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Original Article

# HSV-TK Expressing Mesenchymal Stem Cells Exert Inhibitory Effect on Cervical Cancer Model

Azra Kenarkoohi[1,2], Taravat Bamdad[1*], Masoud Soleimani[3], Hoorieh Soleimanjahi[1], Ali Fallah[4], Shahab Falahi[5]

*1. Department of Virology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.*
*2. Department of Microbiology, Faculty of Medicine, Ilam University of Medical Sciences, Ilam, Iran.*
*3. Department of Hematology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran.*
*4. RNAx Ltd., London, UK.*
*5. Zoonotic Diseases Research Center, Ilam University of Medical Sciences, Ilam, Iran.*

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A growing area of research is focused on cancer therapy, and new therapeutic approaches are welcomed. Mesenchymal stem cell (MSC)-based gene therapy is a promising strategy in oncology. Intrinsic tropism and migration to tumor microenvironment with off lights are attractive features of this type of cell carrier. In this way, suicide genes have also found a good platform for better performance and have shown a stronger anti-tumor mechanism by riding on mesenchymal cells. In this study, we investigated the anti-tumor activity of intratumoral injected MSCs transduced with a lentivector expressing the HSV/TK in a mouse cervical cancer model. Following the injection of MSCs transduced with lentivector carrying TK, MSCs alone or PBS into the mice tumor, ganciclovir was administered intraperitoneally during 14 days, and tumor size, survival time, natural killer (NK) cells and cytotoxic T lymphocyte (CTL) activities were assessed. We demonstrated that combination of suicide therapy and cell therapy leading m,to successful tumor inhibition. Significant reduction in tumor size was detected in test group in comparison with controls. Also, potent antitumor NK and CTL activity was seen in treatment group in comparison with controls. Our data demonstrated that the mesenchymal cells expressing TK had inhibitory effect on cervical cancer model.

**Key words**: Cervical cancer, mesenchymal stem cell, suicide therapy, lentivector, thymidine kinase, cell therapy

Cervical cancer is the fourth most common cancer in women worldwide, with 570,000 new cases and around 311,000 deaths in 2018 (1). Since traditional methods cannot cure cancer, it is necessary to develop new approaches. Suicide gene therapy is a promising method, in which a suicide gene enters tumor cells, and induces apoptosis in cancer cells in the presence of a suitable substrate. In addition to induction of apoptosis, another mechanism involved in this process is bystander effect, where a toxic metabolite produced by transfected cells is transferred to the neighbor cells, resulting in cell death (2-4).

Bystander effect involves the transfer of

*Corresponding author: Department of Virology, School of Medical Sciences, Tarbiat Modares University, Tehran, Iran.
E-mail: bamdad_t@modares.ac.ir





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ganciclovir (GCV) from transduced cell to adjacent cells through gap junction. Monophosphorylated GCV in transduced cells could transit through gap junction's channels to induce cell death in neighbor cells (5). Herpes simplex virus (HSV)- thymidine kinase (TK)/GCV is one of the most frequently used "suicide" gene therapy systems. This enzyme modifies GCV to ganciclovir monophosphate. Then, cellular kinases convert non-toxic monophosphorylated GCV into GCV triphosphate, an analogue of deoxyguanosine triphosphate, which inhibits DNA polymerase and disrupts DNA synthesis (6). The efficient delivery of anti cancer genes and appropriate gene expression is one of the major issues for gene therapy. Recombinant viral vectors and cells are the most powerful carriers for efficient gene transfer (7, 8). Among the viral vectors, lentivectors are potent carriers for gene transfer. These vectors can transduce many dividing and non-dividing cell types (9).

In cancer cell therapy, mesenchymal stem cells (MSCs) have a special place. These cells have a strong tropism to tumor tissue with low immunogenicity without stimulating the host immune system (10). MSCs have also been used to deliver oncolytic viruses into the target tissues (11, 12). Combination of suicide gene therapy and MSCs -based cell therapy has had good results (8, 13). TK expressing MSCs prevented the growth of various tumor cells (14). In addition to inducing apoptosis and the bystander effect mechanism, TK expressing mesenchymal cells were shown to secrete exosomes that contain *TK* mRNA, which is internalized by tumor cells, and cause tumor cell death after GCV administration (15). Here we evaluated the anti-tumor activity of MSCs transduced with HSV-TK genes against the cervical tumor model. Because of the high efficiency, we used lentivectors for HSV-TK gene transfer into MSCs. This is the first study using MSCs transduced with a lentivector expressing HSV-TK gene, injected intratumorally, for the treatment of cervical tumor in mice model.

## Materials and methods

### Production of the pCDH-TK lentiviral vector

HSV TK gene was amplified from plasmid pLOX-GFP-IRES-TK (a kind gift from Dr. Didier Trono, Switzerland) using PCR. The *TK* gene was then inserted into plasmid pCDH-CMV-MCS-EF1-GFP-T2A-Puro.

Lentiviral particles were produced by transient co-transfection of HEK293T cells, human embryonic kidney cells, based on Prof.Trono lab protocol with some modifications, as reported previously (16, 17).

### Mouse MSCs isolation, differentiation and transduction of MSCs

Murine MSCs were harvested from the inguinal adipose tissue of C57/BL6 mice. The plastic-adherent cells were grown in DMEM medium with 10% fetal bovine serum. All differentiation experiments were performed and transduced with pCDH-TK lentiviral vector based on our previous protocol (17).

### Expression of the *TK* gene in transduced cells

RNA extraction and cDNA synthesis from$10^6$ transduced MSCs and non-transduced MSCs were performed using Qiagen kit (Alameda, CA, United States). HSV-TK expression was detected by real time PCR. Beta-2-microglobulin (*B2M)* gene was used as endogenous reference gene. *TK* primers sequence was: forward, 5'-GAGGGCGGCGATG GGATGG-3', Reverse, 5'-GCGGCGGTGGTAAT GACAAGC-3'

### *In vitro* sensitivity of transduced MSCs to GCV

Transduced MSCs were plated in a 96-well cell culture plate in a confluency of $10^4$ cells/well. Twenty-four hours after plating, MSCs were exposed to increasing concentration of GCV 1, 5, 10, 25, 50, 75, 100, and 125 mg/mL, and cells were incubated for 5 days. On the fifth day, MTT assay was performed according to manufacturers' protocol. All experiments were performed at least





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three times.

***In vivo* establishment of mice cervical cancer model and injection schedule**

Six to seven week-old female C57BL/6 mice were purchased from the Pasteur Institute of Iran. All experiments were performed according to the Animal Care and Use Protocol of Tarbiat Modares University. $10^6$ TC-1 cells were suspended in 100 µL phosphate buffered saline (PBS), and subcutaneously injected into the left flanks of the mice. Seven days post inoculation, tumor formation was observed in C57BL/6 mice. One week after the inoculation of the mice with TC1 cells, when tumors reached the average size of 6 mm, 18 C57BL/6 mice were divided into 3 groups. Groups 1 and 2, injected with $2.5\times10^5$ TK-MSCs and MSCs, respectively, and group 3 with 100 $\mu$L PBS followed by GCV injection (100 mg/kg) intraperitoneally, once daily, for 14 days. Mice were monitored daily, and the tumor size for each mouse was measured. Tumor size was calculated using the following formula: $V= (a^2b)/2$. Whereas an equals the largest diameter and b equals the smallest diameter.

**Cytotoxic T cell assay**

Cytotoxic T lymphocyte (CTL) assay was performed by measuring lactate dehydrogenase (LDH) using cytotoxicity assay kit (Cytotoxicity Detection Kit (LDH), Roche, USA) according to the manufacturer's instructions. At 14 days of GCV injection, the mice were sacrificed and their splenocytes isolated. Single cell suspension of splenocyte cells as effector cells and transduced MSCs cells as target cells were mixed at various effector-to-target cell ratios (25: 1, 50: 1 and 100: 1), and were co-cultured in a 96-well plate for 4 h in RPMI1640. After 4 h incubation, the percentage of lysed cells was calculated by measuring LDH release using a LDH cytotoxicity assay kit according to the manufacturer's instructions. The percentage of cytolysis was determined using the following formula: 100×(A–B)/(C– D), where A is the reading of experimental-effector signal value, B equals the effector spontaneous background signal value, C is the maximum signal value from target cells, D equals the target spontaneous background signal value. All experiments were performed at least three times.

**Natural Killer cells cytotoxicity assay**

The killing capacity of Natural killer (NK) cells against tumor cells was determined using a cytotoxicity assay kit. Briefly, single cell suspension of splenocytes was co-cultured with K562 cells for 4 h at different ratios followed by measuring LDH release using a LDH cytotoxicity assay kit according to the manufacturer's protocol (Cytotoxicity Detection Kit (LDH), Roche, USA). Cytotoxicity was calculated as described previously. All experiments were performed at least three times.

**Statistical analysis**

Statistical analysis was performed using SPSS software version 24 and ANOVA test. A P value <.05 was considered statistically significant.

## Results

TK gene was amplified form plasmid PLOX-GFP-IRES-TK by PCR. TK fragment was then inserted into the pCDH-CMV-MCS-EF1-GFP-T2A-Puro plasmid. To confirm cloning, pCDH-TK digestion was performed with Mlu1. Digestion of the recombinant plasmid PCDH-TK with Mlu1 resulted in two fragments of 2400 bp and 6600 bp. Transfection of HEK293T cells with the GFP-expression plasmid PCDH TK and two helper plasmid showed high transfection efficiencies as visualized by the highest proportion of GFP positive cells, as previously reported (17). MSCs were transduced with lentiviral vector carrying *TK*, and then selected with 1.5 µg of puromycin (Figure 1).

Real Time PCR method showed increased expression of TK in the MSCs-TK in comparison with non-transduced cells. The cell viability curve





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of MSCs-TK was graphed in Figure 2. These results indicated that cell viability percent of MSCs-TK cells were 96.37%, 89.5%, 70.8%, 63.95%, 31.25 and 11.65% when exposed to GCV

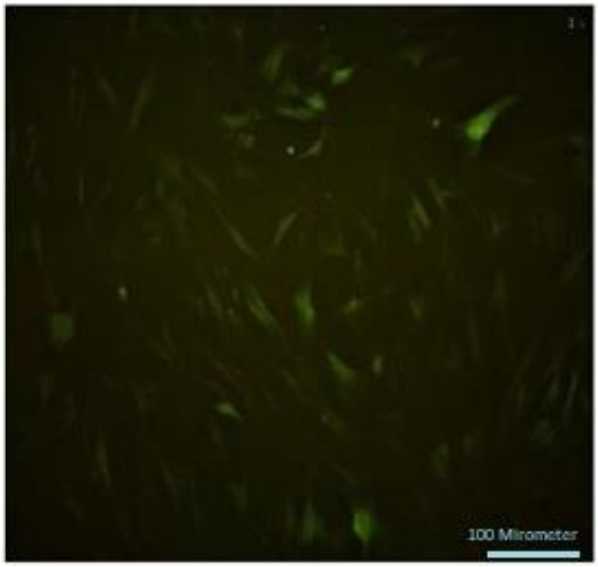


**Fig. 1. Lentiviral transduction of mesenchymal stem cells (MSCs).** MSCs transduced with pCDH-TK at 72 h post transduction. Green cells under fluorescence microscope represent transduced MSCs.

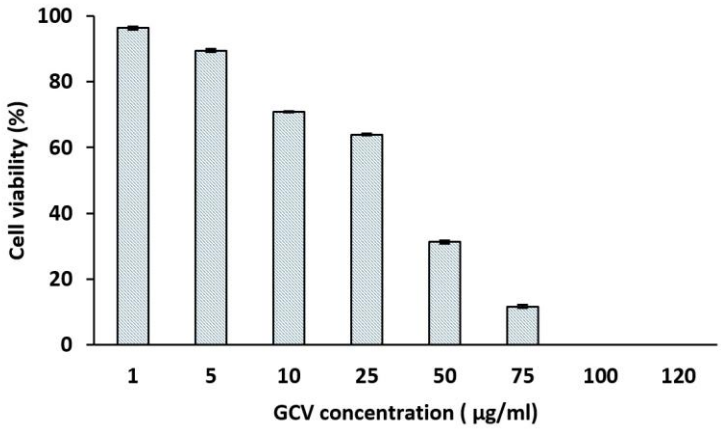


**Fig. 2. The relative survival rate of MSCs-TK cultured with different concentrations of GCV for 5 days.** MSCs-TK were completely killed at 100 µg/mL GCV.

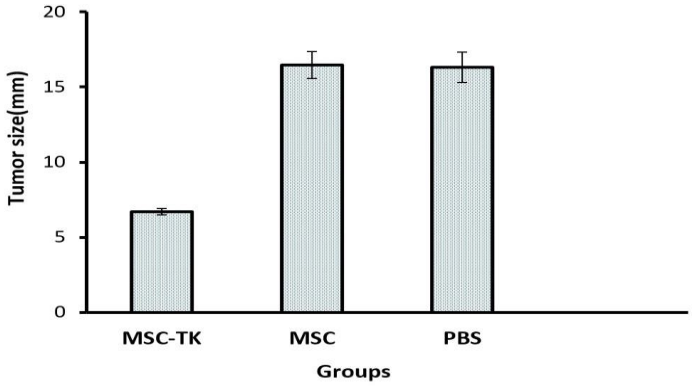


**Fig. 3. The mean tumor size in different groups 10 days after treatment.** The mean tumor size in MSC-TK group was significantly different in comparison with the control groups ($P < 0.05$). The mean tumor sizes were not significantly different between control groups.





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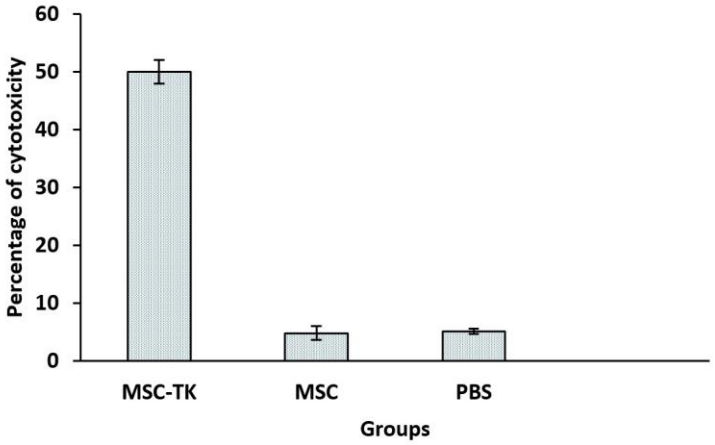


**Fig. 4. Suicide therapy with HSV-*TK* induced cytotoxic T cells in the treated mice group (MSCs-*TK*).** There was a significant difference between the treated mice group and control mice groups (P <0.05).

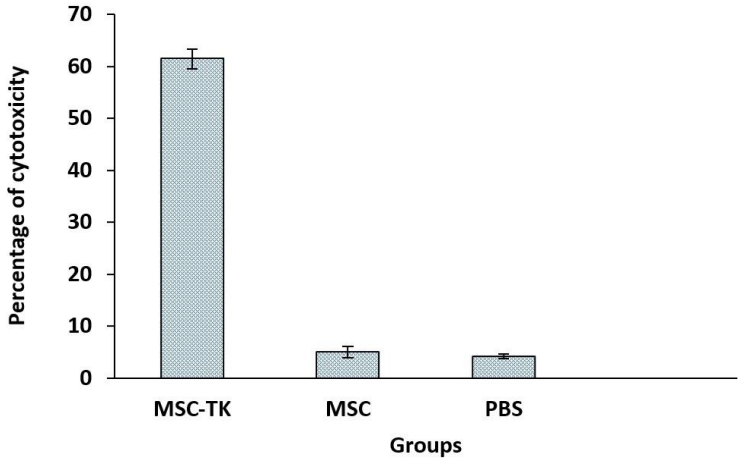


**Fig. 5. Suicide therapy with HSV-*TK* induced NK cell cytotoxicity**. There was a significant difference between the experimental and control groups (P<0.05). The highest percentage of cytotoxicity was seen in the MSCs-TK group.

at the following concentration: 1, 5, 10, 25, 50 and 75 mg/mL, respectively (Figure 2). MSC-TK was completely killed at 100 mg/mL GCV. After the intratumoral injection of transduced MSCs, non-transduced MSCs, and PBS groups, the mice were monitored for tumor size and survival time. After ten days, mice treated with TK expressing MSCs followed by GCV injection showed a significant reduction of tumor size relative to the control groups including non-transduced MSCs and PBS groups, (P<0.05). However, there was no significant difference between the 2 control groups (Figure 3).

At the end of the experiments, the survival time of the mice was evaluated. It was found that the mean survival time was significantly different between experimental and control groups (P <0.05). In control groups, due to the excessive tumor size, we had to kill the mice at week 13. But death was not observed until the 23$^{rd}$ week in mice vaccinated with MSC-TK. CTL assay indicated that *TK* suicide therapy significantly enhanced CD8-T cell-mediated immune responses. As shown in Figure 4, the cytotoxicity percentage is higher in mice treated with MSC-TK in comparison with control groups including mice receiving PBS and MSCs (P <0.05).

The percentage of specific lysis of K562 cell line was determined by LDH measurement (NK cell cytotoxicity assay), and the results were analyzed by ANOVA statistical test. As shown in Figure 5, there was a significant difference between the experimental and control groups (P <0.05). There





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was no significant difference between the 2 control groups (P >0.05).

## Discussion

The low efficiency of gene transfer is one of the main challenges for gene therapy efforts, which can be overcome using viral vectors as gene-delivery vehicles (18). Among the viral vectors, lentivectors are suitable carriers for gene transfer to target cells and tissues, because these vectors can transduce a variety of dividing and non-dividing cell types, and they have the ability to integrate into the cell genome and result in long-term gene expression (4, 19).

Suicide gene therapy using the thymidine kinase against various tumor models is a promising method, where the main strategy is to induce apoptosis. Many studies have been reported based on HSV-TK/GCV system, and have reported acceptable results based on this suicide system on tumor models (4, 8, 13, 20-22).

Some researchers have also inserted the *TK* gene into mesenchymal cells and TK-expressing mesenchymal stem cells have been used for tumor therapy (3, 10, 15, 23).

Intrinsic tropism to tumor tissue, lack of expression of co-stimulatory molecules, low immunogenicity, and easily transduced by viral vectors, are attractive properties of these cells (24-26).

Among viral vectors, lentiviral vectors are efficient to express transgenes in MSCs, and can transduce non-dividing cells, which is important, because 20% of mesenchymal precursors are quiescent (4, 27).

In this project, we constructed a lentiviral vector containing the HSV-TK gene to investigate whether the HSV-TK/GCV suicide system in combination with MSCs could inhibit tumor growth in cervical tumor model. In our experiments, direct injection of $25\times10^4$ mesenchymal cells expressing TK into the tumor, followed by intra-peritoneal GCV administration, resulted in tumor growth inhibition and longer survival time, when compared to control group or mice treated with MSCs and PBS. This was consistent with the study of Zischek et al. in which TK expressing MSCs resulted in a substantial decrease of pancreatic tumor growth (28). Therefore, it seems that a balance of MSC-Tk/TC1 in favor of MSC-Tk was the main reason for efficient tumor eradication. Apoptosis and bystander effects could be a possible reason for the reduction in tumor size relative to control groups.

In addition to apoptosis, the role of immune responses should not be overlooked, dying cells can induce immune responses that inhibit tumor growth (8).

In addition to apoptosis, bystander effect, and immune responses, some part of the reduction in tumor growth may be related to secretory exosomes from mesenchymal cells that contain *TK* mRNA, internalized by tumor cells and triggered cell death (15).

In our study, survival time in control groups receiving MSCs and PBS was 13 weeks, and due to tumor overgrowth, we were forced to kill affected animals. While the group receiving MSCs-TK was followed-up for 23 weeks and no death was observed. In a study conducted by Bak et al., the survival time in mice with glioblastoma receiving MSCs (250000) and the control group, was 48 and 36 days, respectively (29). Previous studies demonstrated that the timing of GCV usage has an important role in response rate and survival time, and it is better to start GCV injection after cell injection and continuing it for more than 3 days (30). Our study was conducted according to this schedule, and led to an increase in survival time.

The results of CTL and NK cell assay showed that cytotoxicity in MSCs-TK group significantly increased in comparison with control groups (MSCs and PBS). Some studies have reported that suicide gene therapy strategy, can induce systemic immune responses and increase the cytotoxicity of NK cells





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in the body (31). TK/GCV system induced rapid cell death and extensive tumor necrosis, which was associated with infiltration of macrophage cells, $T_{CD4}$, $T_{CD8}$, and increased IL-12 concentration in the tumor microenvironment (32).

In conclusion, our results showed that MSCs expressing HSV-TK had an inhibitory effect on cervical cancer in mice.

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**Conflicts of interest**

The authors declare that there is no conflict of interest regarding the publication of this paper.

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