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Wild Type p53 Gene Sensitizes Rat C6 Glioma Cells to HSV-TK/ACV Treatment In Vitro and In Vivo

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Abstract Suicide gene therapy using herpes simplex virus-thymidine kinase (HSV-TK)/ganciclovir (GCV), has been extensively tested for the treatment of glioma. Our previous study showed that exogenous wild type p53 (wt-p53) enhanced the anti-tumor effect of HSV-TK/GCV therapy. However, the use of GCV is hindered by its low penetration to the brain and its toxity when used at higher dose. In the present study, we used another pro-drug, acyclovir (ACV), and examined the therapeutic efficacy of HSV-TK/ACV combining with wt-p53 in C6 glioma cells. We observed that wt-p53 combined with HSV-TK/ACV resulted in the super-additive anti-tumor effect in vitro. Exogenous wt-p53 significantly enhanced the sensitivity of TK positive C6 cells to ACV in vitro. Our in vivo experiment demonstrated that the effect of wt-p53 and HSV-TK/ACV combination therapy was better than that of HSV-TK/ACV alone. The survival time of tumor-bearing rats treated with wt-p53 in combination with HSV-TK/ACV was also significantly prolonged than those treated with HSV-TK/ACV alone. These results suggest that wt-p53 can enhance the therapeutic efficacy of HSV-TK/ACV both in vitro and in vivo. These findings are considerably valuable with the respect of using less toxic ACV as prodrug. This novel strategy could provide benefit to HSV-TK/prodrug gene therapy.

Keywords Glioma · wt-p53 · HSV-TK/ACV · Suicide gene therapy · Combined gene therapy

Abbreviations

HSV-TK	herpes simplex virus-thymidine kinase
GCV	ganciclovir
ACV	acyclovir
wt-p53	wild type p53
BBB	blood-brain barrier
Moi	multiplicity of infections
MST	median survival time
A100	C6 cells transducted by AdCMV-TK at moi of 100
TPA1	C6 cells transducted by AdCMV-p53 and AdCMV-TK at moi of 10 and 100 respectively
TPA2	C6 cells transducted by AdCMV-p53 and AdCMV-TK both at moi of 100

Introduction

The herpes simplex virus-thymidine kinase (HSV-TK) is a nucleoside kinase that can induce cell death when growing cells are exposed to a number of modified nucleosides (pro-drugs),

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such as acyclovir (ACV) and ganciclovir (GCV) [1]. In cells transfected with HSV-TK gene, HSV-TK specifically phosphorylates ACV and GCV to their monophosphates. Thereafter, the monophosphates are further phosphorylated to triphosphates by cellular kinases [2, 3]. The triphosphates are incorporated into the DNA of dividing cells during replication resulting in cell death mainly by apoptosis. This is of particular significance in the brain where normal neurons surrounding the tumor are non-proliferative and therefore not susceptible to toxic metabolites. The therapeutic effect is further strengthened by a bystander effect [4].

GCV has been shown to penetrate the blood-brain barrier (BBB) relatively poorly and is toxic with substantial immunosuppressive, myelosuppressive, and renal-suppressive side effects, resulting in the limited use of GCV clinically [5]. ACV penetrates the BBB more efficiently and is less toxic than GCV [6]. However, it has been reported that GCV is 10 times more potent than ACV as an inhibitor of the growth of HSV-TK gene-transduced cancer cells [7]. So, if the antitumor effect of HSV-TK/ACV could be augmented by some methods to that of HSV-TK/GCV, such an approach would be a better treatment in place of HSV-TK/GCV with fewer side effects.

Because an unrepaired DNA damage caused by triphosphate ACV might induce p53-dependent apoptosis, we speculate that wt-p53 may be able to enhance the antitumor effect of HSV-TK/ACV. Furthermore, our previous studies have demonstrated that exogenous wt-p53 is able to enhance the sensitivity of C6 glioma cells to HSV-TK/GCV therapy and increase the apoptosis of C6 glioma cells after treatment with HSV-TK/GCV in vitro and in vivo [8]. Therefore, in the present study, the therapeutic effect using the recombinant adenovirus-mediated HSV-TK/ACV combined with p53 gene were studied in glioma both in vitro and in vivo.

Materials and Methods

Recombinant Adenoviral Vectors Construction The Ad.CMVβgal, Ad.CMVtk and Ad.CMVp53 containing LacZ gene encoding β-galactosidase(βgal), the HSV-TK gene, wt-p53 gene respectively was construction as reported previously [8]. Recombinant viruses were identified by Southern blot analysis. It was confirmed that AdCMV-TK and AdCMV-p53 only contained HSV-TK gene and p53 gene, respectively. Viral titer was determined by plaque-assay on 293 cells and expressed as plaque formation units (p.f.u.)/ml. The viral titer of AdCMV-TK and AdCMV-p53 was 2.6×10^9 pfu.ml^{-1} and 1.4×10^9 pfu.ml^{-1}, respectively.

Cell Culture Rat C6 glioma cells (C6) and human kidney 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin.

In Vitro Studies C6 cells were seeded at a density of 4×10^3 cells/well in 96-well plates. Cells in triplicate wells were infected with AdCMV-p53 at multiplicity of infections (moi) of 0, 10, 100 as control group, and triplicate wells were infected with AdCMV-p53 at moi of 0 (A100), 10 (TPA1), and 100 (TPA2) first, then with AdCMV-TK at moi of 100 as treatment groups. Cells were incubated in the presence of various concentrations of acyclovir (10^{-3}~10^3 μg/ml). Fresh medium containing ACV was added every other day. The percentage of surviving cells was measured by using 3-(4,5-dime-thylthiazol-2-yl)-2,5 -diphenyltetrazolium bromide (MTT) assay, and the absorbence was measured at 570 nm every 24 h, up to 144 h.

In Vivo Studies Rat C6 glioma model was set up as described earlier [8], Briefly, 5×10^5 C6 cells were injected into the right caudate nucleus of male Sprague Dawley (SD) rats (250–300 g). The SD rats were divided into five groups (ten rats per group). *Blank control group (C)*: 5×10^5 C6 cells were injected; *Empty vector control group (B)*: On day 5 after the implantation of C6 cells, AdCMV-βgal in 50 μl TBS was injected into the tumor. The injection was performed at five points along the needle tract (6 mm, 5.5 mm, 5 mm, 4.5 mm, and 4 mm deep from the dura). Two days later, ACV was intraperitoneally administered at 100 mg/kg/day for 14 days; *p53 group*: On day 5 after the implantation of C6 cells, approximate 1×10^8 p.f.u. of AdCMV-p53 in 50 μl buffer was injected into the tumor; *HSV-TK + ACV treatment group (TA)*: The same procedures were used as B group except replacement of AdCMVβgal with AdCMV-TK. *HSV-TK + p53 + ACV treatment group (TPA)*: On day 5 after the implantation of C6 cells, AdCMV-p53 in 40 μl TBS was injected into the tumors, AdCMV-TK in 40 μl TBS was injected into the tumors the next day. On day 7, ACV was intraperitoneally administered at 100 mg/kg/day for 14 days. The general behavior and the survival of rats in each group, MRI, the HSV-TK and p53 gene expression and cell apoptosis were examined. The tumor volume was estimated by MRI using the formula as described earlier [9].

In Situ Hybridizaiton C6 cells were plated on the coverglass, and then infected with AdCMV-TK at moi of 100 for 24 h. Hybridization was performed in the solution containing digoxigenin-labeled DNA probe (TK cDNA 1.8 kb) at 42°C for 24 h. After hybridization, the slides were blocked and incubated with mouse anti-digoxigenin antibody (Santa Cruz, USA) for 1 h. All slides were stained with NBT/BCIP.

Western Blotting Analysis C6 cells were infected with AdCMV-p53 at moi of 0, 10, and 100 for 24 h, 48 h, and

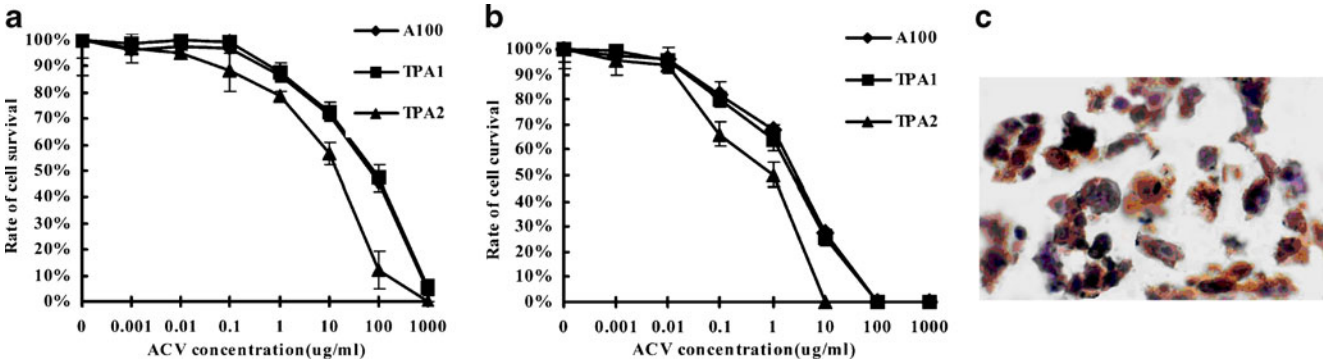

Fig. 1 The survival rate of A100, TPA1 and TPA2 cells. **a** The survival curves at 72 h. The survival rate of TPA2 cells was reduced significantly as compared to TPA1 and A100 cells when exposed to ACV at 10^0 μg/ml, 10^1 μg/ml, or 10^2 μg/ml ($p<0.05$) at 72 h. **b** The survival curves at 144 h. The survival rate of TPA2 cells was reduced significantly as compared to TPA1 and A100 cells when exposed to ACV at 10^0 μg/ml, 10^1 μg/ml, or 10^2 μg/ml ($p<0.05$). **c** TUNEL staining in vitro showed that almost 100% apoptotic C6 cells were found after exposed to 10 ug/ml ACV at 144 h in TPA2 group

72 h. Bicinchoninic acid protein assay kit (Pierce, USA) was used to determine protein concentration. Equal amounts of protein (50 μg) were subjected to SDS-PAGE on 10% acrylamide gel, and transferred to PVDF membrane. Membranes were incubated with a p53 antibody (1:500 dilution) followed by a sheep anti-mouse IgG conjugated with horseradish peroxidase. The p53 protein was detected using a SuperSignal protein detection kit (Pierce, USA). The PVDF membrane was rehybridized with the primary antibody for β-actin (1:500 dilution). The p53 expression was determined after normalization with the expression of β-actin.

TUNEL (Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling) Staining Cells or frozen tissue slides were fixed with 4% paraformaldehyde-PBS, and incubated with TdT and biotinylated dUTP (Santa Cruz, USA) for 45 min. They were then incubated with 50 μl converter-POD and stained with DAB, followed by counterstaining with hematoxylin.

Statistical analysis All data were analyzed with SPSS 10.0 statistics software. The result is presented as mean ± standard error of the mean. Average results were evaluated by one-way analysis of variance (ANOVA) and Bonferroni *t* test. Survival data of tumor-bearing rats were analyzed with Kaplan-Meier survival plot with log rank regression.

Results

Exogenous wt-p53 Sensitizes C6 Glioma Cells to HSV-TK/ACV In Vitro

Western blot analysis demonstrated that the expression of p53 protein was increased to the highest level at 72 h after transfection when moi=10 and 100. MTT assay showed the highest suppressive rate of C6 glioma cells transfected by AdCMV-p53 at moi=100 was approximate 40% at 48–72 h. In situ hybridization showed that 100% of C6 cells expressed HSV-TK gene after transduction with AdCMV-TK at moi=100.

Because C6 glioma cell growth could be inhibited by either wt-p53 or HSV-TK/ACV alone, we would like to examine if combination of wt-p53 and HSV-TK/ACV has an additive or a synergistic effect. In order to differentiate the two possibilities, C6 cells infected with AdCMV-p53 at the same time and moi was used as control. (The cells survival rate of TPA1 or TPA2= A value of TPA1 or TPA2/ A value of C6 cells infected by AdCMV-p53 at moi of 10, moi of 100 respectively at the same time).

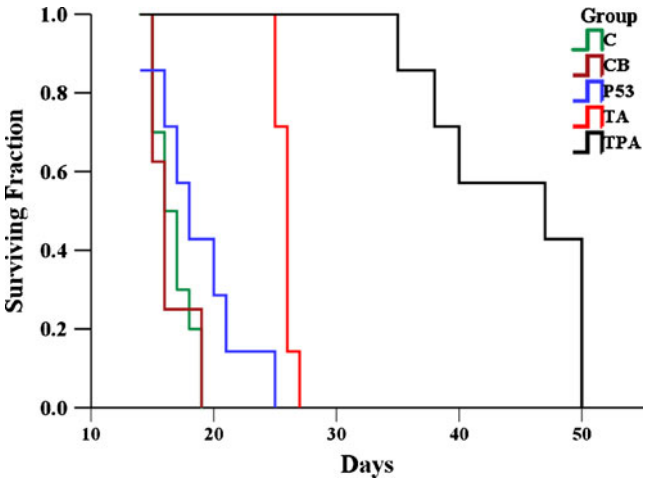

Fig. 2 Kaplan-Meier survival plots of rats bearing C6 glioma treated with wt-p53 combining with HSV-TK/ACV. Rats in TPA group and TA group had a significantly longer survival time as compared to those in two control and p53 groups ($p<0.05$). In addition, rats in TPA group survived significantly longer than those in TA group ($p<0.05$)

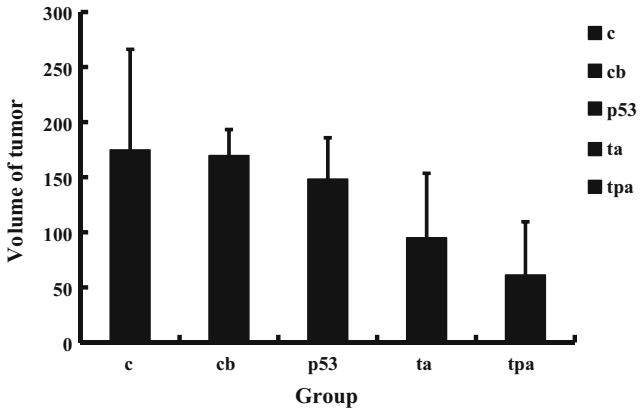

Fig. 3 The brain tumor volume (mm^3) 2 weeks after implantation and treatment. There was no significant difference of tumor volume among p53, TA and two control groups ($p>0.05$). However, the tumor volume in TPA group was significantly smaller than that of p53, TA and two control groups ($p<0.05$)

We demonstrated that wt-p53 combined with HSV-TK/ACV therapy resulted in a super-additive anti-tumor effect. MTT assay revealed that the concentration of ACV for ID_{100} of TPA2 (10 μg/ml) was ten times lower than that for the cells of TK-ACV group (moi=100) at 144 h. The significant anti-tumor effect was demonstrated at 72 h in TPA2 after adding ACV>1 μg/ml ($p<0.05$). The TUNEL staining showed that the apoptosis of almost 100% C6 cells occurred after treatment with 10 μg/ml of ACV at 144 h in TPA2 group (Fig. 1).

Exogenous wt-p53 Sensitizes C6 Glioma Cells to HSV-TK/ACV In Vivo

We demonstrated that the AdCMV-p53 and AdCMV-TK were transfected into C6 cells successfully by Western blotting and In Situ hybridizaiton in vivo. The median survival time (MST) of two control groups was 16 days and the MST of p53 group was 18 days. There was no significant difference among p53 and two control groups ($p>0.05$). Rats in TPA and TA groups had a significantly

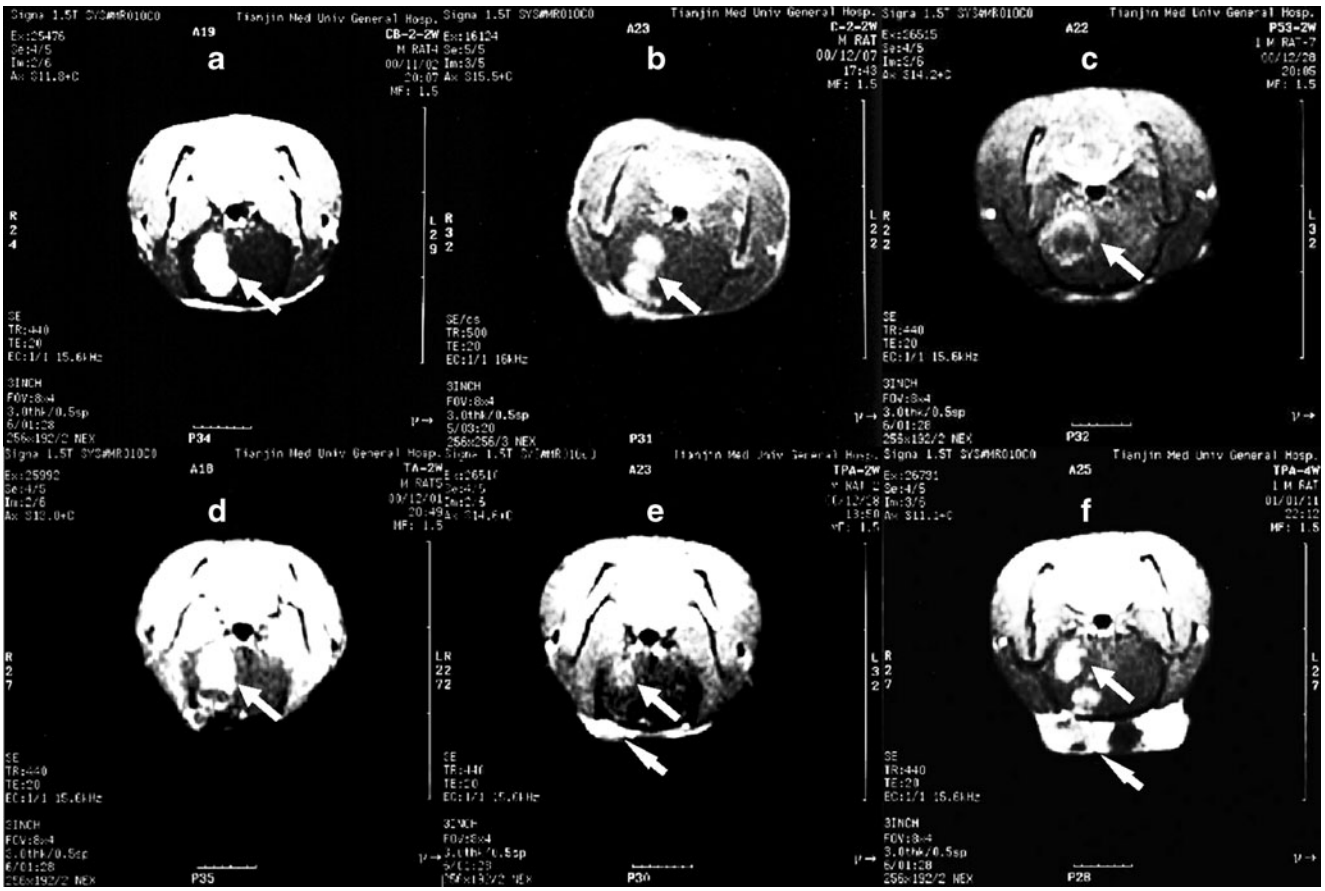

Fig. 4 Enhanced MRI coronal scanning of tumor. **a** Blank control group at 2 weeks. **b** Empty vector control group at 2 weeks. **c** p53 group at 2 weeks. (D) HSV-TK + ACV (100 mg/kg/day) treatment group at 2 weeks. **e** HSV-TK + p53 + ACV(100 mg/kg/day)treatment group (TPA) at 2 weeks. **f** TPA group at 4 weeks. **e**, **f** showed that the untreated subcutaneous C6 glioma (➘) grew more faster than treated intracranial C6 glioma(➘)

longer survival time compared to rats in two control and p53 groups ($p<0.05$). The MST of TA group ($n=9$) was 26 days and the MST of TPA group ($n=9$) was 44 days, indicating that, rats in TPA group survived significantly longer than those in TA group ($p<0.05$) (Fig 2).

The tumor volume was calculated by enhanced MRI at 2 weeks. There were no significant differences in tumor volume among two control, p53 and TA groups ($p>0.05$). However, the tumor volume of TPA group was significantly smaller than that of other groups ($p<0.05$) (Figs. 3 and 4). The number of apoptotic cells in TPA group was much more than that of other four groups.

Discussion

HSV-TK/GCV gene therapy has been demonstrated to be highly effective in animal models of malignant gliomas [10, 11]. However, clinical trials of HSV-TK/GCV therapy in patients with glioma have generally been disappointing [12]. Some factors that contribute to the failure of this approach include the low transfection efficiency of HSV-TK gene, the low penetration of GCV to the brain, and the limited use of GCV dose due to its toxity.

ACV is more lipophilic than GCV and it appears to cross the BBB more efficiently than GCV [6], making it a better choice for treating CNS malignancies using HSV-TK approach. In addition, post-surgery treatment of gliomas with steroids, which is standard treatment in patients, restricts vascular permeability [13] and further impedes the penetration of GCV to the brain. Furthermore, one recent study demonstrated that the bradykinin BBB permeabilizer RMP-7 enhanced GCV toxicity several folds in rats with gliomas [14], suggesting that GCV cannot fully penetrate the BBB. ACV is a less toxic drug and its margin of safety is greater than GCV. Therefore, if GCV could be replaced by ACV in HSV-TK gene therapy, it would be possible to achieve the same therapeutic efficacy without increasing the toxicity.

Some studies tried to improve the therapeutic efficacy of HSV-TK/ACV by IL2, scopadulciol (a diterpenoid), and ponicidin (a naturally occurring diterpenoid isolated from Rabdosia ternifolia) [15–17]. In the present study, we constructed AdCMV-TK and AdCMV-p53 recombinant adenoviral vectors and examined the efficacy of HSV-TK/ACV combining with wt-p53 in C6 cells in vitro. We observed that wt-p53 combined with HSV-TK/ACV resulted in the super-additive anti-tumor effect in vitro. Exogenous wt-p53 significantly enhanced the sensitivity of TK positive C6 cells to ACV (ten times) in vitro. Our in vivo experiment also demonstrated that the effect of wt-p53 and HSV-TK/ACV combination therapy was better than that of HSV-TK/ACV alone. Incorporation of GCVTP into the DNA of dividing cells results in an irreversible DNA damages, meanwhile, the wt-p53 can induce the cells which contain un-repaired DNA damages to apoptosis. It was reported that TK/GCV-induced apoptosis requires p53 [18], therefore, this might be the mechanism of the enhancement of anti-tumor effect of HSV-TK/ACV by wt-p53.

In conclusion, exogenous wt-p53 is able to enhance the sensitivity of C6 glioma cells to HSV-TK/ACV therapy both in vitro and in vivo. Although wt-p53 and HSV-TK/ACV combination therapy cannot eradicate the C6 glioma, we think that there is much room for further improvement of anti-tumor effects by the combination of HSV-TK/ACV system with wt-p53 gene transduction, such as the increase of ACV dose due to the low toxity of ACV.

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