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Killing effects of ganciclovir on human pulmonary adenocarcinoma cell A549 transduced with HSV1-TK gene *in vitro* and *in vivo*

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KEY WORDS HSV1-TK gene; guanosine; gene therapy; lung neoplasms

ABSTRACT

AIM: To observe the killing effects of ganciclovir (GCV) on the human pulmonary adenocarcinoma cell A549 transduced with Herpes simplex virus 1 type thymine kinase (HSV1-TK) gene *in vitro* and *in vivo*. **METHODS**: A retroviral vector containing the TK gene was constructed and transduced into a pulmonary carcinoma cell A549 by electroporation, to observe the sensitivity of the transfected cell to GCV *in vitro* and the bystander effect (MTT assay). Tumor cell apoptosis caused by the TK/GCV system was observed with a flow cell meter (FCM) and a scan electronic microscope (SEM). Recombination and expression of the TK gene were examined with DNA PCR and *in situ* hybridization, respectively. The therapeutic effect of GCV on subcutaneous tumor growth between transfected and parental cells was also compared. **RESULTS**: The sensitivity of the transfected cell to GCV was 46 times higher than that of the parental cell, and the bystander effect was stronger in high cell density than in low cell density. The $subG_0G_1$ peak was shown on the DNA histogram after A549-Tk cell was treated with 50 μmol/L GCV for 3 days by FCM, but not in the A549 cell. A cell cycle analysis showed that the apoptotic cell in the A549-TK and A549 cells were (12.2 ± 1.7) % and (1.3 ± 0.3) %, respectively ($P < 0.01$). The cell apoptosis features of nuclear condensation, apoptotic vesicle, and nuclear showing semimoon feature were found in the A549-TK cell by SEM, but not in the A549 cell. Recombination and expression of the TK gene were positive in the transfected cell. *In vivo*, the growth of tumors formed by the transfected cell was apparently inhibited by GCV, but not in the control group. **CONCLUSION**: The transfected cell obtained sensitivity to GCV and the bystander effect was closely related to intercellular touch. The TK/GCV system killing tumor cell was related to cell apoptosis. GCV inhibited the growth of tumors which were inoculated by A549-TK cell *in vivo*.

INTRODUCTION

Herpes simplex virus I type thymine kinase (HSV1-TK) gene is a drug-sensitive gene. Cells transfected with the gene are sensitive to nontoxic antiviral drugs such as ganciclovir (GCV) or anciclovir (ACV). The mechanism, is that GCV can be transformed by viral thymidine kinase into a monophoshate form, which is subsequently converted by endogenous mammalian kinases to GCV biphosphate and triphosphate. The later competes with normal nucleotides for DNA replication in mammalian cells and causes a premature chain termination when incorporated into the replicating DNA, disrupts cellular proliferation, and causes cell death[1,2]. At present, this gene is widely used to treat many tumors. In order to explore the killing effect of GCV on a pulmonary carcinoma cell A549 transduced with a TK gene, we conducted the experiment *in vitro* and *in vivo*. The results were reported as follows.

MATERIALS AND METHODS

Cell lines The A549 human pulmonary adenocarinoma cell line was purchased from the Institute of Cell Biology, Shanghai. The A549-TK cell (transgene cell) was screened by G418 400 mg/L after the TK gene was transduced into A549 cell with electroporation (a

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retroviral vector containing the TK gene was prepared by ourselves). Both cell lines were cultured in RPMI1640 (Gibco, USA) medium supplemented with 10 % heat inactivated fetal calf serum (Molecular Biological Laboratory, Third Military Medical University), penicillin 100 mL/L as well as streptomycin 100 mL/L.

***In vitro* sensitivity of transduced cell to GCV** Both the A549 and the A549-TK cells were inoculated in a 96-well cell culture plate in a density of 2500/well. Twenty-four hours after inoculation, different concentrations of GCV (Roche Co, Swede) 0.1, 1, 10, 100, 1000, 2000, 2500, 3000, 3500, 4500, and 10 000 μmol/L were added to culture medium and the cells continued to culture for 5 d. Viable cells were counted with an MTT (Sigma Co, USA) assay.

Observation of bystander effects The A549-TK and A549 cells were mixed in different ratios such as 5:95, 10:90, 20:80, 50:50, 80:20, 90:10, and 100:0, and then inoculated in a 96-well cell culture plate in two densities: high density (1×10^4/well) and low density (1×10^3/well). The cells were cultured in RPMI1640 containing GCV 60 μmol/L for 5 d, and live cells were counted with an MTT assay.

GCV inducing apoptosis of A549-TK cells The cell apoptosis of the transduced cells was examined with FCM (flow cell meter, FACSort, Becton Dickinson Co, USA) and SEM (scan electronic microscope, ACAS Ultima 312, Meridian Co, USA) after the A549 and A549-TK cells were treated by GCV 50 μmol/L for 3 – 4 d. Then, the cells were collected after digestion with trysin and washed two times in PBS to reserve the cell sediment by centrifugation. Part of the cells was resuspended in PBS, with a cell concentration of 1×10^6 per mL. Next, the cells were fixed with 70 % ethanol for two hours, stained with fluorescent dye of propidium iodide, and filtered with a nylon net of 180 pores. The fluorescent strength of a single cell was measured and recorded with the FCM. The data were produced using COTTFF software for the cell cycle computer analysis. Using SEM observation, another part of the cells was fixed in 25 % glutaraldehyde-2 % paraformaldehyde in 0.1 mol/L sodium cacodylate buffer and postfixed in 1 % osmium tetroxide. The cells were dehydrated in a graded ethanol series followed by propylene oxide and then embeded in an epoxy resin. The sections were stained with uranyl acetate, lead citrate, and observed on a phillip-EM400 scan electronic microscope.

Examination of recombination and expression of TK gene The DNA of the A549-TK and A549 cells was extracted with a routine method (Taq DNA polymerase chain reaction kit was purchased from Promega Co, USA). The TK gene oligonucletide primers were synthesized in a DNA synthesiser from Shanghai Cell Institute, with sense primer 5′ CCGGCC-CTCACCCTCATCTT 3′ and an antisense primer 5′ TC-CTTCCGTGTTTCAGTTAGCC 3′. A genetic fragment of 681 bp was amplified in a PCR cycler for 35 circles under the cycling conditions: 94 ℃ for 35, 60 ℃ for 50, and 72 ℃ for 1 min. The product of PCR was identified in a 2 % agarose gels electrophoresis. The TK expression of the A549 and the A549-TK cells was examined *in situ* hybridization. The TK gene specific probe was marked with digoxin.

***In vivo* study** *In vivo* study, the A549-TK cells were implanted into the left abdominal walls and the A549 cells in the right abdominal walls at nine nude mice, and each implant inoculated with 2×10^7 cells per 0.2 mL. Six of the nude mice received only one A549-TK or A549 implant in the left or right wall, respectively, but the other three received two implants in each wall. Two weeks after the inoculation, tumors grew up, and their sizes were measured and photos of them were taken. Then, GCV 100 $mg \cdot kg^{-1} \cdot d^{-1}$ was injected into the abdominal cavity of the nude mices. After 14 d in all, the diameter of the tumors was measured and photos were taken before and after treatment.

Statistical analysis Data were presented with $\bar{x} \pm s$ and the statistical analysis was performed using the student paired t test, a value of $P < 0.05$ was accepted as statistical significance.

RESULTS

***In vitro* sensitivity of transduced cell to GCV** Expressed with IC_{50}, the sensitivities of the A549 and the A549-TK cells to GCV were (4157 ± 86) and (91 ± 4) μmol/L, respectively. With IC_{50}, the sensitivity of A549-TK cell to GCV was 46 times higher than that of A549 cell. The growth inhibition curve of A549-TK and A549 cells was graphed in Fig 1.

Observation of bystander effects When mixed cells were inoculated in a higher density (1×10^4/well), with A549-TK cells reaching 20 % in density, a marked growth inhibition, compared to the control group, was observed ($P < 0.01$); when mixed cells were inoculated in a lower density (1×10^3/well), with A549-TK cells reaching 50 % in density, a marked growth inhibition,

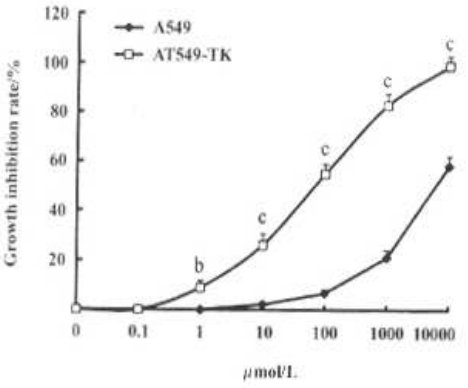

Fig 1. The growth inhibition curve of A549-TK and A549 cells. $n = 4$ experiments. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* A549 cell.

compared to the control group, was observed ($P < 0.05$).

Cell apoptosis examined with FCM and SEM After the A549-TK cell was treated with GCV 50 μmol/L for 3 d, a subG$_0$G$_1$ peak of A549-TK, resulting from apoptotic cells, was shown on the DNA histogram with FCM analysis, but no peak was observed in the A549 cell. The analysis of the cell cycles showed that the numbers of the A549-TK and A549 subG$_0$G$_1$ phase caused by apoptotic cells were (12.2 ± 1.7) % and (1.3 ± 0.3) %, respectively ($P < 0.01$). A DNA histogram of the A549-TK and A549 cells was shown in Fig 2A and Fig 2B.

From the ultrastructure observation of the A549 and the A549-TK cells, cell apoptosis features such as nuclear condensation, apoptotic vesicle, and nuclear showing semimoon were found in the A549-TK cell by SEM, as shown in Fig 3, but not in the A549 cell.

PCR and *in situ* hybridization A specific 681bp fragment of the TK gene was amplified with DNA from the A549-TK cell, but not in the A549 cell (Fig 4).

The results from *in situ* hybridization demonstrated that the expression of TKmRNA was positive in the A549-TK cell, and the stain of mRNA in the cell plasma appeared to be blueish purple, but negative in the A549 cell (Fig 5).

The observation of treatment effect of tumors induced by transfection cells in nude mice The formation of the tumors *in vivo* was shown in Tab 1. The growth curves of the tumors after GCV treatment were shown in Fig 6. The results demonstrated that the tumors could be induced after every point inocculated with 1×10^7 cells in nude mice. The days of tumors formed by A549-TK and

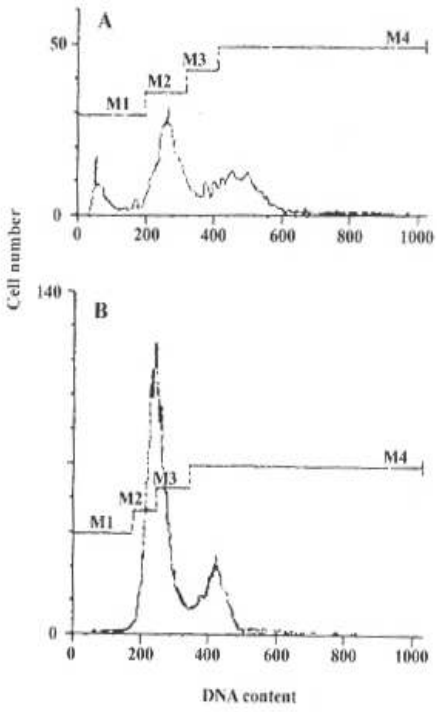

Fig 2. A549-TK (A) and A549 (B) cell DNA histogram.

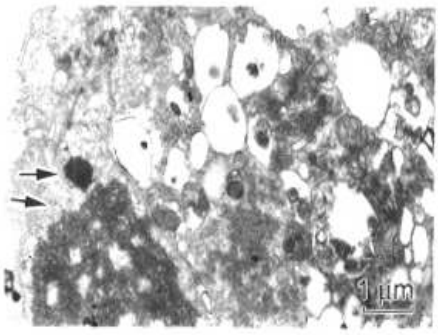

Fig 3. The A549-TK cell was treated by GCV 50 μmol/L for 3 d. Nuclear showed semimoon and apoptotic vesicle which were marked by arrows. × 10000.

A549 cells were not significant different ($P > 0.05$). The volume change of tumors which was induced by A549-TK cells before and after GCV treatment did not show significant increase ($P > 0.05$), but those induced by A549 cells showed a obvious increase in their volume after treatment ($P < 0.01$).

DISCUSSION

Since Moolten[1] first adapted the TK gene to tumor

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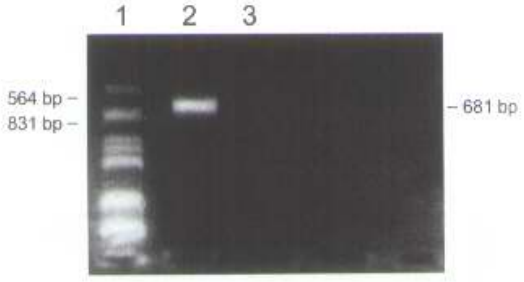

Fig 4. The PCR results of DNA from A549-TK and A549 cells. Lane 1 Marker, lane 2 A549-TK cell, lane 3 A549.

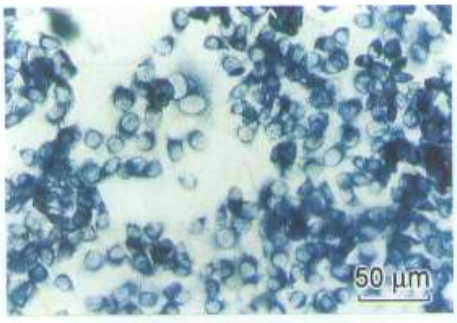

Fig 5. TK mRNA expression of A549-TK cell *in situ* hybridization. × 400.

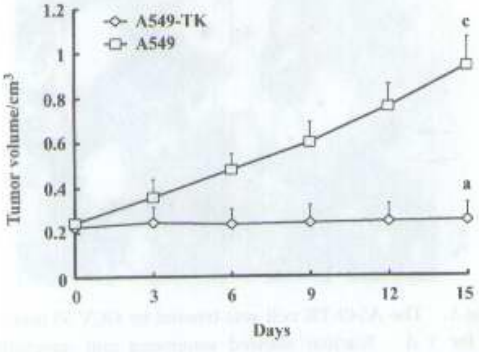

Fig 6. The growth curves of the tumors after GCV treatment. $\bar{x} \pm s$. $n = 12$. $^aP > 0.05$, $^cP < 0.01$ *vs* pretreatment.

treatment in 1986, it has been widely accepted as a treatment for various tumors such as brain glioblastoma, melanoma, and hepatocarcinoma. From research at cell level, to animal experiments and finally, to clinical trials at Phases I and II, its effects and prospects have been known. Experimentally, the retroviral vector was used in our study to transduce the TK gene into a human pulmonary cancer cell A549, and the sensitivity of the transduced cell to GCV was observed *in vitro* and *in vivo*. Our results showed that the sensitivity of A549-TK cell to GCV was only improved 46 times more than that of the A549 cell by computation to IC_{50}. However, much literature previously reported that the sensitivity of transfected cells to GCV was enhanced $10^2 - 10^3$ times than that of parental cells[2]. The difference among transgenic cells was possibly related to cellular biological characters. Many studies demonstrated that bystander effects played a very important role in TK gene killing tumor cells[3,4]. In our study, the phenomenon of bystander effects was observed as well. Our study showed that when mixed cells were inoculated in a high density (1×10^4/well), 60 % of the cells were confluent so that a toxic molecule easily transferred between cells, with a very strong effect. However, when mixed cells were inoculated in a low density (1×10^3/well), cell-cell touch was seen to be stagnant, which apparently weakened the bystander effect. These results demonstrated that the bystander effect was related to intercellular touch. One mechanism of TK/GCV system killing tumor cells was its inducement to cell apoptosis[5-7]. In our study, FCM and SEM were utilized to examine cell apoptosis. The results demonstrated that the A549-TK cell presented an apoptotic change by FCM and some apoptotic character by SEM after it was treated by GCV 50 μmol/L for 3 d, but the A549 cell did not. These concurrent results also supported the inducement of the TK/GCV system to cell apoptosis[8,9]. *In vivo*, many studies reported different results. Some reports demonstrated that tumors could be completely eradicated

Tab 1. Tumors formation and volume change before and after GCV treatment in nude mice. $n = 9$. $\bar{x} \pm s$. $^aP > 0.05$ *vs* A549-TK cell. $^dP > 0.05$, $^fP < 0.01$ *vs* pretreatment. note: each of three of nine nude mice was inocculated two tumors.

Cell	Inoculated number of cells (1×10^7)	Incident of tumor	Time of tumor formed/d	Volume of tumor/mm³ Pretreatment	Posttreatment
A549-TK	1.0	12/12	7 ± 3	224 ± 151	258 ± 167[d]
A549	1.0	12/12	6 ± 2[a]	243 ± 173	953 ± 412[f]

by the TK/GCV system[10,11], some only inhibited the growth of tumors[12,13]. Although our result was not as good as that of previous predictions, but it still demonstrated that growth in the tumors formed from A549-TK cells could be inhibited by the GCV treatment. The mean volume of the tumors from the A549-TK cells increased insignificantly after treatment with GCV compared with pretreatment ($P > 0.05$), but that the mean volume of the control tumors apparently grew after GCV treatment compared with pretreatment ($P < 0.01$). This implied that the A549-TK cell was also sensitive to GCV *in vivo* and could be inhibited or killed by GCV. As a result, our study has contributed towards the application of TK gene in the clinical treatment of tumors.

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更昔洛韦对转染 HSV1-TK 基因的人肺腺癌细胞 A549 的体内外杀伤效应

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关键词 HSV1-TK 基因；鸟苷；基因治疗；肺肿瘤

目的：活体内外观察更昔洛韦(ganciclovir，GCV)对转染 HSV1-TK 基因的人肺腺癌细胞 A549 杀伤效应. **方法**：构建一个包含 TK 基因的逆转录病毒表达载体，用电穿孔法转化人肺腺癌细胞 A549，MTT 法测转基因细胞对 GCV 敏感性和观察旁观者效应；流式细胞仪、扫描电镜检测 GCV 诱导转染细胞凋亡，PCR 和原位杂交分别检测转染细胞 TK 基因整合和表达，活体比较 GCV 对转染细胞、亲代细胞接种裸鼠皮下肿瘤治疗效果. **结果**：转染细胞对 GCV IC_{50} 比亲代细胞提高 46 倍，旁观者效应在高细胞密度较低细胞密度明显. FCM 发现转染细胞经 GCV 作用 3 天后 DNA 直方图呈现亚 G0G1 峰，亲代细胞无该

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峰形成. 细胞周期分析表明亚 G_0G_1 细胞在 A549-TK 和 A549 分别为(12.2±1.7) %和(1.3±0.3) % ($P<0.01$). 扫描电镜发现转染细胞有明显的凋亡特征如核浓缩、凋亡小体和核呈半月征等, 对照细胞无这些变化. PCR、原位杂交表明转染细胞有 TK 基因整合和表达, 体内实验表明转染细胞接种肿瘤受 GCV 治疗抑制生长, 亲代细胞接种肿瘤不受抑制. **结论**: 转 TK 基因细胞获得 GCV 的敏感性, 旁观者效应杀灭肿瘤细胞与细胞间密切接触有关, TK/GCV 杀灭肿瘤与诱导凋亡有关, GCV 活体内抑制转 TK 基因细胞接种肿瘤生长.

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