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Original Article

Recombination plasmid carrying hcg and survivin combination IL-12 generates specific immune responses and anti-tumor effects in a murine breast carcinoma model

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Abstract: The effectiveness of DNA vaccination alone is limited, because it often generates only a weak cellular immune response; therefore, the complementary use of adjuvants may be required to improve vaccine potency and enhance its immune protective effects. The present study aimed to determine the effect of recombinant DNA vaccine-based human hcg and survivin on the development of breast carcinoma in mice and the potential immune mechanisms involved, as well as the adjuvanticity of IL-12. Recombinant plasmids pVAX1-hcg-survivin and pVAX1-IL-12 were constructed, and injected into female mice intramuscularly (i.m.) followed by an electric pulse. The humoral and cellular immune responses after immunization were examined by enzyme linked immunosorbent assay (ELISA) and enzyme-linked immunospot assay (ELISPOT), respectively. To evaluate the anti-tumor efficacy of the plasmids, a mouse model with an hcg-survivin-expressing tumor was designed. Mice vaccinated with the pVAX1-hcg-survivin combination pVAX1-IL-12 plasmid generated the strongest inhibition efficacy on the growth of tumors and prolonged mouse survival. These observations emphasize the potential of IL-12 as an adjuvant for DNA vaccines and of vaccines based on hcg and surviving fusion gene and IL-12 as a promising treatment for breast carcinoma.

Keywords: Breast carcinoma, hcg, surviving, IL-12, anti-tumor, DNA vaccine

Introduction

Breast cancer is the most common malignancy diagnosed in women worldwide. Early detection and improvements in screening have increased the number of premenopausal women diagnosed with breast cancer, while advances in treatment options have contributed to declining breast cancer mortality rates [1]. Recent advances in our knowledge of the biology of the immune system, such as the identification of immunostimulatory genes, combined with improvements in the ability to modify gene expression through genetic engineering, have fostered a new era of tumor immunotherapy [2]. As an active immunotherapy, DNA vaccine can induce strong cellular immune response, which has become one of the hot spots in the study of tumor immunotherapy [3, 4].

Survivin is an apoptosis inhibitor and a member of apoptosis protein (IAP) family, and plays an important role in inhibiting apoptosis. According to the published literatures, survivin cannot be detected in tissues except the testis, thymus, and placenta. Yet it can be overexpressed in several kinds of cancer cells including breast cancer and gastric cancer cells. Therefore, survivin is used as a drug target for novel cancer therapies. Inhibiting the expression of survivin protein can increase the apoptotic rate and inhibit tumor growth [5]. HCG is composed of two distinct noncovalently associated α- and β-subunits and has six major isoforms with different biological properties. The most widely used assays for hCG determination that are practical for use in epidemiological studies are total, intact and free β-hCG (free β-hCG). The total hCG assays quantify both intact hCG and

free β-hCG, which may have distinct associations with risk of breast cancer [6]. So, we believe that these two antigens are ideal targets for immunotherapy of breast cancer.

However, the effectiveness of DNA vaccination alone is limited, because it often generates only a weak cellular immune response; therefore, the complementary use of adjuvants may be required to improve vaccine potency and enhance its immune protective effects [7]. Interleukin 12 (IL-12), which is involved in the differentiation of naïve T cells toward Th1 [8], is an effective adjuvant in increasing the protective immunity. IL-12 co-administration with DNA vaccine priming can induce strong cell-mediated type 1 immune responses [9, 10]. In this study, we used the hcg and surviving antigen with the IL-12 as a adjuvant plasmid to verify the immunogenicity and antitumor activity of this DNA vaccine in tumor bearing mice model.

Material and methods

Construction and expression of DNA vaccine

Based on the coding regions of β-hcg (GenBank: J00117.1) and survivin (GenBank: AY830084.1), we designed a fusion gene containing β-hcg coding region and survivin coding region. The β-hcg gene was connected to the N-terminal of survivin gene by the (G4S)3. The fusion gene was cloned into the eukaryotic expression vector pVAX1 to construct the eukaryotic expression plasmid pVAX1-hcg-survivin. To construct the plasmid expressing IL-12, the mouse IL-12 gene (GenBank: AF128215.1 and AF128213.1) was cloned into pVAX1. Synthesis of the gene was carried out by Invitrogen.

These plasmids were transiently transfected into 293T cells, and expression of hcg -survivin and IL-12 were determined by ELISA with Human hCG ELISA Kit (ab100533, Abcam, USA), Human survivin ELISA Kit (ab183361, Abcam, USA) or Mouse IL-12 p40/p70 ELISA Kit (ab100699, Abcam, USA), respectively.

Mice and cell lines

Female BALB/c mice, 6-8 weeks old, were purchased from Beijing Weitong Lihua Experimental Animal Technology Co. Ltd. (Beijing, China). The mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, Revised 1996). The EMT-6 cell line was purchased from the Shanghai Cell Institute (Shanghai, China).

Animal grouping and vaccination

The experimental mice were randomly divided into 4 groups (n = 6 mice/group): untreated group, pVAX1 group, pVAX1-hcg-survivin group and the pVAX1-hcg-survivin and pVAX1-IL-12 combined group. Beside untreated group, each mouse was injected with 50 μg plasmid and subjected to electroporation. Two-needle array electrodes (BTX, Hollison, USA) were inserted into the muscle (quadriceps muscle of hind legs) immediately after intramuscularly (i.m.) administration of plasmid DNA for electroporation. The distance between the electrodes was 5 mm and the array was inserted longitudinally relative to the muscle fibers. *In vivo* electroporation parameters were as follows: 60 V/mm distance between the electrodes, 50-ms pulse length, 6 pulses with reversal of polarity after each pulse, and administered using a BTX ECM-830 electroporator (Hollison, USA) [13]. On the 10th day and the 20th day after the first immunization, the mice were given an immune boost. The procedure of immune boost is in full agreement with the primary immunization. The mice were sacrificed two weeks after the last immunization, and immunological tests were performed. The number of independent repeat experiments was three.

Assaying antibodies in the serum

To evaluate the humoral response in the mouse model, anti-hcg and survivin antibodies in the sera of immunized mice were detected by enzyme linked immunosorbent assay (ELISA). 96-well microplates were coated with 10 μg/ml recombinant hcg or survivin antigen in bicarbonate buffer and incubated at 4°C overnight. After blocking with 5% powdered milk in phosphate buffer saline (PBS) containing Tween-20, sera taken from the mice 14 days after the final immunization were serially diluted in PBS and incubated on the plates for 2 h at room temperature. After washing with PBS, the plates were incubated with a 1:1000 dilution of a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Zhong Shan Jin Qiao Biological Technology, Beijing, China) at room temperature for 1 h. The reaction was stopped

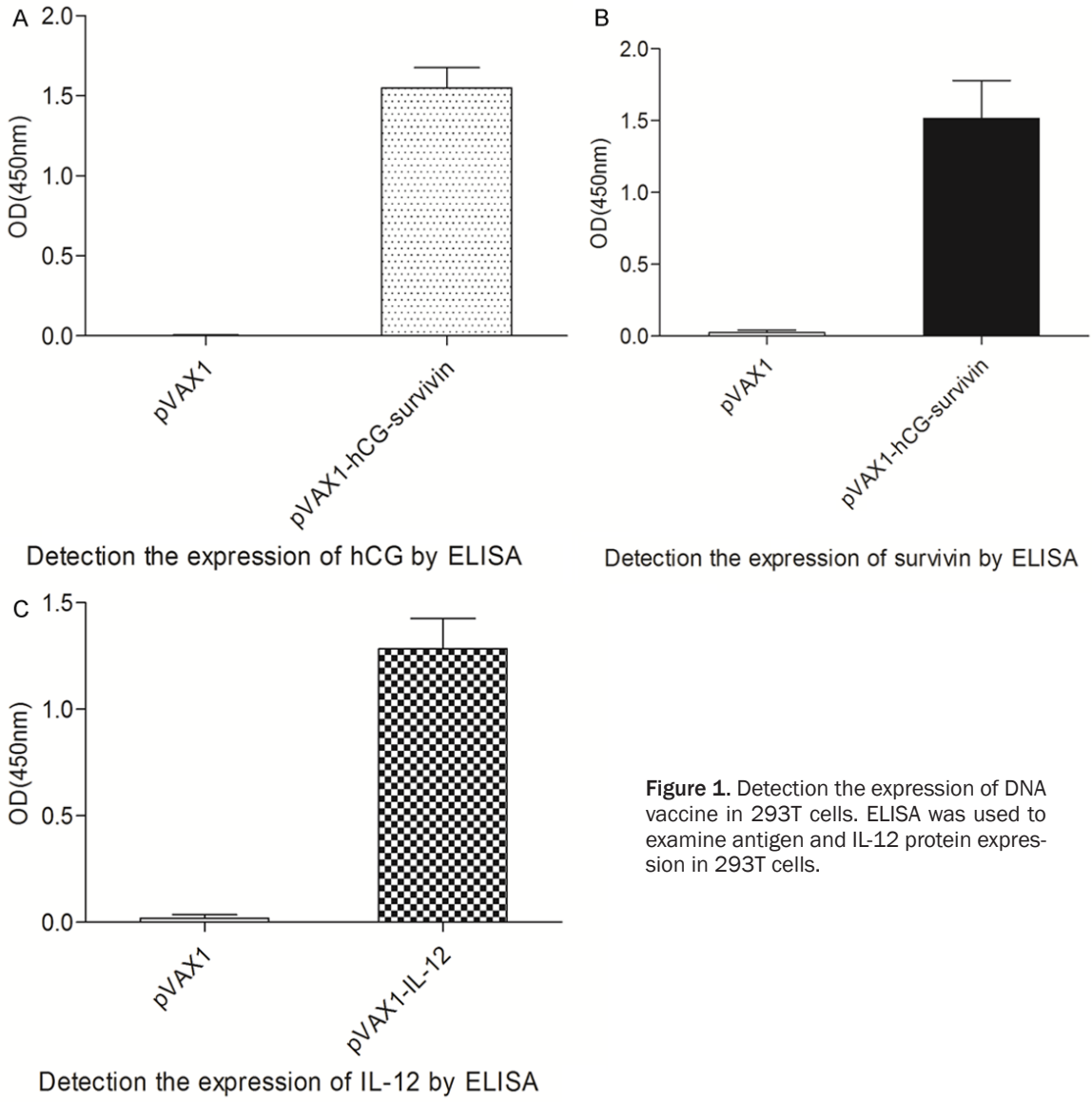

Figure 1. Detection the expression of DNA vaccine in 293T cells. ELISA was used to examine antigen and IL-12 protein expression in 293T cells.

with 2 M H_2SO_4, and the absorbance at 450 nm was measured using an ELISA plate reader.

ELISPOT assay

Splenocytes from control and vaccinated groups of mice were isolated. The IFN-γ enzyme-linked immunospot assay (ELISPOT) was performed according to the manufacturer's protocol (Dakewe Biotech Ltd., Shenzhen, China). In brief, the 96-well plates were coated with anti-mouse interferon (IFN)-γ monoclonal antibody (mAb) at 4°C overnight, and blocked for 1 h at 37°C. The freshly isolated splenocytes (4×10^5 cells/well) from each vaccinated mouse group were added to the wells and incubated with 5 μg/ml recombinant hcg and survivin protein for 48 h. Each test condition was performed in triplicate. The spots were counted and analyzed with the ELISPOT Reader.

In vivo tumor treatment experiments

To test the ability of the DNA vaccine to treat tumors in the mouse model, 6-8-week-old female BALB/c mice were injected subcutaneously in the right flank with 1.5×10^5 EMT-6 cells. Three days after tumor cell injection, ten mice were vaccinated by IM administration with 50 μg of plasmid DNA, and on the 10th and 20th days after the first immunization, the immune was boosted. Tumor development was monitored in individual mice every 4 days, and the tumor volume was calculated according to the following formula: V (mm^3) = 0.5 × long diameters × short diameters^2, and the growth

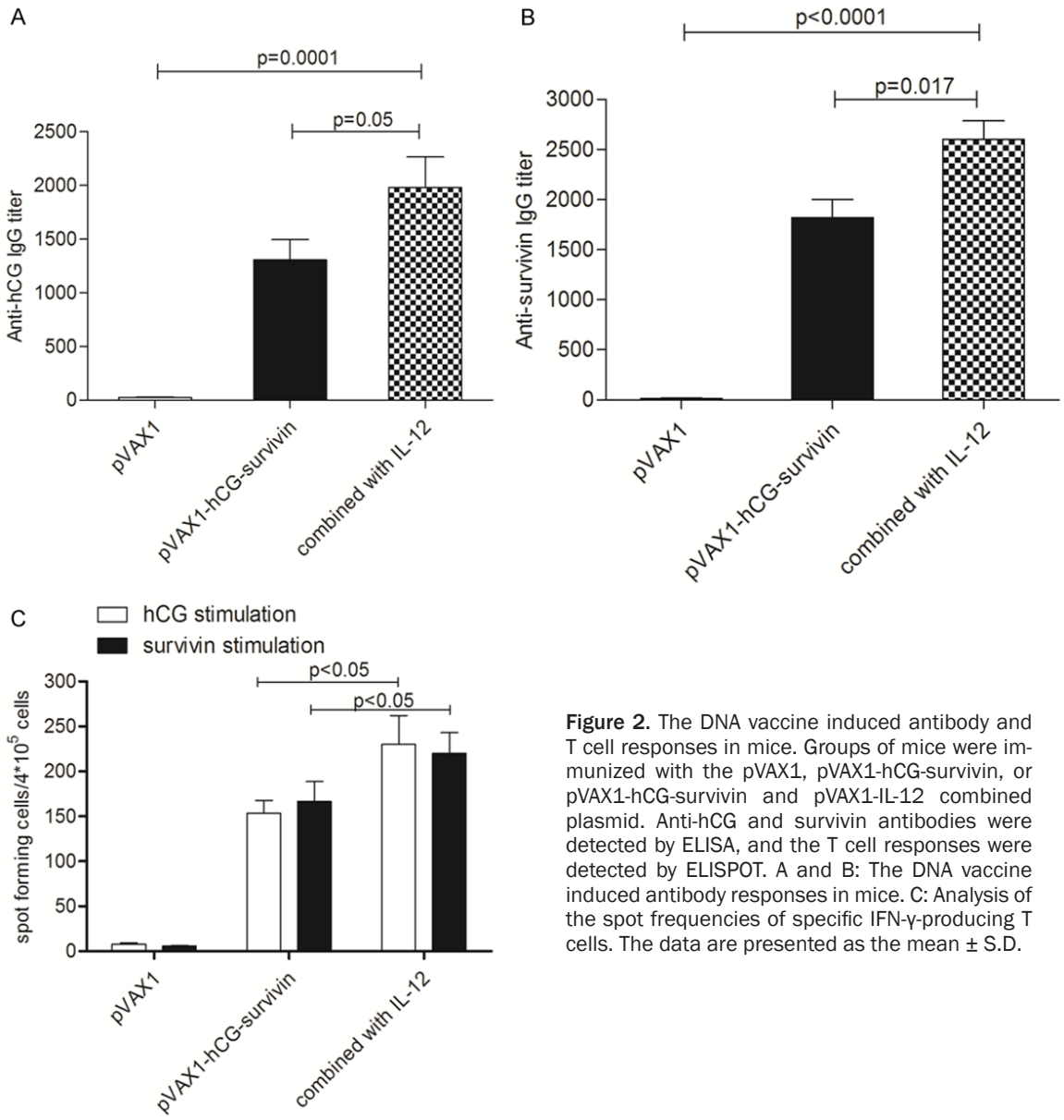

Figure 2. The DNA vaccine induced antibody and T cell responses in mice. Groups of mice were immunized with the pVAX1, pVAX1-hCG-survivin, or pVAX1-hCG-survivin and pVAX1-IL-12 combined plasmid. Anti-hCG and survivin antibodies were detected by ELISA, and the T cell responses were detected by ELISPOT. A and B: The DNA vaccine induced antibody responses in mice. C: Analysis of the spot frequencies of specific IFN-γ-producing T cells. The data are presented as the mean ± S.D.

curves of the tumors were drawn. On the 14th day after last immunization, mice were sacrificed. Tumors were removed and weighed, and the tumor growth inhibition rate was calculated. The calculation formula for tumor inhibition rate was as follows: (average tumor weight in the control group - average tumor weight in the treatment group)/average tumor weight of the control group × 100%.

Statistical analysis

All data are presented as means ± SD. One-way ANOVA followed by Tukey's multiple comparison test was applied to determine the difference between groups using the SPSS 17.0 software. The Kaplan-Meier method was employed to construct the survival curve. For all comparisons, *P* values < 0.05 were considered statistically significant.

Results

Expression of DNA vaccine plasmids in eukaryotic cells

The expression of DNA vaccine plasmids in the 293T cells was verified. 48 h after transfection with the recombinant plasmid pVAX1-hcg-survivin and pVAX1IL-12, cells suspension in 6-well

plates were collected, and the expression of hcg-survivin and IL-12 was detected by ELISA. As shown in **Figure 1A-C**, hcg, survivin and IL-12 could be expressed by the corresponding plasmid in the cells, respectively.

Functional study of the DNA vaccine

In mice vaccinated with pVAX1-hcg-surviving group or combined group, the presence of anti-hcg or survivin antibodies was verified at 2 weeks after the final immunization. As shown in **Figure 2A** and **2B**, both mice in pVAX1-hcg-survivin group or combined group could induce specific antibodies. The combined group induced antibody levels that were approximately 2 times than of the pVAX1-hcg-survivin group. This experiment showed that the IL-12 could improve the antibody response induced by a DNA vaccine.

Two weeks after the final immunization, an IFN-γ ELISPOT assay of spleen lymphocytes was performed to detect the antigen-specific cellular immune response. As shown in **Figure 2C**, IFN-γ secretion was induced in the mice immunized with pVAX1-hcg-surviving group or combined group vaccines. The spot numbers in the two vaccinated groups were significantly higher than that in the empty vector pVAX1 group. Additionally, the number of spots in the mice immunized with combined group vaccine was higher than that in the pVAX1-hcg-survivin group. These results indicate that the antigen-induced release of IFN-γ from spleen cells was significantly enhanced by the use of IL-12 as an immune adjuvant. The results showed that the DNA vaccine could induce both the humoral and cellular immune responses in mice, and the immune response induced by the fusion gene was significantly higher than that of the antigen alone.

In order to study the effect of DNA vaccines on tumor growth, tumor-bearing mice were vaccinated with the DNA vaccine. The effect of immunotherapy was demonstrated by measuring tumor size. Three days after tumor cell injection, mice were vaccinated three times with DNA vaccine plasmids, and the tumor volume was measured every 4 days. Tumor growth in mice treated with pVAX1-hcg-survivin group or combined group was more effectively inhibited compared with that in the pVAX1 vector group (**Figure 3A**). In addition, the combined group vaccine inhibited tumor growth more effectively than pVAX1-hcg-survivin. We also measured tumor weight (**Figure 3B**) and calculated the tumor growth inhibition rate of the different groups of mice (**Figure 3C**) after vaccine treatment. Tumor weight and tumor growth inhibition rates in mice immunized with combined group had a significant difference compared with those in the mice immunized with pVAX1-hcg-survivin.

Discussion

In recent years, immune therapy technology has developed rapidly, and has played an important role in the treatment of many diseases. This is especially true in the treatment of cancer, where immune therapy has shown great potential and therapeutic effect [13]. Anti-tumor immunotherapy using vaccines has become a prominent research topic, and much progress has been made using this approach. In 2012, the world's first anti-tumor therapeutic vaccine, PROVENGE, was approved by the United States FDA (Food and Drug Administration) for prostate cancer treatment [14]. However, many studies have indicated that a vaccine containing only an antigen is not effective enough to induce an anti-tumor immune response, inhibit tumor growth, inhibit recurrence, or inhibit metastasis.

An important area of research is aimed at improving the immunogenicity of DNA vaccines by way of molecular adjuvants. Cytokines are essential for the differentiation and maturation of immune cells and play a key role in the regulation of immune responses. It has been demonstrated that many cytokines themselves have some anti-tumor effect (e.g., GM-CSF, IL-12 and IL-2), and some have developed into a recombinant protein drug that is used as an adjuvant in tumor treatment [15, 16].

In order to increase the immunogenicity and effectiveness of DNA vaccines, there are many studies [17] in which IL-12 is fused with a specific antigen as a molecular adjuvant. It has been found that, in this way, it can significantly enhance the humoral and cellular immune responses, inhibit tumor growth and improve the survival of experimental animals [18].

In this study, to verify whether the immunogenicity and antitumor effect can be enhanced

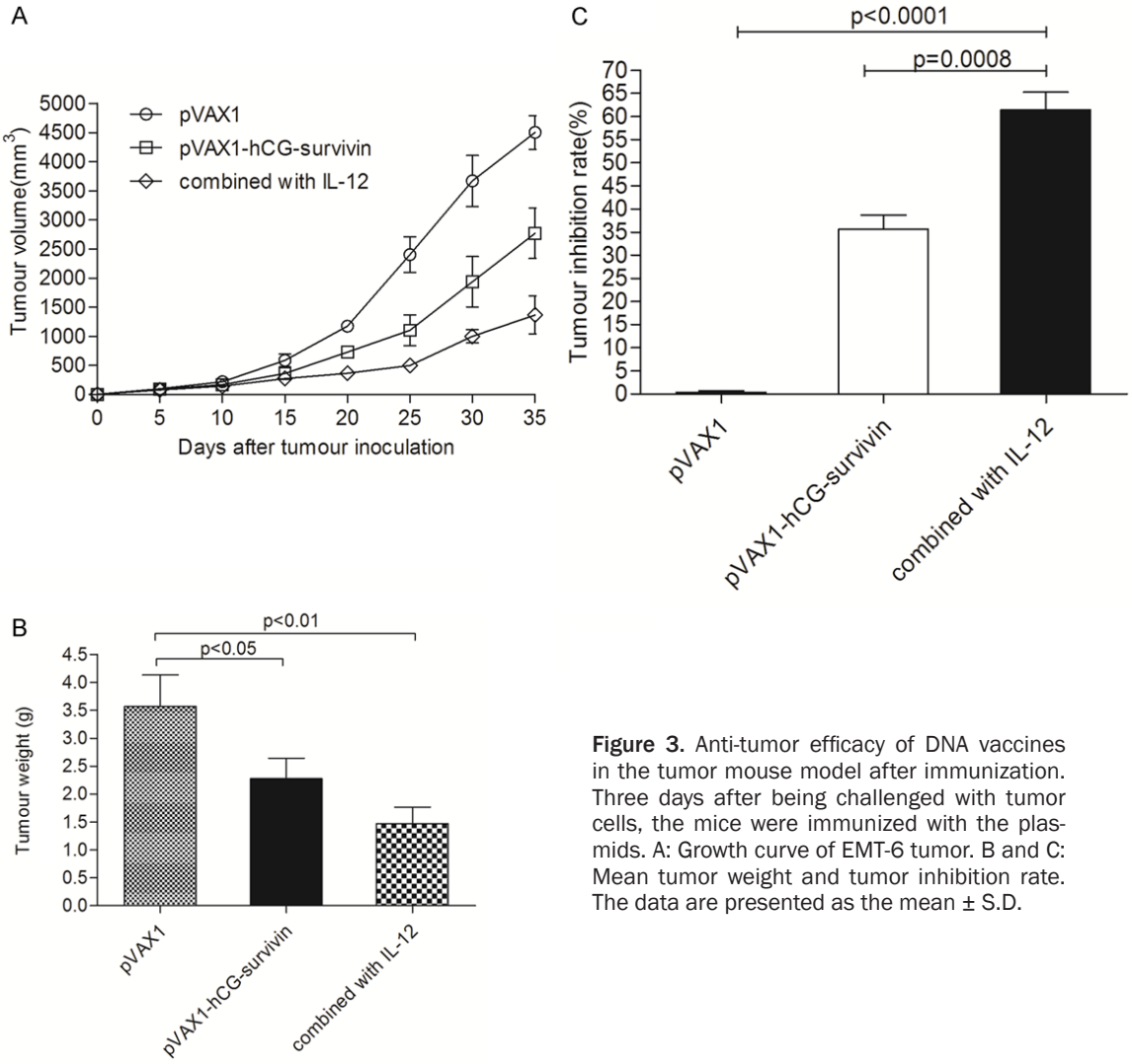

Figure 3. Anti-tumor efficacy of DNA vaccines in the tumor mouse model after immunization. Three days after being challenged with tumor cells, the mice were immunized with the plasmids. A: Growth curve of EMT-6 tumor. B and C: Mean tumor weight and tumor inhibition rate. The data are presented as the mean ± S.D.

through the expression of a fusion of IL-12, we designed and constructed a new type of DNA vaccine that can express hcg-survivin fusion gene and IL-12 as an adjuvant. We demonstrated that vaccination with a DNA vaccine encoding hcg-survivin fusion gene enhanced the B and T cell responses through the use of IL-12 as an adjuvant. Furthermore, this DNA vaccine with IL-12 as adjuvant was able to inhibit tumor growth significantly more than the fusion antigen alone. Therefore, our study suggests that this way may be promising in anti-breast carcinoma therapy.

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Disclosure of conflict of interest

None.

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