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Using Arginine-Glycine-Aspartic Acid (RGD)-Tumor Necrosis Factor (TNF) Modified Phage for Lung Cancer Treatment: Suppressing Tumor Mass Growth and Tumor Marker Expressions in Nude Mouse Model

Uso de Fagos Modificados con Factor de Necrosis Tumoral (TNF) y Ácido Aspártico-Arginina (RGD) para el Tratamiento del Cáncer de Pulmón: Supresión del Crecimiento de la Masa Tumoral y de la Expresión de Marcadores Tumorales en un Modelo de Ratón Nude

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SUMMARY: Currently, the bacteriophages have been applied as the phage therapy for using as a new technology for treating many cancers including lung cancer. This recent study explored the of a double cyclic form of arginine-glycine-aspartic acid with four cysteine residues (RGD) modified phage particles carrying the tumor necrosis factor therapeutic gene to suppress tumor growth. Adult male BALB/c nude mice were injected with A549 lung cancer cells to induce xenograft tumor growth. The control animals were subcutaneously injected with PBS while M13 and RGD-TNF animals were injected with M13 (control phage) and RGD-TNF phage, respectively. At the end of experiment. the tumor mass size was measured. Removed tumor masses and lung tissues of all groups were performed for immunohistochemistry and western blot analysis against Ki67, TNF-α, IL-2, and IFNγ antibodies. The results obviously showed that RGD-TNF phage could suppress the xenograft tumor growth. Additionally, the tissues of tumor mass and lung were localized with all cancer markers. It was revealed that RGD-TNF phage could significantly decrease the expressions of Ki67, TNF-α, and IL-2, but increase that of IFNγ in tumor mass. Only Ki67 and TNF-α expressions were decreased in lung tissue of mice treated with A549 lung cancer cells. It was concluded that RGD-TNF phage is a potential phage therapy for lung cancer treatment to reduce the tumor mass and to regulate the cancer markers.

KEY WORDS: Lung cancer; A 549 cells; Tumor markers; Phage therapy; Xenograft tumor.

INTRODUCTION

The lung cancer is known to be the most common cancer and remains the leading cause of death worldwide. From cancer deaths, it is estimated that 18 percent is of deaths from the lung cancer. Previous reports showed that 2.2 million or 11% were new lung cancer cases and the most common diagnosed lung cancer was in male (Sung *et al*., 2021; Bray *et al*., 2024). Such incidence of lung cancer in 43 countries, reported from 1978 to 2012, demonstrated that 19 countries had significantly declining trends among male patients while 26 countries had increasing trends among female. Interestingly, the highest risks mainly occurred in the population who were born from 1930 to 1950. Recently, it was reported that the new generations have moderately increased risk in some countries (Zhang *et al*., 2021). As estimated, in 2035, the prediction of the incidence of lung cancer in 40 countries will focus on country and sex specific disparities. Globally, the incidence rate of lung cancer among males has been predicted to be decreased by 23 %, while that of females is estimated to be increased by 2 % (Luo *et al*., 2023). Additionally, the numbers of new cases in 40 countries are determined to increase by 65.32% (from 1.31 to 2.17 million) in 2035. In Thailand, particularly in the

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department of respiratory medicine, Phrae hospital, there are lung cancer patients with chronic obstructive pulmonary disease (COPD) and still need to identify the prognostic factors. From 344 COPD patients, it was found that the14 patients developed to be lung cancer during the follow-up period of 6 months. A survival analysis and identified several prognostic factors were associated with lung cancer in COPD patients with age, smoking history, and severity of airflow limitation (Niyomthong, 2022). However, the lack of accessibility to medical doctors or specialists such as oncologists, surgeons, and pathologists, remains a critical problem in Thailand (Reungwetwattana *et al*., 2020). In fact, the important problem of lung cancer is the low survival rate with no symptoms because the patient will meet the doctor when its symptoms appear. When the doctor has diagnosed, the cancer is usually in stage 3 or 4 and often found to already spread to other organs.

Interestingly, a new technology for treating lung cancer called "phage therapy" has been recently developing. Basically, the bacteriophages (or phages) known as viruses that infected bacteria have potential to be used as carriers to deliver drugs and vaccines into cancer cells. Such phages can be modified to target the specific cells and attach drug molecules to their capsids. Moreover, the bacteriophages have been demonstrated to interfere the processes that sustain the tumor growth and spreading (Foglizzo & Marchiò, 2021). A previous study on phage therapy for cancer treatment demonstrated that the combination of doxorubicin with RGD – targeted suicide gene therapy could synergistically destroy the human and murine tumor cells in both 2D and 3D tumor spheres (Tsafa *et al*., 2020). Additionally, a combination of temozolomide with RDG4C-phage-Grp78 targeted gene therapy was also shown to efficiently suppress the proliferation of orthotopic glioblastoma (Przystal *et al*., 2019; Asuvarut *et al*., 2022; Gay *et al.*, 2024). In a murine colorectal cancer model, the GM – CSF phage treatment with radiation therapy was demonstrated to significantly inhibit the tumor growth (Wang *et al*., 2021). Potentially, the recombinant bacteriophages of T7 have been shown to suppress the tumor growth and could be a potential candidate tool for cancer therapy (Hwang & Myung, 2020). Therefore, our study has conducted with the phage therapy by using RGD-phage carrying TNF transgene to inject near to the solid tumor in nude mice inoculated with human lung cancer cell line (A 549 cells). This study attempted to evaluate the efficiency of RGD-TNF phage on tumor size reduction and the metastatic markers of such tumor to the mouse lung.

MATERIAL AND METHOD

Sulforhodamine B Assay (SRB). A549 lung cancer cells were incubated with RGD-TNF phage in 96-well plates (1,000 cells/well) for 6-24 h in triplicate for each condition and the fresh medium or M13 phage was added instead of RGD-TNF phage as a control. Fresh medium was added every 24 h until the SRB staining was performed. Then the cells were fixed at the indicated time points at ºC by adding 50 µl of 10 % cold trichloroacetate before washing with running tap water and drying at room temperature. The SRB staining was done by adding 0.4 % SRB reagent and incubated for 1 hour on a shaker at room temperature. The unbound SRBs were washed for 3 times with 1 % acetic acid and the plates were allowed to air-dry. Bound SRBs were dissolved in 200 µl of 10 mM Tris (pH 10.5) and leaved on shaking platform. Absorbance was read at 490 nm by using an automated microplate reader.

Animals, experimental design, and ethic. Adult male BALB/c nude mice (7 weeks old; 18-20g) were purchased from Nomura Siam International of Thailand. After acclimatization for 7 days, animals were randomized into three groups consisting of no phage control, M13-TNF and RGD-TNF phage groups (n=8/ group). In tumor mass induction period, the control mice were subcutaneously injected with PBS (0.2 ml/ mouse), while M13 and RGD-TNF animals were once injected with A549 lung cancer cells ($1x10^7$ cells in 0.2 ml of PBS) on day 1 before waiting tumor growth for 10 days. In the therapeutic period (Days 10-22), the growing mass on M13-TNF control and RGD-TNF phage mice were injected with M13 or RGD-TNF phage, respectively, at a volume of 20 µL at days 10, 14, 18, and 22. The tumor mass volume was measured at the end of experiment by using a Vernier caliper (Fisher, Pittsburgh, PA, USA) to measure maximal tumor diameter and transverse diameter. On day 23, all mice were anesthetized with thiopental sodium (40 mg/kg BW, Sigma-Aldrich, Inc., USA). before euthanasia by cervical dislocation. Then, the tumor masses were gently removed and weighed. The tumor mass and lung tissue from separated 4 mice were fixed in 10 % formalin to perform immunohistochemical and kept at -80 ºC for the western blot analyses. The animal experiment ethic in this study was approved by the National Research Council of Thailand (recorded code: IACUC-KKU-118/62), Faculty of Medicine, Khon Kaen University, Thailand.

Immunohistochemistry study. Fixed tumor masses formed by A549 cells and lung tissues of all groups were routinely processed for paraffin blocks before sectioning at 5-7 µm thickness by microtome using (semi-automatics, ERM 3,100, Heston, Australia). Tissue sections were stained with Mayer's hematoxylin (Merck KgaA, Germany) and eosin Y aqueous solution (Merck KgaA, Germany). Then, the stained tissue sections were studied under light microscope (Nikon ECLIPSE E200) before capturing with

a digital camera (DXM1200, Nikon, Japan). To observe the tumor protein markers (Ki67, TNF-α, IL-2, and IFNγ) on masses and lung tissues, the deparaffinized sections were performed for immunohistochemistry. In brief, the sections were incubated in citrate buffer (10 mM citric acid, 0.05% Tween-20, pH 6.0) and heated in the microwave (560 Watts, 5 min, 3 times) for retrieving the antigens on the tissue. After colling down at room temperature, the sections were washed with PBS and permeabilized with 0.2 % Triton X-100 (Fluka Chemical Corp., USA) for 10 min. The activities of endogenous peroxidase on tissue section were blocked with 30 % hydrogen peroxide (H_2O_2, Merck, Germany) for 15 min. Then, the non-specific binding proteins were blocked with 3 % bovine serum albumin (Merck, Germany) for 30 min. Subsequently, they were probed with specific primary antibodies: Ki67 (1:2000; AB9260, EMD Millipore Corp., USA.), TNF-a (1:2000; MBS9700322, MyBioSource, Inc., USA), IL-2 (1:2000; MBS2090588, MyBioSource, Inc., USA), or IFNγ (1:2000; MBS2090619, MyBioSource, Inc., USA) for overnight in a moist chamber, but the negative control was omitted for the primary antibody incubation at 4 ºC. All tissue sections were washed with PBS and incubated with their secondary antibodies conjugated with HRP (horseradish peroxidase) at 25 °C for 2 h. Then, the sections were washed and incubated with the 3,3'-di-aminobenzidine substrates (Vector Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin dye for 3 min. Micrographs of positive immunoreactivity on the tissues were captured under a Nikon Light ECLIPSE E200 light microscope linked with a DXM1200 digital camera (Nikon, Tokyo, Japan).

Western blot analysis. Total proteins of the A549 tumor mass and lung tissue were extracted by adding 500 µl of radioimmunoprecipitation assay buffer (Cell signaling Technology Inc., USA) containing protease inhibitor cocktails (Sigma-Aldrich, Inc., USA). Then, the tissues were homogenized by using a glass tissue grinder and further sonicated on ice with a mini-sonicator probe (Ultrasonic Processor, Cole-Parmer Instrument Company, Thailand). The homogenated samples were centrifuged at 14,000 rpm for 10 min at 4 ºC to collect protein supernatant. for determination of the total protein concentration by using the NANO drop ND-100 Spectrophotometer at a wavelength of 280 nm (NanoDrop ND-1000 Spectrophotometer V3.5 User's Manual, NanoDrop Technologes Inc., USA). Subsequently, 100 µg of each total protein sample was loaded onto 10 % SDS-PAGE gel to separate and observe protein profiles. Then, the separated proteins were transferred onto the nitrocellulose membrane (Bio-Rad Laboratories, Inc., Germany) using 100 volts for 150 min. The non-specific binding proteins were blocked with 5 % skimmed milk dissolved in 0.1 % PBST for 60 min. Then, the whole protein membranes were incubated with the specific primary antibody including Ki67, TNF-α, IL-2, or IFNγ (as described in above section 2.3) 4 ºC for overnight. The unbound antibodies on membranes were washed out with 0.05 % PBST and incubated with specific secondary antibody conjugated with HRP for 60 min at room temperature. Then, the antigen-antibody complexes on membrane were detected for the specific immunoreactivity of protein bands by using the enhance chemiluminescence (ECL) substrate reagent kit (GE Healthcare Life Science, USA). The visualized proteins were recorded under Gel Documentation 4 (ImageQuant 400, GH HealthCare, USA).

Statistical analysis. The one-way analysis of variance was used to determine the significant difference among groups. The the means ± SDs were expressed and the significant differences were considered when the p value was less than 0.05.

RESULTS

RGD-TNF phage inhibited the proliferation of A549 lung cancer cells. As shown in Fig. 1, RGD-TNF phage suppressed A549 lung cancer cell growth in a dose-dependent manner compared to control. Results demonstrated that 1.5 x 10^6 TU of RGD-TNF phages maximally inhibited cell growth compared to control. It was suggested that RGD-TNF phage could inhibit A549 lung cancer cells growth i*n vitro* model.

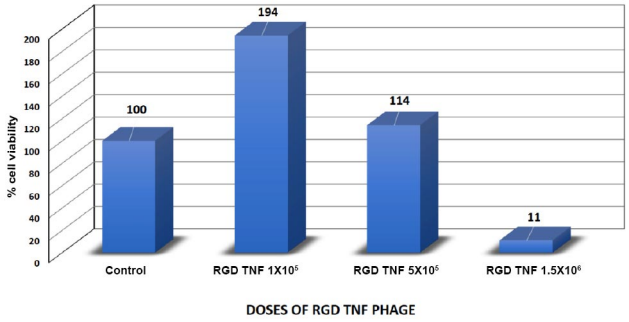

Fig. 1. The percentage of viability or A549 cell death after treating with RGD-TNF phages including 1x10^5, and 1x10^6 tranduction unit (TU), respectively.

The RGD-TNF phage inhibits subcutaneous A549 tumor growth. The results showed that A549 lung cancer cells could form and grow as the tumor mass under subcutaneous of adult nude mice (Fig. 2). The size of lung tumor mass induced was approximately 1 centimeter. Obviously, the A549 tumor size was reduced after treating with RGD-TNF phage as compared to the control M13 phage and no phage control, demonstrated with subcutaneous growth and removal features (Fig. 2).

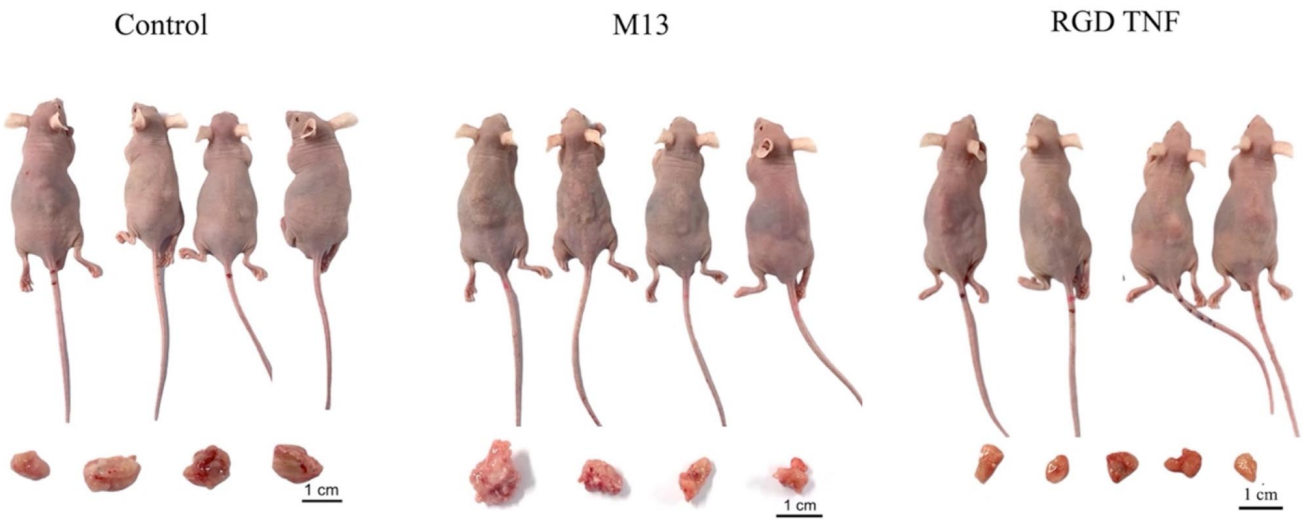

Fig. 2. Showing the subcutaneous tumor growth in nude mice (upper row) and after removing tumor masses (lower role) from control, M13, and RGD-TNF groups, respectively.

Localizations of tumor protein markers in masses and lung tissue. Histological features of A549 induced tumor mass (Fig. 3A) and lung tissue (Fig. 3a) were demonstrated by H&E staining. In general, the intensity of fibroblasts and tumor parenchymal cells was observed in the mass, but the mouse lung tissue seemed to be normal in terms of microstructures. For immunohistochemistry, all tumor protein markers including Ki67, TNF-α, IL-2, and IFNγ were obviously positive to A549 induced tumor mass (Figs. 3B-E). In addition, those proteins were also localized in the lung tissues of nude mice immunized with A549 lung cancer cells (Figs. 3b-e).

Expressions of tumor protein markers in the A549 tumor mass. The protein profiles, expressions, and intensity of Ki67, TNF-α, IL-2 and IFNγ were shown in Figures 4 (A-C). As compared to M13 group, RGD-TNF phage could significantly decrease the expressions of Ki67 and TNF-α, but not IL-2 in the tumor mass. It was found that the expression of IFNγ was significantly increased as compared to that of control and M13 phage group (Figs. 4B-C).

Expressions of tumor protein markers in the lung tissue. Figure 5 showed the protein profiles, expressions, and intensity of Ki67, TNF-α, IL-2 and IFNγ in lung tissues of nude mouse immunized with A549 lung cancer cells. It was revealed that Ki67 and TNF-α, but not IL-2 expressions were significantly decreased in RGD-TNF group as compared to those of control and M13 phage groups (Figs. 5B-C). Significantly, expression of IFNγ was increased as compared to that of only M13 phage group but not in no phage control group (Figs. 5B-C).

DISCUSSION

Recent study has shown the xenograft tumors formed by A549 cell injection in the flank of nude mice as demonstrated in a previous study (Durante *et al*., 2014). The reduction of lung tumor size by treating with RGD-TNF phage and cancer metastasis revealed by some tumor markers into the lung tissue were reported for the first time. Indeed, the tumor cell proliferation determined by Ki-67 expression was significantly decreased in both xenograft tumors and lung tissues of mice treated with RGD-TNF phage. This was associated with the presence of Ki-67 expression in predominant histological subtypes of lung adenocarcinoma (LUAD) as previously described (Li *et al*., 2021). Similar to xenograft tumors, the decreased expression of tumor necrosis factor alpha (TNF-α) was also observed in lung treated with RGD-TNF phage as compared to that of control. It was indicated that RGD-TNF phage has a potential effect to suppress the lung tumor. Since the TNF and its receptor has been reported to widely expressed in non-small cell lung cancer (NSCLC), the other organs that might be migrated from the xenograft tumors of A549 cells in our lung cancer model still needs to be explored. The use of TNF has been proposed to mediate the toxic side effects of immunotherapy and it could be used in the combination with precise therapy of the NSCLC and other cancers (Gong *et al*., 2021). In addition, our RGD-TNF phages also decreased the expression of the interleukin-2 (IL-2), important tumor microenvironment (TME) cytokine inducing the NKs and CD8+ effector T cells to eliminate tumor cells. It was assumed that the cytokine interleukin-2 (IL-2) could also stimulate the regulatory T (Treg) cells and effector immune cells in this study. Therefore, a previous study has suggested spurred interest in using this cytokine for

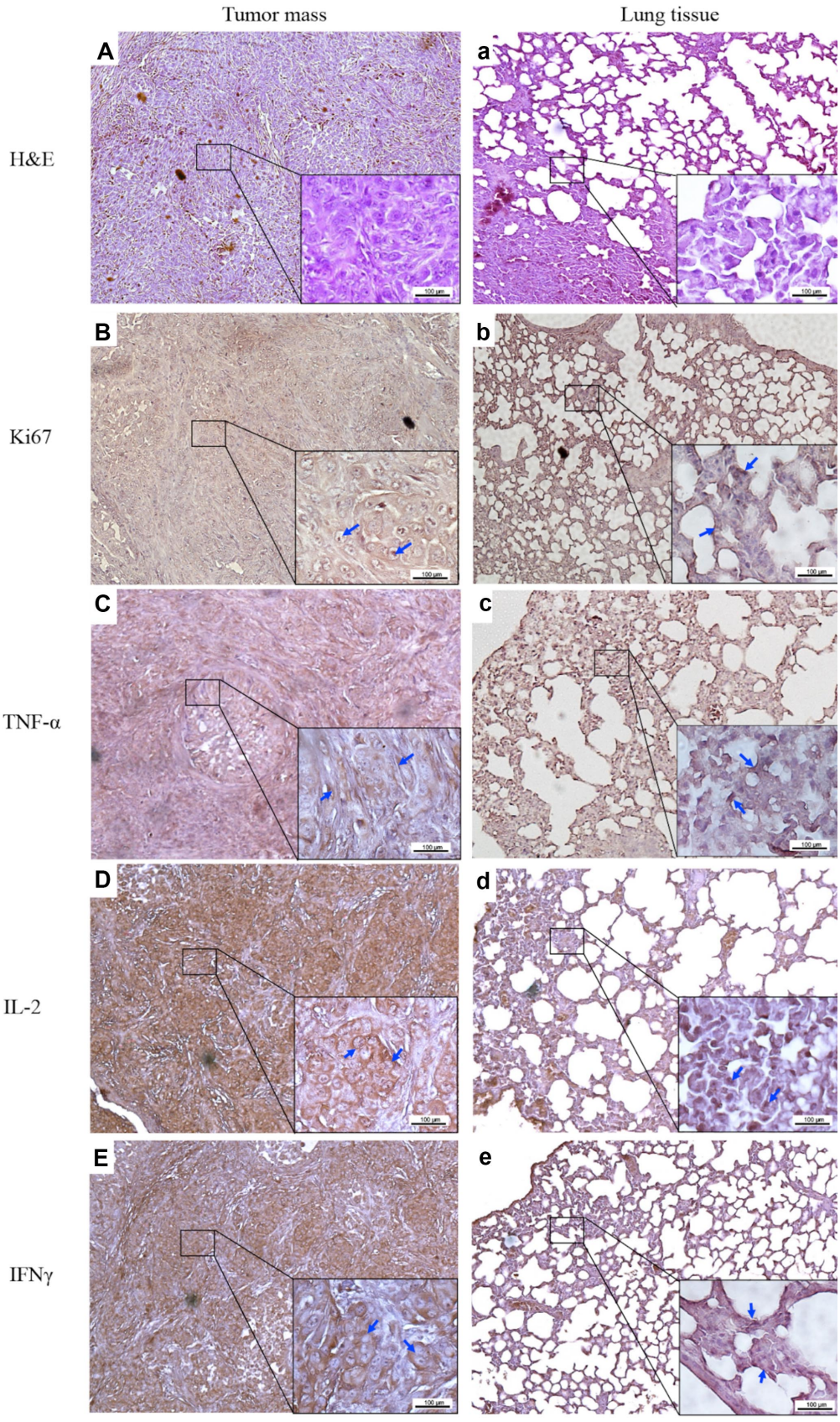

Fig. 3. Localizations of tumor protein markers (Ki67, TNF-α, IL-2, and IFNγ) by using immunohistochemistry approach on the tumor mass induced by A549 lung cancer cells (B-E) and on its lung tissue (b-e). A, a: tissues stained by hematoxylin and eosin (H&E) dyes. Blue arrows indicate positive cell against specific antibody.

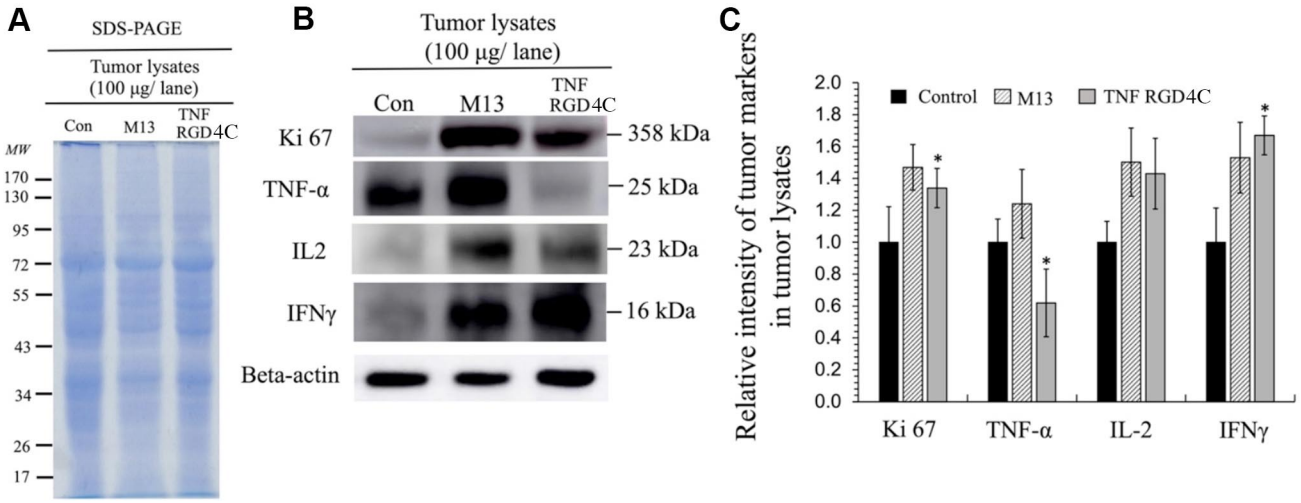

Fig. 4. Representative tumor lysates protein profiles revealed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) (A), western blotting (B), and intensities (C) of Ki67, TNF-α, IL-2 and IFNγ expression using beta-actin as internal control. *p < 0.05, statistically significant difference compared among groups. kDa; kilodalton, MW; molecular weight.

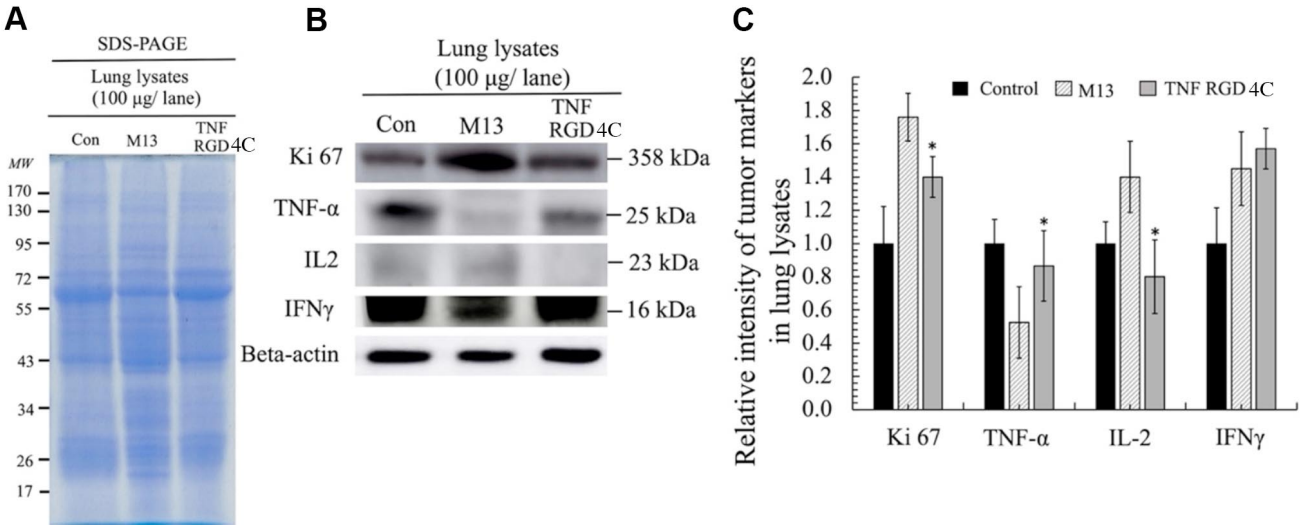

Fig. 5. Representative protein profiles of lung tissue lysates (A), western blotting (B), and intensities (C) of Ki67, TNF-α, IL-2 and IFNγ expressions using beta-actin as internal control. *p < 0.05, statistically significant difference compared among groups. kDa; kilodalton, MW; molecular weight.

immunotherapy of many cancers and autoimmune diseases (Raeber *et al*., 2023). Basically, the interferon-γ (IFN-γ) has an important role in activation of cellular immunity and followed by stimulation of antitumor- immune response. So, the IFN-γ has been proposed as a potential and significant marker in predicting the of patient survivals with NSCLC (Lee *et al*., 2021). In this study, animals treated with RGD-TNF phages showed the significant increase expressions of IFN-γ protein in both tumor mass and lung tissue, indicating actitation activity of this phase on the systemic IFN-γ. Taken together, our study has supported the idea of phage therapy particularly in treating lung cancer since several works also demonstrated its property on suppressing the tumor growth of many cancers (Przystal *et al*., 2019; Hwang & Myung, 2020; Tsafa *et al*., 2020; Foglizzo & Marchiò, 2021; Wang *et al*., 2021).

In conclusion, the RGD-TNF phage could inhibit the lung tumor growth in the xenograft mass and lung of nude mice via regulations of tumor markers including Ki67, TNF-α, IL-2, and IFNγ, respectively. It can also be a potential or alternative treatment for the lung cancer.

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FONGMOON, D.; TAENGOON, C.; NAGONG, M.; LUANGYAI, R.; HAJITOU, A.; SUWAN, K.; BUNSUEB, S. & IAMSAARD, S. Uso de fagos modificados con factor de necrosis tumoral (TNF) y ácido aspártico-arginina (RGD) para el tratamiento del cáncer de pulmón: supresión del crecimiento de la masa tumoral y de la expresión de marcadores tumorales en un modelo de ratón nude. *Int. J. Morphol., 42(6)*:1739-1745, 2024.

RESUMEN: En la actualidad, los bacteriófagos se han aplicado como terapia fágica para su uso como una nueva tecnología para tratar muchos tipos de cáncer, incluido el cáncer de pulmón. Este estudio reciente exploró el uso de una forma cíclica doble de arginina-glicina-ácido aspártico con cuatro residuos de cisteína (RGD) partículas de fago modificadas que llevan el gen terapéutico del factor de necrosis tumoral para suprimir el crecimiento tumoral. Se inyectaron células de cáncer de pulmón A549 a ratones desnudos BALB/c machos adultos para inducir el crecimiento de tumores de xenoinjerto. A los animales control se les inyectó por vía subcutánea PBS, mientras que a los animales M13 y RGD-TNF se les inyectó M13 (fago de control) y fago RGD-TNF, respectivamente. Al final del experimento, se midió el tamaño de la masa tumoral. Las masas tumorales extirpadas y los tejidos pulmonares de todos los grupos se sometieron a análisis de inmunohistoquímica y transferencia Western contra anticuerpos Ki67, TNF-α, IL-2 e IFNγ. Los resultados mostraron obviamente que el fago RGD-TNF podía suprimir el crecimiento de tumores de xenoinjerto. Además, se localizaron los tejidos de la masa tumoral y del pulmón con todos los marcadores de cáncer. Se reveló que el fago RGD-TNF podía disminuir significativamente las expresiones de Ki67, TNF-α e IL-2, pero aumentar la de IFNγ en la masa tumoral. Solo las expresiones de Ki67 y TNF-α disminuyeron en el tejido pulmonar de ratones tratados con células de cáncer de pulmón A549. Se concluyó que el fago RGD-TNF es una terapia de fagos potencial para el tratamiento del cáncer de pulmón para reducir la masa tumoral y regular los marcadores de cáncer.

PALABRAS CLAVE: Cáncer de pulmón; Células A 549; Marcadores tumorales; Terapia de fagos; Tumor de xenoinjerto.

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