

## Basic Study

## Tumor-specific expression of shVEGF and suicide gene as a novel strategy for esophageal cancer therapy

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### Abstract

**AIM:** To develop a potent and safe gene therapy for esophageal cancer.

**METHODS:** An expression vector carrying fusion suicide gene (yCDglyTK) and shRNA against vascular endothelial growth factor (VEGF) was constructed and delivered into EC9706 esophageal cancer cells by calcium phosphate nanoparticles (CPNP). To achieve tumor selectivity, expression of the fusion suicide gene was driven by a tumor-specific human telomerase reverse transcriptase (hTERT) promoter. The biologic properties and therapeutic efficiency of the vector, in the presence of prodrug 5-fluorocytosine (5-FC), were evaluated *in vitro* and *in vivo*.

**RESULTS:** Both *in vitro* and *in vivo* testing showed that the expression vector was efficiently introduced by CPNP into tumor cells, leading to cellular expression of yCDglyTK and decreased VEGF level. With exposure to 5-FC, it exhibited strong anti-tumor effects against esophageal cancer. Combination of VEGF shRNA with the fusion suicide gene demonstrated strong anti-tumor activity.

**CONCLUSION:** The shVEGF-hTERT-yCDglyTK/5-FC system provided a novel approach for esophageal cancer-targeted gene therapy.

**Key words:** Esophageal cancer; Suicide gene; RNA interference; Vascular endothelial growth factor; Nanoparticles

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**Core tip:** Esophageal cancer is a highly aggressive neoplasm with poor prognosis and low survival rates. In this study, an expression vector carrying a fusion suicide gene (yCDglyTK) and shRNA against vascular endothelial growth factor (VEGF) was constructed and delivered into EC9706 esophageal cancer cells by calcium phosphate nanoparticles (CPNP). To achieve tumor selectivity, the expression of the fusion suicide gene was driven by a tumor-specific human telomerase reverse transcriptase promoter. Our results showed that the novel expression vector was efficiently introduced into EC9706 cells by CPNP, leading to cellular expression of yCDglyTK and decreased VEGF level. With exposure to 5-fluorocytosine, it exhibited strong anti-tumor effects against esophageal cancer both *in vitro* and *in vivo*. Combination of VEGF shRNA with the fusion suicide gene demonstrated strong anti-cancer effects. Our study provides a novel approach for esophageal cancer-targeted gene therapy.

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## INTRODUCTION

Esophageal cancer is one of the most prevalent cancers in the world. It has high incidence and mortality rates, especially in Africa, East Asia, and North America<sup>[1]</sup>. Histologically, the most common type of esophageal cancer is esophageal squamous cell cancer (ESCC), which accounts for more than 90% of all cases<sup>[2]</sup>. Despite advances in diagnosis and treatment of ESCC during recent years, postoperative survival rates have not improved in the last decade. The 5-year overall survival rate is still less than 20%<sup>[3,4]</sup>. Novel and effective treatments are urgently needed for esophageal cancer.

Gene therapy has been recognized as a promising strategy for cancer treatment<sup>[5]</sup>. Among various gene therapy approaches, suicide gene therapy holds great promise since suicide gene expression can be manipulated to a particular tissue<sup>[6]</sup>. It transfers prodrug-activating enzyme genes into cancer cells. These genes encode enzymes that can catalyze non-toxic prodrugs into cytotoxic agents to confer drug sensitivity to certain types of cells<sup>[7]</sup>. Cytosine deaminase (CD) and herpes simplex type I thymidine kinase are the two most intensively studied suicide genes. Previous studies showed that a fusion gene (CDglyTK) of yeast cytosine deaminase (yCD) and thymidine kinase (TK) was more effective than either single suicide gene<sup>[8]</sup>. In addition, suicide gene expressed in localized areas of tumor can produce high local concentration of cytotoxic agent [*e.g.*, 5-fluorouracil (5-FU)], thus killing the neighboring tumor cells<sup>[9]</sup>. Expression of yCDglyTK, together with the treatment of prodrug 5-fluorocytosine (5-FC) exhibited targeted therapeutic effects<sup>[10-12]</sup>.

A major limitation for the clinical use of suicide gene therapy for cancer is its proneness to cause side effects due to a lack of tumor specificity. The tissue-specific expression of suicide genes in tumor cells could be achieved by taking advantage of certain tumor-specific transcription regulatory elements, such as promoters and enhancers<sup>[13]</sup>. A number of tumor-specific promoters were employed in previous studies<sup>[14,15]</sup>, and among them, the human telomerase reverse transcriptase (hTERT) promoter demonstrated the greatest tumor specificity<sup>[10]</sup>. The hTERT promoter is inactive in normal somatic cells<sup>[16,17]</sup> and may enable tumor-selective pharmaceutical effects of therapeutic genes.

Angiogenesis plays a vital role in the process of growth and metastasis of solid tumors<sup>[18]</sup>. Vascular endothelial growth factor (VEGF) has been found to be an important angiogenesis factor in cancer development<sup>[19]</sup>. It exerts various biological effects on the growth and spread of tumors, including induction of proteinases, cell mitogenesis, cell migration, increase of vascular permeability, and survival maintenance of newly formed blood vessels. Overexpression of VEGF

has been found in about 60% of esophageal carcinoma cases<sup>[20,21]</sup>. Downregulating VEGF may be a potential targeted treatment strategy for esophageal cancer<sup>[22]</sup>.

Another key consideration for developing a successful gene therapy system is improving transfection efficiency while minimizing toxicity and enhancing stability<sup>[23,24]</sup>. A number of viral vectors and non-viral plasmids have been developed for gene therapy. However, viral vectors have some serious drawbacks, such as triggering immune response<sup>[25]</sup>, severe hepatic inflammation<sup>[26]</sup>, random chromosomal integration<sup>[27]</sup>, and cytotoxicity to host cells<sup>[28]</sup>. Non-viral vectors are considered more promising as gene delivery vehicles because they are safe, easy to synthesize, cost-effective, and have a low degree of immunogenicity<sup>[29]</sup>. In our previous work, we developed the calcium phosphate nanoparticle (CPNP) as a novel non-viral tool for efficient gene delivery<sup>[11,12,30]</sup>. CPNP-delivered suicide genes mediated tumor specific cytotoxicity in gastric cancer and colon cancer<sup>[11,12]</sup>.

In this study, tumor-specific shVEGF-yCDglyTK expression cassette was delivered using CPNP into human esophageal cancer cells and xenograft esophageal carcinoma. The therapeutic efficacy of this novel gene therapy system was evaluated, and the results suggest it was specific and effective for esophageal carcinoma.

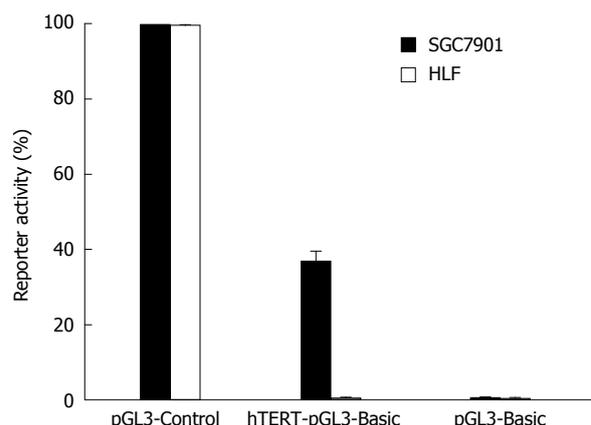
## MATERIALS AND METHODS

### Cell lines and cell culture

Human esophageal squamous cell cancer cell line EC9706 and normal human lung fibroblast cell line HLF were obtained from the Central Laboratory of Xiangya Hospital, Central South University. A human cervical adenocarcinoma cell line HeLa was acquired from the Cancer Research Institute, Central South University. Cells were cultured in Roswell Park Memorial Institute 1640 medium (Hyclone, Logan, UT, United States) supplemented with 10% heat-inactivated fetal bovine serum and maintained in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37 °C.

### Construction of tumor-specific shVEGF-yCDglyTK expression cassette

Human hTERT promoter was amplified by polymerase chain reaction (PCR) from HeLa genomic DNA by using the following primers: forward primer 5'-GCGACGCGTGATTTCGCGGGCACAGACG-3' and reverse primer 5'-AAACTCGAGCCACGTGCGCAGCAGGAC-3'. The product was cloned into the Mlu I and Xho I sites of the pGL3-Basic vector. pcDNA3.1(-)-CV-yCDglyTK<sup>[11]</sup> and pGenesil-shVEGF<sup>[31]</sup> were constructed in previous studies. pcDNA3.1(-) hTERT-yCDglyTK was made by replacing the carcinoembryonic antigen (CEA) promoter with the hTERT promoter. An expression cassette pcDNA3.1(-)-shVEGF-hTERT-yCDglyTK, which expressed shVEGF and yCDglyTK, was constructed as



**Figure 1 Human telomerase reverse transcriptase promoter activities in cancerous and normal cells.** EC9706 and HLF cells were transfected with hTERT reporter vector (hTERT-pGL3 basic). pGL3-Control and pGL3 basic were transfected as positive and negative controls, respectively. hTERT: Human telomerase reverse transcriptase.

shown in Supplementary Figure 1. All constructs were confirmed by sequencing.

### Transcriptional activity analysis of the hTERT Promoter

EC9706 and HLF cells were seeded in 24-well plates with  $1 \times 10^5$  cells per well. Three plasmids, hTERT-pGL3 Basic, pGL3-Basic, and pGL3-control, were transfected when the cell monolayer reached 80%-85% confluence. The activities of luciferase and beta-galactosidase ( $\beta$ -gal) were detected 48 h post transfection. The luciferase activity was normalized against  $\beta$ -gal activity and expressed as relative luciferase activity.

### Transfection efficiency of CPNP-DNA complexes

CPNP was produced according to previous studies<sup>[11,12,30]</sup>. The characteristics of CPNP have been described previously<sup>[11]</sup>. Two micrograms of pEGFP-N1 DNA were mixed with 20  $\mu$ g of CPNP to generate the CPNP-pEGFP-N1 complexes. EC9706 cells were seeded in 12-well plates at a density of  $2 \times 10^5$  cells per well. When the cell monolayer reached 80%-85% confluence, the CPNP-pEGFP-N1 complexes were added to the cells. The pEGFP-N1 DNA-liposome complexes (10  $\mu$ g:2  $\mu$ g) were used as a positive control. Transient transfection efficiency after 48 h was determined by examining GFP expression using flow cytometry and fluorescence microscopy.

### Stable transfection

EC9706 cells were seeded in 6-well plates at a density of  $2 \times 10^5$  cells each well. When the cell reached 70%-80% of confluence, pcDNA3.1(-) null, pGenesil-shVEGF, pcDNA3.1(-)-hTERT-yCDglyTK, and pcDNA3.1(-)-hTERT-shVEGF-yCDglyTK were mixed with CPNP (2  $\mu$ g of DNA: 20  $\mu$ g of CPNP), respectively<sup>[30]</sup>. At 48 h after transfection, G418 was added to cell culture medium, the final concentration

of which was 600 µg/mL. The G418-resistant colonies were picked 16 d later and kept in 96-well plates. These cells were maintained in selective medium containing 200 µg/mL of G418.

#### **Reverse transcription-PCR and Western blot analysis**

Total RNA from parental EC9706 cells and stable transfected cells was prepared by using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's protocol. Reverse transcription (RT)-PCR of  $\gamma$ CDglyTK and VEGF were carried out with AMV reverse transcription kit (Promega, Madison, WI, United States). Primers for  $\gamma$ CDglyTK were: forward primer 5'-GGGAGATTAGAGGGCAAAGTGT-3', reverse primer 5'-ACGGCGTCGGTCACGGCATAA-3'. The  $\gamma$ CDglyTK PCR product was 707 bp. A VEGF PCR product of 112 bp was produced by forward primer 5'-TCTTCAAGCCATCCTGTGTG-3' and reverse primer 5'-ATCCGCATAATCTGCATGGT-3'. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control, and a PCR product of 101 bp was produced by forward primer 5'-CCTGTTTCGACAGTCA GCCG-3'; reverse primer 5'-CGACCAAATCCGTTGACTCC-3'. The amplified fragments were separated in 2% agarose gels for visualization.

For Western blot assay, 30 µg protein from each sample was loaded for 10% sodium dodecyl sulfate gel electrophoresis and transferred to polyvinylidene fluoride membrane. The membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline with tween (TBST) buffer at room temperature for 1 h. The membranes were then incubated overnight at 4 °C with anti-CD antibody (QED Bioscience Inc., San Diego, CA, United States). After washing three times with TBST buffer, blots were incubated for 2 h at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Sigma, St. Louis, MO, United States). Signals were detected by enhanced chemiluminescence with Western blot detection system (Amersham, Piscataway, NJ, United States). GAPDH was used as a control to ensure equal protein loading.

#### **Cytotoxicity assay**

Parental and stable EC9706 cells were seeded at a density of 5000 cells per well in 96-well plates and incubated at 37 °C for 24 h. 5-FC was added into the culture medium, and the final concentration was 200 µg/mL. Methylthiazole tetrazolium (MTT) assays were conducted after incubation for 24, 48, 72, and 96 h, respectively. At the end of the incubation period, 20 µL of MTT stock solution (5 mg/mL, Sigma) was added per well, and the supernatant was carefully removed 4 h later. The formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Promega). Optical density (OD) was determined by using a multi-well plate reader by measuring absorbance at 570 nm with a 690

nm reference wavelength. The background absorbance of medium was subtracted. All samples were assayed in triplicate, and the mean value for each experiment was analyzed. Cell growth curves were plotted with culture time on the horizontal axis and OD570 values on the vertical axis.

#### **Hoechst staining**

Nuclear morphology changes of apoptotic cells were detected by staining with Hoechst 33258. Parental EC9706 cells and stable cells transfected with empty vector, shVEGF,  $\gamma$ CDglyTK, and shVEGF- $\gamma$ CDglyTK were cultured in complete medium supplemented with 5-FC (200 µg/mL). After 48 h, cells were washed three times with phosphate buffered saline (PBS) and stained with 5 µg/mL Hoechst 33258 for 30 min in the dark. Stained nuclei were visualized using a fluorescence microscope with a wavelength of excitation at 355 to 366 nm.

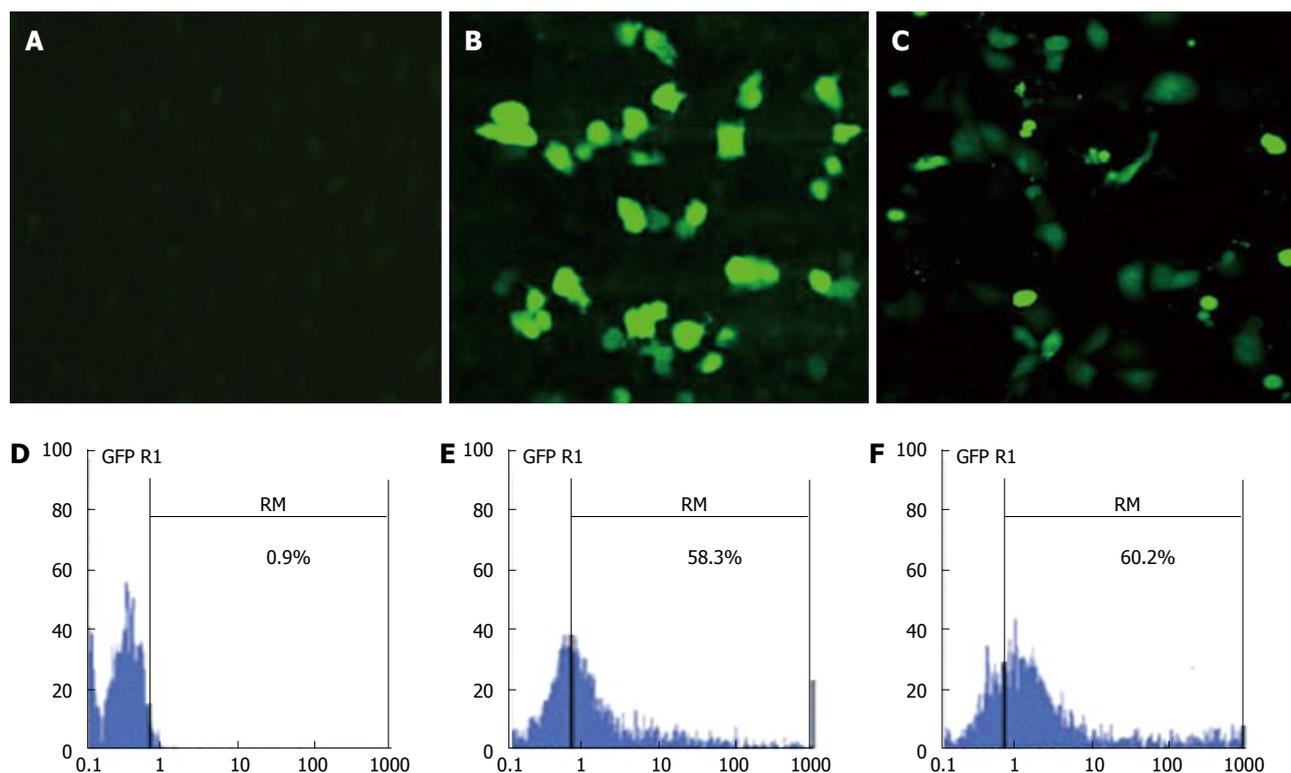
#### **Flow cytometry analysis of cell apoptosis**

Flow cytometry was performed as previously described<sup>[11]</sup>. EC9706 cells stably expressing shVEGF,  $\gamma$ CDglyTK, and shVEGF- $\gamma$ CDglyTK and untransfected EC9706 cells were seeded in 10 cm dishes, respectively. 5-FC was added to the culture medium at a concentration of 200 µg/mL when the cells reached 90%-95% confluence. Cells were pelleted after 48 h, washed with PBS, and then resuspended in staining buffer (HEPES supplemented with 2.5 mmol/L CaCl<sub>2</sub>). Flow cytometry was employed for detecting cell apoptosis after addition of fluorescein isothiocyanate-labeled annexin V and incubation for 15 min at 4 °C.

#### **Animal experiments**

The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Central South University. Twenty-five female BALB/c nude mice (4-6 wk old, 18-20 g) were acquired from Slac Laboratory Animal of Shanghai Co. Ltd, China. A suspension of  $5 \times 10^6$  EC9706 cells was inoculated subcutaneously into the right flank of nude mice. After 10 d, when transplanted tumors reached a volume of 100-200 mm<sup>3</sup>, the mice were randomized into five groups, named A, B, C, D, and E, with five mice in each group. Group A was the non-treating control group; Group B received intratumoral injection of the CPNPs/pcDNA3.1(-)null complex; Group C received intratumoral injection of the CPNPs/pGenesil-shVEGF complex; Group D received intratumoral injection of the CPNPs/pcDNA3.1(-)-hTERT- $\gamma$ CDglyTK complex; and Group E received intratumoral injection of the CPNPs/pcDNA3.1(-)-shVEGF-hTERT - $\gamma$ CDglyTK complex.

The intratumoral injections of CPNP/DNA complexes were carried out every other day and repeated three times in total. One day after the first intratumoral injection, mice in groups B, D, and E received daily



**Figure 2** Transfection efficiency of calcium phosphate nanoparticles. A-C: Images of transfected EC9706 cells acquired by fluorescence microscope at magnification  $\times 200$  after 48 h of transfection. Negative control (A), CPNP-GFP complex (B), Liposome/GFP complex (C); D-F: Qualitative analysis of transfection efficiency by flow cytometric assay. Negative control (D), CPNP-GFP complex (E), Liposome/GFP complex (F). CPNP: Calcium phosphate nanoparticles; GFP: Green fluorescent protein.

intraperitoneal injection of 5-FC (500 mg/kg) for 14 consecutive days. The body weights of mice were recorded twice every week, and symptoms of side effects including change in behavior and food withdrawal were closely monitored. The longest (L) and shortest (W) perpendicular tumor diameters were measured every 3 d with calipers. To estimate the tumor volume; the following formula:  $V = (1/2)W^2 \times L$  was used to calculate the three-dimensional volume of the xenograft. The growth curve was drawn according to the tumor volume. Animals were euthanized if the tumor diameter reached 1.5 cm or weight loss was more than 20% during the experiment. All surviving mice were euthanized by CO<sub>2</sub> inhalation and cervical dislocation at the end of the experiment.

#### Immunohistochemistry assays

The esophagus cancer xenograft tissue was cut at 4- $\mu$ m thickness. The sections were incubated overnight at 4 °C with primary polyclonal antibody (anti-CD). The anti-rabbit antibody (Abgent, San Diego, CA, United States) was used as secondary antibody and incubated with sections for 30 min at 37 °C. Color development was performed with the streptavidin-peroxidase system (Sigma-Aldrich). The chromogen was 3,3-diaminobenzidine tetrahydrochloride. Nuclei were lightly counterstained with hematoxylin. Microvessel counts (MVC) were analyzed to evaluate the degree of

angiogenesis. Sections were immunostained with anti-CD34 antibody and examined at 200  $\times$  magnification. At least three microscopic images were collected and further analyzed with Axion Vision Rel 4.6 software to obtain MVC.

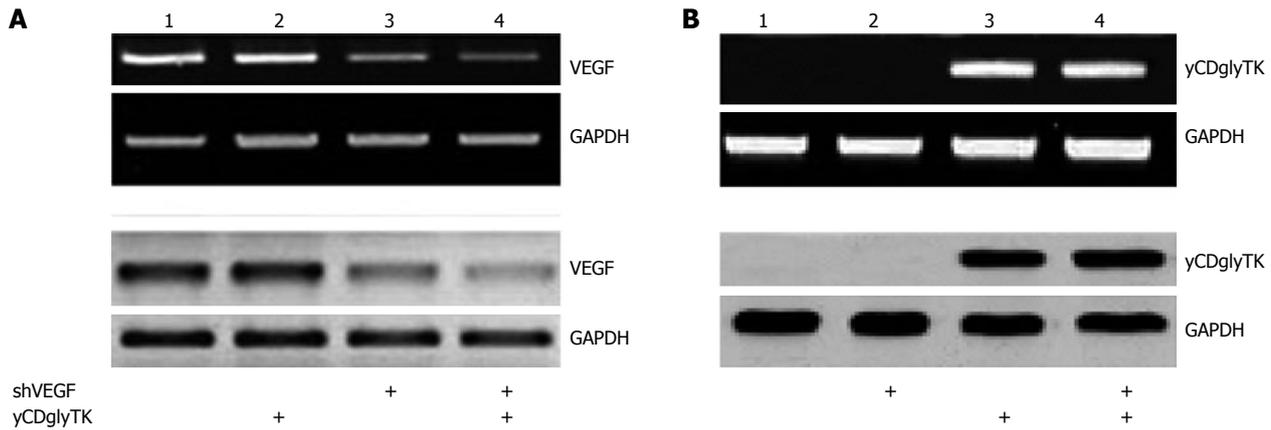
## RESULTS

### Assembly of tumor-specific shVEGF-yCDglyTK expression vector

In order to develop a more effective treatment for esophageal cancer, a novel expression plasmid, which could express a shRNA for VEGF and a fusion suicide gene yCDglyTK (Supplementary Figure 1), was constructed. The expression of shVEGF was under the control of the U6 promoter, a pol III promoter; the expression of yCDglyTK was controlled by the tumor-specific hTERT promoter.

### Specific transcriptional activities of the hTERT promoter in esophageal carcinoma cells

Activities of the hTERT promoter were determined by luciferase assay in EC9706 esophageal cancer cells and HLF normal lung fibroblast cells. The pGL3-Control vector containing SV40 promoter and enhancer sequences was used as a positive control. Luciferase activity driven by the hTERT promoter was much higher in EC9706 cells than in the HLF cells (Figure



**Figure 3** Changes of vascular endothelial growth factor and yCDglyTK expression in established stable cell lines. A: Representative VEGF mRNA and protein expression were analyzed by RT-PCR (top panel) and western blot (bottom panel), respectively. GAPDH was used as an internal control. Lane 1, EC9706/null; lane 2, EC9706/yCDglyTK; lane 3, EC9706/shVEGF; lane 4, EC9706/shVEGF-yCDglyTK; B: Representative yCDglyTK mRNA and protein expression were analyzed by semiquantitative RT-PCR (top panel) and western blot (bottom panel), respectively. GAPDH was used as an internal control. Lane 1, EC9706/null; lane 2, EC9706/shVEGF; lane 3, EC9706/yCDglyTK; lane 4, EC9706/shVEGF-yCDglyTK. GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; RT-PCR: Reverse transcription polymerase chain reaction; VEGF: Vascular endothelial growth factor.

1). The results indicated that tumor specificity driven by the hTERT promoter could be achieved in EC9706 esophageal carcinoma cells.

#### Transfection efficiency of CPNP on esophageal cancer cells

EC9706 cells were transiently transfected with CPNP/green fluorescent protein (GFP) complex to evaluate the transfection efficiency of CPNP. EC9706 cells were assessed 48 h after transfection for GFP expression by using fluorescence microscopy and flow cytometry analysis. As shown in Figure 2, the proportion of GFP expressing cells transfected with CPNP was 58.3%, similar to that in liposome-mediated transfection group (60.2%). These data suggested that CPNP is an efficient strategy to deliver DNA into EC9706 esophageal cancer cells.

MTT assay was carried out to test the toxicity. Compared to the control group, liposome inhibited the cell growth by about 21%, while CPNP-DNA group had little effect on the cell growth (Supplementary Figure 1).

#### Changes in expression of VEGF and yCDglyTK by introducing the expression construct

RT-PCR and western blot were used to determine the expression of VEGF and yCDglyTK. Compared with cells transfected with pcDNA3.1(-), EC9706 cells transfected with pcDNA3.1(-)-shVEGF-hTERT-yCDglyTK showed significantly decreased expression of VEGF at both the mRNA and protein levels. No significant changes of VEGF expression were observed in EC9706 cells transfected with pGenesil-shVEGF and pcDNA3.1(-)-shVEGF-hTERT-yCDglyTK (Figure 3A). yCDglyTK expression was not present in cells transfected with pcDNA3.1(-). Its expression was substantially increased at both the mRNA and protein level by introduction of pcDNA3.1(-)-shVEGF-hTERT-

yCDglyTK into EC9706 cells (Figure 3B).

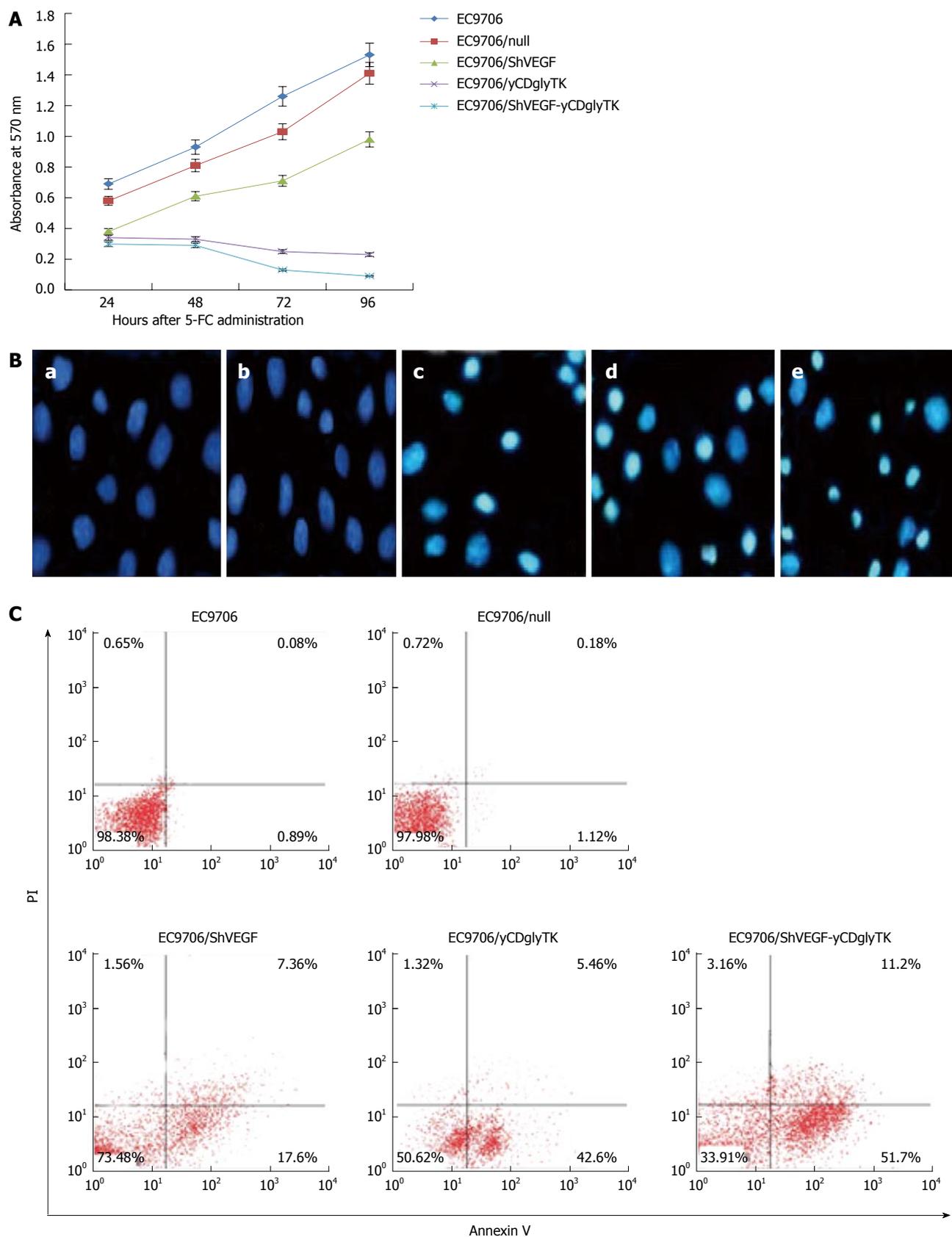
#### Effects of shVEGF-yCDglyTK/5-FC system on cell growth and apoptosis in EC9706 cells

MTT assay was performed to assess cytotoxicity of the shVEGF-yCDglyTK/prodrug system in EC9706 cells. Parental and stable EC9706 cells were treated with 200  $\mu$ g/mL of 5-FC. Cells were harvested at 24, 48, 72, and 96 h after 5-FC administration and subjected to MTT assay. As shown in Figure 4A, 96 h after 5-FC treatment, cells growth in the shVEGF group, yCDglyTK group, and shVEGF-yCDglyTK group was inhibited by 36%, 85%, and 94%, respectively.

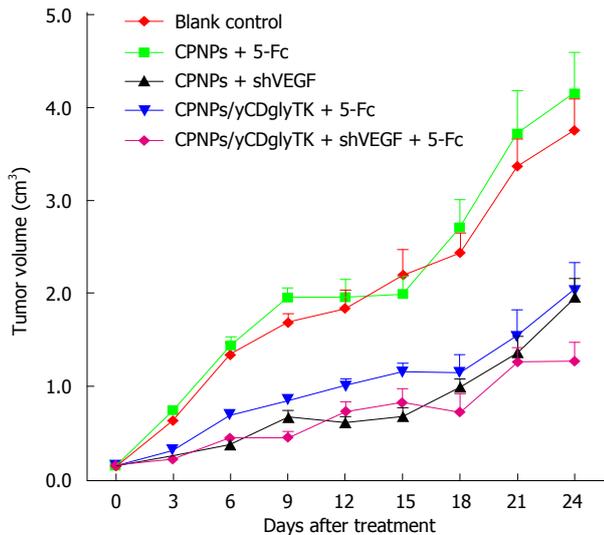
Forty eight hours after 5-FC exposure, cell apoptosis were determined by Hoechst staining and flow cytometry, respectively. Hoechst staining showed that 5-FC induced a significant increase in apoptosis in EC9706 cells stably modified with the pcDNA3.1(-)-shVEGF-hTERT-yCDglyTK (Figure 4B). In contrast, apoptotic cells were barely found in parental EC9706 cells and cells carrying empty vector. Consistent results were found by the flow cytometry analysis (Figure 4C). Apoptotic population (early and late apoptotic) in EC9706 cells expressing shVEGF, yCDglyTK, and shVEGF together with yCDglyTK were 24.96%, 48.06%, and 62.9%, respectively. The percentage of apoptotic cells in untransfected EC9706 cells and EC9706 null cells transfected with an empty vector was only 0.97% and 1.3%, respectively. These results showed that both shVEGF and yCDglyTK expression could induce apoptosis and that combining them had the strongest effect on apoptosis induction.

#### Anti-tumor activity of shVEGF-yCDglyTK/5-FC system in vivo

The anti-tumor activity of the shVEGF-yCDglyTK/5-FC system was investigated with EC9706 xenograft



**Figure 4** Effects of shVEGF-yCDglyTK/5-FC system on EC9706 cells. A: Cell viability of parental and stable EC9706 cells were determined with MTT assay at various time points after 5-FC treatment. The results shown are representative of three independent experiments; B: Representative images of Hoechst 33258-stained nuclei at magnification  $\times 200$ . Apoptotic nuclei are condensed or fragmented; parental EC9706 cells (a); EC9706/null (b); EC9706/shVEGF (c); EC9706/yCDglyTK (d); EC9706/shVEGF-yCDglyTK (e); C: Representative dot plots of flow cytometry analysis. The numbers represent the percentage (%) of cells. Upper right quadrant, late apoptosis; lower right quadrant, early apoptosis; lower left, live cells. 5-FC: 5-fluorocytosine.



**Figure 5** shVEGF-yCDglyTK/5-FC system inhibited tumor growth in the EC9706 xenograft model. Twenty-five BALB/C nude mice bearing EC9706 xenografts were randomized into five groups. The CPNPs/null, CPNPs/shVEGF, CPNPs/yCDglyTK or CPNPs/shVEGF-yCDglyTK complexes were delivered by intratumoral injection every other day, and the injection was repeated three times in total. 5-FC (500 mg/kg) was administered daily for 14 consecutive days. Tumors were measured every 3 d. All of the mice were sacrificed at day 36 after inoculation.

models. Tumors receiving CPNPs/null injection grew at a similar rate to that of the blank control group. On day 24, when compared with blank control group, CPNPs/yCDglyTK + 5-FC group and CPNPs + shVEGF group showed about 46% and 48% tumor growth inhibition. When combining CPNPs/yCDglyTK + 5-FC and shVEGF, the tumor growth was inhibited by 66% at day 24. These results demonstrated the potential benefit of the shVEGF-yCDglyTK/5-FC system for treating esophageal cancer (Figure 5).

#### Immunohistochemistry staining for yCDglyTK and VEGF

The expression and distribution of yCDglyTK and VEGF in the tumor xenograft were visualized by using immunohistochemistry staining. As shown in Figure 6A, tumor tissue expression of yCDglyTK was detected in mice injected with CPNPs/yCDglyTK or CPNPs/shVEGF-yCDglyTK. Meanwhile, it was not present in other groups, including non-treatment, sham control, and CPNPs/shVEGF groups. VEGF expression was quantified and represented by integrated optical density (IOD) values (Figure 6B). VEGF levels were significantly decreased in tumor tissues receiving injection of CPNPs/shVEGF or CPNPs/shVEGF-yCDglyTK, when compared with those of other groups ( $P < 0.01$ ). No significant difference in VEGF expression was found between these two groups ( $P > 0.05$ ). Since VEGF is an important angiogenesis factor, neovascularization was assessed by quantification of MVD. CPNPs/shVEGF and CPNPs/shVEGF-yCDglyTK groups showed lower MVD as compared to other groups (Figure 6C). The results suggested that the intratumoral injection of

CPNPs/shVEGF-yCDglyTK could effectively inhibit neovascularization by down-regulating VEGF expression.

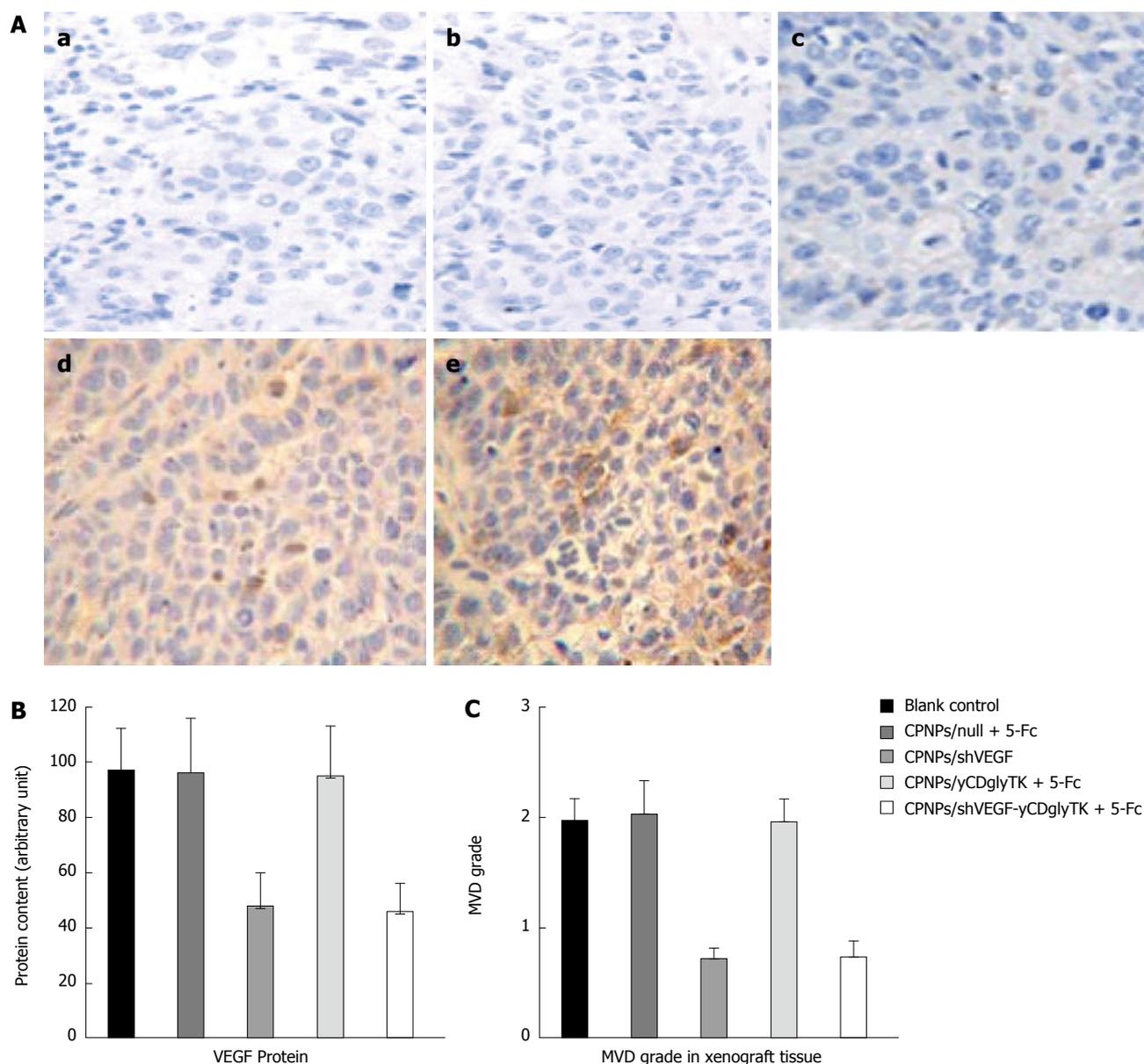
## DISCUSSION

Esophageal carcinoma is a common cause of death globally. Traditional remedies for esophageal cancer, such as surgery, chemotherapy, and radiotherapy, all have drawbacks. For example, surgery is applicable only if the tumor is diagnosed at an early stage. Chemotherapy and radiotherapy have serious side effects as a result of lacking tumor specificity. Therefore, novel methods are required for the treatment of esophageal cancer. Gene therapy, especially suicide gene therapy, has been studied extensively for cancer treatment<sup>[32]</sup>. For suicide gene therapy, the therapeutic transgenes can convert a non-toxic pro-drug, which easily penetrates the tumor cell membrane, into a cytotoxic drug<sup>[33]</sup>. However, present suicide gene therapies have limited success due to lack of tumor specificity and an effective gene delivery tool.

One key point for successful gene therapy is the development of a safe and effective gene delivery system. Viral vectors are the most widely investigated delivering system because of their high transfection efficiency. However, viral vectors have some serious drawbacks, such as triggering immune response, severe hepatic inflammation, and random chromosomal integration<sup>[25-27]</sup>. Among non-viral vectors, cationic lipids could cause toxic effects when repeatedly used and induce potent anti-inflammatory activity *in vivo*; calcium phosphate precipitation had low transfection efficiency. The use of nanoparticles has become one of the most promising vectors because of their high transfection efficiency and low toxicity. In previous studies, we developed a new gene delivery system using either CPNP or calcium carbonate nanoparticles (CCNP)<sup>[11,12,30,31,34]</sup>. In this study, CPNP successfully delivered the suicide gene into EC9706 esophageal cells, with low toxicity.

Inserting a tumor-specific promoter upstream of suicide genes has been proven to be a successful strategy to achieve targeted expression of suicide genes in tumor tissues. Previously, we successfully used CEA promoter for targeting gastric cancer and colon cancer<sup>[11,12]</sup>. In this study, the specificity against esophageal cancer cells was achieved using the hTERT promoter.

VEGF stimulates tumor cell proliferation and angiogenesis in tumor tissue, and overexpression of VEGF has been found in the majority of human cancers<sup>[35,36]</sup>. Silencing VEGF with RNA interference (RNAi) could inhibit tumor growth and metastasis<sup>[37,38]</sup>. In this study, a novel fusion gene vector carrying a suicide gene (yCDglyTK) and a VEGF shRNA was developed. This expression vector was transfected into EC9706 esophageal cells by CPNP. Increased



**Figure 6** Immunohistochemistry analysis for yCDglyTK and vascular endothelial growth factor in EC9706 xenograft sections. A: Histological expression and distribution of yCDglyTK at magnification  $\times 200$ ; no-treatment control group (a); CPNPs/null + 5-Fc (b); CPNPs/shVEGF (c); CPNPs/yCDglyTK+5-Fc (d); CPNPs/shVEGF-yCDglyTK +5-Fc (e); B: Integrated optical density (IOD) values of VEGF expression in EC9706 xenografts. Anti-VEGF antibody was used for immunohistochemistry assay; C: Quantification of angiogenesis by microvessel counts (MVC) in EC9706 xenografts. Anti-CD34 was used for microvessel staining. CPNPs: Calcium phosphate nanoparticles; VEGF: Vascular endothelial growth factor; 5-Fc: 5-fluorocytosine.

expression of yCDglyTK and decreased expression of VEGF were confirmed at both the mRNA and protein level in EC9706 cells.

*In vitro* antitumor activity of this novel system in the presence of prodrug was tested by MTT assay, Hoechst staining, and flow cytometry. The antitumor effect of the CPNP/shVEGF-yCDglyTK/5-Fc system was further evaluated *in vivo* by using EC9706 cell xenograft model. Both yCDglyTK/5-Fc and shVEGF successfully inhibited the tumor growth, and the combination of the two showed the strongest anti-tumor activity. Subsequent immunohistochemistry staining confirmed the expression of yCDglyTK, the knockdown of VEGF,

and decreased neovascularization in the cancer tissue.

In summary, the CPNP/shVEGF-hTERT-yCDglyTK/5-Fc system developed in this study may overcome several challenges for cancer gene therapy. The use of CPNP increased delivery efficiency, and insertion of hTERT promoter improved tumor specificity. VEGF-targeted shRNA further enhanced the anti-tumor effect. This combination of gene therapies exerted more potent anti-esophageal cancer activity *in vitro* and *in vivo*. The present study provides a novel and promising strategy for esophageal cancer treatment. We will try to optimize this approach to make it more feasible in the future clinical trials.

## COMMENTS

### Background

Esophageal cancer is one of the most prevalent cancers in the world. It has high incidence and mortality rates. Novel and effective treatment options are urgently needed for esophageal cancer.

### Research frontiers

In recent years, gene therapy, especially suicide gene therapy, has been recognized as a promising strategy for cancer treatment.

### Innovations and breakthroughs

In this study, tumor-specific shVEGF-yCDglyTK expression cassette was delivered using calcium phosphate nanoparticle (CPNP) into human esophageal cancer cells. The therapeutic efficacy of this novel gene therapy system was evaluated *in vitro* and *in vivo*, and results showed that it was an effective strategy for esophageal carcinoma treatment.

### Applications

The present study provides a novel and promising strategy for esophageal cancer treatment.

### Terminology

CPNP is a novel non-viral tool for efficient gene delivery.

### Peer-review

This study investigated a novel technique for targeted gene therapy in an esophageal cancer model. The authors report transfection rates and altered gene expression profiles. The study is well conducted and well described. The techniques are appropriate and the experiments are clearly written. The study topic is interesting and relevant.

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