

# Therapeutic effect of recombinant plasmid-encoded human interleukin-12 in tumor-bearing mice

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**Abstract.** The aim of this study was to investigate the effects of gene therapy using a recombinant plasmid encoding human interleukin-12 (rIL-12, pcDNA6-p70) on transplanted tumors in mice. Tumor-bearing mice were transplanted with sarcoma-180 (S-180) cells and randomly divided into three groups of 10 mice with each group receiving a separate treatment. Following this, pcDNA6-p70 (dissolved in purified water; 100  $\mu$ g/mouse), cyclophosphamide (dissolved in 0.9% saline; 40 mg/kg) or 0.9% saline (100  $\mu$ l/mouse) was directly injected into the tumors on the 4th, 7th, 10th, 14th and 17th days following transplantation of the S-180 cells. Mice survival time was monitored and surviving mice were sacrificed on the 21st day. In addition to survival time, tumor volume, NK cell activity, spleen lymphocyte proliferation and IFN- $\gamma$  production were investigated. The mice were also monitored for any adverse effects regarding the administration of pcDNA6-p70. Our results demonstrated that pcDNA6-p70 prolongs the survival time of tumor-bearing mice, decreases tumor size ( $P < 0.01$ ) and increases the proliferative response of spleen cells, the activity of NK cells and the serum level of IFN- $\gamma$ . There were no significant adverse effects caused by the administration of pcDNA6-p70. The results of the present study support the hypothesis that gene therapy using the rIL-12 plasmid exerts a therapeutic effect in tumor models by triggering antitumor cellular immunity.

## Introduction

Cancer is a leading cause of mortality in humans. According to the International Agency for Research on Cancer approximately

18.7% of individuals worldwide are likely to develop some form of cancer in their lifetime, and 11.2% of individuals worldwide may succumb to the disease (1). In the last 20 years, advances in cancer research and the consequent development of more effective therapies have significantly increased the survival times associated with certain types of cancer (1). However, there have been few breakthroughs that are useful in all patients, particularly those with advanced stage cancer. One of the most favorable new approaches to cancer treatment currently being developed is tumor biotherapy. In particular, targeting cytokines has been shown to induce the proliferation of immunocompetent cells that have antitumor activity (2-4).

One of the strongest and most broad-ranging immunocyte-stimulating cytokines is interleukin-12 (IL-12), which is known to have powerful antiviral and antitumor activity, and is also a core regulating factor in natural immunity (4-6). However, the potential therapeutic effects of administering recombinant IL-12 are limited due to the toxicity and side-effects. In addition, the expression and purification of recombinant IL-12 is expensive, the half-life of IL-12 *in vivo* is short and certain individuals generate antibodies against IL-12 (7,8). Despite these shortcomings, progress has been made towards developing novel methods of IL-12 delivery by combining recombinant IL-12 with an appropriate carrier. The most significant attempts have involved a gene therapy approach to delivery of IL-12 for which several positive results in animal models of tumors and in phase I clinical trials have been produced (9-11).

The construction of a plasmid containing human recombinant IL-12, IL-12 pcDNA6-V5-His-p70 (pcDNA6-p70), has been described and it has been demonstrated that this plasmid has biological activity *in vitro* and *in vivo* (12). The aim of this study was to investigate the therapeutic effects of administering the IL-12-encoding plasmid pcDNA6-p70 to mice bearing transplanted tumors and to determine the safety profile of the plasmid *in vivo* in tumor-bearing mice. Results from this study may form the basis for further investigation of the potential of gene therapy using pcDNA6-p70 in humans.

## Materials and methods

**Reagents.** The recombinant plasmid pcDNA-p70, which codes for human IL-12, was constructed as previously described (12). Methyl thiazolyl tetrazolium (MTT) and lactate dehydrogenase (LDH) were obtained from Sigma (St. Louis, MO, USA),

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**Abbreviations:** MTT, methyl thiazolyl tetrazolium; NBT, nitroblue tetrazolium; LDH, lactate dehydrogenase; DMSO, dimethyl sulfoxide; NK, natural killer; NAD<sup>+</sup>, nicotinamide adenine dinucleotide; DAB, diaminobenzidine; MHC, major histocompatibility complex

**Key words:** recombinant human IL-12 plasmid, antitumor effect, gene therapy

cyclophosphamide from the 12th Pharmaceutical Factory (Shanghai, China), RPMI-1640 complete medium from Gibco (Carlsbad, CA, USA), and the ELISA detection kits for mouse IFN- $\gamma$  from the Jingmei Bioengineering Co., Ltd (Shenzhen, China).

*Laboratory animals and S-180 cells.* Kunming mice were provided by the Center for Laboratory Animals in Qingdao Drug Identification Office (Shandong, China). The S-180 cells were obtained from the Institute of Materia Medica of the Shandong Academy of Medical Science (Shandong, China). The study was approved by the ethics committee of Shandong Medical College.

*Cell modification.* The S-180 cell line was transfected with pcDNA6-p70 by polyethyleneimine (PEI; Sigma). The modified cell line (S-180/IL-12) was selected in 10% FBS RPMI-1640 with Blasticidin S HCl (final concentration 10 mg/ml; Invitrogen, Carlsbad, CA, USA) for 14 days and cultured in maintenance solution (Blasticidin S HCl, 2 mg/ml). The modified cell line (S-180/IL-12;  $2 \times 10^5$ ) was cultured in complete medium for 48 h. The expression level of pcDNA6-p70 in the supernatant was detected using a human IL-12 ELISA kit.

*S-180 tumor-bearing mouse model and the administration of pcDNA6-p70.* S-180 cells were injected into the peritoneal cavity and the resulting ascites were extracted, washed with physiological saline and the density of the cells within the fluid was adjusted to between  $2 \times 10^7$  and  $6 \times 10^7$  cells/ml before 0.2 ml was injected into the right armpit of the mice ( $n=30$ ). The majority of the tumors were formed by the 4th day following S-180 cell transplantation. The mice were randomly divided into three groups ( $n=10$  in each). The first group received pcDNA6-p70 (dissolved in purified water; 100  $\mu$ g/mouse), the second cyclophosphamide (dissolved in 0.9% saline; 40 mg/kg), and the third 0.9% saline (100  $\mu$ l/mouse). The compounds were directly injected into the tumor on the 4th, 7th, 10th, 14th and 17th days following transplantation of the S-180 cells. On the 20th day, the mice were weighed and blood samples were collected from the tail vein. The mice were sacrificed on the 21st day and the tumor, spleen and thymus of each mouse was removed and weighed. The ratio of tumor suppression was calculated by dividing the weight of the tumors obtained from the mice administered pcDNA6-p70 or cyclophosphamide by the weight of the tumors removed from the mice administered physiological saline. The spleen and thymus indices were calculated by dividing the respective organ weight by body weight  $\times 1000$ .

*MMT assay used to measure the proliferation of spleen cells.* To measure the proliferation of the spleen cells, cell suspension was constructed from each mouse spleen where the number of viable cells was  $>95\%$  (staining by trypan blue). The cell suspension was adjusted to  $2 \times 10^6$  cells/ml with RPMI-1640 nutrient medium containing 10% solcoseryl. The cells were transferred to a 96-well plate (100  $\mu$ l/well;  $n=3$  wells for each mouse spleen sample) and 5  $\mu$ l Concanavalin A (ConA) was added to each well. Control wells for the assay contained 5  $\mu$ l RPMI-1640 nutrient medium alone. The cells were incubated at 37°C in 5% CO<sub>2</sub> and 95% humidity for 72 h. Shortly before

the incubation time was complete, 20  $\mu$ l MTT (5 mg/ml) was added to each well. Following completion of the incubation period, 150  $\mu$ l dimethyl sulfoxide (DMSO) was added to each well, the contents were thoroughly mixed and the absorbance was read at 570 nm using ELISA (Jingmei Bioengineering Co., Ltd.). The amount of lymphocyte proliferation was calculated by subtracting the absorbance reading of the control wells from that of the sample wells.

*LDH assay used to measure the cytotoxic activity of natural killer (NK) cells.* In order to measure the activity of NK cells, an effector cell suspension containing mouse spleen cells diluted to a density of  $1 \times 10^7$  cells/ml with RPMI-1640 culture medium and a target cell suspension of S-180 cells with a density of  $2 \times 10^5$  cells/ml were prepared. The two cell suspensions were added together in a 96-well plate with a ratio of effector to target cells of 50:1 in each well (sample mixture,  $n=3$  wells for each mouse spleen sample). At the same time, two control mixtures were set up; one to indicate the maximum release of LDH possible (100  $\mu$ l S-180 cells + 100  $\mu$ l 2% Triton X-100), and the other to indicate the level of spontaneous release of LDH from the target cells (100  $\mu$ l S-180 cells + 100  $\mu$ l RPMI-1640 culture media). The sample and control mixtures were incubated at 37°C in 5% CO<sub>2</sub> and 90% humidity for 2 h before 100  $\mu$ l of the supernatant from each well was transferred into another 96-well plate and heated at 37°C for 10 min. Next, 100  $\mu$ l fresh LDH detection mixture [0.032 mg NBT, 0.08 mg NAD<sup>+</sup>, 0.008 mg PMS and 4  $\mu$ l sodium lactate (1 mol/l)] was added to each well, incubated for 10 to 15 min at room temperature in the dark, and then the reaction was terminated with 30  $\mu$ l citric acid (1 mol/l). The absorbance of each reaction mixture was measured using ELISA (ELISA Equipment, Source). Cytotoxic activity of NK cells was calculated using the formula: (absorbance of sample mixture - absorbance of spontaneously released LDH) / (absorbance of maximum LDH - absorbance of spontaneous released LDH)  $\times 100\%$ .

*Detection of IFN- $\gamma$  in tumor-bearing mice.* IFN- $\gamma$  was detected using ELISA. A 40  $\mu$ l sample of blood collected from the caudal vein of each mouse's tail was mixed with 100  $\mu$ l 0.9% saline and centrifuged for 10 min (800 rpm). The resulting supernatant was then subjected to ELISA using an ELISA kit. The absorbance of the reaction mixture was measured at 490 nm and the IFN- $\gamma$  content of the mouse serum was calculated (pg/ml).

*Immunohistochemistry.* Immunohistochemistry was performed on 4  $\mu$ m sections. The primary antibody (anti-human IL-12 monoclonal antibody; R&D Systems, Minneapolis, MN, USA) was incubated overnight. Sections were visualized with 3,3'-diaminobenzidine (DAB)-chromogen and lightly counterstained with haematoxylin. Diluted goat serum was used as the negative control for the primary antibody.

The samples were immunohistochemically stained for IL-12 and were assessed without knowledge of the clinicopathological features. At least 500 carcinoma cells were examined in 10 randomly selected fields ( $\times 200$ ) within the same section under light microscopy to determine the staining status of IL-12. Samples were considered positive when the unequivocal

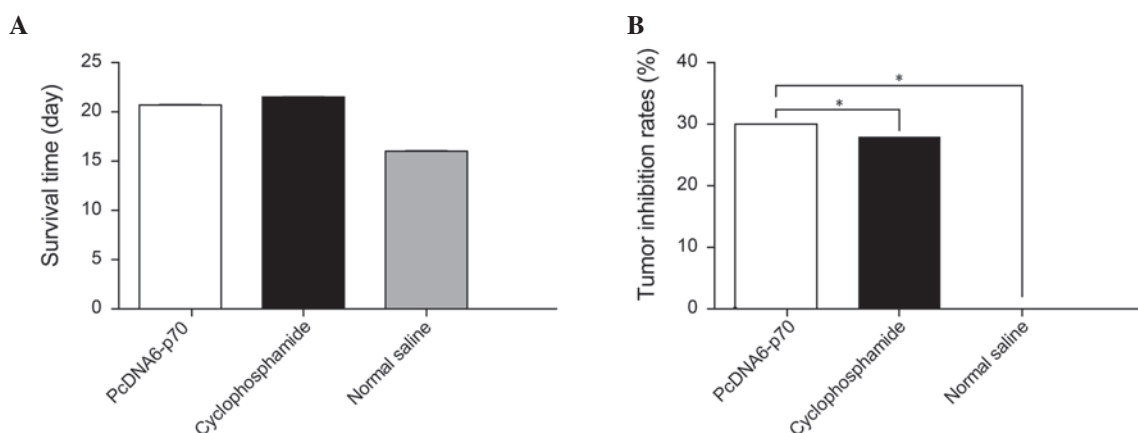


Figure 1. The inhibitory effects of pcDNA6-p70 on tumor growth in tumor-bearing mice (n=10 each group), and the effect on (A) the survival time (day) and (B) the tumor inhibition rates (%). \*P<0.01, compared with normal saline.

staining of the cytoplasm and/or nuclear compartment was observed in >10% of the tumor cells.

**Safety evaluation for pcDNA6-p70 in normal mice.** In addition to the main study groups, two further groups of normal mice were administered pcDNA6-p70 (n=10) or 0.9% saline (n=10) and were monitored for adverse effects relating to the drug treatment. pcDNA6-p70 (100  $\mu$ l/time, 5  $\mu$ g/g) or physiological saline (100  $\mu$ l/time) were injected hypodermically into the right armpit of each respective group of mice, and temperature, weight and general state of health (including appetite, fur color, response to stimulation and locomotor activity) were recorded on the 1st, 2nd, 3rd, 7th and 14th day following the first injection.

**Statistical analysis.** Statistical differences between the outcomes of each measurement of the therapeutic activity of pcDNA6-p70 were analyzed using the one-way ANOVA test and SPSS 14.0 statistical software. P $\leq$ 0.05 was considered to indicate a statistically significant difference.

## Results

**IL-12 expression determined by ELISA.** The expression of S-180/IL-12 in the modified cell line was 0.8-1.4 ng/ml (assessed in the supernatant from  $2 \times 10^5$  cells grown for 48 h in 2 ml of medium) measured by ELISA (data not shown), indicating that pcDNA6-p70 was successfully expressed in S-180/IL-12.

**Expression of IL-12 in the tumor.** Tumor sections were scanned under low-power magnification (x200) to select the most intense areas; >40% of the tumor cells was considered positive. A strong positive expression was considered in interstitial substance mononuclear cells.

**Inhibitory effect of pcDNA6-p70 on the growth of S-180 tumors in mice.** Compared with the tumor-bearing mice administered 0.9% saline, the mice treated with pcDNA6-p70 demonstrated 30% inhibition of the growth of the transplanted tumor cells (P<0.01) (Fig. 1). The tumors on the mice administered pcDNA6-p70 were visibly smaller (Fig. 2), and the mice survival time was also prolonged compared to the saline

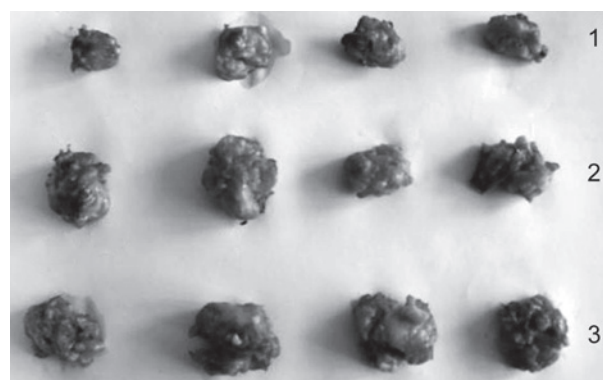


Figure 2. The effects of pcDNA6-p70 on the tumor volume in tumor-bearing mice in the 1) pcDNA6-p70 group, 2) cyclophosphamide group and the 3) normal saline group.

control group (Fig. 1). Notably, the inhibition of tumor growth and prolongation of survival time observed with pcDNA6-p70 was similar to that observed with administration of the clinical chemotherapeutic agent cyclophosphamide.

**Effects of pcDNA6-p70 on the immune function of tumor-bearing mice.** Measurements of the relative size of the spleen and thymus, the number of lymphocytes, the activity of NK cells and the IFN- $\gamma$  content of the serum in tumor-bearing mice indicated that pcDNA6-p70 had a significant effect on immune function. Compared with the tumor-bearing mice administered 0.9% saline, the mice administered pcDNA6-p70 had higher spleen and thymus indices, more proliferative lymphocytes, higher NK cell activity and increased IFN- $\gamma$  serum content (P<0.01) (Fig. 2). These results varied from the effects of cyclophosphamide where lymphocyte proliferation was significantly lower compared with the mice treated with pcDNA6-p70 as in the case of IFN- $\gamma$  serum content and the mouse spleen and thymus indices (P<0.05) (Fig. 3).

**Safety of pcDNA6-p70 in normal mice.** The only adverse reaction observed following the administration of pcDNA6-p70 was the development of anorexia in a few mice during the first

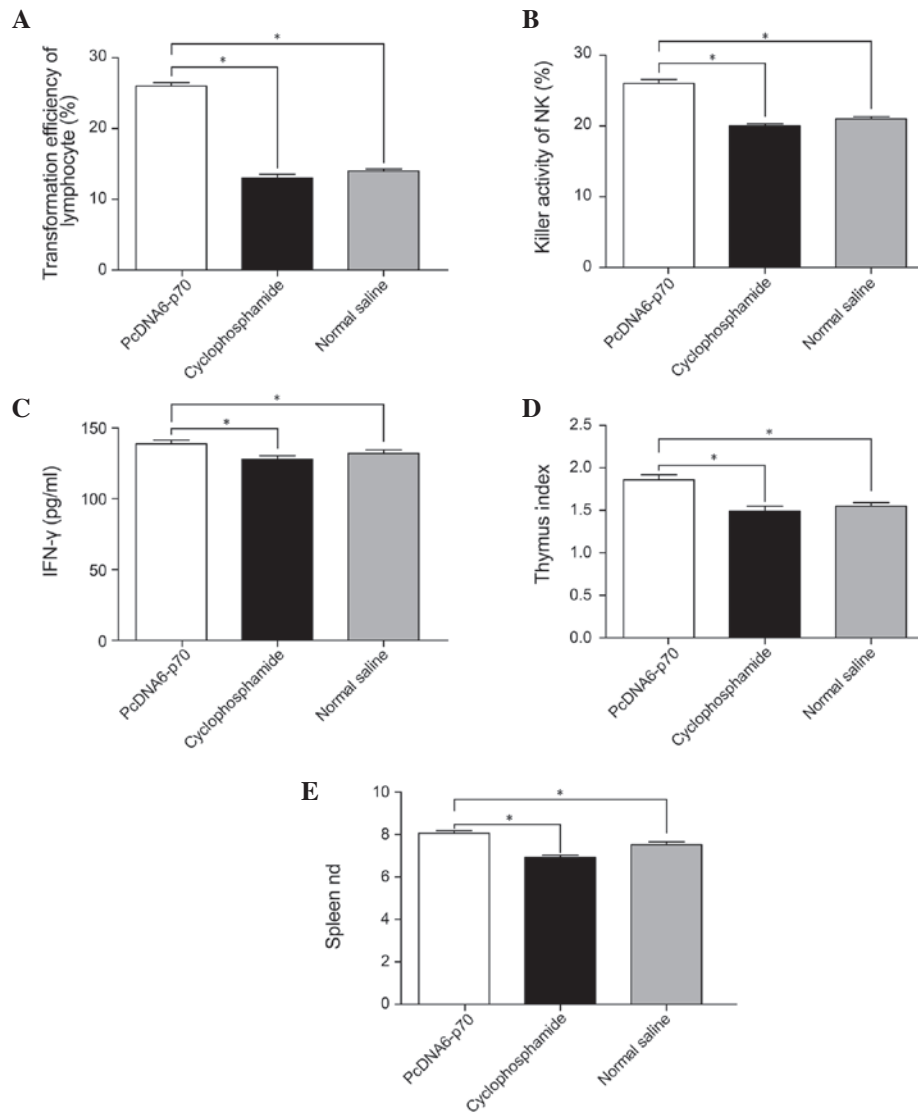


Figure 3. Effect of pcDNA6-p70 on the cell measures of immune function in tumor-bearing mice (n=10 each group) including (A) the transformation efficiency of lymphocytes (%), (B) the killer activity of NK (%), (C) IFN- $\gamma$  (pg/ml), (D) the thymus index and (E) the spleen Nd. \*P<0.01, compared with normal saline and cyclophosphamide.

3-5 days following injection. This effect quickly disappeared and the mean body temperature and weight demonstrated no significant deviation or differences between the mice administered pcDNA6-p70 and the mice administered 0.9% saline (Fig. 4).

## Discussion

Although the mechanisms of tumor development are complicated, malfunction of immune surveillance is considered to be one of the most significant factors in the process (13,14). This suggests that a key approach in antitumor therapy should reinforce the ability of the immune system to recognize tumor antigens as 'foreign' and the enhancement of the activity of specific cellular immunity. A number of immune cells types play a role in the correlation between tumor development and immune function. The first line of antitumor defense is the non-specific NK cells, which are followed by more specific T cells (13,14). IL-12, secreted by antigen-presenting cells (e.g.,

monocytes, macrophages and dendritic cells), is an important antitumor and immune regulatory factor (15), which has several antitumor effects. IL-12 stimulates the differentiation and proliferation of T cells and NK cells, strengthens the cytotoxicity of cytotoxic T lymphocytes, NK cells and macrophages, and induces the secretion of IFN- $\gamma$  (16). The induction of the proliferation of type I T helper (Th1) cells by IL-12 results in the secretion of cytokines, including IL-12 and IFN- $\gamma$ , and a consequent increase in the expression of MHC (17). However, the anti-tumor effect of IL-12 is mostly determined by the secondary generation of IFN- $\gamma$  and administration of an antibody against IFN- $\gamma$  blocks the antitumor effects of IL-12 (16). The secretion of IFN- $\gamma$  produces antitumor effects through various mechanisms, including stimulation of the cytotoxic cytokine TNF- $\beta$ , proliferation of lymphocytes, induction of nitrous oxide and suppression of the growth of blood vessels within tumors (18). The induction of NK cells and cytotoxic T cells by IL-12 also results in the production of a large quantity of IFN- $\gamma$  (18,19).

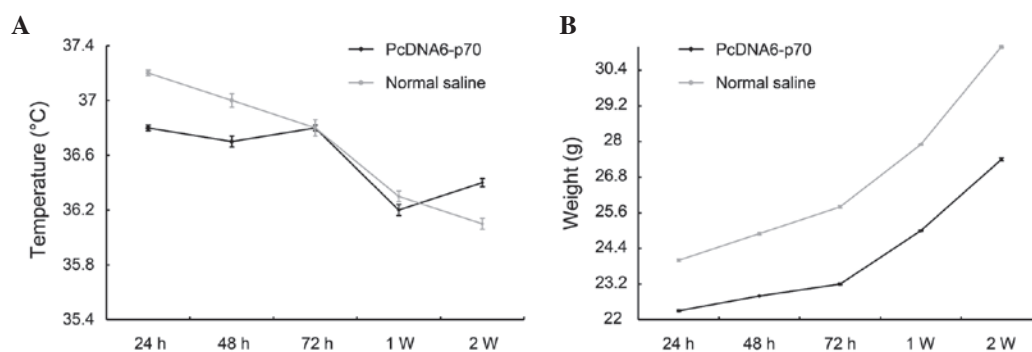


Figure 4. Safety profile of pcDNA6-p70 in mice (n=10 each group) and the effect on (A) temperature (°C) and (B) weight (g).  $P>0.05$ , compared with normal saline.

Various experiments have indicated that any one of these mechanisms alone can have specific antitumor activity. For example, IL-12 has been demonstrated to inhibit the growth of tumors in a mouse model where NK cells are inactive. By contrast, the antitumor effect of IL-12 was lower in a nude mouse model indicating that T cells are involved in the anti-tumor mechanism (19). In addition to lymphocyte-mediated antitumor mechanisms, IL-12 also activates non-lymphocyte pathways, including those regulated by IP-10, an important chemokine that exerts an antitumor effect through inhibition of tumor vascularization (20). Injection of adenovirus expressing IL-12 (Adcmv-IL-12) into mice carrying RenCa tumors resulted in the cell infiltration of macrophages and neutrophilic granulocytes into the tissues surrounding the tumor blood vessels (8). Similarly, in a tumor model lacking CD4<sup>+</sup>, CD8<sup>+</sup> or NK cells, there was infiltration of numerous non-lymphocytes and activation of Kupffer cells, indicating that the antitumor effect of non-lymphocytes is correlated with the activation of IP-10 (20). Thus, the antitumor effect of IL-12 is exerted by direct and indirect actions of lymphocyte and non-lymphocyte components of the host immune system.

In this study, it was demonstrated that a number of the antitumor effects of endogenous IL-12 can be replicated with the administration of pcDNA6-p70 in tumor-bearing mice. pcDNA6-p70 increases the weight of the spleen and thymus, and also increases the level of IFN- $\gamma$ . It also promotes the activation of cytotoxic lymphocytes, stimulates the secretion of silent or activated peripheral T cells, promotes mouse spleen cell proliferation and activity of NK cells. From these findings, it can be concluded that pcDNA6-p70 controls the growth of tumors and kills tumor cells by regulating the immune system and stimulating T and NK cells. In the cyclophosphamide control group, although cyclophosphamide extended the survival time of the mice, the immune function was slightly decreased, as expected. Cyclophosphamide is a widely used antitumor drug. Its antitumor effects are mediated by its activated form, phosphamide chlormethine, which is produced through hydrolysis by excess phosphatase in the liver or tumor *in vivo*. Cyclophosphamide is able to inhibit various types of tumor, but is particularly effective in malignant lymphoma, acute or chronic lymphocytic leukemia and multiple myeloma. However, the toxic side-effects of cyclophosphamide are evident in clinical treatment and include moderate to severe immunosuppression. Other common side-effects include

gastrointestinal reaction, inhibition of bone marrow, alopecia and sterile cystitis (21). The findings from this study suggest that recombinant IL-12 is able to exert a greater antitumor effect by activating endogenous antitumor pathways compared with the exogenous effects of chemotherapeutics, such as cyclophosphamide, and that the use of pcDNA6-p70 is capable of avoiding the side-effects of chemotherapeutics. Numerous studies in animals have demonstrated that recombinant IL-12 has a greater therapeutic effect in over 20 types of tumors, with the greatest effect in lung neoplasms and lymph neoplasms (8-12). Translation of this antitumor activity into patients, however, is limited by the side-effects of the direct application of recombinant IL-12.

In this study, we also demonstrated that the direct injection of pcDNA6-p70 produced a high and continuous local cytokine density in the tumor, compared to a low density in the blood. This type of action corresponds with the physiological functions of autocrine and paracrine cytokines. The high density of cytokines in the local environment can increase antigen expression (including MHC) in the tumor cells and recruit immunocytes (including T cells, B cells and NK cells). Furthermore, it can induce other cytokines. All these factors reinforce the immune functions in different ways, thereby effectively enhancing the antitumor immunity.

Due to the side-effects caused by the direct use of IL-12, research into gene therapy using a carrier system combined with recombinant IL-12 is a promising treatment modality. In this study, normal mice treated with pcDNA6-p70 demonstrated no significant adverse effects compared to control mice treated with 0.9% saline. This included no significant differences in temperature, weight or general state of health (including appetite, fur color, response to stimulation and locomotor activity). Similarly, the safety of plasmids bearing recombinant IL-12 has also been revealed by other studies. Wolff *et al* demonstrated that there was no integration of recombinant plasmids into the host genome by screening more than 1800 plasmids after *Escherichia coli* containing recombinant plasmid DNA were injected into mice. The methylating pattern of the plasmid DNA in the injected *Escherichia coli* remained the same for 19 months in the muscles of mice, indicating that there was no plasmid replication (22). Similarly, a study by Jiao *et al* in primates found that there was no anti-DNA detected even after reinjection (23). Imboden *et al* also reported that there were no abnormalities in organ histology and serum biochemical

markers in mice administered the recombinant plasmid (pNGVL-3-mIL12) at a dose of 0.5 to 5  $\mu$ g. Levels of serum IFN- $\gamma$  were also normal, demonstrating that recombinant plasmids containing IL-12 are safe *in vivo* and could be used in gene therapy (24).

In conclusion, results of this study have shown that the *in vivo* delivery of recombinant IL-12 using a plasmid vector (pcDNA6-p70) has a significant antitumor effect, and with further development and testing may be useful in the clinic.

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