

Effective inhibition of tumor *in vivo* with a novel DNA vaccine targeting chimeric G250

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Abstract. – **OBJECTIVE:** To develop a promising approach for tumor immunotherapy with G250 antigen-based DNA vaccine and to investigate its anti-tumor response in mice with renal cell carcinoma.

MATERIALS AND METHODS: G250 derived from human, monkey and mouse were prepared by PCR. The heterogeneous chimeric G250 gene was obtained by integrating different gene fragments of three species. Then, the chimeric G250 was inserted into a eukaryotic expression plasmid pVAX1-IRES-GM/B7 to obtain DNA vaccine (named pVAX1-tG250-GM/B7) which could express chimeric G250 antigen and immune adjuvants simultaneously. By transfecting into Cos7 cells, the expression of chimeric G250 antigen was tested using flow cytometry and immunofluorescence assay. The immunological response and protection against tumor were evaluated *in vivo*.

RESULTS: Recombinant plasmid DNA vaccine was constructed successfully through identification of PCR and gene sequencing. The chimeric G250 antigen was well expressed in Cos7 cells. A strong immune response can be detected through ELISPOT and ELISA induced by pVAX1-tG250-GM/B7. The mice vaccinated with pVAX1-tG250-GM/B7, balb/c showed significant inhibition of tumor and a longer time of survival compared with control group.

CONCLUSIONS: The experimental results of this study exhibited that the DNA vaccine based on heterogeneous chimeric antigen can produce efficient anti-tumor effect *in vivo*, and they represent a promising strategy for tumor immunotherapy.

Key Words:

Immunotherapy, DNA vaccine, G250.

animal immunity through recombinant expression vectors containing gene fragments encoding heterologous proteins¹. The expression product of DNA vaccine is presented and combined with the major histocompatibility complex (MHC), which furtherly induces the corresponding humoral or cellular immune responses². Many animal and human experiments have proved that DNA vaccine could effectively enhance humoral immune response and cellular immune response³.

The tumor-associated antigen (TAA) plays an important role in the design of therapeutic DNA vaccines against tumor, which is expected to induce efficient anti-tumor effect. However, homologous TAA easily escapes immune surveillance in most cases, causing tumor cells to become unresponsive to immune system. Heterologous TAA from different species that has similar biological characteristics, could be used to improve specific immune response against tumor. The immunological cross-reaction between human and other species is considered to be the main factor in this process. Therefore, heterologous antigen is an ideal candidate to break immune tolerance, which could promote the cross-reaction immunological response through genetic homology between different species⁴. It is reported that DNA vaccine encoding heterologous tumor antigen (HER-2/neu) induced a more powerful immune response than the homologous tumor antigen *in vivo*⁵. Therefore, the DNA vaccine targeting heterologous antigens has become more popular in the field of tumor immunotherapy.

Recently, we designed and constructed a novel plasmid DNA vaccine targeting G250, a tumor-associated antigen widely expressed in renal cell carcinoma⁶. In this DNA vaccine, the chimeric G250 gene was composed of different sequence encoding corresponding domain of human G250, mouse G250, and monkey G250. In addition, the

Introduction

DNA vaccine, also known as gene vaccine or nucleic acid vaccine, is a new type of vaccine developed in the 1990s. It achieves human or

vaccine also contained some gene fragments that can simultaneously express immune molecules as adjuvant, e.g., GM-CSF (Granulocyte-macrophage Colony Stimulating Factor), B7.1 (co-stimulatory molecule). Finally, we performed animal experiments with Renca-bearing balb/c mice immunized with heterologous chimeric DNA vaccine to demonstrate better effect of treatment *in vivo*.

Materials and Methods

Design of Chimeric G250 Gene

The gene sequences encoding extracellular domain of human G250, monkey G250 and mouse G250 were found in Genebank respectively. Then, the fragments encoding antigen epitopes were identified through BLAST analysis or published literature⁷. The human G250 antigen epitopes encoding sequence (named hG250) was retained, the rest were replaced with partial monkey G250 sequence (named moG250) and partial mouse G250 sequence (named mG250). Then, maG250, hG250 and mG250 were linked together sequentially to form chimeric G250 gene (named tG250) (Figure 1), which has 89% of homology with the gene segment encoding extracellular domain of human G250.

Construction and Expression of Recombinant Plasmids

Teasy-G250 plasmid containing the full-length human G250 gene, the eukaryotic expression vector pCI-Fc-GPI containing the gene sequence of human IgG1-Fc and GPI (glycosyl phosphatidyl inositol), pVAX1-IRES-GM/B7 containing fragment of IRES (internal ribosome entry site), human GM-CSF, and B7.1 were constructed by our laboratory previously. In this study, we constructed the DNA vaccine

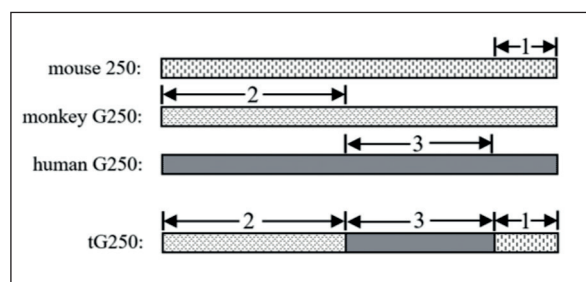


Figure 1. The schematic figure of gene segment encoding chimeric G250. 1: moG250; 2: mG250; 3: hG250.

containing chimeric G250 and adjuvant molecule which was named pVAX1-tG250-GM/B7. In brief, the fragment of moG250, hG250 and mG250 was obtained by PCR respectively. The 3' end of hG250 and the 5' end of mG250 were linked to form the fusion fragment hmG250. The 3' end of moG250 and the 5' end of hmG250 were linked to obtain tG250. Then, tG250 gene fragment was inserted into pCI-Fc-GPI vector. Subsequently, the fusion fragment tG250-Fc-GPI was obtained by double enzyme digestion from pCI-tG250-Fc-GPI, and was inserted into pVAX1-IRES-GM/B7. pVAX1-tG250-GM/B7 was composed of signal peptide, tG250, Fc-GPI, IRES, GM-CSF and B7.1 (Figure 2). The recombinant plasmid was identified by PCR and sequence analysis, then were extracted by Endotoxin free Giga kit (Qiagen, Shanghai, China). Cos7 cells were transfected with pVAX1-tG250-GM/B7, and the expression of chimeric antigen tG250 was identified by flow cytometry and immunofluorescence respectively.

Cell Lines and Animals

Cos7 cells were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). Balb/c mice renal cell carcinoma cell line Renca/G250, which expressed human G250 antigen stably, was established previously in our laboratory. All cells were cultured with Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS) culture medium. They were incubated at 37°C in an atmosphere containing 5.0% CO₂ and saturating humidity. The culture medium was changed every 2-3 days.

Female balb/c mice (6 weeks old) were provided from Beijing Experimental Animal Center (Beijing, China) and were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health publication No. 85-23, Revised 1996). Animal experiment procedure in this study was approved by the Animal Ethics Committee of Beijing Institute of Basic Medical Sciences.

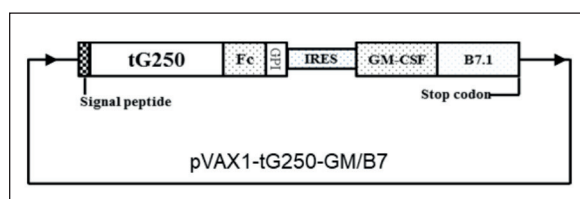


Figure 2. The schematic figure of pVAX1-tG250-GM/B7.

Immunization in Balb/c

Six-week-old female balb/c mice were randomly divided into 3 groups (15 mice per group). Group A was vaccinated with blank vector pVAX1; group B was vaccinated with adjuvant (pVAX1-IRES-GM/B7); group C was vaccinated with DNA vaccine pVAX1-tG250FcGB. Renca/G250 cells in logarithmic growth phase were harvested to prepare single cell suspension. Cell number was calculated, and cells were diluted to 4×10^6 cells / ml with PBS (phosphate buffered saline) (0.09 mol/L). Then, 100 μ l of cell suspension was inoculated subcutaneously into the right side of balb/c mice's dorsal. Three days later, each group of mice was immunized by intramuscular injection into the quadriceps femoris of mice with 100 μ l pVAX1 (0.1 μ g/ μ l), 100 μ l pVAX1-IRES-GM/B7 (0.1 μ g/ μ l) and 100 μ l pVAX1-tG250FcGB (0.1 μ g/ μ l) respectively, and immunization was repeated 14 days after first immunization. Five days after the last vaccination, bloods and spleens of 5 mice in each group were collected for the evaluation of immune activity. The other 10 mice of each group were reserved for observation of tumor growth and survival.

IFN- γ ELISPOT Assay

To evaluate cellular immune responses to specific antigen in mice, splenic lymphocytes were tested for interferon- γ (FN- γ) secretion using enzyme-linked immunospot assay (ELISPOT). First, spleen cells were collected five days after the last immunization. Then, RBC were lysed and the splenic lymphocytes were isolated through density gradient centrifugation method with lymphocyte isolation liquid (Dakewe Biotech Ltd, Shenzhen, Guangdong, China). The isolated lymphocytes were suspended in 10% FCS RPMI-1640 and were adjusted to the concentration of 5×10^6 cells/ml. 100 μ l lymphocytes cell suspension (5×10^5 cells) was added to each well of the 96-well precoated IFN- γ ELISPOT plate (Dakewe Biotechnology Ltd, Shenzhen, Guangdong, China). The lymphocytes were stimulated with purified recombinant G250 protein (1 μ g/well), then were cultured for 48 h in humidified incubator at 37°C, 5% CO₂. After removal of cells and washing with PBST for three times, biotinylated rat anti-mouse INF- γ antibody was added and the plate was incubated for 1 hour at 37°C. Then, the plate was washed and 100 μ l AEC (3-amino-9-ethycarbazole) was added per well, followed by incubation for 45

minutes at 37°C. Finally, the plate was washed for six times and the spots representing IFN- γ secretion were counted by the ImmunoSpot analyzer.

Detection of Antibody and Cytokine

IL-2, IL-4, IL-10 and anti-G250 antibodies were detected by enzyme-linked immune sorbent assay (ELISA) respectively. 96-well plates were coated with G250 protein purified previously (0.25 mg/well) for 12 hours at 4°C. The serum from venous blood was diluted at 1:100 and incubated in the G250 protein precoated plates for 2 hour at 37°C. Then, the plates were washed with Phosphate Buffered Saline and Tween-20 (PBST; 0.05% Tween 20 in PBS) and blocked with 1% bovine serum albumin for 1 hour at 37°C. HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a was added into the plates (100 mL/well) respectively, and the plates were incubated for 1 hour at 37°C. Finally, the antibody titers were detected using TMB system and the OD values were measured at 492 nm by a Bio-Rad plate reader. Similarly, the supernatant of splenic lymphocytes culture was also harvested to detect IL-2, IL-4, and IL-10 with corresponding antibody respectively according to the manufacturer's protocol (BD Biosciences Pharmingen, San Diego, CA, USA).

Anti-Tumor Efficacy Evaluation

After inoculation with Renca cell and vaccination described above, we recorded the time of tumor formation of mice when the nodules of tumor can be touched. Following, the length and width of tumor were measured with vernier caliper every 2 days. The volume of tumor was calculated according to the formula: tumor volume (mm³) = $0.5 \times \text{length} \times (\text{width})^2$. Tumor growth in mice was observed until time point when the first death occurred among mice. Besides, the survival time was observed and survival curve was drawn. The anti-tumor efficacy of our vaccine was eventually evaluated by tumor growth and survival analysis.

Statistical Analysis

GraphPaD 8.0 software (GraphPad Software Inc., San Diego, CA, USA) was used for data analysis and graph. The statistical differences between groups were determined by One-Way ANOVA method, and Bonferroni test was used for post-hoc test of pairwise comparison. In all analysis, $p < 0.05$ was considered statistically significant.

Results

Identification and Expression of DNA Vaccine

The sequence of hG250 (384 bp), moG250 (513 bp), mG250 (144 bp) and chimeric tG250 (1131 bp) was identified correctly as expected by PCR (Figure 3). As follows, the gene fragment of tG250 was further confirmed by sequencing analysis. The recombinant plasmid pVAX1-tG250-GM/B7 was successfully constructed by double digestion. In the recombinant plasmid we constructed, the carboxy terminus of the tG250 gene was fused with a segment gene encoding IgG Fc, which can be used as a label to detect the expression of the fusion protein. FITC-labeled goat anti-human IgG antibody was used to incubate with Cos7 cells for 48 hours after transfection. Flow Cytometry showed the recombinant plasmid group had a positive expression rate of 11.58% (Figure 4). The protein was expressed successfully and were mainly distributed on the cell membrane with the help of GPI (Figure 4). The results of transfection demonstrated that the heterologous chimeric tG250 antigen can be efficiently expressed in eukaryotic cells.

ELISPOT Assay

The level of IFN- γ secretion represents the strength of the antigen-specific cellular immune response. The results of ELISPOT assay are

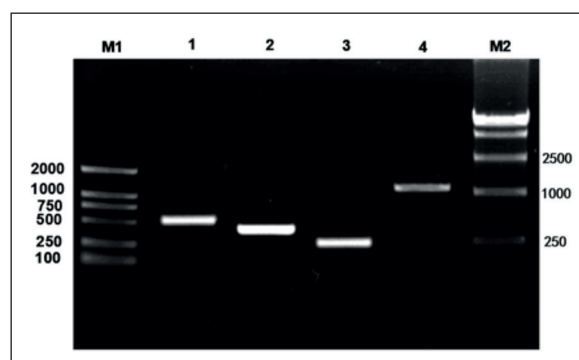


Figure 3. Identification of recombinant plasmid pVAX1-tG250-GM/B7 by PCR. M1, M2: DNA maker; lane 1: PCR product of moG250; lane 2: PCR product of hG250; lane 3: PCR product of mG250; lane 4: PCR product of tG250.

shown in Figure 5A. IFN- γ secretion of lymphocytes can be detected in the mice of each group. The numbers of spot in mice vaccinated with pVAX1-tG250FcGB was significantly higher than those in mice vaccinated with pVAX1-IRES-GM/B7 and pVAX1 ($p < 0.05$). The IFN- γ secretion was also detected in both the pVAX1-IRES-GM/B7 group and pVAX1 group. However, there was no significant difference between pVAX1-IRES-GM/B7 group and pVAX1 group, which indicated that IFN- γ secretion of control group was caused by no-specific stimulation secretion.

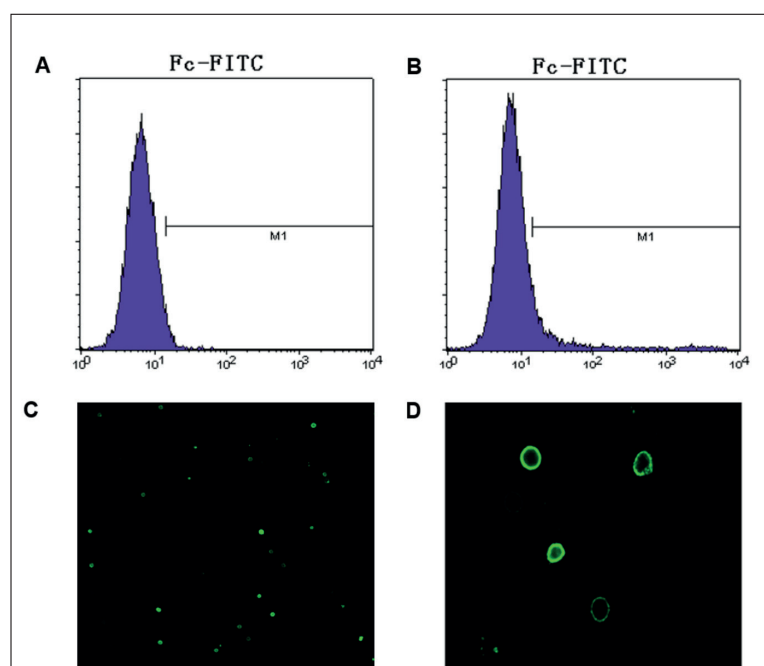
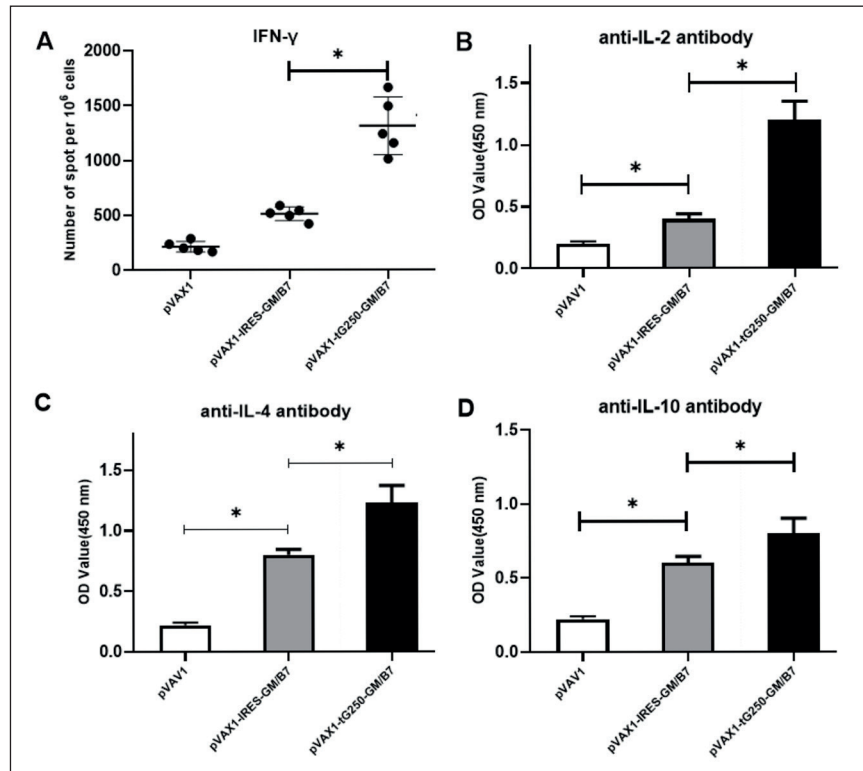


Figure 4. Identification and expression of chimeric G250 antigen by flow cytometry and immunofluorescence. (A) Negative group (transfected with pVAX1); (B) positive group (transfected with pVAX1-tG250-GM/B7); (C) positive group (magnification, $\times 10$); (D) positive group (magnification, $\times 40$).

Figure 5. Splenic lymphocytes of each group were harvested and cultured. The release of IFN- γ from T cells were analyzed by ELISPOT (A). Cytokines (IL-2, IL-4 and IL-10) were detected from the culture supernatant of splenic lymphocytes by ELISA (B, C, D). Asterisk (*): The significant difference with $p < 0.05$.



Cytokines Assay

To determine the cytokines level changes in the anti-tumor process after vaccination, we conducted the ELISA with the culture supernatant of spleen lymphocytes. As shown in Figure 5B, C and D, the cytokines of IL-2, IL-4 and IL-10 were all increased significantly in spleen lymphocytes of mice vaccinated with pVAX1-tG250FcGB. The level of cytokines in the adjuvant group was significantly higher than those in the blank control, however, it was significantly lower than that in the vaccine group. The release of these cytokines indicated that the mice vaccinated with chimeric recombinant plasmid had evident advantages over others.

Total IgG and IgG1/IgG2a Isotypes Analysis

The 96-well plate was coated with purified G250 protein, and the mice serum was added to incubate together. Then, the serum IgG, IgG1, and IgG2a was detected in each group. Relevant data were analyzed as shown in the Figure 6. Mice IgG could be detected in the serum of the DNA vaccine group and the adjuvant group, and there was significant difference of OD450 value between the two groups ($p < 0.05$). Figure 6A showed the differences in IgG subtypes between

each group, which indicated DNA vaccines can induce intense humoral immunity. However, the ratio of IgG1 and IgG2a was up to 4:5 (Figure 6B), therefore the Th1-type cells may predominate during the immune response induced by DNA vaccine.

Protection Against Tumor

After inoculation with Renca and immunization, the tumor forming time and tumor volume of the mice were recorded. The tumor forming time of the mice vaccinated with pVAX1-tG250-GM/B7 was significantly longer than that of others ($p < 0.05$) (Table I). Tumor growth in mice was significantly inhibited by pVAX1-tG250-GM/B7. In addition, pVAX1-IRES-GM/B7 also showed a positive effect on inhabitation of tumor, which was confirmed by the significant difference between group B and group A ($p < 0.05$) (Figure 7A). The survival of mice in each group was shown in Figure 7B. All mice both in group A and group B died within 60 days after tumor challenge with Renca, however, 60% of mice vaccinated with pVAX1-tG250-GM/B7 were still alive at the same time. The survival rate of the mice in group A was significantly higher than those in the other groups ($p < 0.05$).

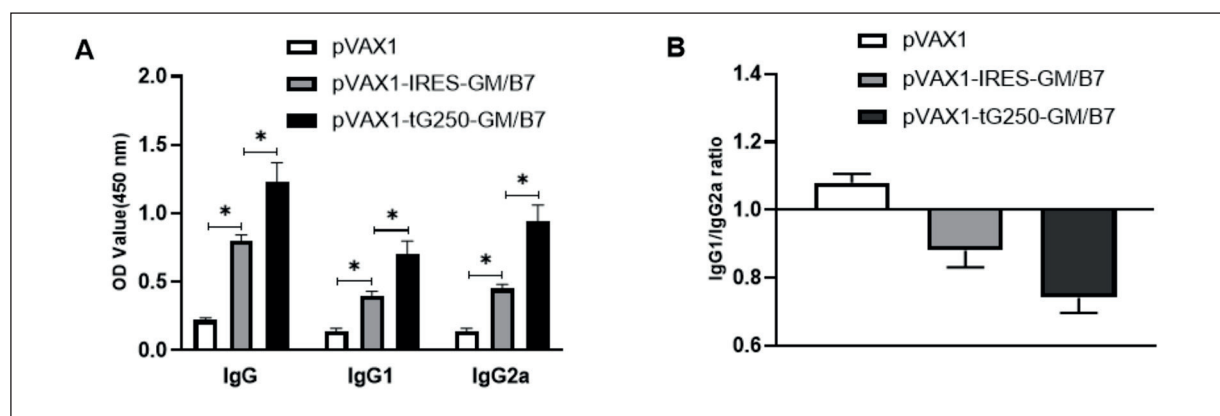


Figure 6. The sera were collected at 5 days after the last vaccination. **A**, The antigen-specific antibody and IgG1/IgG2a isotype were analyzed by ELISA. **B**, The ratio of IgG1/IgG2a. Asterisk (*): The significant difference with $p < 0.05$.

Table I. The tumor formation time of each group vaccinated with different plasmids in mice.

Groups	Tumor formation (days)
pVAX1	5.80 ± 0.93
pVAX1-IRES-GM/B7	$7.40 \pm 1.21^*$
pVAX1-tG250-GM/B7	$9.70 \pm 1.18^*$

Asterisk (*): the significant difference with $p < 0.05$.

Discussion

Recently, DNA vaccines are developing rapidly in the field of immune gene therapy, which show great potential in the prevention and treatment of infectious diseases and tumors⁸. However, the main weakness of DNA vaccine is the low immune efficacy. It is difficult to produce a strong immune response due to lower immunogenicity of endogenous tumor antigens. To stimulate the

body to produce stable and high levels of humoral and cellular immune responses, the strategy of DNA vaccine design must be optimized.

Tumor-associated antigen G250 (carbonic anhydrase IX) is widely expressed in almost all clear-cell renal cell carcinoma and most other types of renal cell carcinoma (RCC), while it is rarely or not detected in normal kidney or most other tissues⁹. Therefore, G250 is considered as an ideal choice for immunotherapy against renal cell carcinoma. In our study, extracellular domain of G250 was selected as the target to induce anti-tumor response. In order to break the immune tolerance to endogenous G250 antigen, partial G250 gene fragments from monkey, mouse and human was integrated together to form a heterogeneous chimeric gene, which had 89% of homology with the human gene segment. We presume that the chimeric G250 can be expressed *in vivo* to induce immune cross-reactivity, which promote immune system in the body to recognize the en-

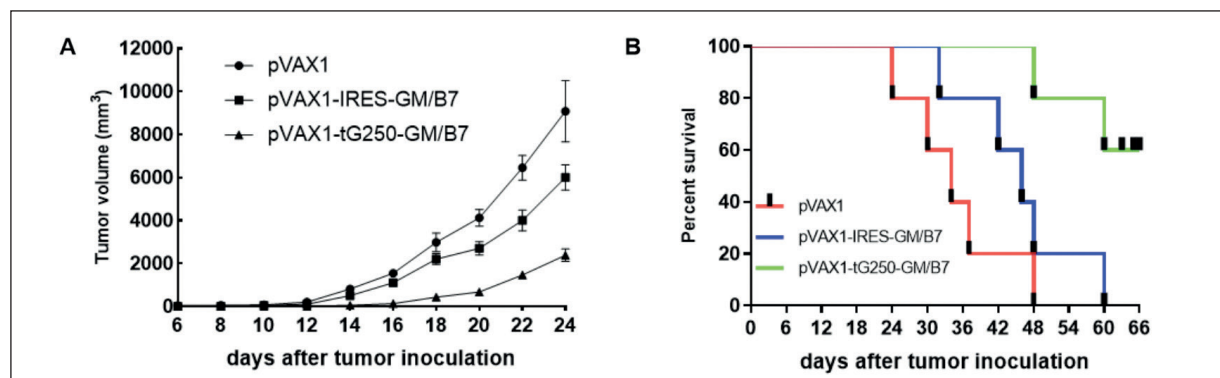


Figure 7. The protection effect of pVAX1-tG250-GM/B7 against tumor was detected by challenging with Renca cells in mice. **A**, The tumor growth curves in each group after tumor inoculation. **B**, The survival curves after tumor inoculation.

ogenous G250 antigen expressed on the surface of RCC cells, thereby inducing specific immune killing reaction. Studies¹⁰⁻¹² have confirmed that vaccines targeting heterologous antigens have a better therapeutic effect.

The recombinant plasmid DNA vaccine we constructed involved the chimeric G250 described above. Besides, some molecular adjuvants were also employed in the construct. We fused the chimeric G250 with the Fc fragment of human IgG (IgG Fc), which plays an important role of immune adhesion effect. By binding with Fc receptor of phagocyte and NK cell, IgG Fc mediates immunoregulation, antibody dependent cell-mediated cytotoxicity, pinocytosis, etc., and it can also be used as a detection label for expression identification, especially in the absence of commercialized antibody of the chimeric G250 protein¹³. In addition, Glycosyl-phosphatidyl-inositol (GPI)-anchored protein was introduced to fuse with IgG Fc, which can make the chimeric G250 protein be presented on the cell membrane and recognized by APCs¹⁴. In the present strategy, GM-CSF and B7.1 were also co-expressed *via* IRES (internal ribosome entry site) in our recombinant plasmid DNA vaccine. GM-CSF and B7.1 are both widely used molecular adjuvants in tumor vaccine research, which have been proved to have great immune synergistic effect¹⁵⁻¹⁷. GM-CSF activate dendritic cells and other specialized antigen-presenting cells. B7.1 promotes proliferation of T cells, as well as the secretion of chemokines^{18,19}. The co-expression of TAA and immune adjuvant simultaneously may create a suitable microenvironment for antigen-presenting cells²⁰. Considering safety, we used the eukaryotic expression vector pVAX1 which is approved by FDA and can be applied to human body.

Then, we investigated the potential antitumor mechanism of the recombinant plasmid DNA vaccine by series of immunological detection. ELISPOT assay was used to detect the secretion of IFN- γ in mice splenic lymphocytes after immunization. The formed spots were counted by the pre-coated IFN- γ monoclonal antibody. The results confirmed that mice in the DNA vaccine group could produce specific IFN- γ secretion of lymphocytes. As is known, IFN- γ is a proinflammatory cytokine which is mainly produced by Th1 lymphocytes and is closely related to T-mediated CTL cytotoxicity²¹. IL-2, IL-4, IL-10, total IgG and IgG1/IgG2a isotypes were assessed by ELISA. The levels of the cytokines IL-2, IL-4, and IL-10 were significantly increased in mice

vaccinated with pVAX1-tG250-GM/B7 compared with other groups. We also found higher antibody titers of IgG and a mixed IgG1/IgG2a response with predominance of IgG2a production. The immunological parameters above indicated that the DNA vaccine can elicit a predominant Th1 immune response which plays a crucial role in tumor immunotherapy. Furthermore, the significant difference of survival periods and tumor inhibition was consistent with immunological characteristic in each group. Considering the low expression of antigen G250 in normal renal, we did not perform renal histologic examination of the immunized mice in this research. The specificity of the immune response induced by our vaccine was needed to be verified by further histology analysis of tumor and other non-target organs, such as kidney, liver, heart, and brain.

Conclusions

The present study demonstrated that the recombinant plasmid DNA vaccine based on heterologous chimeric G250 could induce efficient immune response *in vivo*, and then, significantly inhibit tumor growth and prolong the survival of tumor-bearing mice. These results suggest that heterologous chimeric antigen combined with immune adjuvant molecules had great potential in the field of DNA vaccine for antitumor therapy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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