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*Original Article*

# Study on the effect of BMSCs-EGFP-tk as mediator of HSV1-tk/GCV suicide gene therapy directed against A549 in vitro

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**Abstract:** This study aims to observe the expression of HSV1-tk in mouse bone marrow mesenchymal stem cells (BMSCs-EGFP-tk) and detect the inhibition and killing effects of BMSCs as mediator of HSV1-tk/GCV on A549 cells in vitro, which can provide the experimental basis for gene therapy of lung cancer. We constructed the recombinant plasmid Vector pDON-AI-2 Neo-HSV1-tk-IRES2-EGFP with genetic engineering methods. Then we obtained the virus-like particles with infection ability after packaging the virus. The recombinant plasmid was transfected into mouse bone marrow mesenchymal stem cells in vitro. The expressions of EGFP in cells were observed by fluorescence microscopy and HSV1-tk gene was detected with RT-PCR. At last, the A549 cells and BMSCs-EGFP-tk cells were co-cultured with in vitro contact method, and the effect of BMSCs-EGFP-tk/GCV system was determined by MTT. Results indicated that the biological characteristics of BMSCs-EGFP-tk were consistent with those of BMSCs and fluorescent light expression and HSV1-tk gene expression can persist at least 15 days. The A549 cells and BMSCs-EGFP-tk cells were co-cultured and BMSCs-EGFP-tk:A549 = 2:1, adding 1 μg/mL GCV, the theory mortality is 58.44%, but actually the mortality is 90%. There is almost no difference between BMSCs-EGFP-tk and BMSCs cells in biological characteristics. The growth of A549 cells have an obviously inhibition and the bystander effect is outstanding in vitro after co-culture and this experiment lays solid foundation for the future research.

**Keywords:** Lung cancer, BMSCs, HSV1-tk, co-culture, bystander effect

## Introduction

Lung cancer is a kind of malignant tumor and poses a serious threat to human health. 80%~90% patients among them are non-small cell lung cancer (non-small cell lung cancer, NSCLC). The overall 5-years survival rate of lung cancer is only 10%~13% [1] and the proportion of adenocarcinoma is rising. At present, lung adenocarcinoma is usually treated by means of operation, radiation, chemotherapy, immune, Chinese medicine, biological therapy and so on. There is still lack of effective methods. However, its diagnosis is difficult due to no obvious clinical symptoms in the early. Many patients had developed to advanced adenocarcinoma when the diagnosis can be done. Lung adenocarcinoma is not sensitive to the radiotherapy so the overall clinical efficacy is poor. The suicide gene therapy of lung adenocarcinoma became the research hotspot in recent years with the development of tumor biological treatment [2, 3].

Suicide genes mainly include the thymidine kinase (TK) gene of herpes simplex virus (HSV) and varicella zoster virus (VZV), nitroreductase gene and GPT gene of Escherichia coli, cytosine deaminase gene. HSV1-tk gene is one of the most studied and widely used genes. The HSV1-tk/GCV treatment system was firstly used for the treatment of cancer by Mootlen in 1986 and it was confirmed to have significant direct tumoricidal effect [4].

"Bystander effect" is one of the important ways to solve the problem of low transfection efficiency in gene therapy in vivo, while the vector selection of gene transfection and expression is another important way to solve this problem.

There is a big effect on transfection efficiency with different vectors. At present, there are two commonly used vector systems for tumors' gene therapy: viral and non-viral vector [5].

Bone marrow mesenchymal stem cells (BMSCs) have the advantage of good mobility and tumor tissue tropism, being easy to be transfected and long-term stable expression [6, 7]. Taking it as a gene carrier for cancer treatment is one of the stem cells and tissue engineering research hotspots [8-11].

In this study, we packaged the retroviral particles carrying HSV1-tk gene into the BMSCs, BMSCs can be as a "companion vector" of viral vector involved in tumor gene therapy. The non-toxic prodrug ganciclovir (GCV) was changed into the cytotoxic phosphorylation GCV and we studied the inhibition with lung adenocarcinoma A549 cells in vitro. This can provide reference for further research on its inhibitory/killing effect on lung adenocarcinoma in vivo.

## Materials and methods

### *Construction of recombinant plasmid pDON-AI-2-Neo-HSV1-tk-IRES2-EGFP*

The HSV1-tk cDNA fragments were obtained by polymerase chain reaction (PCR). Two restriction sites *Bgl*II and *Sal*I were added to each of the primer respectively. The sequences of primer were as follows: HSV1-tk F: 5'-GGAAGATCTATGGCTTCGTAC-3', R: '-ACGCGTCGACCGTGTTTCAGTTAG-3'. Then we constructed the recombinant plasmid pHSV1-TK/18T with pMD18-T cloning kit (Takara Biotechnology Co., Ltd, Dalian). The recombinant plasmid pHSV1-TK/18T and pIRES2-EGFP plasmid vector (Takara Biotechnology Co., Ltd, Dalian) were digested with the restriction enzyme *Bgl*II and *Sal*I (Thermo Fisher Biochemical Product Co., Ltd, Beijing) respectively. After that they were purified and ligated with T4 DNA ligase (Thermo Fisher Biochemical Product Co., Ltd, Beijing), therefore we got the recombinant plasmid pHSV1-tk-IRES2-EGFP. In the same way, the recombinant plasmid pHSV1-tk-IRES2-EGFP and retroviral vector pDON-AI-2 Neo were digested with the restriction enzyme *Bgl*II and *Hpa*I (Thermo Fisher Biochemical Product Co., Ltd, Beijing) respectively to construct the recombinant plasmid pDON-AI-2-Neo-HSV1-tk-IRES2-EGFP.

### *Package the virus*

Briefly, the logarithmic growth phase 293T cells were digested with trypsin (Sigma, USA) then adjusted the cells' density and re-culture them, they can be used for transfection when the cells' density was 70%~80%. The packaging plasmid mixture composed of pDON-AI-2 Neo-HSV1-tk-IRES2-EGFP, pGP and pE-eco were co-transfected into 293T cells. Then discard the medium containing the transfection mixture after cultured for 8 h and added new culture medium, the cell supernatant was collected after 48 h, centrifuge at 4°C, 4000 g for 10 min, remove the cell debris, then use the 0.45 μm PVDF film to filter the supernatant and harvest the packaged virus particles.

### *Isolation of BMSCs*

SPF, three-weeks male BALB/C mice with weight from 15 to 16 g were used for the isolation of BMSCS. Took the femoral bone marrow under aseptic conditions and put it into DMEM medium containing 15% FBS low sugar (Gibco, USA), then pipetted it repeatedly to form a single cell suspension and centrifuged at 1000 r/min for 5 min. After discarded the supernatant, the sediment was re-suspended, then cultured them in the culture flask at 37°C with 5% $CO_2$.

### *Transfected BMSCs*

The recombinant plasmid pHSV1-tk-IRES2-EGFP was transfected into BMSCs in vitro with lipofectamine transfection reagent and retroviral vector respectively. Liposome mediated recombinant plasmids were transfected into BMSCs cells in accordance with the manual of Lipofectamine™ 2000 kit (Invitrogen, USA). The other transfection method was that the recombinant retroviral supernatant was added into the BMSCs culture flask and cultured at 37°C with 5% $CO_2$. The EGFP green fluorescent protein and the expression of HSV1-tk mRNA were detected after transfection in these two methods. We found 10 living cells in an optical microscope view and then counted the fluorescence expression cells number under fluorescence microscope, we counted 10 views continuously to calculate the transfection efficien-

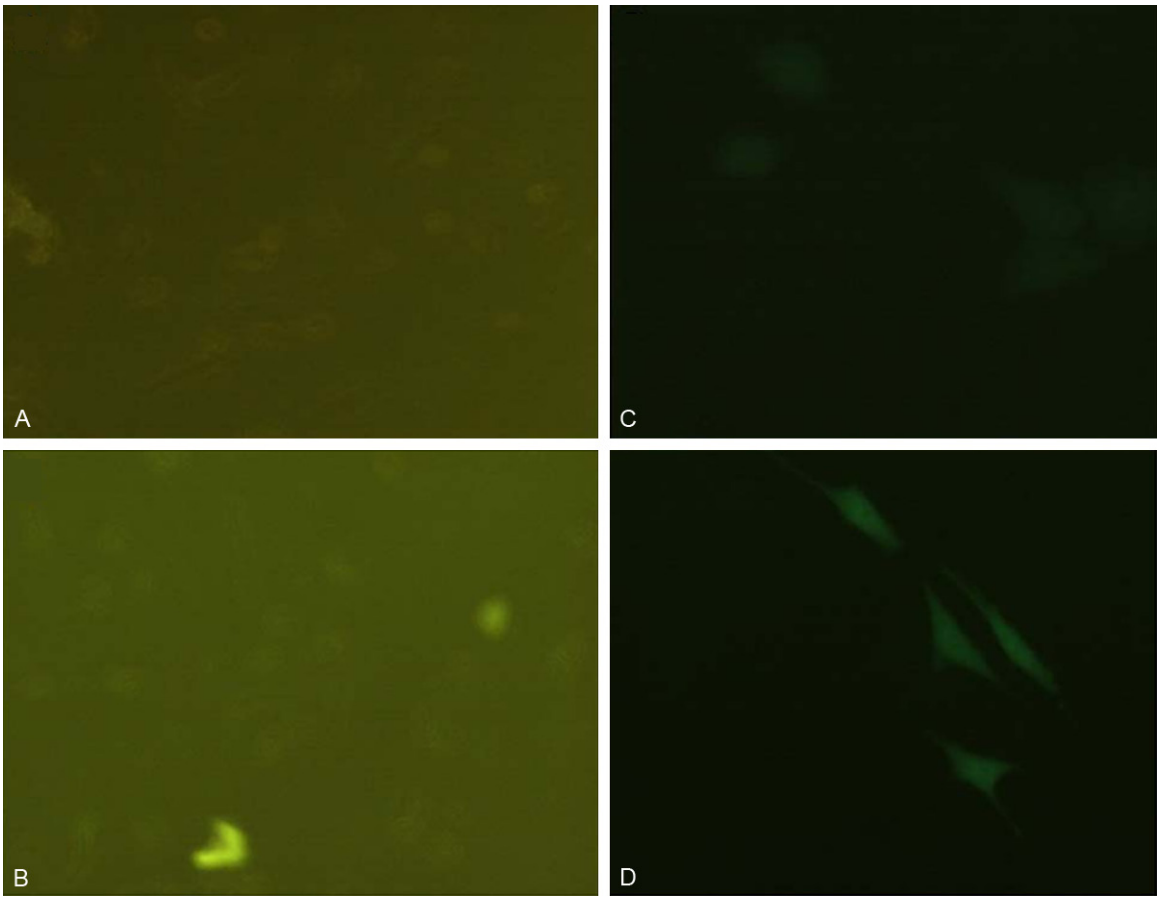


**Figure 1.** After transfected BMSCs for 48 h with Liposome or retrovirus method, the expression of EGFP under the fluorescence microscope (*100). A: Control of Liposome; B: Control of retrovirus; C: Positive expression of Liposome method; D: Positive expression of retrovirus method.

cy. The transfection efficiency = fluorescence expression cells number *10/100.

*The stability of long-term cultured BMSCs-EGFP-tk cells*

After transfection for 6 h, 12 h, 24 h, 36 h, 48 h, 72 h, 9 d and 15 d, we observed cellular morphology and growth status of BMSCs and BMSCs-EGFP-tk cells. At the same time, the total mRNA was isolated and detected the expression of HSV1-tk mRNA with RT-PCR. The statistical analysis of the data was done with SPSS16.0 software.

*The inhibition and killing effects of BMSCs-EGFP-tk cells on A549 cells in vitro*

A549 and BMSCs-EGFP-tk cells were cultured in 25 $cm^2$ flask with complete medium (10% FBS and 1% L-Glutamine DMEM or L-DMEM) at 37°C with 5% $CO_2$ incubator. Then they were co-cultured in 24 well plates with different mixing ratio. After culturing for 24-48 h, 1 ug/ml GCV were added into them. The inhibition and killing effects of BMSCs-EGFP-tk cells on A549 cells were determined by MTT. The cells were seeded in 96 well plates according to the experimental group and the amount was 4000 in each well. They were detected with MTT after treatment at 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, 168 h. The absorbance (A value) was the average of 8 wells in each group at 570 nm. The experiments were repeated three times.

## Results

We successfully constructed the pDON-AI-2-Neo-HSV1-tk-IRES2-EGFP recombinant plasmids and packaged cell 293T/tk with recombinant retroviral vector containing HSV1-tk gene. The genome of packaged virus containing HSV1-tk gene were confirmed by RT-PCR.

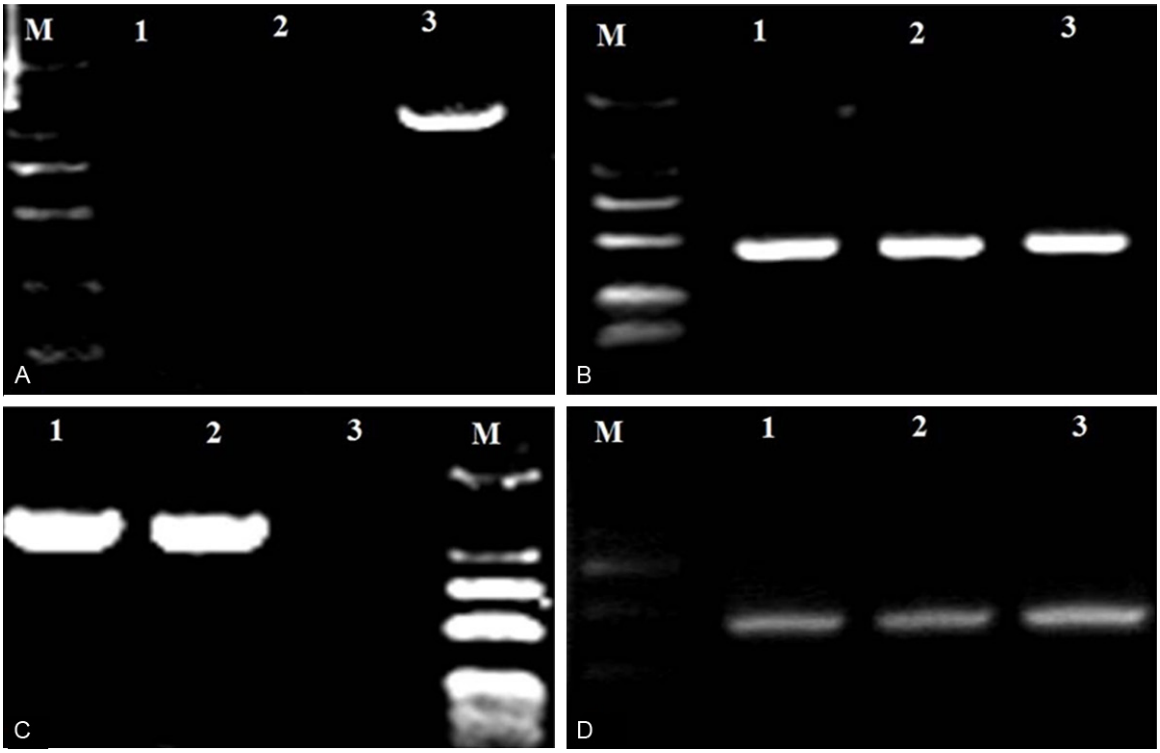


**Figure 2.** The expression of HSV1-tk mRNA in BMSCs after Liposome transfection (A, B) and retroviral transfection (C, D). (A) HSV1-tk gene, M: DL2000 marker, 1: control, 2: pIRES2-EGFP Vector, 3: pHSV1-tk-IRES2-EGFP; (B) β-actin gene, M: DL2000 marker, 1: control, 2: pIRES2-EGFP Vector, 3: pHSV1-tk-IRES2-EGFP; (C) HSV1-tk gene, M: DL2000 marker; 1, 2: pDON-AI-2 Neo-HSV1-tk-IRES2-EGFP 3: Control; (D) β-actin gene, M: DL2000 marker; 1, 2: pDON-AI-2 Neo-HSV1-tk-IRES2-EGFP 3: Control.

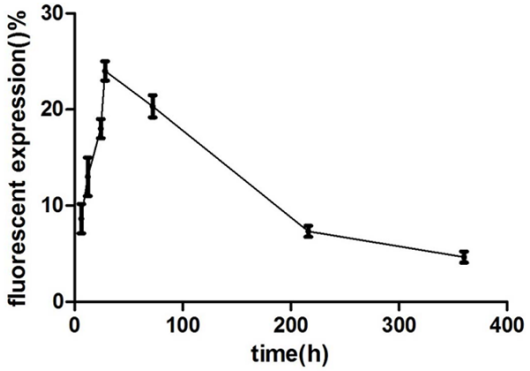


**Figure 3.** The fluorescence expression of BMSCs infected with pDON-AI-2 Neo-HSV1-tk-IRES2-EGFP.

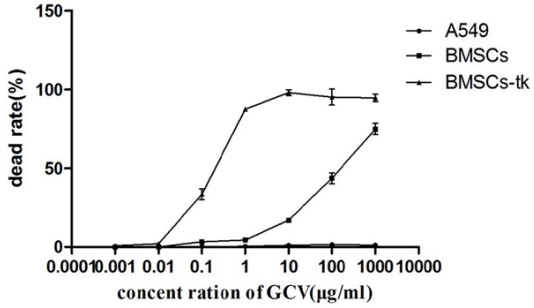


**Figure 4.** The sensitivity of A549 cell, BMSCs, BMSCs-EGFP-tk cell lines to GCV.

The EGFP green fluorescent protein and the expression of HSV1-tk mRNA were detected after transfection at the same time. It was confirmed that both lipofectamine transfection reagent and retroviral vector methods could transfect the BMSCs. However, the transfection efficiency of retroviral vector and lipofectamine transfection was 13.0% and 24.0% respectively, the transfection efficiency of retroviral vector was obviously higher than that of the lipofectamine transfection reagent ($P < 0.05$) (**Figures 1** and **2**).

The long-term cultured BMSCs-EGFP-tk cells were long shuttle shape and only few cells were round. The cellular morphology was uniform and the cells grew in good condition. The biological characteristics of BMSCs-EGFP-tk cells were consistent with those of BMSCs. The transfected BMSCs cells had the strongest fluo-

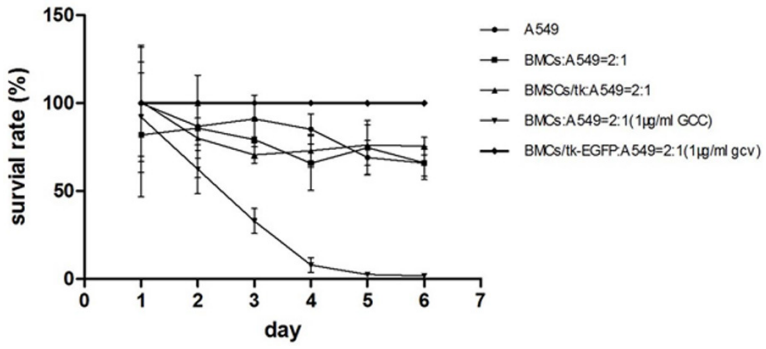


**Figure 5.** The growth curve changes of A549 cells by different treated ways.

rescent expression after 48 h and it could last for 15 days (**Figure 3**). We could detect HSV1-tk mRNA gene after transfected 15 days. However there were no fluorescent expressions or HSV1-tk mRNA gene in normal BMSCs cells.

BMSCs-EGFP-tk is more sensitive to GCV than BMSCs and A5% and ha549 cells. When the concentration of GCV is 1 μg/ml, it has little cytotoxicity to BMSCs and A549 cells while has large cytotoxicity to BMSCs-EGFP-tk which mortality reached more than 8s significant dose-correlation (**Figure 4**). So we selected 1 μg/ml as the test dose.

When the concentration of GCV is 1 μg/ml, the mortality of BMSCs-EGFP-tk cells cultured alone was 87.42% and A549 cells cultured alone was 0.48%. So the theoretical mortality of co-cultured BMSCs-EGFP-tk and A549 cells was A*[0.8742*a+0.0048*(1-a)]/A×100%. A = the total cell number of each well, a = the ratio of BMSCs-EGFP-tk and A549 cells. The actual mortality is higher than the theoretical mortality of co-cultured cells. When BMSCs-EGFP-tk:A549 = 1:10, the theoretical mortality was 8.34% while the actual mortality was 14.88%. The bystander effect was not obvious. When BMSCs-EGFP-tk:A549 = 2:1, the theoretical mortality was 58.44% while the actual mortality was almost 90%, the bystander effect was obvious. So we took the mixing ratio of BMSCs-EGFP-tk:A549 = 2:1 as the co-cultured optimal proportion. **Figure 5** showed the growth curve changes of A549 cells by different treated ways. We can find in BMSCs-EGFP-tk:A549 = 2:1, 1 μg/ml GCV group, on the fourth day, the survival rate of A549 cells has a lower level of 20.96%, it has significant inhibited effect on the growth of A549 cells.

## Discussion

HSV1-tk/GCV suicide gene treatment system is widely used and the results showed that it is effective in the treatment of prostate cancer, ovarian cancer, melanoma and other tumors [12-14]. Enhanced green fluorescent protein gene (EGFP) is a reporter gene that can be expressed in the cells and does not require other exogenous substrates involved in [15, 16], it is widely applied in the regulation of gene expression, transgenic animal, protein positioning in the cell and other research on the gene function [17]. We constructed the pHSV1-tk-IRES2-EGFP recombinant plasmid successfully in this study. Then we used recombinant retrovirus vector pDON-AI-2 Neo to carry HSV1-tk gene into packaging cell line 293T and completed the assembly with the complementary role of defective virus vector and packaging cells. At last we got the pDON-AI-2 Neo-HSV1-tk-IRES2-EGFP recombinant retroviral particles with the ability of infecting host cells.

We infected BMSCs instantaneously and got BMSCs-EGFP-tk cells using retroviral mediated method. The basic morphological characteristics of both BMSCs-EGFP-tk cells and normal BMSCs are almost the same which showed that the exogenous HSV1-tk gene had no effect on the physiological function of BMSCs. EGFP and HSV1-tk mRNA expression could still be observed in BMSCs and with stable traits after they were long-term normal cultured for 15 days.

The co-culture system is to co-culture two kinds of mixed cells in vitro culture conditions which simulates in vivo environment. It can make one kind of cells have stable morphology and function and maintain for a long time, the interactions between cells and cells can be observed [18]. The commonly used co-culture methods include: contact and non-contact [19]. It is widely used in the study of invasiveness, metastasis and experimental treatment of tumor [20], angiogenesis and nutrition supply and the mechanism of tumor central necrosis etc [21, 22]. Li et al. [23] co-cultured BMSCs and myocardial cells to study the effects of

myocardial cells on BMSCs. Cheng et al. [24] co-cultured BMSCs and embryonic stem cells and found that some cytokines secreted by BMSCs can improve the survival rate of the embryonic stem cells and BMSCs can be used as feeder layer cells of embryonic stem cells. However, there was little study on the co-culture of lung cancer cells and BMSCs.

We used a direct contact co-culture method in this study. The two kinds of cells can be fully contacted and interacted completely with this co-culture method, and it is more effective to observe the role of HSV1-tk/GCV suicide gene treatment system than non-contact co-culture method. BMSCs-EGFP-tk cells and A549 cells were co-cultured and the pro-drug GCV was added at the same time. The actual mortality of co-culture cells is higher than the theory mortality, and the mortality rate increased gradually with the increase of the proportion of BMSCs-EGFP-tk. When the proportion of BMSCs-EGFP-tk was 67% (BMSCs-EGFP-tk:A549 = 2:1), the theory mortality was 58.44% and the actual mortality was close to 90%, it had obvious bystander effect. So we selected BMSCs-EGFP-tk:A549 = 2:1 as the optimal co-culture proportion. After adding 1 ug/ml GCV for 96 h, the mortality reached 87.42% (theoretical mortality should be 24%), it showed that most of the BMSCs not being transfected with TK gene were killed and it confirmed the bystander effect. Only BMSCs or BMSCs-EGFP-tk cells did not have significant inhibitory effect on the growth of A549 cells, while we observed a significant inhibitory effect on the growth of A549 cells in the 1 ug/ml GCV group. So the application of BMSCs-EGFP-tk/GCV system in vitro on lung adenocarcinoma cell line A549 showed good tumor suppressor, cytotoxic effect and bystander effect.

In conclusion, this study successfully constructed and packaged recombinant retroviral particles pDON-AI-2 Neo-HSV1-tk-IRES2-EGFP and infected mice BMSCs instantaneously with it. BMSCs-EGFP-tk cells expressing HSV1-tk gene were cultured successfully. Their killing effect on lung adenocarcinoma A549 cells was verified in vitro. It provided a solid foundation for the feasibility of suicide gene therapy of lung cancer.

## Disclosure of conflict of interest

None.

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