Dual Expression of shAkt1 and Pdcd4 Suppresses Lung Tumorigenesis in *K-ras^{LA1}* Mice

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Abstract. *Background/Aim: Lung cancer has the highest mortality rate among cancers and current therapies are not efficient. Therefore, novel therapeutic methods are urgently needed. Here, we examined the effectiveness of simultaneous Akt1 inhibition and Pdcd4 over-expression using a dual expression system in suppressing tumorigenesis in K-ras^{LA1} mice (a lung cancer model). Materials and Methods: An shRNA targeting Akt1 (shAkt1) and cDNA of programmed cell death protein 4 (Pdcd4) were inserted into a dual expression vector (shAkt1+Pdcd4). A sorbitol diacrylate-polyethylenimine (SDA-PEI) carrier was used because of low toxicity and high transfection efficiency. Aerosolized SDA-PEI/shAkt1+Pdcd4 complex was delivered to the mice twice a week for 4 weeks using a nose-only exposure inhalation chamber. Results: Simultaneous Akt1 inhibition and Pdcd4 over-expression synergistically induced potent antitumor effect. Analysis revealed significant reduction in lung tumor number. Conclusion: Dual expression of shAkt1 and Pdcd4 effectively suppresses lung tumorigenesis.*

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Cancer statistics show that lung cancer has the highest mortality rate in both men and women (1, 2). Aerosol delivery has the advantages of uniform distribution, fewer systemic side effects and access to a larger bronchial epithelial surface area (3-6).

Because various genes are associated with tumor progression, a dual expression vector is an effective approach for cancer therapy through simultaneous expression of two genes (7). A dual expression vector has two distinct promoters: a cytomegalovirus (CMV) promoter and a U6 promoter. In this study, expression of Pdcd4 and shAkt1 were controlled by the CMV promoter and the U6 promoter, respectively.

Pdcd4 was first recognized as a gene that is up-regulated during apoptosis (8). It is also associated with cancer cell invasion (9). In contrast, serine/threonine-protein kinase B (Akt1) activation is associated with cell growth, proliferation and survival and plays an important role in cancer cell growth by activating the anti-apoptotic pathway (10-11). Therefore, blockade of Akt downstream signaling could be an effective method for cancer therapy.

In this study, a short-hairpin RNA targeting *Akt1* (shAkt1) and *Pdcd4* cDNA (shAkt1+Pdcd4) were inserted into a dual expression vector to inhibit lung tumorigenesis and cell proliferation.

Because cellular uptake of naked DNA has low efficiency, an effective and safe carrier is necessary for successful cancer gene therapy. Cationic polymers have been used for gene therapy due to their stability, ease of modification and high biocompatibility (12-15). Polyethylenimine (PEI) is a highly efficient cationic carrier owing to its pH buffering effect, however, it is also known to be toxic (16-18).

In a previous study, sorbitol diacrylate-polyethylenimine (SDA-PEI) was developed as an alternative non-viral carrier with high efficiency and biocompatibility (19). In this study, the DNA binding ability of SDA-PEI was analyzed by agarose gel retardation assay. Toxicity and *in vivo* transfection efficiency were confirmed after aerosol delivery. Furthermore, the synergistic therapeutic effect of Akt1 inhibition and Pdcd4 over-expression in *K-ras*^{LA1} mice was investigated after aerosol delivery of SDA-PEI/shAkt1+Pdcd4 complexes.

Materials and Methods

Materials. The monoclonal antibody against Pdcd4 was produced *via* a general method (20). The BLOCK-iT™ U6 RNAi Entry vector kit was purchased from Invitrogen (Carlsbad, CA, USA) and Akt1 antibody was purchased from Cell Signaling (Boston, MA, USA). GAPDH antibody was purchased from AbFrontier (Seoul, Korea).

Cloning of shRNA of Akt1 and over-expression of Pdcd4. Short-hairpin RNA (shRNA) sequence targeting mouse *Akt1* mRNA was designed (5'- GAAGGAGGTCATCGTCG-3'). shAkt1 was synthesized according to the above sequence and was cloned into BLOCK-iT™ U6 entry vector (Invitrogen). The cassettes containing U6 promoter and shAkt1 sequences were generated by following the manufacturer's instructions (BLOCK-iT™ U6 RNAi Entry vector system; Invitrogen). For over-expression of Pdcd4, total RNA was purified from the lung tissue and cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR). The following set of primers was used for the RT-PCR: Forward primer (5'-ATAAGAATGCGGCCGCATGG ATATAGAAAATGAGCAG-3') and a reverse primer (5'-ATAGTTA GCGGCCGCTCAGTAGCTCTCAGG TTTAA-3'). The PCR product and shAkt1 were then inserted into the pRFP-C-RS vector (Origene Technologies Inc., Rockville, MD, USA) to generate the shAkt1 and Pdcd4 dual expression vector. The final construct was verified by restriction enzyme analysis and sequencing.

Preparation of SDA-PEI/shAkt1+Pdcd4 complex. SDA-PEI was synthesized following the standard procedure described earlier (19). To confirm the DNA condensation capability, agarose gel retardation was checked at various SDA-PEI/DNA ratios as described previously (21).

In vivo aerosol delivery study. K-ras^{LA1} lung cancer model mice were obtained from the Human Cancer Consortium-National Cancer Institute (Frederick, MD, USA) and were cared according to the regulations and policy for the care and use of laboratory animals published by the Seoul National University. Animals were maintained in the laboratory animal facility under a 12h light/dark cycle. Temperature was controlled at 23˚C±2˚C and 50%±10% humidity.

For aerosol gene delivery, mice were exposed to the aerosol in a nose-only exposure chamber following previously established methods (21). To investigate the efficiency of SDA-PEI as a gene delivery carrier, mice were exposed to aerosol containing a complex of red fluorescent protein (RFP) expression vector with PEI or SDA-PEI. Two days after inhalation, mice were sacrificed, lung tissues were fixed and embedded in Tissue-Tek OCT (Sakura, Torrance, CA, USA) for the detection of RFP signal. Tissue cryosection was performed with a microtome (Leica, Nussloch, Germany) and 10 μm sections were mounted on slides. The slides were observed under a confocal laser scanning microscope (Carl Zeiss, Jena, Germany).

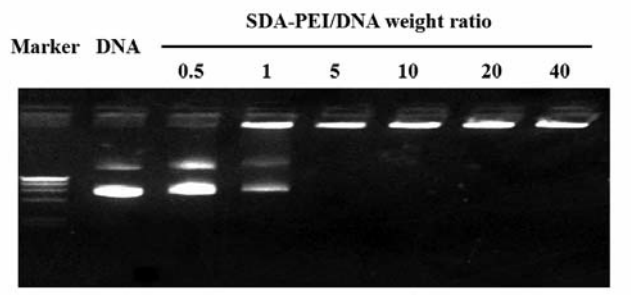

Figure 1. *Agarose gel electrophoresis of SDA-PEI/shAkt1+Pdcd4 complexes at various weight ratios (0.5-40). SDA-PEI, sorbitol diacrylate-polyethylenimine; Akt1, serine/threonine-protein kinase B (Akt1); shAkt1, small hairpin RNA of Akt1; Pdcd4, programmed cell death protein 4.*

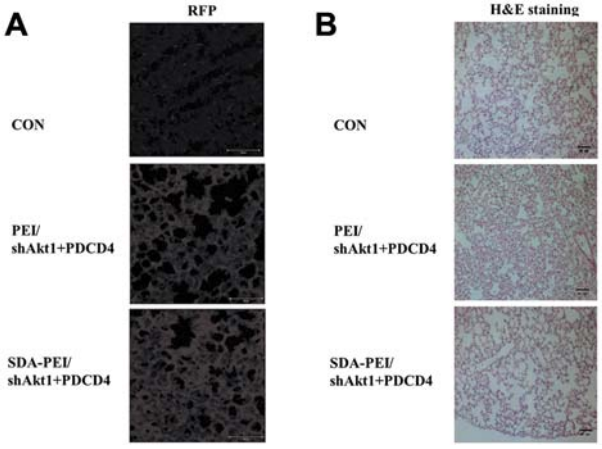

Figure 2. *In vivo confirmation of transfection efficiency after aerosol delivery. (A) RFP expression analysis (Magnification: 200X, scale bar represents 100 μm). (B) Histopathological study of the lungs-hematoxylin and eosin (H&E) staining (Magnification: 200X, scale bar represents 50 μm). CON, control group; RFP, red fluorescence protein.*

Twelve *K-ras*^{LA1} mice were divided randomly into 4 groups (3 mice/group). Control mice were left untreated and the SDA-PEI only group was exposed to aerosol containing 4 mg of SDA-PEI in distilled water. PEI/shAkt1+Pdcd4 and SDA-PEI/shAkt1+Pdcd4-treated mice were exposed to aerosol containing 0.4 mg of *shAkt1+Pdcd4* DNA with 4 mg of PEI or SDA-PEI in distilled water, respectively.

The aerosol was delivered 8 times (twice a week for 4 weeks). Tumors on lung surfaces were counted using a microscope following the established method (21). For histopathological examination, the lungs were fixed in 10% neutral buffered formalin. All methods used in this study were approved by the Animal Care and Use Committee at Seoul National University (SNU-130117-2).

Western blot analysis. The protein concentrations of homogenized lung lysates were measured using a Bradford kit (Bio-Rad, Hercules, CA, USA). Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Pharmacia, Cambridge, UK). The membranes were blocked in 5% skim milk in

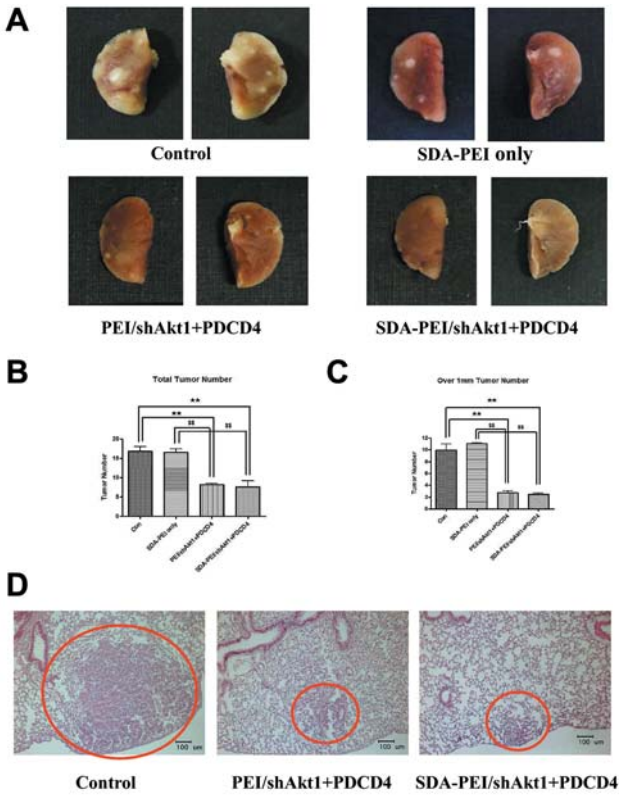

Figure 3. *Suppression of lung tumorigenesis in SDA-PEI/shAkt1+Pdcd4 complex treated K-ras^{LA1} mice. (A) Lung tumor lesions. (B) Lung tumor number (n=3, $^{**}p<0.01$ compared with control; $^{\$\$}p<0.01$ compared with SDA-PEI only). (C) Number of tumors larger than 1 mm (n=3, $^{**}p<0.01$ compared with control; $^{\$\$}p<0.01$ compared with SDA-PEI only). (D) Histopathological examination of the lungs in control and treated mice (Magnification: X100, scale bar represents 100 μm).*

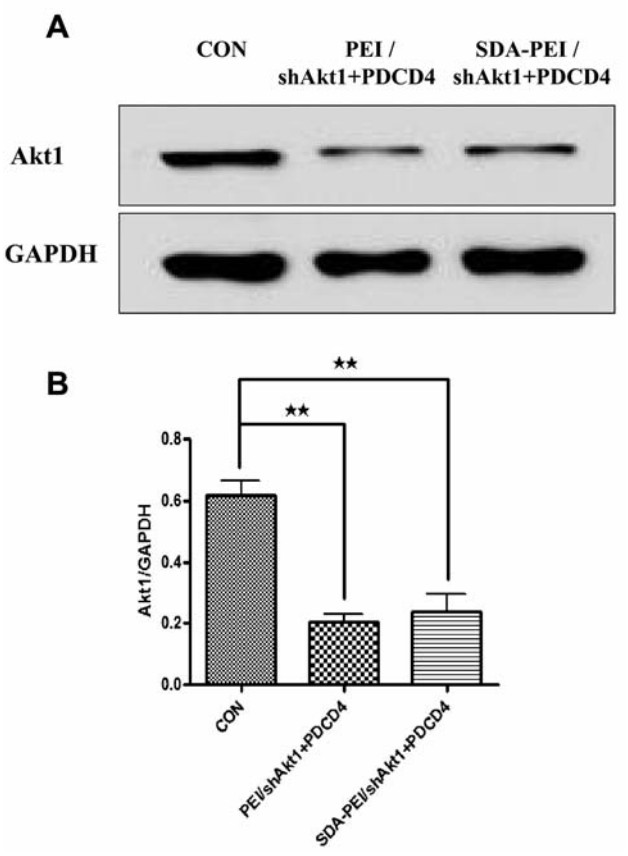

Figure 4. *Significant decrease of Akt1 protein expression in SDA-PEI/shAkt1+Pdcd4 treated mice. (A) Western blot analysis and (B) Densitometric analysis of Akt1. Each bar represents the mean±SEM (n=3, $^{**}p<0.01$ compared with control).*

TTBS (Tris-Buffered Saline+Tween 20) for 1 h and incubated overnight with corresponding primary antibodies. Next, the membranes were incubated for 3 h at 4˚C with secondary antibodies conjugated to horseradish peroxidase (Invitrogen). After incubation, the membranes were washed with TTBS for 30 min and bands were detected using the luminescent image detector Ez-Capture MG (ATTO, Tokyo, Japan). Densitometric analysis was performed using the CS Analyzer software (ATTO).

Histopathological analysis. For histological analysis, lung tissues were fixed in 10% neutral buffered formalin and paraffin embedded. Sectioned tissues (4 μm) were stained by hematoxylin and eosin (Sigma-Aldrich, MO, USA) and mounted with cover slips using Permount (Fisher Scientific, Waltham, MA, USA) solution. Slides were observed using a light microscope (Carl Zeiss).

Statistical analyses. Data are presented as mean values±standard error of three different experiments. Statistical significance between the two populations was analyzed by the Student's *t*-test using Microcal Origin (Microcal Software; Northampton, MA, USA). The statistical significances of differences is presented in terms of probability values ($p<0.05$ (*) was considered significant and $p<0.01$ (**) was highly significant) compared with corresponding values.

Results

Characterization of SDA-PEI/shAkt1+Pdcd4 complexes. The condensation ability of the SDA-PEI/shAkt1+Pdcd4 complex was confirmed by agarose gel electrophoresis. SDA-PEI/shAkt1+Pdcd4 complexes at six different weight ratios (0.5, 1, 5, 10, 20 and 40) of the polymer solution and plasmid DNA (shAkt1+Pdcd4) were prepared. In agarose gel electrophoresis, the sample with a weight ratio 5 showed considerable retardation (Figure 1).

In vivo aerosol delivery study. The dual expression vector harboring RFP (Figure 6) was delivered to the mice along with the SDA-PEI complex for analyzing transfection efficiency *in vivo*. Mice treated with the SDA-PEI/shAkt1+Pdcd4 complex

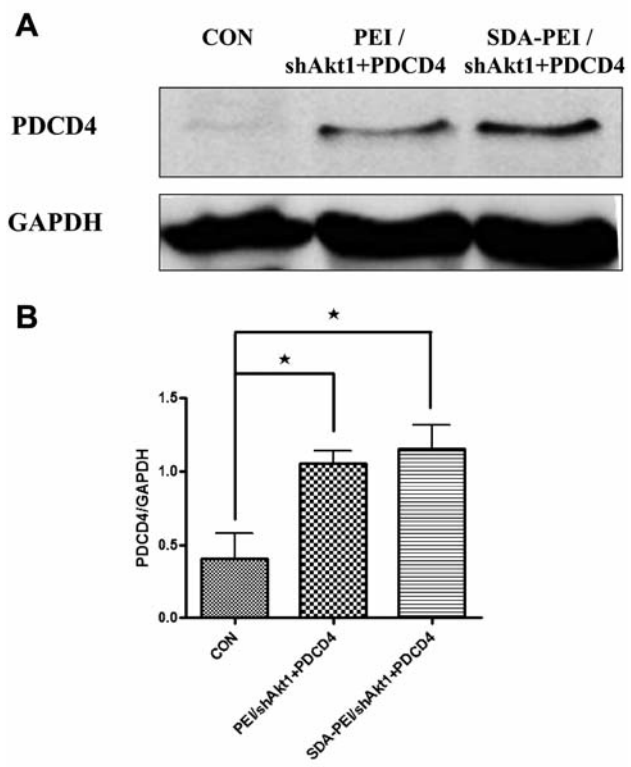

Figure 5. *Significant increase of Pdcd4 expression in SDA-PEI/shAkt1 +Pdcd4 treated mice. (A) Western blot analysis and (B) densitometric analysis of Pdcd4. Each bar represents the mean±SEM (n=3, $^{*}p<0.05$ compared with control).*

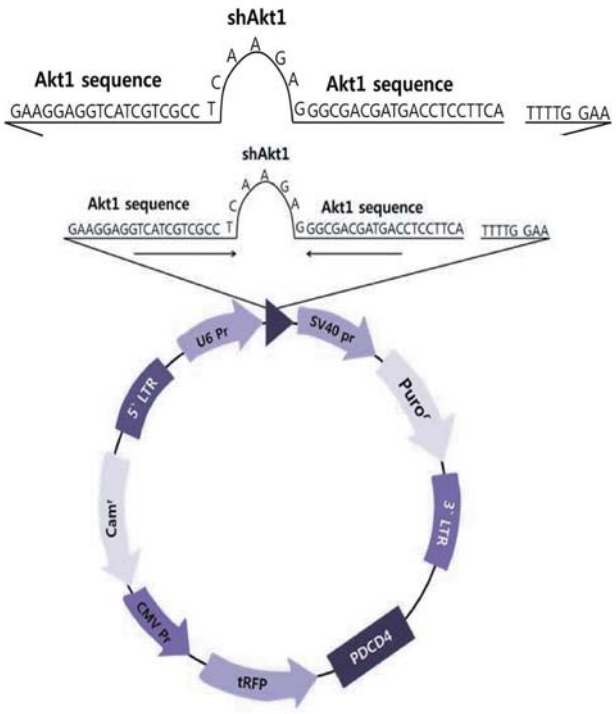

Figure 6. *Map of the dual expression vector with shAkt1 and Pdcd4.CMV pr, cytomegalovirus promoter; U6 pr, U6 promoter; LTR, long terminal repeat; Cam^r, chloramphenicol resistence; Puro^r, puromycin resistence; SV40 pr, simian virus 40 promoter; RFP, red fluorescence protein.*

exhibited higher red fluorescence than the control group did (Figure 2A). Moreover, H&E staining showed no evidence of inflammatory lesions in the lungs of the SDA-PEI/shAkt1+Pdcd4 treated mice (Figure 2B).

Aerosol delivery of shAkt1 and over-expression of Pdcd4 with SDA-PEI suppresses lung tumorigenesis. Aerosol delivery of the SDA-PEI/shAkt1+Pdcd4 complex significantly reduced the total tumor number (Figure 3A). The anti-tumor effect of the SDA-PEI/shAkt1+Pdcd4 complex is presented as in terms of total tumor number and number of tumors larger than 1 mm tumor (Figures 3B and 3C). The total tumor number (n=3, $^{**}p<0.01$ compared with control; $^{\$\$}p<0.01$ compared with SDA-PEI only) and over 1 mm tumor number (n=3, $^{**}p<0.01$ compared with control; $^{\$\$}p<0.01$ compared with SDA-PEI only) were significantly decreased in the SDA-PEI/shAkt1+Pdcd4 treated group. The anti-tumor effect of SDA-PEI/shAkt1+Pdcd4 was also confirmed by H&E staining (Figure 3D).

Confirmation of protein expression changes in the lung induced by aerosol delivery of shAkt1 and Pdcd4 with SDA-PEI. To confirm the transfection efficiency of the SDA-PEI/shAkt1+Pdcd4 complex, changes in the expression of Akt1 and Pdcd4 were analyzed by Western blot. Repeated aerosol delivery of SDA-PEI/shAkt1+Pdcd4 significantly suppressed the Akt1 protein expression (Figure 4) (n=3, $^{**}p<0.01$ compared with control) and significantly increased Pdcd4 expression (Figure 5) (n=3, $^{*}p<0.05$ compared with control).

Discussion

Pdcd4 expression is down-regulated in various tumors and this down-regulation is associated with apoptosis. Furthermore, Pdcd4 inhibits neoplastic transformation and loss of this protein is related to poor prognosis in cancer (22, 23). Akt promotes cellular proliferation and inhibits apoptosis (11). Moreover, Akt pathway activation is observed in most non-small cell lung cancers (24). Aerosol delivery of shAkt1 with a cationic carrier was effective in lung cancer suppression (25). Furthermore, dual expression vectors are promising tools in biological research (7). Given these facts, we explored the therapeutic utility of dual expression of shAkt1 and Pdcd4 in the *K-ras*^{LA1} murine lung cancer model.

At a weight ratio over 5, the SDA-PEI/DNA complex showed retarded migration in agarose gel electrophoresis (Figure 1). This result suggested that the SDA-PEI

completely binds DNA (shAkt1+Pdcd4) and inhibits its migration in gel electrophoresis.

Transfection efficiency of aerosol-delivered SDA-PEI was confirmed by fluorescence expression in the lungs. The fluorescence intensity was much higher in the lungs of SDA-PEI/shAkt1+Pdcd4-treated mice than in the control group (Figure 2A). Moreover, no inflammatory lesion was found in the lungs of SDA-PEI/shAkt1+Pdcd4-treated mice. These data demonstrate that SDA-PEI is biocompatible and an efficient carrier for aerosol gene delivery.

Finally, we showed that aerosol delivery of SDA-PEI/shAkt1+Pdcd4 significantly decreased the total tumor number and the number of tumors larger than 1 mm, thereby confirming the therapeutic efficiency of the complex (Figure 3). In addition, the efficiency of SDA-PEI/shAkt1+Pdcd4 complex was also verified by Western blot analysis of Akt1 and Pdcd4. The Akt1 expression was significantly reduced, whereas Pdcd4 expression was increased in the SDA-PEI/shAkt1+Pdcd4 treated group (Figure 4, 5). Taken together, these data demonstrate that the SDA-PEI/shAkt1+Pdcd4 complex effectively inhibits lung cancer progression through suppression of Akt signaling and up-regulation of Pdcd4 in K-ras^{LA1} mice.

Our findings also suggest that repeated aerosol delivery of a dual expression vector could be a promising approach for effective lung cancer therapy.

Acknowledgements

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