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Original Article

Cell-killing effect of MAR regulating HSV-tk/GCV suicide gene system on ECA109 cells

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Abstract: This study aimed to investigate the cell-killing effect of HSV-tk/GCV suicide gene system driven by β-globin matrix attachment region (MAR) on ECA109 cells and its bystander effect. The integration and expression of lentivirus vectors HSV-tk and MAR-HSV-tk in ECA109 were identified using RT-PCR and Western blot; the cell survival rate and cell-killing bystander effect were detected in different time periods by MTT assay after HSV-tk and MAR-HSV-tk virus infection. Compared with the uninfected cells, the survival rates were significantly decreased with the presence of GCV after infected with HSV-tk and MAR-HSV-tk viruses for 24 h, 36 h, 48 h, 60 h and 72 h, respectively; Moreover, under the same conditions, compared with HSV-tk cells, the survival rate of cells infected with MAR-HSV-tk virus was decreased more significantly and the bystander effect on tumor cell killing was more obvious. Based on the effect of HSV-tk suicide gene system, MAR could enhance the expression of HSV-tk gene. Its killing effect on esophagus cancer cells and bystander effect were more significant, suggesting that the MAR driving HSV-tk/GCV suicide gene system could more efficiently inhibit the proliferation and promote the apoptosis of tumor cells.

Keywords: Esophageal cancer, matrix attachment region, HSV-tk/GCV suicide gene system, bystander effect

Introduction

Esophageal cancer is one of the common malignant tumors of the digestive tract. According to statistics, the morbidity ranks eighth in the world [1]. In China, the morbidity and mortality of esophageal cancer are highest worldwide, ranks fourth in the death cause of cancer, which seriously threaten to people's health [2]. In recent years, with the development of tumor molecular biology, gene therapy has been attracting more and more attention as a new means of cancer therapy. Of which, the suicide gene therapy is most familiar among numerous gene therapy strategies [3]. Suicide gene therapy is to transfect some drug sensitivity enzyme genes into the tumor cells. After the corresponding enzymes were produced by the tumor cells, the low-toxicity or nontoxic prodrug were transfected into cytotoxicity product, so as to achieve the tumor cells killing effect [4, 5]. There are many kinds of suicide gene therapy systems. The research on HSV-tk/GCV system is relatively clear, which has been gradually used in the treatments of liver cancer, gastric cancer, cervical cancer and other tumors [6-8]. The stable expression of gene expression vector plays a key role in the gene therapy [9]. Research showed that matrix attachment region (MAR), as a cis-acting element, it could increase the transcription activity by combining with chromatin and improve the expressions of exogenous genes [10, 11]. The recombinant retroviral vector carrying MAR and HSV-TK genes was used to infect the human esophagus cancer cell line ECA109 in the study. The killing effect of MAR driving HSV-tk/GCV system on esophagus cancer and bystander effect were detected by the cytotoxicity experiment in vitro.

Materials and methods

Lentivirus packaging

The constructed lentivirus recombinant plasmids pLOX-HSV-tk (15 mg), pLOX-MAR-HSV-tk (15 mg), helper plasmids psPAX2 (9.6 mg) and Pmd2.G (5.4 mg) were added in 800 ml Opti-MEM respectively. 60 ml PEI was added in 800 ml Opti-MEM, stood for 5 min, mixed, incubated at room temperature for 20 min, added in ECA109 cells and transfected for 6 h. The liquid was replaced; the sample was centrifuged 36 h later. The supernatant was removed, and then

the viruses were filtrated and collected. The partial cells were removed for genomic PCR and Western blot identification.

RT-PCR

$1*10^5$ MAR-HSV-tk and HSV-tk cells were collected. The total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was reversely transcribed into cDNA as template according to the instruction of reverse transcription kit (Invitrogen, Carlsbad, CA, USA). The RT-PCR primers were designed according to the target gene sequence. HSV-tk (Forward): 5'-GCAAGAAGCCACGGAAGTCC-3', HSV-tk (Reverse): 5'-CCCCGATATGAGGAGCCAGA-3'; MAR-HSV-tk (Forward): 5'-GAGTGAGTGTCAGGCAGGTG-3', MAR-HSV-tk (Reverse): 5'-CCGTCTATATAAACCCGCAG-3'. The sizes of both PCR products were about 300 bp. Meanwhile, β-actin was taken as the internal reference. β-actin (Forward): 5'-CCTGTTCCTCCCTGGAGAAG-3', β-actin (Reverse): 5'-ACATGGTGGTACCACCAGAC-3'. The amplified fragment was 200 bp. RT-PCR reaction system: 10 μL 2*SYBR Green qPCR Master Mix (Bio-Rad, Hercules, CA, USA), 1 μL cDNA, 1 μL upstream and downstream primer (10 μmmol•L-1) respectively. The double distilled water was used to complement until the total volume was 20 μL. PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s. The HSV-tk and MAR-HSV-tk mRNA were directly obtained by RT-PCR. Meanwhile, the uninfected ECA109 cells were taken as the control.

Western blot

5*SDS-loading buffer was added in the equivalent ECA109 cells after infected with MAR-HSV-tk and HSV-tk viruses, then boiled for 15 min in 100°C metal bath. The SDS-PAGE electrophoresis and membrane transferring were performed. 5% skim milk was used to close for about 30 min. The sample was incubated overnight using 1:1000 diluted Anti-TK rabbit polyclonal antibody (Santa-Cruz, CA, USA), washed for 3 times in the next day, 10 min/time, incubated using the diluted 1:5000 sheep-anti-rabbit IgG second antibody (Huaan Biotechnology Co., Hangzhou, China) at room temperature for 1 h, washed using PBST for 3 times. ECL was used for coloration. The β-actin (Huaan Biotechnology Co., Hangzhou, China) was taken as the internal reference.

MTT detection

The MAR-HSV-tk and HSV-tk in logarithmic growth period as well as uninfected ECA109 cells were inoculated with about $8*10^3$ in 96-well plates, cultured at 37°C, 5% CO_2 for 24 h. The final 10 mg/ml GCV (Abcam, Cambridge, UK) was added and continued to culture for 24 h, 36 h, 48 h, 60 h and 72 h, respectively. Three multiple holes were established for the samples in three groups at each time period. After 5 time periods, 20 ml MTT (1 mg/ml) (Beyotime Institute of Biotechnology, Shanghai, China) was added in each hole respectively and continued to culture for 4 h. The culture solution was removed. 150 ml DMSO was added and shaken for about 10 min until the purple crystals were fully dissolved. Finally, the OD value in each hole was measured at 570 nm using enzyme-linked immunosorbent assay.

Bystander effect detection of MAR on HSV-tk/GCV system

The equivalent MAR-HSV-tk^+ and HSV-tk^+ cells were respectively mixed with the uninfected ECA109 cells with different ratios and inoculated in 96-well plates, so that the proportions of the uninfected cells accounting for the total number of cells were 20%, 40%, 60% and 80%, respectively. Three multiple holes were established for each ratio. After cultured for 24 h, the culture medium was replaced into 10 mg/ml GCV. The sample was continued to culture for 48 h. The survival rate of cells was calculated by MTT method according to the above steps.

Statistical analysis

All the data were analyzed using SPSS19.0 statistical software (SPSS Inc, Chicago, IL, USA). The count data were expressed using the rate (%). The comparison between groups was tested using χ^2 test. $P<0.05$ indicated that the difference was statistically significant.

Results

MAR-HSV-tk and HSV-tk mRNA expression by RT-PCR

The amplified fragment sizes of MAR-HSV-tk and HSV-tk were about 300 bp. The agarose gel electrophoresis detection found that there were highlight and specific target bands near 300 bp band but no band in the uninfected ECA109 control cells, suggesting that MAR-HSV-tk and HSV-tk genes had been effectively

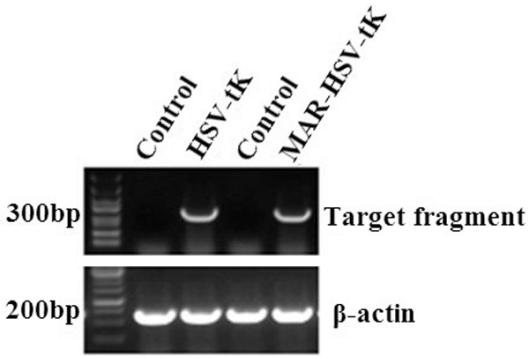

Figure 1. Detection of MAR-HSV-tk and HSV-tk mRNA expression by RT-PCR.

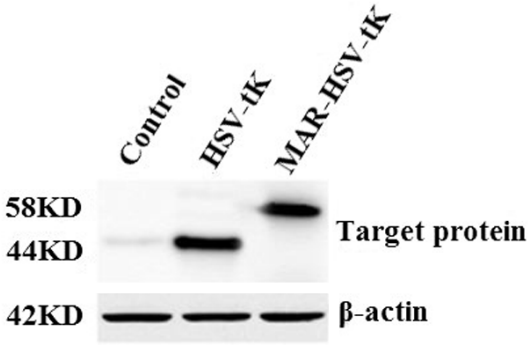

Figure 2. Detection of MAR-HSV-tk and HSV-tk protein expression by Western blot.

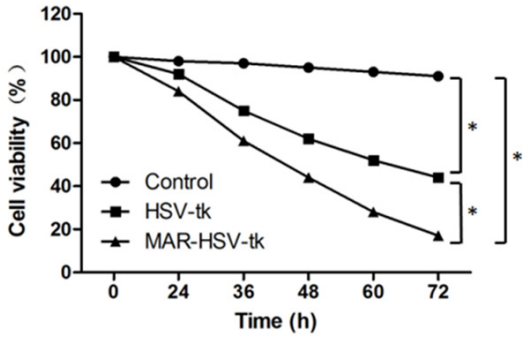

Figure 3. Effect of MAR on killing effect of HSV-tk/GCV suicide gene system in vitro. *: P<0.05.

integrated into the genome of ECA109 cells and had higher mRNA expression (**Figure 1**).

MAR-HSV-tk and HSV-tk protein expression by Western blot

After the ECA109 cells were infected with MAR-HSV-tk and HSV-tk viruses, partial cells were collected and Western blot was performed. The result found that compared with the uninfected ECA109 cells, HSV-tk and MAR-HSV-tk were highly expressed in the cells, suggesting that the lentiviral vector was successfully constructed and could be used for the subsequent killing in vitro and bystander effect experiment (**Figure 2**).

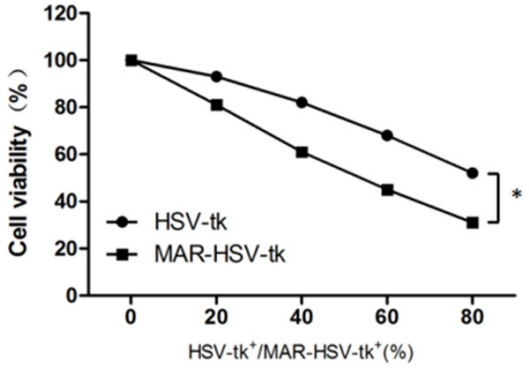

Figure 4. Effect of MAR on the bystander effect of HSV-tk/GCV suicide gene system. *: P<0.05.

Effect of MAR on the killing effect of HSV-tk/GCV system in vitro

MTT assay result was shown in **Figure 3**. The ECA109 cells were infected with MAR-HSV-tk and HSV-tk viruses had more sensitive reaction on GCV. Moreover, with the extension of GCV time, the growth inhibition was more obvious, namely, the cell survival rate was lower. While GCV had no obvious killing effect on the uninfected ECA109 cells; In addition, the survival rates of MAR-HSV-tk$^+$ and HSV-tk$^+$ cells were compared. The result found that the survival rates of MAR-HSV-tk$^+$ at all time periods were significantly lower than those of HSV-tk$^+$ cells ($P<0.05$), suggesting that the cis-acting element MAR significantly enhanced the killing effect of GCV on HSV-tk$^+$ cells.

Effect of MAR on the HSV-tk/GCV system bystander effect

The results showed that after GCV was added, with increase of HSV-tk$^+$/HSV-tk$^-$ and MAR-HSV-tk$^+$/HSV-tk$^-$, the growth inhibition rate of ECA109 cells was increased, while the survival rate was decreased gradually. Moreover, compared with the HSV-tk$^+$/HSV-tk$^-$ mixed cells, the survival rates of MAR-HSV-tk$^+$/HSV-tk$^-$ mixed cells were decreased in all time periods ($P<0.05$) (**Figure 4**). This showed that affected by MAR regulation, HSV-tk/GCV suicide system had more significant bystander effect on ECA109 cells.

Discussion

At present, the morbidity and mortality of esophageal cancer show a downward trend in China. However, affected by many obstacles in the prevention and control work, its mortality is still at higher level and its overall treatment effect is unsatisfactory. So it is necessary to seek a new treatment for efficiently inhibiting the tumor growth and proliferation [12]. In recent years, gene therapy has gradually become a new tumor therapy. Moreover, among many ways, it is most popular and well-known to treat the tumor for suicide gene system.

Herpes simplex virus thymidine kinase (HSV-tk/GCV) suicide gene system is the most common and efficient suicide gene system, which has been used in the anti-tumor experiment [13]. The principle is that after HSV-tk gene is input into the tumor cells, it will encode an enzyme. This enzyme can phosphorylate the nonpoisonous prodrug GCV (Ganciclovir), eventually; the phosphorylated GCV is phosphorylated into triphosphate GCV (GCV-TP) poisonous substance under the action of kinase. The poisonous substance can cause the termination of DNA synthesis and chromosome degeneration by inserting DNA synthesis chain, which ultimately can result in the apoptosis of tumor cells [14-16]. In addition, another advantage of HSV-tk/GCV suicide gene system is the bystander effect, namely, after the prodrug GCV is given, the cells of transfecting HSV-tk/GCV suicide gene are killed, and the uninfected adjacent cells are also killed. The suicide effect of HSV-tk/GCV system should be further amplified [17].

Although HSV-tk/GCV suicide gene system has been better verified in many experiments in vitro and in vivo, there is still a certain gap away from the ideal clinical cancer therapy due to the input specificity of exogenous gene and low expression of viral vector. MAR is a DNA sequence binding the nuclear matrix or nuclear skeleton specificity in the eucaryon chromatin. It is involved in multiple biological functions of the cells, including the involvement of DNA replication and transcriptional regulation. It can significantly enhance the expression of exogenous gene and effectively overcome the transgene silencing [18, 19]. In view of these functions, as a cis-regulatory element, it is also used in the cancer therapy [20, 21]. Studies showed that the fusion of MAR sequence and some suicide genes could significantly enhance the expression of suicide gene. So it could effectively inhibit the proliferation of cancer cells and promote the apoptosis [22]. At present, there was no study on MAR sequence enhancing HSV-tk/GCV suicide gene expression system or targeted therapy of esophageal cancer. MAR was fused with HSV-tk/GCV suicide gene by lentiviral vectors and transfected into the human esophageal cancer cell line in this study, in order to explore the killing effect in vitro of MAR on HSV-tk/GCV and bystander effect.

Our study found that compared with the uninfected esophageal cancer ECA109 cells, both cells infected with HSV-tk and MAR-HSV-tk viruses occurred to significant apoptosis under the presence of GCV. And with the extension of time, the survival rate of ECA109 cells showed a continuously and significantly decreased trend. In addition, compared with HSV-tk$^+$ cells, the apoptosis of MAR-HSV-tk$^+$ was more obvious and the cell survival rate was decreased more significantly. This showed that GCV had no significant toxic effect on the cells uninfected with HSV-tk virus. And MAR could significantly enhance the expression of HSV-tk gene, so as to achieve more efficient tumor killing effect.

Similarly, in the bystander effect experiment, with the decrease of HSV-TK$^-$ cells ratio, the survival rates of HSV-tk$^+$ and MAR-HSV-tk$^+$ mixed cells were decreased significantly. Moreover, when HSV-tk$^-$ cells and MAR-HSV-tk$^+$ were mixed, the killing effects was more significant. The cell survival rate was only 28% after reacted with 10 mg/ml GCV for 48 h. However, HSV-tk$^-$ cells and HSV-tk$^+$ cells were mixed, still 57% of the cells survived under the same conditions. Therefore, MAR could significantly enhance the expression of HSV-tk, so as to translate the non-toxic GCV into CV-TP, to enhance its bystander effect and to accelerate the apoptosis rate of tumor cells. Previous studies showed that the bystander effect of HSV-tk/GCV suicide system might be related to the communicating function of intercellular gap junction [7, 8]. If the GCV concentration was increased, the killing effect of MAR on the tumor cells and bystander effect would be more obvious.

At present, although HSV-tk/GCV suicide gene system has been applied in the gene therapy of many tumors, there are many problems in the

treatment system, such as low targeting, low vector expression and potential pathogenicity [23]. Related researches showed that some traditional treatments, such as radiotherapy and chemotherapy could promote the killing effect of HSV-tk/GCV system on tumor cells [24, 25]. Therefore, exploring all kinds of safe and effective strategies, enhancing the targeting and expression of HSV-tk/GCV system will greatly promote the effect of suicide gene in tumor therapy.

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Disclosure of conflict of interest

None.

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