

# Plasmid mediated kallistain gene expression via intramuscular electroporation delivery *in vivo* for treatment of NCI-H446 subcutaneous xenograft tumor

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**Abstract:** Kallistatin (KAL) is a novel anti-tumor protein with anti-angiogenic activity. The aim of this study was to investigate whether intramuscular injection of KAL plasmid DNA by electroporation could inhibit NCI-H446 subcutaneous xenograft tumor growth in mice. The tumor model of BALB/c nude mice was induced by subcutaneous inoculation of  $5 \times 10^6$  NCI-H446 cells into the mice right flank. The next day, naked plasmid pEGFP or pKAL was electrotransferred into the skeletal muscle of nude mice (n=6 for each group), with the optimized electroporation conditions. Tumor cells migration were assessed by E-cadherin staining; proliferation was determined by anti-Ki-67 staining; and apoptosis was assayed via TUNEL, tumor microvessel density (MVD) was examined by anti-CD34 staining to evaluate the angiogenesis of tumor. Compared to the pEGFP treating group, tumor growth was inhibited by 85% (pEGFP group:  $486 \pm 187$  mm<sup>3</sup>, pKAL group:  $71 \pm 33$  mm<sup>3</sup>) at day 42, the MVD of tumor tissues was significantly decreased, and tumor cellular proliferation was also inhibited. The results indicate that this therapeutic strategy might serve as a promising approach for cancer clinical therapy.

**Keywords:** Kallistain, xenograft tumor, electroporation, gene therapy.

## INTRODUCTION

Lung Cancer is a major cause of malignancy-related death worldwide, and the mortality rates have been rose up to 54% from total cancer deaths in 2013 (Siegel *et al.*, 2013). The incidence of cancer is rising each year, as there are no appropriate strategies available for cure. At present, the main therapies include surgical intervention, chemotherapy and radiotherapy. Therefore, looking for novel yet effective therapeutic strategies merit great clinic significance.

KAL is a unique serpin (serine proteinase inhibitor), which was first extracted from normal human serum as a tissue kallikrein-binding protein (Chao *et al.*, 1986). Pharmacological studies have demonstrated that KAL possesses anti-inflammatory (Wang *et al.*, 2005), anti-fibrosis (Shen *et al.*, 2008) and anti-tumor effects (Lu *et al.*, 2007). The foundations of successful gene therapy include safe and efficient gene delivery, using viral and nonviral vectors (Prud'homme *et al.*, 2006). Viral vectors could deliver gene high efficiency, but the toxicity and immunogenicity discovered in clinic trials served as the roadblocks for viral vector development. Therefore, plasmid DNA was considered as a safe gene delivery vector (Taniyama *et al.*, 2012 and Cukjati *et al.*, 2007). However, low gene transfer efficiency has limited the clinical application of naked plasmid DNA compared with the efficiency that viral vector did achieve. Along with the introduction of electroporation technique, the

discrepancy in efficiency has been substantially reduced, the transfection efficiency of naked plasmid DNA can be increased from 100-1000 folds (Zhu *et al.*, 2008). Electroporation approach could effectively deliver genes to specific tissues, including skin, muscles, liver, kidney, lung, brain, arteries, retina, spinal cord, cornea, tumors, etc (McMahon *et al.*, 2004).

Earlier studies have indicated that KAL inhibited the tumor growth by suppressing angiogenesis (Miao *et al.*, 2002). This study includes the investigation whether plasmid DNA via intramuscularly electroporation can inhibit the growth of NCI-H446 subcutaneous xenograft tumor.

## MATERIALS AND METHODS

### Materials

The NCI-H446 cell line was purchased from American Type Culture Collection (ATCC), and cultured in RPMI-1640 Medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 100 units/mL penicillin and 100 µg/mL streptomycin (Amersco, USA). Six to eight week old male BALB/c nude mice were purchased from Slaccas Company (Shanghai, China), the EndoFree Plasmid Giga Kit was purchased from QIGEN (Hilden, Germany), DNA Delivery Device was purchased from TERESA Healthcare Sci-Tech Company (Shanghai, China); Antibodies against E-Cadherin, Ki-67 and CD34 were purchased from Abcam (Hong Kong, China), and In Situ Cell Death Detection kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

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### **Plasmid DNA**

The recombinant expression plasmid pEGFP and pKAL were constructed as described previously (Diao *et al.*, 2007). Plasmids were extracted and purified with the EndoFree Plasmid Giga Kit as per the protocol. The plasmid DNA prep was diluted to 1mg/mL with physiological saline and kept frozen.

### **Subcutaneous xenograft tumor model**

Xenograft tumor model was established by subcutaneous inoculation of  $5 \times 10^6$  NCI-H446 cells in 100 $\mu$ l PBS (pH 7.4) into the dorsal right flank of BALB/c nude mice using 25-G needles. Next day, pEGFP or pKAL was injected by intramuscular electrotransfer, six mice in each group. After inoculation of NCI-H446 cells, the largest and shortest diameters of tumor growth were measured every week. Tumor volume was calculated according to the formula  $0.52 \times a \times b^2$ , where a is the largest diameter and b is the shortest diameter.

### **In vivo electroporation**

The mice were anesthetized by intraperitoneal injection of pentobarbital sodium at a dose of 30 mg/kg body weight. The plasmid DNA was injected into mice skeletal muscle using the TERESA DNA delivery device, with the optimized electroporation condition of 60 V, 10ms pulse width, 12 pulse times, the longitudinal electric field direction, plasmid concentration of 1.0mg/ml, dose volume of 100 $\mu$ l (Diao *et al.*, 2007). The mice were sacrificed at day 42, during which time the tumor volume was more than 5% of the total body weight.

### **Histopathology assay**

To evaluate the pathological changes, the xenograft tumor tissue specimens were fixed immediately with 4% paraformaldehyde after having removed from nude mice, then washed in PBS, dehydrated in graded ethylalcohol, embedded in paraffin, cut into 5  $\mu$ m sections, deparaffinized, rehydrated in descending concentrations of ethylalcohol and stained with hematoxylin eosin (H&E) on one or two slides of each sample.

### **Immunohistochemistry and TUNEL assay**

The sections along the H&E staining slides were processed for immunohistochemistry assay. Antigen was retrieved by boiling in 10 mM sodium citrate buffer (pH 6.0) in microwave for 10 minutes, then left in the buffer and cooled at room temperature. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide for 20 minutes at room temperature, then the sections were incubated with blocking buffer (1% BSA in PBS) for 30 minutes, incubated with anti-CD34, anti-E-cadherin and anti-Ki-67 primary antibody for 60 minutes, then incubated with the appropriate HRP-conjugated secondary antibody for 60 minutes. All incubations were conducted at room temperature. Finally, the sections were stained with freshly prepared 3, 3'-diaminobenzidine

(DAB), counterstained with hematoxylin. The sections were washed 3 times with PBS between each step.

TUNEL (Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling) assay was performed in accordance with manufacturer's instructions using In Situ Cell Death Detection kit.

### **Statistical analysis**

The results were presented as mean  $\pm$  standard deviation (SD). The data were subjected to Student's t-test. Comparison was considered statistically significant if the P value was less than 0.05.

## **RESULTS**

### **KAL inhibits the growth of NCI-H446 subcutaneous xenograft tumors in vivo**

Tumor volume was monitored every week in all the nude mice. Tumor formation was significantly reduced in the pKAL group ( $71 \pm 33$  mm<sup>3</sup>) compared with the pEGFP group ( $486 \pm 187$  mm<sup>3</sup>) at 42 day (fig.1).

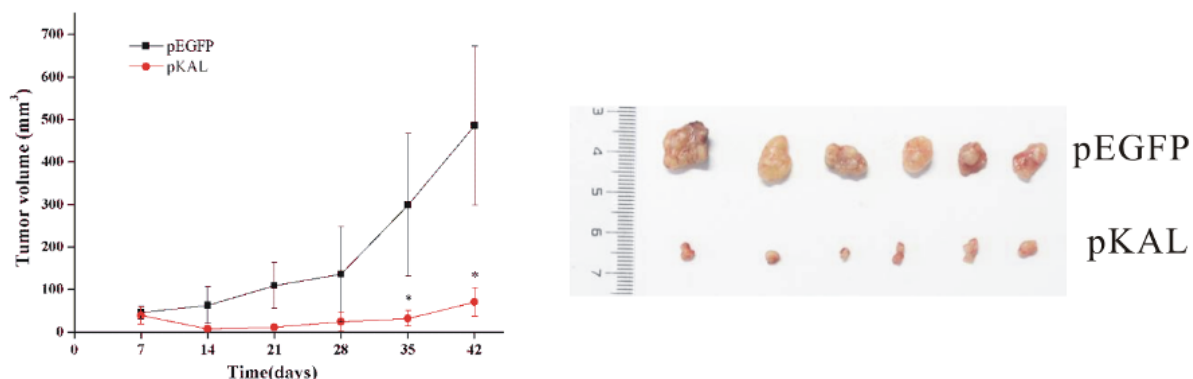
### **Histopathology, immunohistochemistry and TUNEL assay**

The paraffin sections of tumor tissue were subjected to H&E or immunohistochemical staining with anti-CD34, E-cadherin and Ki-67 antibodies respectively. Tumor cell apoptosis was assayed by TUNEL assay (magnification,  $\times 200$ ). MVD (CD34 positive cells) is the marker of tumor angiogenesis, E-cadherin and Ki-67 is the characterization of tumor cells migration and proliferation, respectively. TUNEL is a staining method of choice for rapid detection of the apoptotic cells in tissue sections.

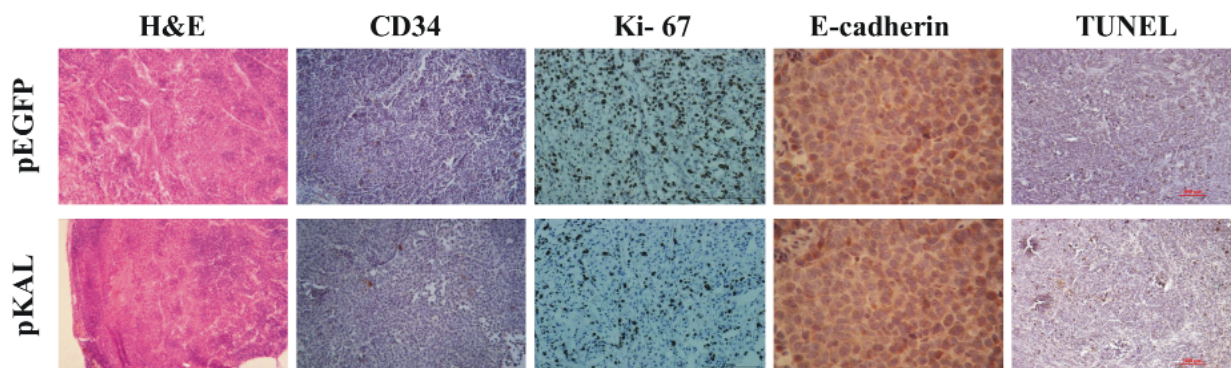
The histopathological examination of the tumor tissue by H&E staining demonstrated that pathological tumor necrotic area was reduced in pKAL group. Immunohistochemistry staining showed MVD was significantly inhibited in tumor tissues treated with pKAL, and the cellular proliferation (Ki-67 positive cells) also decreased in tumors when compared with the pEGFP control group, and TUNEL assay indicated that the apoptosis of tumor cells is indiscriminatory (fig. 2).

## **DISCUSSION**

Plasmid DNA gene therapy by electroporation has advantages in terms of simplicity, efficiency and safety (Murakami *et al.*, 2011). It has been successfully employed in clinical trials related to cancer treatment. Previous studies have indicated that the deliver efficiency of the naked plasmid can be improved by intramuscular electroporation, the approach doesn't cause electroporation-associated muscle damage (Diao *et al.*, 2011).



**Fig. 1:** The growth curves of subcutaneous xenograft tumors. Tumor volumes of the pKAL group versus the pEGFP group on the indicated days (mean±SD). Tumor growth in pKAL group was significantly retarded compared with the pEGFP control group (\*P < 0.05).



**Fig. 2:** Histopathology and immunohistochemical examination of tumor effected tissues.

In conclusion, the present results demonstrated that pKAL effectively inhibited the growth of the tumor by inhibiting the proliferation and angiogenesis of tumor cells in NCI-H446 subcutaneous xenograft mice models. This intramuscular electroporation therapeutic strategy may provide a promising and novel approach based anti-angiogenesis for anti-cancer treatment.

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