Targeted gene therapy of the *HSV-TK/hIL-12* fusion gene controlled by the hSLPI gene promoter of human non-small cell lung cancer *in vitro*

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Abstract. The incidence of lung cancer and lung cancer-associated mortality have markedly increased worldwide, and gene-targeted therapy has emerged as a promising treatment strategy. The present study aimed to explore the targeted antitumor effect of the herpes simplex virus-thymidine kinase/human interleukin-12 (*HSV-TK/hIL-12*) fusion gene regulated by the human secretory leukocyte protease inhibitor (*hSLPI*) promoter of human non-small cell lung cancer (hNSCLC). There were four recombinant eukaryotic expression vectors: pcDNA3.1-CMV-TK, pcDNA3.1-CMV-TK/hIL-12, pcDNA3.1-phSLP-TK and pcDNA3.1-phSLP-TK/hIL-12. These were constructed and transfected into the A549, SPC-A1 and HepG2 cell lines *in vitro*. The expression of the *HSV-TK/hIL-12* fusion gene was detected with reverse transcription-polymerase chain reaction (RT-PCR), and the content of *hIL-12* was measured using an ELISA. The antitumor effect of the fusion gene on the A549, SPC-A1 and HepG2 cell lines was determined using an MTT assay. Analysis of the experimental data demonstrated that genes regulated by the cytomegalovirus promoter were expressed at the same level in three different tumor cell lines. Genes regulated by the *hSLPI* promoter were expressed in the A549 and SPC-A1 cell lines, but not in the HepG2 cell line. Coincidentally, the *hIL-12* expression levels were similar to those observed in previous RT-PCR findings. In the Pcmv-TK/Pcmv-TK-hIL-12 group for all three cell lines, as well as in the PSLPI-TK/PSLPI-TK-hIL-12 group for the A549 and SPC-A1 cell lines, the cell survival rate declined significantly and the fusion gene transfection group indicated a lower cell survival rate, when compared with single gene transfection group. The present study indicated that the fusion gene regulated by the *hSLPI* promoter had a targeted antitumor effect on hNSCLC, and that the combined suicide gene and immune gene therapy had a stronger antitumor effect, compared with single gene therapy.

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Introduction

In the last decade, the incidence and mortality of lung cancer have markedly increased globally (1). Of the diagnosed histological types of lung cancer, >80% are non-small cell lung cancer (NSCLC), which is frequently diagnosed at an advanced stage (2). The efficacy of traditional treatment, including surgery, radiotherapy and chemotherapy, is limited, and effective treatments that inhibit tumor recurrence and lower the mortality rate among patients with late-stage lung cancer and distant metastases are still inadequate (3). With recent developments in the molecular etiology and gene therapy of lung cancer, molecular targeted therapies are considered the most promising approach to overcome difficulties with the treatment of lung cancer (4).

The suicide gene therapy caused by herpes simplex virus-thymidine kinase (HSV-TK) has received significant attention due to its direct killing effect and bystander effect (BSE) (5); however, the efficacy of HSV-TK/GCV treatment in treating cancer is limited, due to insufficient gene transfection and the insufficient induction of host immunity (6-8). Previous studies have demonstrated that the combination therapy of *HSV-TK* and immune genes has an improved response when compared with therapy using the simple *HSV-TK* gene; additionally, combination therapy has been demonstrated

to improve survival time in animals and to promote tumor regression (9,10).

Interleukin (IL)-12 is produced by monocytes, macrophages and B cells. It has multiple physiological and pathological functions and can be used for treating infection, autoimmune disease and tumors (11). A previous study reported that the systemic administration of recombinant IL-2 in combination with HSV-tk gene therapy exhibited an enhanced antitumor effect in a murine bladder cancer model (MBT-2) (12). Also, combination gene therapy with HSV-tk+GCV and IL-12 increased the expression of interferon-γ-dependent *Fas* and *FasL*, contributing to tumor cell apoptosis in murine prostate cancer models (RM-1 and RM-9) (13,14); however, the majority of studies regarding the combined gene therapies of *HSV-TK* and *IL-12* have only applied to the mouse (*m*)*IL-12* gene (13,14). Therefore, there are a limited number of studies on the co-operative antitumor effect of the human (*h*)*IL-12* gene (15). The present study further explored the co-operative antitumor effect of the *HSV-TK* and *hIL-12* genes.

The cytomegalovirus (CMV) promoter is frequently used in tumor gene therapy due to its efficient transcription activity in mammalian cells; however, its lack of tumor specificity is a limitation (16,17). *In vivo* host cells transfected with the CMV promoter can be killed using the metabolites of precursor drugs, which have side effects in normal tissues (16,17); therefore, further studies are required in order to understand how to improve the specificity of the *TK* gene, thus reducing the side effects.

The human secretory leukocyte protease inhibitor (*hSLPI*), an 11.7 kDa non-glycosylated, serine protease inhibitor with 107 amino acids (18), is highly active in human cancer cells, including hNSCLC (19), pancreatic (20) and ovarian cancer (21), but not in normal differentiated human cells, including the liver, the endocrine glands and the blood system (22); therefore, *hSLPI* was selected as a tumor-specific promoter.

In the present study, the *hSLPI* promoter was cloned, which regulated the *HSV-TK*/*hIL-12* gene-targeted expression in hNSCLC. To the best of our knowledge, the present study was the first to establish a eukaryotic expression vector containing *HSV-TK*/*hIL-12* and *hSLPI*, and to transfect this into target cells by liposome-mediated gene transfection. The aim of the present study was to investigate the killing effect of the *HSV-TK*/*hIL-12* gene regulated by *hSLPI*, in order to explore a novel strategy for the molecular targeted therapy of hNSCLC.

Materials and methods

Cell lines and plasmids. Human lung adenocarcinoma cell lines (A549 and SPC-A1) and a hepatoblastoma cell line (HepG2) (23) were obtained from the Jilin Tumor Research Institute (Changchun, China) and the Academy of Military Medical Sciences (Changchun, China), respectively. Plasmid pcDNA3.1(+) was procured from the Central Laboratory of China-Japan Union Hospital of Jilin University (Changchun, China).

Cell culture. A549, SPC-A1 and HepG2 cell lines were cultured in 10-cm plastic culture dishes using Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% Gibco fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The culture conditions were: 37˚C, 5% CO_2 and saturated humidity. The medium was changed every two days, and the cells were passaged using trypsin/ethylenediaminetetraacetic acid medium (Gen-View Scientific, Inc., El Monte, CA, USA) at 37˚C for 5 min when the confluence was ~100%.

Construction of vectors. The hSLPI promoter gene sequence was amplified by polymerase chain reaction (PCR) using the forward primer, 5'-TTCGACGCGTCTCACTGCAGCCTC AAAC-3'; and the reverse primer, 5'-TTCTAGCTAGCGGTG AAGGCAGGAGTGAC-3'; and the two restriction enzymes *Mlu*I and *Nhe*I, which were added to each of the primers. After replacing the CMV sequence of pcDNA3.1(+) with *hSLPI* using the *Mlu*I and *Nhe*I enzymes, the pcDNA3.1-hSLPI vector was obtained. HSV-TK-IRES and IL-12 sequences were also cloned from the pGT60-hIL-12 vector, provided by Professor Sun Ying from the King's College London (London, UK). The sequences of the primers were as follows: HSV-TK-IRES forward, 5'-AAA CCGGAATTCGCCATCATGGCCTCGTAC-3'; and reverse, 5'-TATGCGGCCGCGGTATTATCGTGTTTTTC-3'; and two restriction sites *Eco*RI and *Not*I were added to each primer. *hIL-12* forward, 5'-AAATATGCGGCCGCTAAGCCACCA TGGGTCAC-3'; and reverse, 5'-CCGCTCGAGCGTTAGGAA GCATTCAGATAG-3'; and two restriction sites *Not*I and *Xho*I were added to each primer. Vectors containing HSV-TK-IRES and *hIL-12* sequences, under the transcription control of the *hSLPI* promoter, were constructed by connecting the HSV-TK-IRES and *hIL-12* sequences to the pcDNA3.1-PSLPI vector, particularly the fusion gene eukaryotic expression vector pcDNA3.1-phSLPI-TK/hIL-12. Vectors containing the fusion gene *HSV-TK*/*hIL-12* regulated by the CMV promoter were also constructed by connecting the HSV-TK-IRES and *hIL-12* sequences to the pcDNA3.1(+) vector, particularly pcDNA3.1-CMV-TK/hIL-12. Similarly, by connecting the HSV-TK-IRES to the pcDNA3.1-PSLPI or the pcDNA3.1(+) vectors, a single gene eukaryotic expression vector was produced, either pcDNA3.1-phSLP-TK or pcDNA3.1-CMV-TK.

Liposome-mediated gene transfection. Plasmid pcDNA3.1(+), pcDNA3.1-CMV-TK/hIL-12, pcDNA3.1-CMV-TK, pcDNA3.1-phSLP-TK/hIL-12 and pcDNA3.1-phSLP-TK were extracted using a Endo-Free Plasmid Mini kit (Promega Corporation, Madison, WI, USA). The content and purity of the extracted plasmids were determined using a NanoDrop 2000 (Thermo Fisher Scientific, Inc.). Logarithmic growth phase cells of the A549, SPC-A1 and HepG2 cell lines were seeded into 24-well plates at a density of $5x10^4$ cells/well and cultured for 18-24 h, at 37˚C in an atmosphere containing 5% CO_2, until the cells reached ~80% confluency.

Following this, cells were transfected using Lipofectamine® 2000, according to the manufacturer's instructions (Invitrogen; Thermo Fisher Scientific, Inc.). Solution A was prepared by diluting 0.4 μg plasmids with 25 μl DMEM without FBS. Solution B was prepared by diluting 1 μl liposomes with 25 μl DMEM without FBS. A mixture of solutions A and B was incubated for 30-45 min at room temperature, and then DMEM without FBS was added to attain a volume of 400 μl. The cells were incubated with the prepared mixture for 5 h; after adding 400 μl DMEM with 20% FBS, the mixture

was incubated again. The transfected cells were selected using G418 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at different concentrations (800 mg/l for A549 and SPC-A1; 400 mg/l for HepG2).

The cells were sorted into groups as follows: Control group, cells without transfection; pcDNA3.1(+) group, cells with pcDNA3.1(+); Pcmv-TK-hIL-12 group, cells with pcDNA3.1-CMV-TK/hIL-12; Pcmv-TK group, cells with pcDNA3.1-CMV-TK; PSLPI-TK-hIL-12 group, cells with pcDNA3.1-phSLP-TK/hIL-12; and PSLPI-TK group, cells with pcDNA3.1-phSLP-TK.

HSV-TK/hIL-12 fusion gene expression analysis using reverse transcription (RT)-PCR. Total RNA was extracted from three cell lines in the control, Pcmv-TK-hIL-12 and PSLPI-TK-hIL-12 groups using an RNA Purification kit (Promega Corporation). Total RNA was subjected to cDNA synthesis using a Reverse Transcription System (Promega Corporation). The primers of human *β-actin* were as follows: Forward, 5'-GAGCTACGA GCTGCCTGACG-3'; and reverse, 5'-CCTAGAAGCATTTGC GGTGG-3'. The primers of *HSV-TK* and *IL-12* are as aforementioned. The RT-PCR performed comprised 35 thermal cycles at 95˚C for 5 min, 94˚C for 30 sec, 55˚C for 45 sec, 72˚C for 100 sec and 72˚C for 10 min. The PCR product of 5 μl/lane was visualized with ethidium bromide dye on 1% agarose gel and the imaging was observed by JS 680D gel imaging system (Shanghai Peiqing Science and Technology Co., Inc., Shanghai, China). The negative control used was water. The gray value of DNA per lane was analyzed by ImageJ 1.43b (National Institutes of Health, Bethesda, MD, USA).

hIL-12 gene expression levels analysis using an ELISA. The cells in the logarithmic growth phase from the control, pcDNA3.1(+), Pcmv-TK-hIL-12 and PSLPI-TK-hIL-12 groups were seeded in 100-ml culture flasks, with each containing $6x10^6$ cells. The supernatant (0.5 ml) was collected after 24, 48 and 72 h. The IL-12 concentration of the supernatant was determined using the Human IL-12 ELISA kit (cat. no. DRE10282; Solarbio, Beijing, China). Each group underwent three repetitions.

Lymphocyte proliferation assay. The supernatants of the control, pcDNA3.1(+), Pcmv-TK-hIL-12 and PSLPI-TK-hIL-12 group A549 cells, after culture for 72 h, were collected and added to the lymphocytes isolated from healthy human peripheral blood samples obtained at the Second Hospital of Jilin University in May 2014. The present study was conducted with the approval of the Institutional Ethics Committee of The Second Hospital of Jilin University (approval no. 139). Based on the type of supernatant, the lymphocytes were grouped as follows: Control group, pcDNA3.1(+) group, Pcmv-TK-hIL-12 group and PSLPI-TK-hIL-12 group. Each group comprised two subgroups: The experimental well and the control well. Human lymphocytes were plated in a 96-well plate at a density of 1x105 cells/well. The corresponding supernatant (100 μl) and phytohaemagglutinin (PHA, 2 μl, 5 mg/ml; Sigma-Aldrich; Merck KGaA) were added to the cells within the experimental wells to generate a final concentration of 50 μg/ml, and 200 μl of the corresponding supernatant without PHA was added to the cells within the control wells. Each well had a total volume of 200 μl. Following this, lymphocytes were cultured in DMEM containing 10% FBS with 100 U/ml penicillin and 100 μg/ml streptomycin, at 37˚C in 5% CO_2 humidified atmosphere and an MTT assay was performed after 24, 48 and 72 h. Dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was used to dissolve the purple formazan and the wavelength used to measure the formazan was 490 nm. With OD indicating the optical density, the lymphocyte proliferation rate was calculated as follows: Cell proliferation rate (%) = (OD value of experimental well - OD value of control well)/(OD value of control well) x 100%. Each group underwent three repetitions.

Analysis of the direct antitumor effect of the fusion gene using an MTT assay. Three types of cells were divided into six groups, as follows: Control group, pcDNA3.1(+) group, Pcmv-TK-hIL-12 group, Pcmv-TK group, PSLPI-TK-hIL-12 group and PSLPI-TK group. The cells in the logarithmic phase were collected and plated at a density of $5x10^3$ cells/well in a 96-well plate. GCV at a concentration of 10 μg/ml, and lymphocytes at an effector-target ratio of 20:1 were added into the experimental wells, and the DMEM medium (200 μl/well) without GCV or any lymphocytes, was added into the control wells. Each well had a total volume of 200 μl, and each group underwent three repetitions of the assay. The MTT assay was performed after culturing for 72 h at 37˚C with an atmosphere containing 5% CO_2. The cell survival rate was calculated as follows: Cell survival rate (%) = OD value of experimental well/OD value of control well x 100%.

BSE of fusion gene analysis using an MTT assay. TK^+ cells obtained from the Pcmv-TK, PLPI-TK, Pcmv-TK-hIL-12 and PSLPI-TK-hIL-12 groups were cultured together with TK- cells where TK^+ cells were in the proportion of 0, 10, 25, 50, 75 and 100% of the total cells. Mixed cells were plated in 96-well plates at a density of $5x10^3$ cells/well, with GCV at a final concentration of 10 μg/ml and lymphocytes at a 20:1 effector-target ratio for the experimental wells, and DMEM medium (200 μl/well) without GCV or lymphocytes for the control wells. Each well had a total volume of 200 μl, and each group had three repetitions. The MTT assay was performed 72 h later. The cell survival rate was calculated as follows: Cell survival rate (%) = OD value of experimental well/OD value of control well x 100%.

Statistical analysis. The SPSS version 13.0 software (SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis. The quantitative data are expressed as the mean ± standard deviation. Student's t-test was used to analyze differences in gene expression and cell survival rate between two groups. A one-way ANOVA followed with least significant difference for post hoc were performed to analyze intergroup differences for hIL-12 gene expression, lymphocyte proliferation rate and cell survival rate. A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

The HSV-TK/hIL-12 fusion gene eukaryotic expression vectors regulated by hSLPI are constructed successfully. The *hSLPI* promoter gene sequence (1,250 bp) was amplified from the

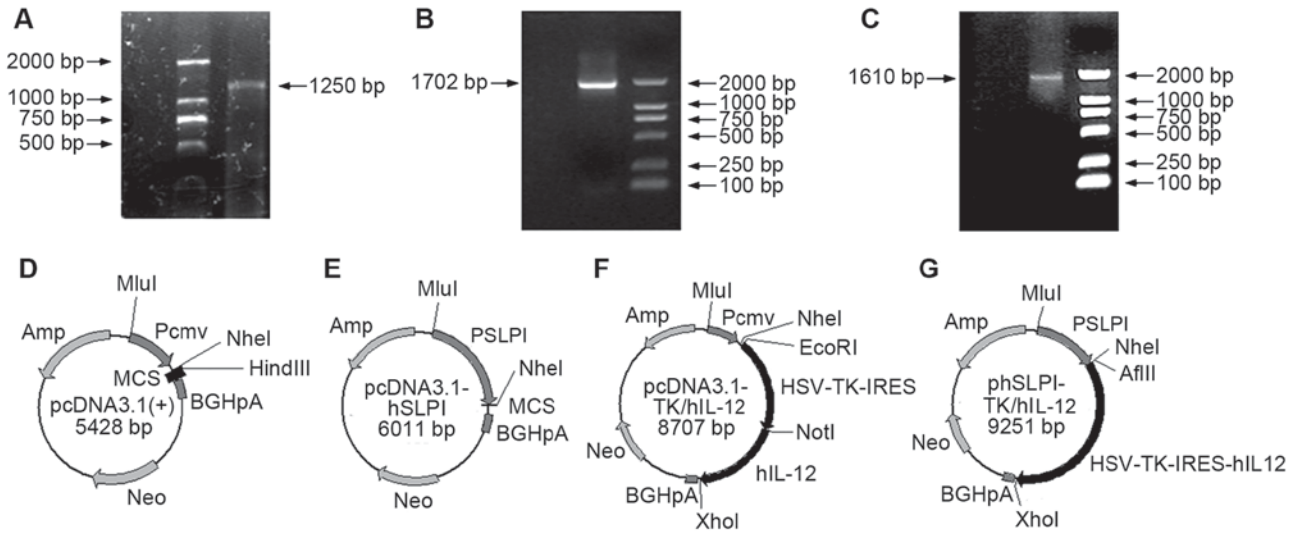

Figure 1. Construction of vectors. (A) Agarose gel electrophoresis of the PCR product of the *hSLPI* promoter. (B) Agarose gel electrophoresis of the PCR product of HSV-TK-IRES. (C) Agarose gel electrophoresis of the PCR product of *hIL-12*. (D) The frame of the PCDNA3.1(+) vector. (E) The frame of the pcDNA3.1-PSLPI vector. (F) The frame of the pcDNA3.1-CMV-TK/hIL-12 vector. (G) The frame of the pcDNA3.1-phSLPI-TK/hIL-12 vector. PCR, polymerase chain reaction; hSLPI, human secretory leukocyte protease inhibitor; hIL-12, human interleukin 12; BGHpA, bovine growth hormone polyadenylation signal; HSV-TK-IRES, herpes simplex virus-thymidine kinase-internal ribosome entry site; MCS, multiple cloning site. CMV, cytomegalovirus.

genome DNA of human mononuclear cells in the peripheral blood using PCR (Fig. 1A). HSV-TK-IRES (Fig. 1B) and IL-12 (Fig. 1C) sequences were cloned from the pGT60-hIL-12 vector. Replacing the CMV sequence of pcDNA3.1(+) with *hSLPI*, using *Mlu*I and *Nhe*I enzymes, enabled the pcDNA3.1-PSLPI vector to be obtained (Fig. 1D and E). After connecting the HSV-TK-IRES and *hIL-12* sequences with the pcDNA3.1(+) vector, pcDNA3.1-CMV-TK/hIL-12 was obtained (Fig. 1F). Following connecting the HSV-TK-IRES and *hIL-12* sequences with the pcDNA3.1-PSLPI vector, pcDNA3.1-phSLPI-TK/hIL-12 was obtained (Fig. 1G).

HSV-TK/hIL-12 fusion gene regulated by hSLPI promoter is successfully expressed in hNSCLC

HSV-TK/hIL-12 fusion gene expression is demonstrated using RT-PCR. The expression of *HSV-TK/hIL-12* mRNA among various groups and three different cell lines was analyzed by RT-PCR. Three electrophoresis bands were observed: *β-actin* (416 bp), *hIL-12* (1,610 bp) and *HSV-TK* (1,137 bp). The control groups for all three cell lines, and the PSLPI-TK-hIL-12 group for HepG2 cells did not express the *hIL-12* or *HSV-TK* gene. On the contrary, the PSLPI-TK-hIL-12 group for the A549 and SPC-A1 cell lines, and the Pcmv-TK-hIL-12 group for all three cell lines expressed the *hIL-12* and *HSV-TK* genes (Fig. 2A). Evaluation of the OD of the electrophoresis bands indicated no significant differences in the *hIL-12* to *β-actin* ratio in the Pcmv-TK-hIL-12 group among the A549, SPC-A1 and HepG2 cells (0.39±0.06 vs. 0.37±0.04 vs. 0.42±0.04; P>0.05). No significant differences were observed in the PSLPI-TK-hIL-12 group between the A549 and SPC-A1 cells (0.18±0.03 vs. 0.21±0.02; P>0.05) either; however, the ratio in the PSLPI-TK-hIL-12 group was lower than that calculated for the Pcmv-TK-hIL-12 group Between A549 and SPC-A1 cells (P<0.01; Fig. 2B). For the *HSV-TK* and *β-actin* ratio, no significant differences were determined between A549, SPC-A1 and HepG2 cells within the Pcmv-TK-hIL-12 group (0.34±0.07 vs. 0.38±0.05 vs. 0.33±0.03; P>0.05). No significant differences in the *HSV-TK* and *β-actin* ratio were demonstrated in the PSLPI-TK-hIL-12 group between A549 and SPC-A1 cells (0.17±0.04 vs. 0.19±0.04; P>0.05); however, the ratio was lower in the PSLPI-TK-hIL-12 group than in the Pcmv-TK-hIL-12 group between A549 and SPC-A1 cells (P<0.01; Fig. 2C). The results indicated that the genes regulated by the CMV promoter were expressed in three types of tumor cell lines at the same expression level, without cell specificity. The genes regulated by the *hSLPI* promoter were expressed in two types of lung cancer cell lines in a targeted manner, the exception being the HepG2 cells.

hIL-12 gene expression in protein level is demonstrated using an ELISA. An ELISA was performed to detect *hIL-12* gene expression. The results indicated that all three cell lines infected with Pcmv-TK-hIL-12 expressed *hIL-12*, and that no significant differences were determined among the A549 [(16.52±0.11 ng)/10^6/72 h], SPC-A1 [(16.45±0.22 ng)/10^6/72 h] and HepG2 cells [(16.57±0.14 ng)/10^6/72 h] (P>0.05). No *hIL-12* expression was observed in the PSLPI-TK-hIL-12 group of the HepG2 cells. The expression level of *hIL-12* within A549 [(4.32±0.15 ng)/10^6/72 h] and SPC-A1 cells [(4.52±0.28 ng)/10^6/72 h] infected with PSLPI-TK-hIL-12 was significantly lower than that in the Pcmv-TK-hIL-12 group (P<0.01). The expression levels of each group increased as the culture time increased (P<0.01; Fig. 3). These results indicated that the gene regulated by the CMV promoter expressed *hIL-12* in all three cell lines at the same level, and without cell specificity. The gene regulated by the *hSLPI* promoter expressed *hIL-12* in the two types of lung cancer cell lines, and not in HepG2 cells, with specificity for lung cancer tissue (P<0.01).

hIL-12 promotes lymphocyte proliferation. It was observed via optical microscopy that lymphocytes cultured for 3 days in the control wells of each of the four groups had a round

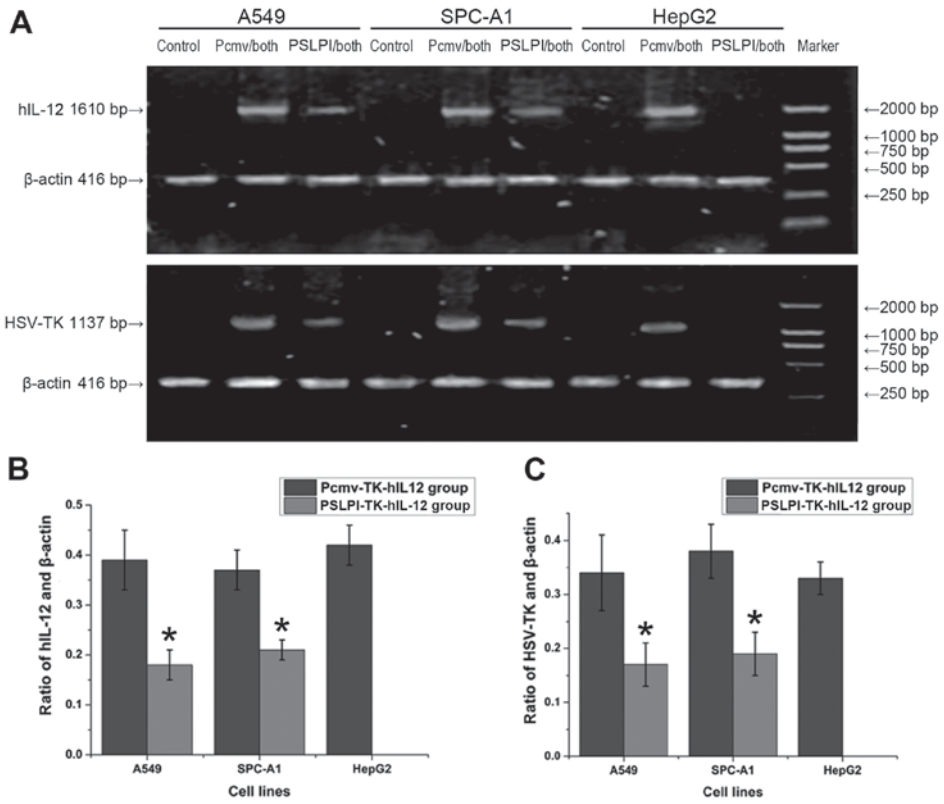

Figure 2. *HSV-TK/hIL-12* fusion gene expression analysis using RT-PCR. (A) The control groups in three cell lines and the PSLPI-TK-hIL-12 group in HepG2 did not express the *hIL-12* or *HSV-TK* gene. By contrast, others expressed the *hIL-12* and *HSV-TK* genes. (B) With β-actin as an internal control, the *hIL-12* expression was lower in the PSLPI-TK-hIL-12 group than in the Pcmv-TK-hIL-12 group (*P<0.01). (C) With β-actin as an internal control, the *HSV-TK* expression level was lower in the PSLPI-TK-hIL-12 group than in the Pcmv-TK-hIL-12 group (*P<0.01). HSV-TK, herpes simplex virus-thymidine kinase; hIL-12, human interleukin-12.

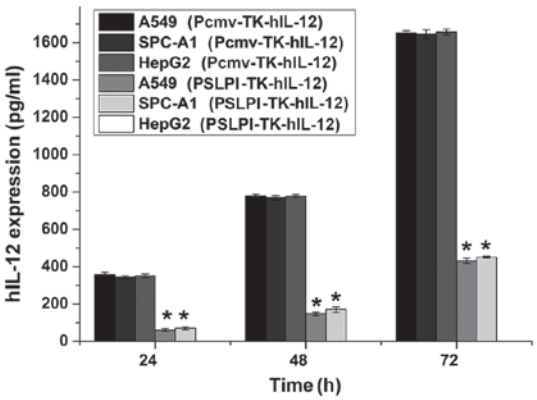

Figure 3. *hIL-12* gene expression levels analysis using an ELISA. Following transfection, the *hIL-12* expression in the Pcmv-TK-hIL-12 group (A549, SPC-A1 and HepG2 cells; no significant difference among the three cell lines) was demonstrated to be higher than that in the PSLPI-TK-hIL-12 group (A549 and SPC-A1 cells; no significant difference between the two cell lines) by ELISA (P<0.01). No *hIL-12* expression was observed in the PSLPI-TK-hIL-12 group of HepG2 cells. The expression of each group increased as the culture time increased (P<0.01). The expression of *hIL-12* in the PSLPI-TK-hIL-12 group was lower than that in the Pcmv-TK-hIL-12 group (*P<0.01). hIL-12, human interleukin-12; TK, thymidine kinase.

appearance. Conversely, following stimulation with PHA, the lymphocytes increased in volume, their sizes and shapes became irregular and they frequently transformed into lymphoblasts (Fig. 4A-D). The results demonstrated lymphocyte proliferation in all experimental wells, as compared with in the control wells of all of the groups. Compared with the control group, the cell proliferation rate did not change significantly in the pcDNA3.1(+) group; though it increased significantly in the Pcmv-TK-hIL-12 and PSLPI-TK-hIL-12 groups (P<0.01). The increased expression level in the PSLPI-TK-hIL-12 group was still lower than that in the Pcmv-TK-hIL-12 group (P<0.01; Fig. 5). The results indicated that the *hIL-12* expressed by the fusion gene eukaryotic expression vectors had notable proliferation activity to the lymphocytes that were stimulated with PHA. The activity increased with the amount of *hIL-12* expression and the duration of exposure (P<0.01).

Specific and cooperative killing effect of the HSV-TK/hIL-12 fusion gene regulated by the hSLPI promoter in hNSCLC in vitro

Direct antitumor effect of the HSV-TK/hIL-12 fusion gene. The killing effect of lymphocytes at a 20:1 effector-target ratio combined with GCV at a final concentration of 10 μg/ml was evaluated using an MTT assay after 72 h of culture. For all three cell lines, the cell survival rate in the pcDNA3.1 groups did not change significantly, as compared with in the control groups (P>0.05). In the PSLPI-TK and PSLP-TK-hIL-12 groups of the two lung cancer cell lines, cell survival rate declined significantly, compared with that in the control groups (P<0.01), while no significant difference was noted with the HepG2 cell line (P>0.05). In the Pcmv-TK and Pcmv-TK-hIL-12 groups of the three cell lines, cell survival rate declined significantly,

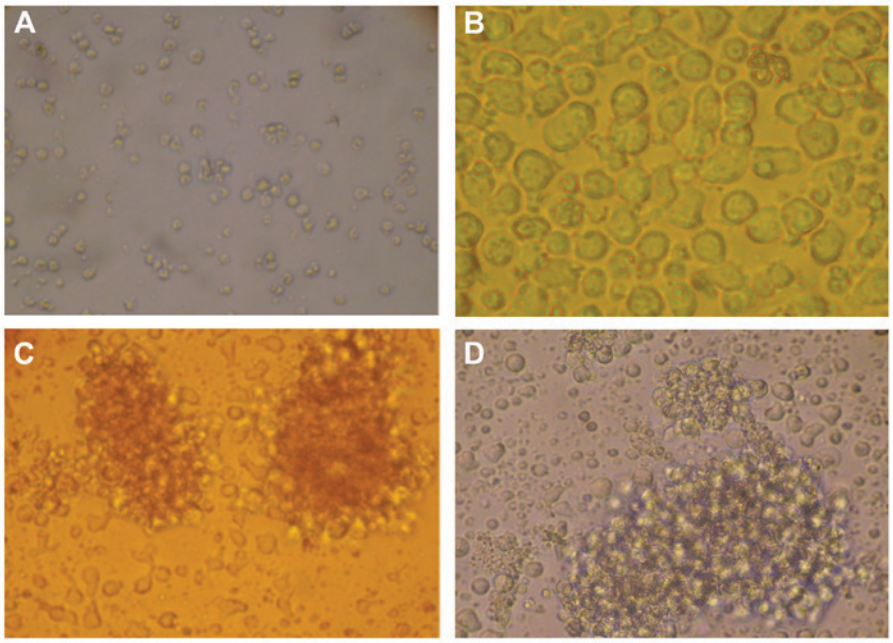

Figure 4. The lymphocyte proliferation treated with the supernatant of A549 cells. (A) Control group: the majority of the lymphocytes appeared round, with part of them containing uneven edges and necrosis (magnification, x400). (B) pcDNA3.1(+) group: a number of the lymphocytes frequently became lymphoblasts. They increased in volume, and their size and shape became irregular (magnification, x800). (C) Pcmv-TK-hIL-12 group and (D) PSLPI-TK-hIL-12 group: the lymphoblasts markedly increased in number and frequently grouped together to form clusters (magnification, x400). TK-hIL-12, thymidine kinase-human interleukin-12.

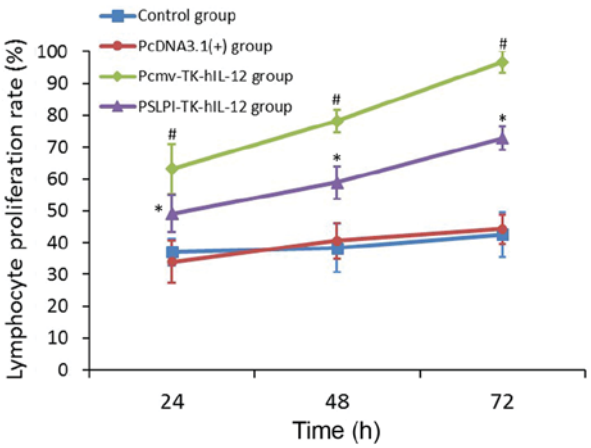

Figure 5. The lymphocyte proliferation rate treated with the supernatant of A549 cells. The cell proliferation rate did not change significantly in the pcDNA3.1(+) group, compared with the control group; however, it increased in the Pcmv-TK-hIL-12 and PSLPI-TK-hIL-12 groups (P<0.01). The lymphocyte proliferation rate in the PSLPI-TK-hIL-12 group was higher than that in the pcDNA3.1(+) and control groups (*P<0.01). The lymphocyte proliferation rate in the Pcmv-TK-hIL-12 group was higher than that in the PSLPI-TK-hIL-12 group (#P<0.01). TK-hIL-12, thymidine kinase-human interleukin-12.

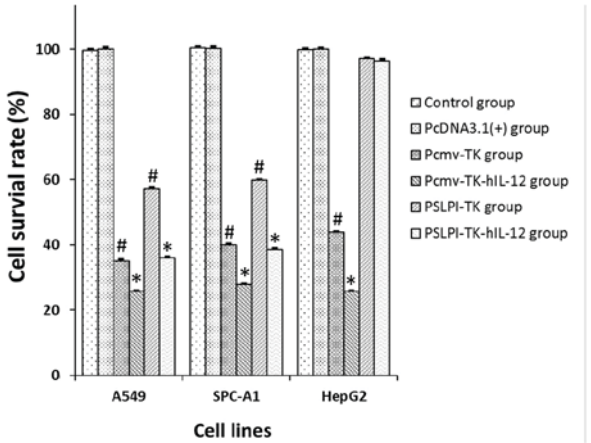

Figure 6. Effects of ganciclovir and lymphocytes on the survival rate of the three cell lines. In the control and PcDNA3.1 groups of all three cell lines, the cell survival rate was 100%. In the PSLPI-TK/PSLPI-TK-hIL-12 groups (A549 and SPC-A1 cells) and Pcmv-TK/Pcmv-TK-hIL-12 groups (A549, SPC-A1 and HepG2 cells), the cell survival rate declined significantly, compared with that in the control groups (P<0.01), and the fusion gene indicated a lower cell survival rate, compared with single gene. The cell survival rate in the Pcmv-TK group/PSLPI-TK group was lower than that in the pcDNA3.1(+) and control groups (#P<0.01). The cell survival rate in the Pcmv-TK-hIL-12 group/PSLPI-TK-hIL-12 group was lower than that in the Pcmv-TK group/PSLPI-TK group (*P<0.01). TK-hIL-12, thymidine kinase-human interleukin-12.

compared with that in the control groups (P<0.01; Fig. 6). These results indicated that *TK/GCV* gene regulated by the *hSLPI* promoter had a notable killing effect on the lung cancer cells specifically (P<0.01). Conversely, the CMV promoter had no tissue specificity.

The present study compared the killing effect between single gene and fusion gene treatments. In the CMV control group, the fusion gene therapy had a lower cell survival rate in A549, SPC-A1 and HepG2 cells (25.12, 25.77 and 25.82%, respectively), compared with single gene therapy (35.01, 40.29 and 43.91%, respectively; P<0.01). In the *hSLPI* control group, the cell survival rate in the fusion gene groups of A549 and SPC-A1 cells (35.99 and 38.54%, respectively) was also lower than that in the single gene groups (57.29 and 59.90%, respectively; P<0.01). These results indicated that the *HSV-TK/hIL-12* fusion gene induced cooperative killings.

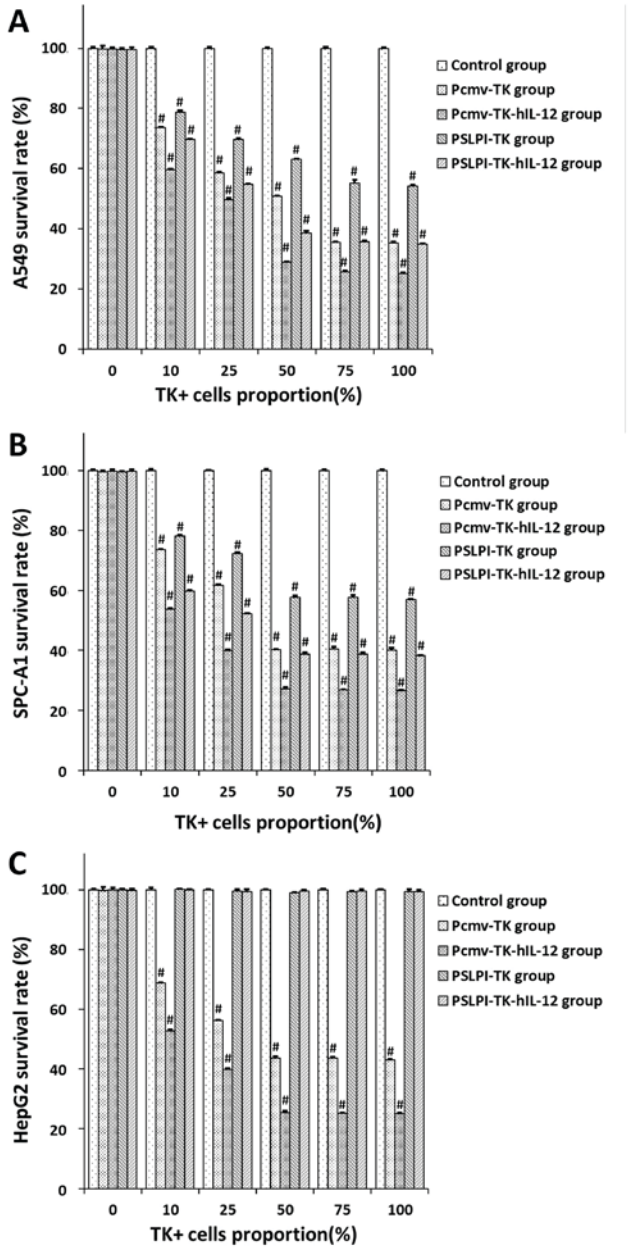

Figure 7. Bystander effects on three cell lines. (A) A549 and (B) SPC-A1 cell lines: the cell survival rate in the Pcmv-TK, Pcmv-TK-hIL-12, PSLPI-TK and PSLP-TK-hIL-12 groups declined significantly from 10% TK$^+$, compared with that in the control groups (P<0.01). (C) HepG2 cells: the cell survival rate in the Pcmv-TK and Pcmv-TK-hIL-12 groups declined significantly from 10% TK$^+$, compared with that in the control groups (P<0.01), while no significant difference was noted with the PSLPI-TK and PSLP-TK-hIL-12 groups (P>0.05). The cell survival rate declined significantly, compared with the control group, from 10% TK$^+$ ($^{\#}$P<0.01). TK-hIL-12, thymidine kinase-human interleukin-12.

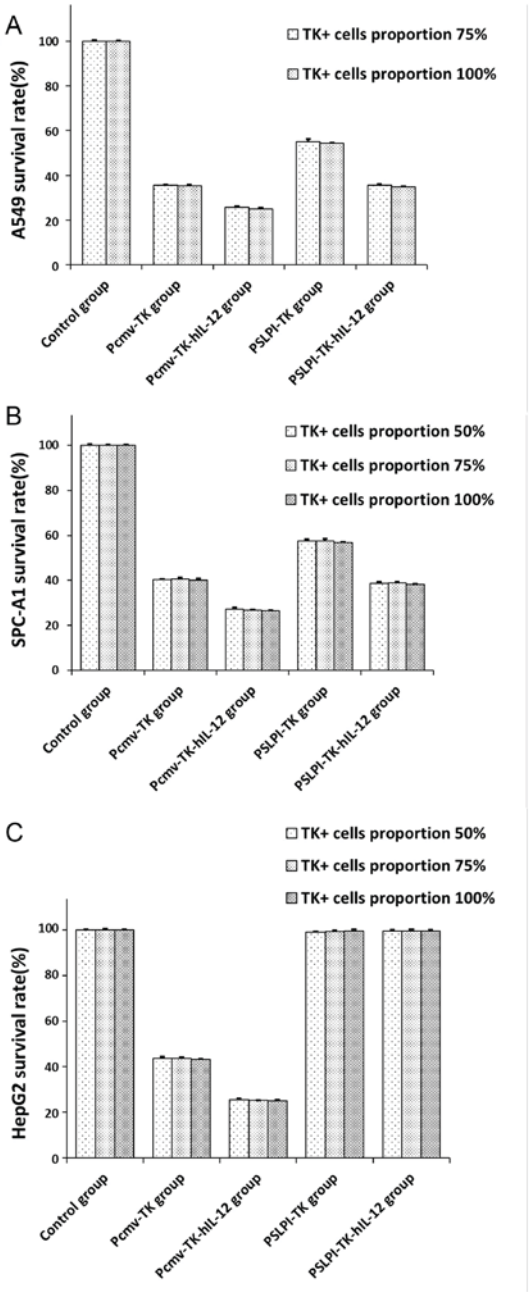

Figure 8. Bystander effects on three cell lines. (A) A549 cells: at 75% TK$^+$, the survival rate in the control, Pcmv-TK, Pcmv-TK-hIL-12, PSLPI-TK and PSLPI-TK-hIL-12 groups did not change significantly, compared with 100% TK$^+$ (P>0.05). (B) SPC-A1 cells and (C) HepG2 cells: at 50% TK$^+$, the survival rate in the control, Pcmv-TK, Pcmv-TK-hIL-12, PSLPI-TK and PSLPI-TK-hIL-12 groups did not change significantly, compared with 75 and 100% TK$^+$ (P>0.05). TK-hIL-12, thymidine kinase-human interleukin-12.

BSE of the HSV-TK/hIL-12 fusion gene. GCV at a final concentration of 10 μg/ml and lymphocytes at 20:1 effector-target ratio were added into tumor cells, of which TK$^+$ cells accounted for 0, 10, 25, 50, 75 and 100%. An MTT assay was performed 72 h later, and the cell survival rate was calculated. For A549 and SPC-A1 cell lines, the cell survival rate in the Pcmv-TK, Pcmv-TK-hIL-12, PSLPI-TK and PSLP-TK-hIL-12 groups declined significantly from 10% TK$^+$, compared with that in the control groups (P<0.01; Fig. 7A and B). For HepG2 cells, the cell survival rate in the Pcmv-TK and Pcmv-TK-hIL-12 groups declined significantly from 10% TK$^+$, compared with that in the control groups (P<0.01), while no significant difference was noted with the PSLPI-TK and PSLP-TK-hIL-12 groups (P>0.05; Fig. 7C). For the A549 cells at 75% TK$^+$ the survival rate in the control, Pcmv-TK, Pcmv-TK-hIL-12, PSLPI-TK and PSLPI-TK-hIL-12 groups did not change significantly, compared with 100% TK$^+$ (P>0.05; Fig. 8A). For the SPC-A1 cells and HepG2 cells at 50% TK$^+$ the survival rate in the control, Pcmv-TK, Pcmv-TK-hIL-12, PSLPI-TK and PSLPI-TK-hIL-12 groups did not change significantly, compared with 75% TK$^+$ and 100% TK$^+$ (P>0.05; Fig. 8B and C). These results indicated

that the *HSV-TK*/GCV system had a BSE on tumor cells, and that the phSLPI-TK/GCV system had a targeted killing effect on lung cancer cells.

Discussion

In recent years, gene therapy has emerged as a promising strategy for the treatment of cancer (24); however, a number of limitations are associated with its clinical application, particularly the reduced specificity in delivering functional therapeutic genes into tumor cells (25). Therefore, prior studies have been focused on developing targeting strategies (26,27). Tissue-specific promoters represent one of the primary methods of gene therapy (28,29).

hSLPI is produced by cells in the respiratory tract and genital mucosa epithelium, and is highly expressed in hNSCLC (19); by contrast, it has low expression in other normal tissues, particularly in the liver (22). This indicated potential therapeutic inhibitory effects mediated by the expression of tumor necrosis factor (TNF)-associated apoptosis-inducing ligand, TNF-α, death receptor (DR)-4, DR-5 and TNF receptor-I, which lead to the activation of the apoptosis pathway via caspase-2, -8 and -9 (30). Previous studies indicated that *SLPI* was significantly overexpressed in ovarian cancer samples, compared with in matched normal samples (31). Additionally, studies used the *hSLPI* promoter to regulate the suicide gene therapy of cervical (32) and ovarian cancer (33) had achieved an antitumor effect. In 2004, Maemondo *et al* (34) used this promoter in the therapy of hNSCLC (squamous cell carcinoma HS-24 and adenocarcinoma A549 and H358 cells) and achieved a potent antitumor effect *in vivo* and *in vitro*. In the present study, the eukaryotic expression vector of *HSV-TK*/*hIL-12* fusion gene regulated by the *hSLPI* promoter was constructed, specifically expressed in lung cancer cells so as to improve the safety and effectiveness of lung cancer gene therapy. To further investigate the specificity of the *hSLPI* gene promoter in lung cancer, the HepG2 cell line was selected as the negative control, which had already been constructed in the lab to prove the effectiveness of gene targeting therapy. The *HSV-TK*/*hIL-12* gene expression at the mRNA level was investigated using RT-PCR. The results indicated that genes regulated by the CMV promoter were expressed in three types of tumor cell lines at the same level ($P>0.05$), without cell specificity. The genes regulated by the *hSLPI* promoter were expressed in the two types of lung cancer cell lines, and not in HepG2 cells. Coincidentally, an ELISA was preformed to detect the *hIL-12* expression, and the results were similar to the RT-PCR findings. The MTT assay also revealed that the *HSV-TK*/*hIL-12* gene regulated by the *hSLPI* promoter had a notable and specific killing effect on the lung cancer cells ($P<0.01$). These results demonstrated that the genes regulated by the *hSLPI* promoter had an effect on lung cancer cells in a targeted manner.

Currently, two commonly used vector systems exist for tumor gene therapy: Viral and non-viral vectors (35). Nanni *et al* (36) transferred the *mIL-12* gene into tumor cells using retroviral vectors. The administration of this product as a vaccine led to tumor regression in 80-90% of the tumor-burdened mice. The output of mIL-12 reached 400-2, 500 pg/l0^6/24 h. Loskog *et al* (37) used the adenovirus vector with the *mIL-12* gene in bladder cancer therapy and obtained an antineoplastic effect. The production of mIL-12 reached 6-11 ng/ml after the cells were cultured for 48 h *in vitro*; however, viral vectors have a number of disadvantages (38-42). The major problem is the generation of neutralizing antibodies, which are formed due to immunogenicity and inflammatory toxicity (41). Ring *et al* (42) determined that when the suicide gene, regulated by the ERBB2 promoter, was transferred into cells using viral vectors, the promoter lost its transcriptional activity and specificity, and its specific antitumor effect was weakened. In comparison, cationic lipids as a non-viral vehicle have several advantages, including low toxicity, no immunogenicity, simple operation, good reproducibility and applicability to mitotic and non-mitotic cells *in vivo* and *in vitro* (26). Through continual improvement, the transfection efficiency of liposomes has been increased up to ~90% (43,44); therefore, in the present study, Lipofectamine® 2000 was used, which could provide high transfection efficiency and high levels of transgene expression in a range of mammalian cell types *in vitro* (45). The present study indicated that the production of the *hIL-12* gene, regulated by the CMV promoter, was 16.52±0.11 ng/10^6/72 h, 16.45±0.22 ng/10^6/72 h and 16.57±0.14 ng/10^6/72 h, in A549, SPC-A1 and HepG2 cells, respectively. The data were consistent with those of previous studies.

Since the first application of the *TK* gene in tumor treatment studies by Moolten in 1986 (46), it had been extensively studied in various human tumor types (9,10,47,48); however, a number of basic and clinical studies indicated that the combination therapy of the *HSV-TK* gene along with immune-associated genes had a greater success than single *TK* gene therapy (9-14). Ramesh *et al* (49) demonstrated that the *TK*/*GCV* gene did not inhibit tumor growth effectively in nude mice without immunity, with the transfection rate being 100%. On the contrary, when the transfection rate was 50%, the tumor inhibition rate could reach 100% in mice with normal immunity. The results indicated that the integrity of the host immune system is essential for *TK* gene therapy. In the present study, by comparing the killing effect of the single gene with the effect of the fusion gene, it was demonstrated that in CMV control groups (A549, SPC-A1 and HepG2) and *hSLPI* control groups (A549 and SPC-A1), the fusion gene had a lower cell survival rate, compared with the single gene. The results indicated that the *HSV-TK*/*hIL-12* fusion gene induced the cooperative antitumor effect on hNSCLC cell lines *in vitro*.

Previous studies demonstrated that in the *HSV-TK*/*GCV* gene antitumor system not only are the cells transfected with the *TK* gene killed, but the adjacent non-transfected tumor cells are also (46,50,51). This phenomenon is known as a BSE (52). The BSE can expand the killing effect of the *TK*/*GCV* gene significantly (52); therefore, the present study explored the BSE of the TK/GCV system on A549, SPC-A1 and HepG2 cells. The results of the MTT assay indicated that, at 10% TK^+, the cell survival rate in the Pcmv-TK/Pcmv-TK-hIL-12 groups (A549, SPC-A1 and HepG2) and the PSLPI-TK/PSLPI-TK-hIL-12 groups (A549 and SPC-A1) declined significantly, compared with the control group ($P<0.01$). In A549 cells, at 75% TK^+, it could achieve the efficiency of the 100% TK^+ group. For the SPC-A1 and HepG2 cells, at 50% TK^+, it could achieve the

efficiency of the 100% TK^+ group. These results indicated that the *HSV-TK*/GCV system had a BSE on tumor cells.

In conclusion, the present study selected the *hSLPI* promoter as a target for *hNSCLC* gene therapy. The data indicated that the fusion gene regulated by the *hSLPI* promoter had targeted expression in hNSCLC, and combined suicide gene therapy with immune gene therapy generated significantly stronger therapeutic antitumor effects, compared with single gene therapy. The present study provided evidence to warrant preclinical studies of this lung cancer treatment, and may present the theoretical basis for a novel therapeutic strategy.

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