A DNA Vaccine Targeting the Fetal Liver Kinase-1 (Flk-1) can Activate the Special CD8+ T Cell and Inhibit the Metastasis of Orthotopic Lewis Lung Cancer Model

Xin Liu[#], Yan Chen[#], Zhi Ping Wu, Cong Guo Jin, Xiao Qun Chen, Jia Li, Yong Chun Zhou and Xi Cai Wang[*]

Tumor Institute of Yunnan Province, the Third Affiliated Hospital of Kunming Medical University (Tumor Hospital of Yunnan Province), 519 Kunzhou Road, Kunming, Yunnan Province, 650118, P.R., China

Abstract: Lung cancer is the leading cause of cancer related deaths and need new more effective therapies. In this study, we investigated the anti-tumor effect of recombinant orally DNA vaccine delivered by attenuated *S.typhimurium* strain SL3261 (aroA mutant) targeting vascular endothelial growth factor receptor (VEGFR-2), also known as fetal liver kinase-1 (Flk-1) in mouse. The cDNA of extracellular domains (ECD) of VEGFR-2 (Flk-1_{ECD}) was amplified by RT-PCR and cloned into the pcDNA3.1 (+) vector, then transformed to the attenuated *S.typhimurium* strain to construct the oral DNA vaccine. Then pcDNA3.1-Flk-1_{ECD} was successfully transfected into COS-7 cells and the recombinant protein was detected by Western blot. The effect of the oral DNA vaccine was analyzed by flow cytometry (FCM) analysis and cytotoxicity assay. For mimic the local and regional growth pattern seen in lung cancer patients, the effect of the oral DNA vaccine on tumor growth and metastasis was analyzed by orthotopic cancer cells challenge *in vivo*. The results demonstrated that the oral DNA vaccine can overcome peripheral immune tolerance, and generated Flk-1- specific $CD8^+$ cytotoxic T cell response. Moreover, this oral DNA vaccine could effectively reduce tumor growth, metastasis and increase the survival. It indicated that the oral VEGFR2 DNA vaccine encoding Flk-1_{ECD} delivered by *salmonella* might act a potential strategy for immunotherapy of lung cancers.

Keywords: Lung cancer, VEGFR-2, oral vaccine, angiogenesis, metastasis, orthotopic.

INTRODUCTION

Currently, lung cancer is the leading cause of cancer-related deaths in men and women worldwide [1], and about 80% of lung cancers are non-small cell lung carcinoma (NSCLC). The overall 5-year survival rate of patients with NSCLC remains at 14% [2]. The effect of traditional cytotoxic chemotherapy on lung cancer is very small. Clearly, novel treatment strategies are desperately needed.

In contrast to conventional chemotherapy, targeted gene therapy approaches are aimed at selectively inducing cancer cell death without affecting the normal functions of the organism, and are a promising strategy to cure cancer patients. The progression of the majority of non-small cell lung cancer (NSCLC) is strongly associated with angiogenesis [3-7]. Vascular endothelial growth factor (VEGF) and its receptor tyrosine kinases play vital roles in angiogenisis [8]. Some studies have shown that immunization with plasmid DNA encoding angiogenesis-related antigens can inhibit tumor associated angiogenesis [9-12]. The transfer of expression plasmids from bacteria to mammalian cells has been established [13, 14] and strains of *Salmonella* has been utilized to deliver a variety of therapeutic genes to cancer cells for antitumour activity evaluation *in vivo* [15-17]. And many reports shows that recombinant *salmonella* vaccine can overcome immune tolerance and generate specific immune response to inhibit tumor angiogenesis and growth [18-19].

We constructed an oral Flk-1-based DNA vaccine pcDNA3.1-Flk-1_{ECD} delivered by attenuated *S.typhimurium* strain SL3261 (aroA mutant) and our previous data showed that this DNA vaccine could inhibit tumor growth in heterotopic lung cancer mice model [20]. However, there are a lot of differences between heterotopic cancer model and clinical practice.

To further estimate the effect of the DNA vaccine, we detected T cell immune response activated by an oral DNA vaccine targeting Flk-1 and observed the treatment of the DNA vaccine in mouse orthotopic Lewis lung cancer model which closely mimic the patterns observed for the natural progression of primary lung cancer [21]. The effect of the vaccine was assessed both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Bacterial Strains, Cell Lines and Animals

The attenuated *S. typhimurium* strain SL3261 (aroA mutant) was kindly provided by Dr. Ji Fen Bian (The

*Address correspondence to this author at the Tumor Institute of Yunnan Province, the Third Affiliated Hospital of Kunming Medical University (Tumor Hospital of Yunnan Province), 519 Kunzhou Road, Kunming, Yunnan Province, 650118, P.R., China; Tel: 86-0871-68101793; Fax: 86-0871-68101793;
E-mail: wangxc2005323@126.com

[#]Xin Liu and Yan Chen contributed equally to this study and regarded as co-first authors.

ISSN: 1927-7210 / E-ISSN: 1927-7229/13

Experimental Center of Medical Molecular Biology in Shandong University, China) and bacteria were routinely grown at 37℃ in Luria-Bertani (LB) broth or on agar plates, when required for selection, with 100mg/mL ampicillin. The African green monkey kidney fibroblast-like cell line COS-7 was purchased from the cell bank of Chinese Academy of Science. The murine Lewis lung carcinoma cell line (3LL) was purchased from Shanghai Institute of Thoracic Tumors, Shanghai Chest Hospital Shanghai, China. The mouse pancreatic islet endothelial cell (MS1) was a gift from Professor Marie C. Lin (Department of Chemistry, Hong Kong University, Hong Kong). Female C57BL/6J mice, aged 6 to 8 weeks, were purchased from Vitalriver Inc, Beijing, China, and maintained in our animal laboratory facility. The animal certificate number is SCXK (Beijing) 2002-0003. All animal were housed under specific-pathogen-free conditions and studies were performed in accordance with the guidelines for animal care established by Kunming Medical University's Animal Care and Use Committee including the use of anesthetic and euthanasia etc.

Construction and Identification of Plasmid Vector

The construction of the plasmid vector encoding sequence of the extracellular domains (ECD) of VEGFR-2 has been described [21]. The Flk-1_{ECD} cDNA was amplified by RT-PCR from C57BL/6 mice embryo and cloned into the *Kpn1-Xbal* sites of pcDNA3.1 (+) (Invitrogen, USA). The recombinant plasmid was identified by restriction enzyme analysis (Kpn1/Xba1) and confirmed by sequencing.

Detecting Flk-1_{ECD} Expression by Western Blotting

To detect the expression of Flk-$1_{ECD,}$ COS-7 cells were transfected with the newly constructed plasmid pcDNA3.1-Flk-1_{ECD} using lipofectmin 2000, according to the manufacture's protocol, in parallel with the original pcDNA3.1 (+) vector as a negative control. 48hr after transfection, cell lysates were tested by Western blotting. Protein levels were determined using the Bradford protein assay. Total cell lysates were loaded on a 10% SDS- polyacrylamide gel electrphorisis and transferred on to activated PVDF membranes. The monclonal antibody goat anti-mouse VEGFR-2 (R&D) was used to detect the Flk-1_{ECD} products followed by a secondary rabbit anti goat antibody labeled with horseradish peroxidase. ECL plus western blotting dectection system (Amersham Biosciences, USA) was employed for detection.

Transformation and Detection of *S. typhimurium*

Attenuated *S. typhimurium* was transformed as described [22]. The plasmid includes pcDNA3.1(+), pcDNA3.1-EGFP and pcDNA3.1-Flk-$1_{ECD.}$ Two groups of C57BL/6J mice were administered by oral with a single dose of 10^8 CFUs of *S.typhimurium* harboring either pcDNA3.1-EGFP or pcDNA3.1 (+) vectors respectively. The animals were sacrificed 7 days and 14 days after the immunization and the small intestines and spleens were collected. Single cells suspensions of five Peyer's patches and the spleen from each mouse were homogenized and serially diluted in PBS. EGFP-expressing cells were detected by flow cytometry.

Detection Peripheral Blood T Cells by Flow Cytometry

Three groups of C57BL/6J mice were administered by oral with saline or a dose of 10^8 colony-forming units (CFUs) of *S.typhimurium* harboring either pcDNA3.1-EGFP or pcDNA3.1 (+) vectors respectively. Blood were collected in heparinized tubes from mice before immunity and 2 weeks after immunity (before challenged). The percentage of $CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells was determined by fluorescence activated flow cytometry analysis using a combination of PC5-labeled anti-mouse CD3 antibody, RD1-labeled anti-mouse CD4 antibody, ECD-labeled anti-mouse CD8 antibody (Beckman Coulter Inc., Fullerton, California, USA) as described [21]. Samples were analyzed in a Beckman Coulter EPICS XL flow cytometer (Beckman Coulter Inc., Fullerton, California, USA).

In Vitro Cytotoxicity Assay

For the determination of the possible Flk-1 specific cytotoxicity mediated by cytotoxic T-lymphocytes (CTLs), a 4-hour standard ^{51}Cr release assay was performed as described [23]. Briefly, Flk-1-positive murine pancreatic islet endothelial cells (MS1) were used as target cells. Splenocytes obtained from the immunized or control mice were prepared by passing through cell strainers and treated with ammonium chloride to deplete erythrocytes. 100 µl effector cells and ^{51}Cr-labeled target cells were assigned at different E: T (effector-target) ratios to each well of microtiter plates and were incubated at 37°C for 4 hours. The percent cytotoxicity was calculated using the following: (experimental release - spontaneous release)/ (maximum release - spontaneous release)×100.

Construction of Orthotopic Lewis Lung Cancer Model

To further investigate the efficacy of the DNA on tumor growth and metastasis *in vivo*, we constructed an orthotopic challenge assay as described and incorporated a number of changes [20]. In brief, Matrigel was thawed on ice to avoid gel formation. 3LL cell suspensions for thoracic injections were prepared of equal volumes of cells in PBS and Matrigel stock, mixed before injection. Cells suspended in Matrigel were injected into the left lung and produced solitary lesions. Mice anesthetized with sodium pentobarbital (50mg/kg body weight) were placed in the resupine position. Sterilized with 75% alcohol and made a 5mm incision in the skin of left lateral thorax, at the lateral ventral axillary line, 1.5cm above the lower rib line to exposure the muscle. One-ml insulin syringes with 30-gauge hypodermic needles were used to inject the cell inoculum into the left lateral thorax. The needle was quickly advanced 3mm into the thorax and was stay 5 seconds after the injection of cell suspension, then quickly removed. Sewed up the incision and observed the mice until they were revival.

Immunization, Challenge and Necropsy

According to the reference [20], mice were immunized for 3 times at a week interval with 100ul saline containing 1×10^8 clone formation units (CFUs) of *S.typhimurium* transformed with pcDNA3.1-Flk-1_{ECD}. The negative control includes pcDNA3.1 (+) vector or saline. Mice were challenged in left lung 2 weeks after immunization. Because the orthotopic model of lung

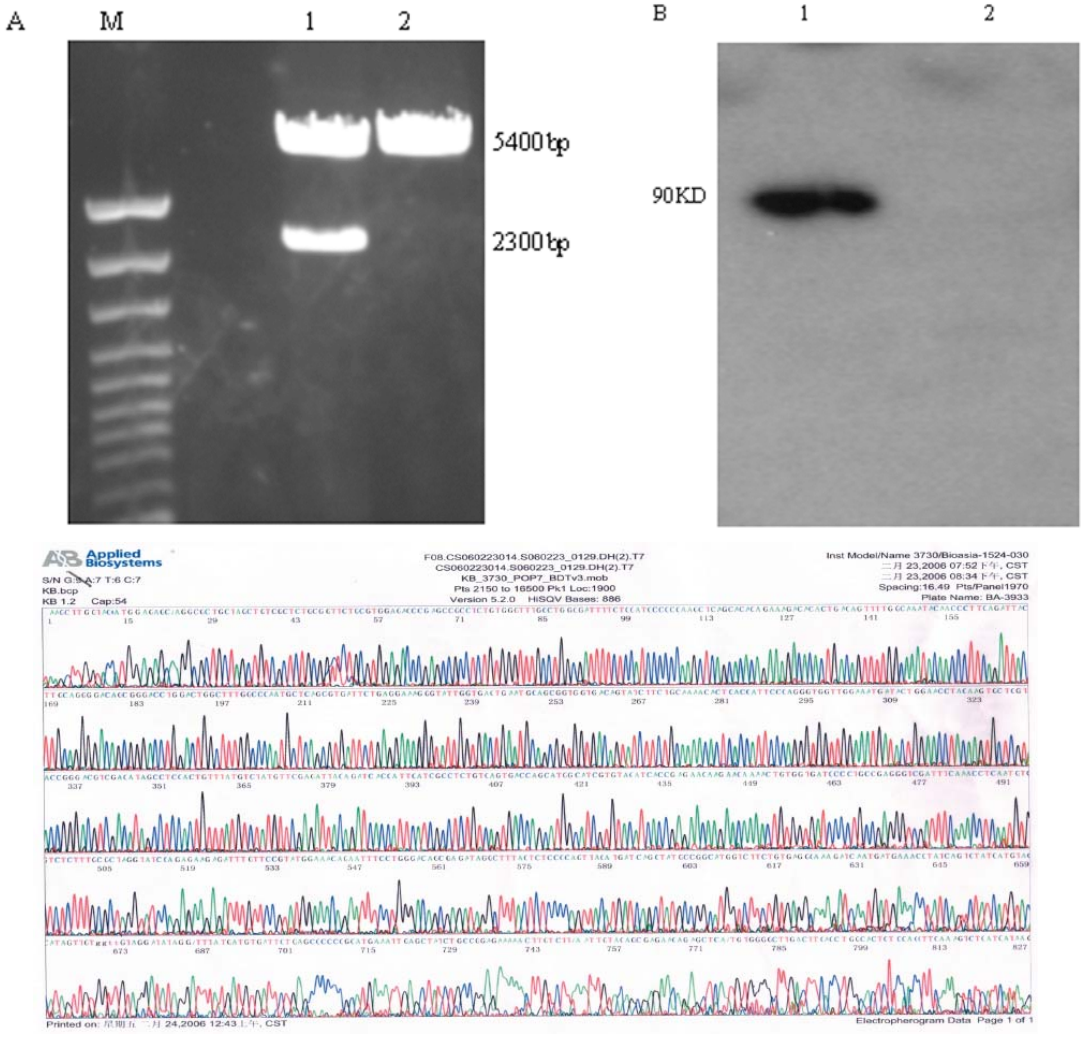

Figure 1: Construction and expression of the plasmid pcDNA3.1-Flk-1_{ECD} *in vitro*. (**A**) Identification of pcDNA3.1-Flk-1_{ECD} by restriction enzyme. M: DNA marker; Lane1:pcDNA3.1-Flk-1_{ECD}/Xba1+Kpn1; Lane2: pcDNA3.1-Flk-1_{ECD}. (**B**) Identification of Flk-1_{ECD} with western blotting. Lane1: Flk-1_{ECD} (90 kDa); Lane2: pcDNA3.1 (+) (negative control). (**C**) The result of sequencing.

cancer can not measure accurately. So the effect of treatment was assessed by the matastasis in the extrathoracic lymph nodes and the contralateral lung. For estimate the effect on metastasis, a group of the mice were sacrificed when one of them died. Subsequent to operation, the thoracic cavity was inspected. Thoracic organs were then removed, including all of the lymph nodes and tumors. Other visceral organs were removed and inspected for presence of metastases. Orthotopic tumors were removed, washed in PBS. For H&E staining procedures, one part of the tumor was fixed in formalin and embedded in paraffin. Another experiment was designed to estimate the effect on survival in orthotopic transplanted mice. Mice were challenged and immunizated using the same method.

Statistics

Statistical significance was analyzed by Student's t-test and ANOVA. Survival curves were constructed according to the Kaplan–Meier method and statistical significance was determined by the log-rank test. All statistical tests used the SPSS 10.0 software.

RESULTS

Plasmid Construction and Expression *In Vitro*

2.3kb Flk-1_{ECD} cDNA was isolated from murine embryo by RT-PCR and was cloned into pcDNA3.1 (+) to construct plasmid pcDNA3.1-Flk-1_{ECD}. Correct DNA ligation was detected by restriction enzyme analysis

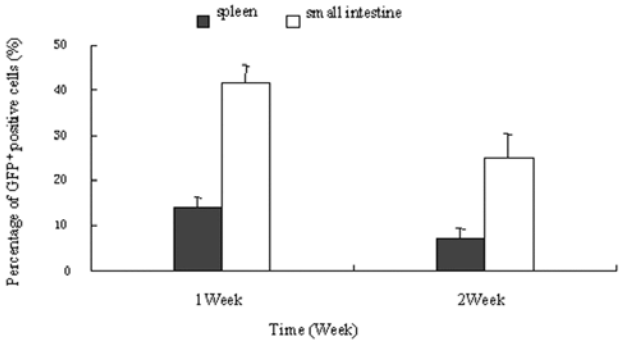

Figure 2: EGFP expressed in Peyer's patches and splenocytes. After oral administration of 10^8 CFUs attenuated *S.typhimurium* SL3261 transformed with pcDNA3.1-EGFP or pcDNA3.1 (+) vector, mice were sacrificed at time 7 days, 14 days and fresh specimens of small intestine and spleens were removed for analysis after thoroughly washing with PBS. Fluorescence expression of EGFP was detected by flow cytometry. The EGFP expression of pcDNA3.1-EGFP group was higher obviously compared to pcDNA3.1 (+) vector group. There were EGFP expression both in Peyer's patches and spleen in pcDNA3.1-EGFP group and GFP positive rate in Peyer's patches (□) was higher than that in splenocyte (■) (P<0.05). The percentage of GFP positive cells in splenocyte and Peyer's patches were decreased gradually with prolongation of immunization time.

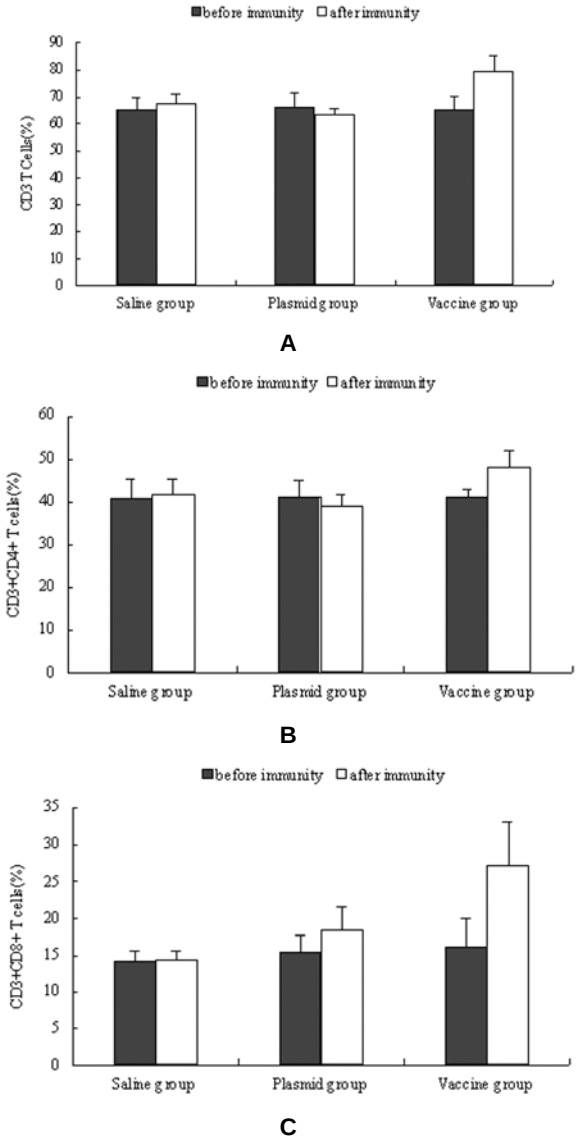

Figure 3: Activation of peripheral T cells. Mice were vaccinated with pcDNA3.1- Flk-1_{ECD}, pcDNA3.1-EGFP or saline 3 times in 1-wk intervals. Blood were harvested 3 weeks after immunity. The percentage of $CD3^+CD4^+$ T-cells and $CD3^+CD8^+$ T-cells of peripheral blood were measured by flow cytometry analysis. After immunization, the amount of T cells in pcDNA3.1- Flk-1_{ECD} group was increased compare with that in pcDNA3.1 (+) vector and Saline group. In pcDNA3.1- Flk-1_{ECD} group, the percentage of $CD3^+CD4^+$ T-cells and $CD3^+CD8^+$ T-cells after immunity (□) were increased compared with the percentage before immunization (■) (**A**) The percentage of $CD3^+$ T-cells increased in mice vaccinated with pcDNA3.1- Flk-1_{ECD} compared with pcDNA3.1-EGFP and Saline group (**B**) The percentage of $CD3^+CD4^+$ T-cells increased in mice vaccinated with pcDNA3.1- Flk-1_{ECD} compared with pcDNA3.1-EGFP and saline group. (**C**) The percentage of $CD3^+CD8^+$ T-cells significantly increased in mice vaccinated with pcDNA3.1- Flk-1_{ECD} compared with pcDNA3.1-EGFP and Saline group (P<0.01).

Table 1: Activation of Peripheral T Cells

T cells	Detection time	saline	plasmid	vaccine
$CD3^+$	Before immunity	65.2±4.80	66.31±5.63	65.11±5.27
	After immunity	67.32±3.78	63.35±2.56	79.38±6.01
$CD3^+CD4^+$	Before immunity	40.84±4.36	41.10±3.91	41.09±1.93
	After immunity	41.60±3.88	39.06±2.73	48.06±4.12
$CD3^+CD8^+$	Before immunity	14.27±1.34	15.40±2.46	16.17±3.76
	After immunity	14.43±1.30	18.33±3.34	27.29±5.82

The percentage of $CD3^+CD4^+$ T-cells and $CD3^+CD8^+$ T-cells of peripheral blood were measured by flow cytometry analysis. After immunization, the amount of T cells in pcDNA3.1- $FLK1_{ECD}$ group was increased compare with that in pcDNA3.1 (+) vector and Saline group. In pcDNA3.1- $FLK1_{ECD}$ group, the percentage of $CD3^+CD4^+$ T-cells and $CD3^+CD8^+$ T-cells after immunity were increased compared with the percentage before immunization.

(Figure **1A**). Sequencing analysis (Figure **1C**) showed that the reading frame was correct. Expression of plasmid pcDNA3.1-Flk-1_{ECD} in transient transformation of cos-7 cells was detected by Western blot and the expected protein bonds (90 kDa) were shown (Figure **1B**).

Gene Expression *In Vivo* Mediated by *S. typhimurium*

We detected the expression of the gene delivered by *S.typhimurium in vivo* by flow cytometry. Mice were fed with the SL3261 strain harboring either pcDNA3.1-EGFP or pcDNA3.1(+) vectors. We found that there were GFP positive cells in both splenocyte and Peyer's patches 1 week after vaccination with pcDNA3.1-EGFP. Furthermore, the percentage of GFP positive cells in Peyer's patches was higher than that in splenocyte. The percentage of GFP positive cells in splenocyte and Peyer's patches decreased gradually with prolongation of immunization time (Figure **2**).

Activation of Peripheral T Cells

To estimate the activation of T cells of mice after immunization, we detected the percentage of $CD3^+CD4^+$ T-cells and $CD3^+CD8^+$ T cells by flow cytometry. After immunization, the total amount of T cells in pcDNA3.1-Flk-1_{ECD} group increased compare with that in pcDNA3.1(+) and Saline group. More important, the percentage of $CD3^+CD4^+$ T-cells and $CD3^+CD8^+$ T-cells before immunization in pcDNA3.1-Flk-1_{ECD} group increased compared with the percentage after immunization, especially the $CD3^+CD8^+$ T-cells (Figure **3**, Table **1**). These results demonstrated that cell immunity response in mice had been activated after pcDNA3.1-Flk-1_{ECD} immunization.

^{51}Cr Release Assay

Furthermore, splenocytes from mice were tested in a 4-h ^{51}Cr release assay for CTL activity against Flk-1-positive. Significantly higher level of CTL activity against the Flk-1-positive endothelial cell line (MS1) in pcDNA3.1-Flk-1_{ECD} immunized mice was showed compared with that in control groups (Figure **4**). These results indicated that vaccination with pcDNA3.1-Flk-1_{ECD} can result in induction of antigen-specific CTL responses in mice.

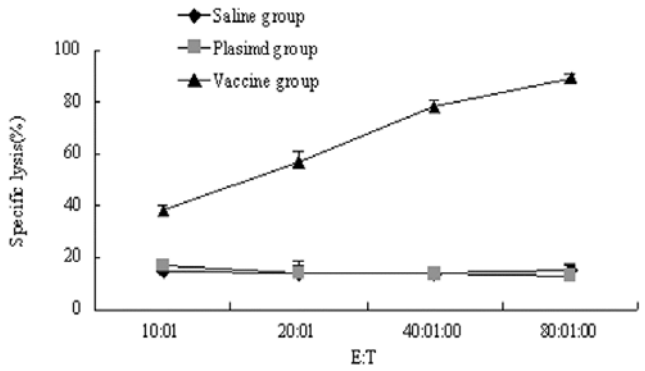

Figure 4: Cytotoxic T-lymphocyte (CTL) activity of splenocytes. Cytotoxic T-lymphocyte (CTL) activity of splenocytes against Flk-1-positive endothelial cells (MS1) was assessed in a standard 51Cr release assay. Higher CTL activity was detected in pcDNA3.1- Flk-1_{ECD} immunized mice compared with pcDNA3.1 (+) vector and Saline group (P<0.05). Results indicated that CTLs which specific to Flk-1 antigen could be induced in pcDNA3.1- Flk-1_{ECD} immunized mice (P<0.05). Error bars indicate s.d.

Evaluation of Antitumor Effect

To further evaluate the antitumor effect of the DNA vaccine by oral administration, we constructed the orthotopic mice model of lung cancer. When one mouse died 2 weeks after challenge, all mice were sacrificed and dissected to exposure the lungs. Lung tissues were collected for the subsequent histology assay. Our data showed that all mice had the tumors formed as a single focus at the site of injection into the lung. In control groups, tumors grew progressively within the injected lung and spread to regional and extrathoracic lymph nodes, and the contralateral lung

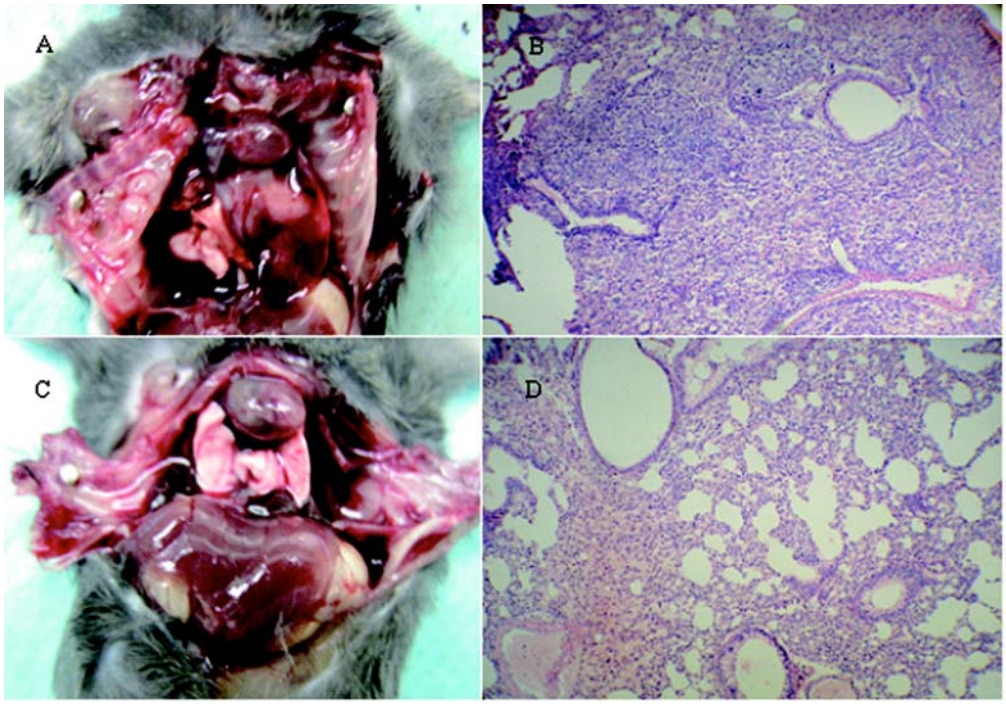

Figure 5: Growth and metastasis of orthotopic lung cancer in different group. Mice were immunized for 3 times at a week interval. Mice were challenged in left lung 3 week after immunization. When one mouse died 2 weeks after challenge, all mice were sacrificed and dissected to exposure the lungs. Lung tissues were collected for the subsequent histology assay. Results showed that all mice had the tumors formed as a single focus at the site of injection into the lung. In control groups, tumors grew progressively within the injected lung and spread to regional and extrathoracic lymph nodes, and the contralateral lung. (**A**) representative lung tumor nodules and (**B**) histology of lung tumor nodules (100 × magnification) in control groups. While in vaccine group, the tumor volumes were smaller and there were no macroscopic metastasis in contralateral lung and lymph node. (**C**) representative lung tumor nodules and (**D**) histology of lung tumor nodules (100 × magnification) in vaccine group.

(Figure **5A**, **B**). In contrast, the tumor volumes in vaccine group were smaller and there were no macroscopic metastasis in contralateral lung and lymph node (Figure **5C**, **D**).

At the same time, another experiment (6 mice in each group) was designed to observe the effect of the oral DNA vaccine on the survival time. In comparison with the pcDNA3.1(+) vector control, pcDNA3.1-Flk-1_{ECD} vaccination significantly increased the survival duration ($p<0.05$). However, there is no significant difference between the vector control and Saline control group (Figure **6**). These data suggested that pcDNA3.1- Flk-1_{ECD} also could inhibit tumor growth and metastasis to prolong the survival time.

DISCUSSION

In the clinical situation, effective vaccination protocols are needed. Numerous vaccination strategies against tumor have yielded encouraging results in mice. Among them, DNA vaccine has emerged as a promising approach to developing effective vaccines. DNA vaccine can transfer by different methods, includes inject naked DNA or transfer by different vector. Most researchers belived that gene transfer need an efficient and relevant vector. These vectors are classified into chemically compounds, viral vectors and bacteria vectors [24]. Bacteria-based gene delivery vehicles would have several potential advantages compared to recombinant viral vectors. Apart from the

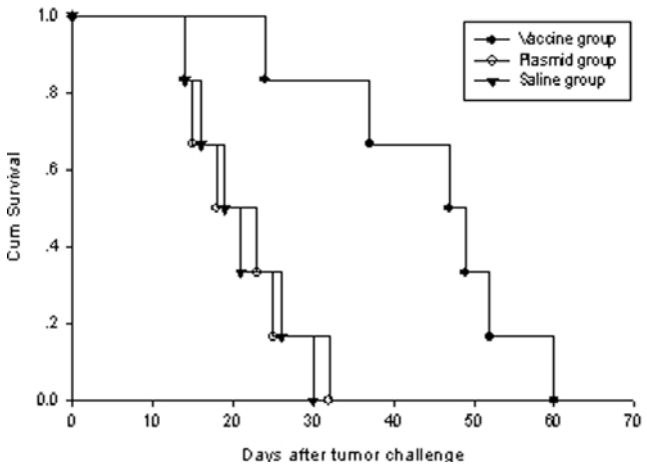

Figure 6: Survival of C57BL/6 mice which immunized by oral DNA vaccine (■), in comparison with the vector control, pcDNA3.1- Flk-1_{ECD}. vaccination significantly increased the survival duration ($P<0.05$).

possibility to be produced cheaply, bacteria could replicate and invade a high number of tumour cells due to their motility properties, as well as allowing for immediate termination of treatment with antibiotic if necessary. As a cancer gene therapy vector, attenuated *salmonella* offers several advantages compared to other bacterial species, including the relative ease of attenuation and genetic manipulation, the ability to grow in both low and well-oxygenated environments and the possibility to administered either i.t., i.p. or i.v. and even orally [25]. Attenuated *salmonella* vaccines have been shown to efficiently elicit both humoral and cellular immune responses against a variety of antigens [18, 19, 21, 26, 27]. In our case, 1 week after immunity, *Salmonella* vaccine can be detected in spleen and small intestine, GFP positive cells in small intestine were higher than spleen. It was uniform with the metabolism of *salmonella*. *Salmonella* can be phagocyted by intestinal macrophages and DCs in the intestinal submucosa and rapidly disseminate through the bloodstream and lymphatic system to the liver and spleen [28, 29]. The percentage of the GFP positive cells decreased 2 weeks after immunity. It was perhaps because the expression of foreign antigens in bacteria, which it does not require or use, places a metabolic burden on the organism that can affect growth rate and select for plasmid loss in the case of plasmid transformed bacterias or bacteria have undergone mutations to silence antigen expression [30]. The problem perhaps could be overcome by repeated immunizations and need be approved in future.

Murine VEGF receptor-2 (VEGFR2, also known as Flk-1), binds VEGF, shows a restricted expression on endothelial cells and is upregulated once these cells proliferate during angiogenesis in the tumor vasculature [8]. The full length Flk-1 gene was about 4kb, encoding for a protein of approximately 190kDa [31], which are likely to be introduced mutation during vaccine production. Some studies have shown that antibodies against the extracellular region of VEGFR-2 could inhibit tumor angiogenesis [32, 33], and other studies suggested that the DNA vaccine encoding peptides from self-VEGFR-2 extracellular region could induce the specific cellular immune response to Flk-1 [34]. We prepared a DNA vaccine encoding the complete extracellular domain of Flk-1 gene carried by attenuated *S. typhimurium*. In our present work, we detected the ability of the vaccine to induce anti-Flk-1 immune responses. The data showed that DNA vaccines can induce cellular immune response. The vaccination procedure induced T cells increased obviously, especially $CD8^+$ T cells. The reason perhaps was *salmonella* delivered Flk-1 gene to antigen presenting cells (APCs). such as dendritic cells. At the same time, *salmonella* elicited many pathogen-associated molecular patterns which stimulate APCs to mature and migrate to secondary lymph nodes [35]. The dendritic cells presented the Flk-1 antigen and provided the activated signal to $CD8^+$ T cells so that the T cell had the proliferation activity and Flk-1 special cytotoxicity against the targeted cells. The results shows that the DNA vaccine can activate the immune system of the mice and let the mice have ability to prevent the cancer challenge.

Despite progress in many areas of oncology, lung cancer remains one of the most deadly cancers. In most cases, lung cancer patients are diagnosed with advanced inoperable disease, and the only therapeutic option is systemic chemotherapy. Most of the research and development of novel therapeutic for lung cancer still relies upon s.c. tumor models, which are potentially less clinically relevant because organ microenviroment influences the phenotype of tumor cells [36]. Study showed that in contrast to tumors growing s.c., tumors in the lung are less susceptible to treatment with paclitaxel, suggesting that orthotopic models are more relevant to evaluate therapy strategy for human lung cancer. The pattern of spread of lung cancer within the thorax is similar to that observed clinically in patients with lung cancer, suggesting that the model is clinically relevant. Therefore it may be prudent to first screen active agents in s.c. tumor models then screen active agents in orthotopic models before the initation of clinical trials [20]. So after we had completed the screen of the effect of pcDNA3.1-Flk-1_{ECD} on s.c. tumor model, now is the orthotopic model. In our case, tumors formed as a single focus at the site of injection into the lung in pcDNA3.1-Flk-1_{ECD} group. While in control group tumors grew progressively within the injected lung and spread to regional and extrathoracic lymph nodes, and the contralateral lung. The absence of clinically apparent distant metastasis is most likely due to the aggressive pattern of locoregional spread of cancer cells with mice dying of diffuse thoracic disease before development of significant distant metastasis. So in our previous study, we had injected the 3LL cells into the tail vein to construct the metastasis disease. Our data showed that the Flk-1 recombinant *salmonella* vaccine can low down the growth of the cancer and decrease the metastasis obviously. But it can not inhibit the tumor growth completely.

In summary, we have developed a Flk-1 recombinant *salmonella* vaccine. Our data showed that

the vaccine could induce activation of immune system and specific T cell immunity against Flk-1 positive cells. What's more, it could inhibit the tumor growth and metastasis in orthotopic Lewis lung cancer model. But it could not inhibit the tumor growth completely. Therefore, further work need to explore how to improve the anti-tumour effect of the DNA vaccine.

ACKNOWLEDGEMENT

This work was supported by National Natural Sciences foundation of China (No. 81060177), The Applied and Basic Research Foundation of Yunnan Province (No. 2007C0024R, No. 2011FZ133 and No. 2012FB067), and Yunnan Provincial Technology Project of Health (No. 2010NS079 and No.2010NS080).

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Received on 15-10-2013 Accepted on 11-11-2013 Published on 19-11-2013

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