cell-line (Figure 3B).

## Discussion

Nuclear transport system connects the cytosol and the nucleus in eukaryotic cells. Importin 7 nuclear import receptor binds its cargo and allows nuclear transport (18). Importin 7 is significantly overexpressed in colon cancer compared to

normal tissues (19, 20). In our study, the expression level of importin 7 is increased in lung adenocarcinoma compared to normal lung tissues. Moreover, the expression of importin 7 in lungs of *K-ras*$^{LA1}$ mice is significantly increased compared to wild-type C57BL/6 mice (Figure 1). Overexpression of importin 7 in human lung adenocarcinoma and *K-ras*$^{LA1}$ lung tissues led us to study the effect of importin 7 down-regulation for lung cancer treatment.

We showed the therapeutic effect of importin 7 knock-down in *K-ras*$^{LA1}$ lung cancer model mice. Repeated aerosol delivery of GPT-SPE/shImportin 7 suppressed lung tumorigenesis. The number of total tumor, larger than 1 mm and smaller than 1 mm, was significantly decreased in GPT-SPE/shImportin 7 complex delivered group (Figure 2).

Akt (also known as protein kinase PKB) plays an important role in cancer by inhibiting apoptosis and stimulating proliferation (21). Moreover, Akt is overexpressed in non-small cell lung cancers compared to normal lung tissues (22). Previous study showed that partial depletion of importin 7 induces tumor suppressor p53 activation and p53-dependent growth arrest (23). Given these facts, we confirmed the mechanism of importin 7 down-regulation in LA-4 murine lung cancer cell-line. Expression of Akt is decreased and p53 is increased in importin 7-down-regulated stable cell-line (Figure 3B).

Growth inhibition and apoptosis in cancer cells are key factors for cancer treatment. To study whether deficiency of importin 7 induces apoptosis, we investigated the expression of apoptosis proteins, including Bcl-xL, Bax and PARP. Our data show that down-regulation of importin 7 increased Bax and cleaved PARP, whereas decreased anti-apoptotic proteins, such as Bcl-xL (Figure 3C).

In conclusion, our data suggest that down-regulation of importin 7 suppresses lung tumorigenesis *via* suppressing Akt activity and inducing apoptosis (Figure 4). Therefore, importin 7 could be an emerging target for lung cancer treatment.

## Acknowledgements

Ah Young Lee and Seong-Ho Hong were supported by the Research Institute for Veterinary Science, Seoul National University and BK21 PLUS Program for Creative Veterinary Science Research. The biospecimens (human lung tissues) for this study were provided by the Korea University Guro Hospital of National Biobank, a member of the National Biobank of Korea.

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*Received March 31, 2017*
*Revised April 10, 2017*
*Accepted April 11, 2017*