Inhibition of prostate cancer by suicide gene targeting the FCY1 and HSV-TK genes

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Abstract. Prostate cancer is one of the most prevalent tumors. The switch of androgen signal dependence makes therapy more complex. Although reports on introduction of a single suicide gene exist, double suicide gene therapy has not been reported yet. In the current study, two suicide genes were constructed in the pIRES plasmid driven by PSMA promoter. 5-FC and GCV combination *in vitro* led to a higher growth inhibition on prostate cancer compared to a single prodrug. Retarded xenograft tumor growth was observed in castrated nude mice after double suicide gene activation. Furthermore, decreased metastasis was observed with double suicide gene treatment. These findings suggest that specific double suicide gene strategy could be a potential option for the therapy of prostate cancer.

Introduction

The incidence of prostate cancer has been increasing in China (1-3). It is a slow growing cancer, explaining in part its low sensitivity to chemotherapy that mostly targeted proliferating cells. Surgery is the most efficient therapy, early detection is not easy and therefore a number of patients lose the opportunity of surgery (4). New strategies of therapy are thus needed (5). Interruption of androgen signaling is an efficient approach (6). However, most patients will get lower efficacy (7). The mechanisms regulating the switch are mainly unknown (8,9).

Gene directed enzyme pro-drug the therapy (GDEPT) was developed for treatment of cancer. This therapy is based on administration of a non-toxic pro-drug, which can be processed into a cytoxic inhibitor of tumor cells, inducing apoptosis and/or necrosis, resulting from suicide gene expression triggered by a tumor cell-specific promoter (10). Therefore, it was developed for many different types of cancer, including glioma, prostate cancer and colon cancer (11-13).

Thymidine kinase of herpes simplex virus (HSV-TK) and the cytosine deaminase (CD) genes, which are both non-toxic to mammalian cells, were used as single suicide genes in prostate cancer. The TK gene can activate pro-drugs such as ganciclovir (GCV) to block the synthesis of RNA in tumor cells (14,15). The CD gene can turn 5-fluorocytosine (5-FC) into 5-fluoro-2,4(1H,3H) pyrimidinedione (5-FU). The latter is an inhibitor of RNA and thymine in cancer cells (16). The CD gene is less effective than its counterpart from yeast (17,18). Although the FCY1 gene from yeast was once developed for prostate cancer (19), there is no combination employment of CD and TK suicide gene in this type of cancer up to now (20).

Prostate specific antigen (PSA) and prostate specific membrane antigen (PSMA) are major promoter candidates for GDEPT (19). It is reported that PSA activity is regulated by androgen (21). Although PSA was once used in GDEPT and could inhibit prostate cancer cells, it cannot generate sufficient transcription when the androgen signals are absent (22). This would mean that when loss of androgen signal dependence occurs during advanced stages, GDEPT becomes less effective. PSMA is less dependent on androgen signal (23). Therefore, it has been the choice in many studies (24).

In this study, the CMV promoter in eukaryotic pIRES vector was successfully replaced by PSMA to simultaneously express the suicide genes FCY1 and HSV-TK. Both suicide genes were efficiently expressed in the prostate cancer cells LNCaP and PC-3. Combination of 5-FC and GCV significantly inhibited tumor cell growth *in vitro*. In xenograft tumor models, prostate carcinoma growth was inhibited in castrated nude mice. In addition, metastasis was inhibited after double suicide gene strategy could be a potential option for the therapy of prostate cancer.

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Figure 1. Double suicide gene expression driven by the PSMA promoter in prostate cancer cells. (A) Suicide gene transcripts in transfected LNCaP cells; (B) suicide gene products in transfected LNCaP cells displayed by Western blot; (C) suicide transcripts in transfected PC-3 cells; (D) suicide gene products in transfected PC-3 cells; (D) suicide ge

Materials and methods

Promoter replacement and plasmid construction. The PSMA promoter was amplified by PCR from human hyperplastic prostate tissue. Primers for amplification of this sequence were: 5'-GCAGATCTAGTCACTATTATTAGCCATCTC-3' and 5'-TAGAGCTCTCCAGTTTCTCCACCACA-3'. After an initial denaturation step at 94°C for 5 min, all reactions were subjected to 35 cycles of annealing at 55°C for 30 sec, extension at 72°C for 90 sec and denaturation at 94°C for 30 sec.

The pIRES plasmids were digested with *Bgl*II and *Sac*I to remove the CMV fragment. Then purified PSMA promoter fragments from PCR products were cleaved with the same enzymes and subcloned into pIRES plasmids, generating the pPSMA-IRES plasmids. The authenticity of the fused genes was confirmed by DNA sequencing.

The pPSMA-IRES and pDC312D-FCY1 plasmids were digested by *Eco*RI and *Nhe*I. Then the recovered FCY1 fragment was inserted into pPSMA-IRES plasmid and named pPSMA-IRES-FCY1. Recovered TK fragment from the pLTKcSN plasmids were cloned into pPSMA-IRES-FCY1 plasmid after *Xho*I and *Sal*I treatment. The new recombinants were named pPSMA-IRES-FCY1-TK.

Cell transfection. LNCaP, PC-3 and HeLa cells (CTCC, Beijing, China) were cultured in RPMI-1640 medium. The cells at ~80% confluence were transfected with 1 μ g pPSMA-IRES-FCY1-TK or control plasmid using Lipofectamine 2000 (Invitrogen, CA, USA). The transfected LNCaP cells were cultured in the presence of 350 μ g/ml neomycin (G418, Gibco, MD, USA) to obtain stable expression (25). Both transiently transfected (48 h) and stably transfected cells were subjected to reverse transcription PCR and Western blotting.

RT-PCR assay. Total RNA was extracted from transfected cells with 1 ml TRIzol reagent (Gibco) and reverse transcribed

into cDNA using random primers (Promega, WI, USA). The cDNA was amplified by PCR using the following specific oligonucleotide primers: 5'-GAATGGCAAGCAAGTGG GAT-3' and 5'-CACAACAACCTCGTGACCT-3' for the FCY1 mRNA; 5'-CCAGGTCGCAGATCGTCGGTAT-3' and 5'-ACATCGACCGCCTGGCCAAA-3' for the TK mRNA.

Western blot assay. The transfected cells were lysed in RIPA buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM MgCl₂, 0.5% NP-40, 1 mg/ml bovine serum albumin (BSA), and 0.1 mM PMSF]. The protein concentration was determined using the BCA protein assay reagents (Pierce, IL, USA). The samples were analyzed by SDS-PAGE (12% gel), blotted to PVDF membranes, and probed by the following antibodies: rabbit polyclonal IgG antibodies to FCY1 (Santa Cruz, CA, USA), HSV-TK (Santa Cruz), the monoclonal anti-β-actin antibody (Sigma, MO, USA). The secondary antibody was HRP-conjugated goat anti-mouse or anti-rabbit IgG antibodies. The membrane was developed using chemiluminescent reagents (Pierce, CA, USA).

MTT assay. The transfected cells (1x10⁵/well) were plated into 96-well plates. Different dosage of GCV and 5-FC was administered for 72 h. Then the MTT (5 mg/ml, Sigma, MO, USA) was employed for another 4 h. In the end, 150 μ l DMSO (Sigma, MO, USA) was added into each well of the plate, and the plate was incubated at 37°C for 10 min, followed by the measurement of the optical value using the microplate reader (25). Growth inhibition ratio (GIR) was calculated using the formula: GIR%=(1-OD_{treated}/OD_{control})%.

Coefficient of drug interactionon (CDI) count. CDI was obtained with the following formula; CDI=AB/A+B. AB represents cell survival ratio of two different agent combinations and A or B means cell survival ratio of a single agent administration. Synergistic effects were recorded to exist, when the value of CDI was no more than one.



Figure 2. Growth inhibition of transfected prostate cancer cells after prodrugs administration. (A) Growth inhibition of suicide gene in transfected LNCaP cells; (B) growth inhibition of suicide gene in transfected PC-3 cells; (C) CDI of LNCaP cells in 5-FC and GCV combination; (D) CDI of PC-3 cells in 5-FC and GCV combination. Bars represent means \pm SD. R, pPSMA-IRES-FCY1-TK vector; P, pIRES vector; F, 5-FC; G, GCV. *P<0.05, n=6.



Figure 3. Cell cycle analysis in transfected prostate cancer cell lines after pro-drug administration. (A) LNCaP cell ratio in different cycle phases; (B) PC-3 cell ratio in different cycle phase. R, vector pPSMA-IRES-FCY1-TK; P, pIRES vector; F, 5-FC; G, GCV. Bars represent means ± SD. *P<0.05, n=4.

Cell cycle assay. The transfected cells were collected after GCV and 5-FC treatment. After fixation with 95% alcohol and staining by propidium iodide, samples were analyzed by flow cytometry (BD, CA, USA).

Cancer cell inoculation in nude mice. Male nude mice were castrated by surgery. All the animals were maintained in SPF environment. Three weeks later after castration, stable LNCaP cells (5x10⁶) were subcutaneously injected. Two weeks later, pro-drugs 5-FC and GCV were administered. Then tumor growth was monitored every 2 days by measuring length (L) and width (W) with a sliding caliper. The tumor size was calculated as LxS²x0.51. All animal experimental protocols were approved by the Research Animal Administration of the Fourth Military Medical University.

PSA detection. After the stably transfected cancer cells were injected, blood was drawn twice from the mice through the caudal vein to get serum for PSA detection. The PSA level was assessed by ELISA.

Histology analysis. Tissues from mouse tumors were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned at 10 μ m, and stained with H&E, and microscopic images were captured (Olympus, Japan).

Statistical analysis. The significance of the difference between different groups was statistically analyzed by SPSS 10.0 using



Figuer 4. Xenograft prostate cancer growth in nude mice. (A) tumor growth curve after different treatments; (B) gross tumor profile in nude mice after different treatments; (C) mouse survival after different treatments; (D) PSA levels in tumor bearing mice after different treatments. F, 5-FC; G, GCV; NT, pIRES vector; T, pPSMA-IRES-FCY1-TK vector. Bars represent means ± SD. *P<0.05, n=10.

5

10

Davs

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Student's t-test and χ^2 analysis. At P<0.05, the differences were regarded as statistically significant.

Results

D

PSA concentration (µg/I)

5

0

Double suicide gene expression in prostate cancer cell lines. For more specific delivery, the CMV promoter in the pIRES plasmids was replaced by the PSMA promoter (19). The eukaryotic vector, pPSMA-IRES-FCY1-TK, was constructed by inserting the PSMA promoter, the FCY1 fragment and the HSV-TK gene. The vector was introduced into LNCaP, PC-3 and HeLa cell lines by liposome. As shown in Fig. 1A, LNCaP cells transfected with the pPSMA-IRES-FCY1-TK vector specifically transcripted FCY-1 (381 bp) and HSV-1K (457 bp) fragments. The cells transfected with control vector and HeLa cells transfected with pPSMA-IRES-FCY1-TK vector did not show the transcripts. Western blot analysis showed that 14 and 17 kDa bands only appeared in the lane derived from recombinant vector transfected LNCaP cells (Fig. 1B). When the transfection was applied to the PC-3 cells, which does not depend on androgen, both RT-PCR and Western blot analysis demonstrated that expression was present (Fig. 1C and D). These data indicate that the PSMA promoter can specifically trigger the transcription and expression of FCY1 and TK suicide gene in prostate cancer cells *in vitro*.

LNCaP and PC-3 cell growth was inhibited in vitro after double suicide gene activation. Driven by the PSMA promoter, the FCY1 and HSV-TK suicide genes can be expressed in the androgen-dependent LNCaP cells (19,26). We further investigated whether the recombinant plasmids can inhibit the growth of prostate cancer cells mediated by pro-drugs *in vitro*. As shown in Fig. 2A, growth inhibition induced by 5-FC in LNCaP cells was 30%, while GCV made 40% cell growth inhibition at most, similar to that of untransfected LNCaP cells (data not shown). However, when both compounds were administrated, growth inhibition reached ~70%.

Since PSMA promoter activity is less dependent on androgen, we explored the inhibition ratio of the less-dependent androgen cells, PC-3 (26). Growth inhibition in PC-3 cells by 5-FC was ~50%, while GCV inhibited only 40%, similar to that of untransfected PC-3 cells (data not shown). When both compounds were administered, inhibition of growth increased to ~60% (Fig. 2B).



Figure 5. Liver metastasis in nude mice after different treatments. F, 5-FC; G, GCV; NT, pIRES vector; T, pPSMA-IRES-FCY1-TK vector. Bars represent means ± SD. *P<0.05, n=10. Magnification, x400.

To further confirm the combined administration result, the coefficient of drug interactionon (CDI) was evaluated. As shown in Fig. 2C and D, the CDI of 5-FC and GCV was ≤ 0.7 when they were employed to LNCaP and PC-3 cells respectively, which means that these two agents could lead to synergistic effects in prostate cancer cells. Altogether, these data indicate that the combination of 5-FC and GCV induced a more pronounced inhibition than single pro-drug administration *in vitro*.

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Cell cycle disorder induced by double suicide gene activation in LNCaP and PC-3 cells. The effect of suicide gene activation on the cell cycle of prostate cancer cells was tested. LNCaP cells transfected by the recombinant plasmids and treated with 5-FC and GCV combination, were more efficiently blocked at S phase as compared to the control cells (51 vs. 45%, Fig. 3A). A similar result was found in PC-3 cells (52 vs. 43%, Fig. 3B). These data demonstrate that LNCaP and PC-3 cell growth inhibition after double suicide gene activation may be linked with cell cycle disorder.

Suppressed xenograft tumor growth in castrated nude mice. More than 80 passages of these cells can decrease androgen dependence *in vivo* (26). LNCaP cells that underwent >80 passages were employed to observe tumor formation efficiency in the castrated nude mice. LNCaP cells gave rise to more efficient tumor formation in castrated mice when compared to control mice (95 vs. 75%, data not shown). To monitor tumor growth *in vivo*, the stable LNCaP cell line was used. RT-PCR and Western blotting confirmed the double suicide gene expression in stably transfected LNCaP cell line (data not shown). The modified LNCaP cells could efficiently develop into solid subcutaneous tumors, similarly to non-transfected cells. Decreased tumor growth was observed when the pro-drugs were combined (Fig. 4A and B). The LNCaP cells without suicide gene activation grew quicker in mice (Fig. 4A and B). Further supportive evidence for this was the survival rate observation, as shown in Fig. 4C. Enhanced survival was observed in mice that received 5-FC and GCV combined treatment, as compared to the other groups.

Since PSA level is a reflection of prostate cancer progression, it was measured in the inoculated mice. Consistent with the growth profile, a decreased PSA level was observed after treatment with both 5-FC and GCV (Fig. 4D). These data indicate that the combination of 5-FC and GCV induced a more pronounced inhibition than single pro-drug administration *in vivo*.

Inhibition of liver metastasis by double suicide gene expression. Metastasis is another feature of prostate cancer (27). Histology analysis was performed to detect metastasis. Liver, kidney, intestine and omentum were resected for sectioning and H&E staining. Liver metastasis was absent in the group of mice that received 5-FC and GCV combination treatment, while this was present in the controls (Fig. 5). Single pro-drug treatment was not able to prevent liver metastasis (Fig. 5). Other internal organs were not invaded (data not shown). These data indicate that double pro-drug treatment, but not single treatment, was able to inhibit prostate cancer metastasis to the liver.

Discussion

For efficient suicide gene therapy, a higher specific promoter is necessary to prevent normal tissue impairment. In the current study this requirement was met by the use of the PSMA promoter. PSA promoter was inferior to the PSMA promoter because it heavily depends on the androgen signal (19,21). It is well known that prostate cancer undergoes a switch in androgen signal dependence (7). Therefore, the PMSA promoter is a better choice for suicide gene therapy.

The transmembrane protein PSMA is specifically expressed in prostate tissue with a lower dependence on the androgen signal (19,21). Furthermore, blockade of androgen can decrease PSMA levels in plasma (28,29). Consistent with earlier data (19), PSMA specifically drove suicide gene activation and inhibited LNCaP cell growth. Although the PC-3 cell line was considered to lack PSMA, the FCY1 and HSV-TK genes were expressed in these cells and were able to inhibit its growth. The mechanism is not clear, but it was reported to be linked with methylation and homozygous deletion (30,31).

Although the use of single suicide genes, assisted by PSMA has been developed for prostate cancer, the inhibition was not so good (18,19,32). The double suicide gene approach employed in this study was found to be more effective. The double suicide gene approach is regarded to be a promising approach (10-12). Also, we found that prostate cancer growth inhibition mediated by the double suicide genes results from the arrest of the S phase.

LNCaP cells were chosen in this study due to its dependence on androgen at earlier stages (9,19,26). More efficient tumor formation was observed in castrated nude mice when high-passage LNCaP cells were injected. The castration also modeled the situation in prostate cancer patients. As expected, the 5-FC and GCV combination slowed tumor growth *in vivo*, consistent with other studies of suicide gene therapies (32).

The use of two pro-drugs prevented internal organ metastasis, which is important for prostate cancer therapy. Up to now no method can stop metastasis (34). As a result, specific double suicide gene therapy may be expected to make a difference.

In summary, prostate cancer undergoes an androgen signal dependence switch and is therefore not easy to treat. Suicide gene therapy is a potential candidate because artificial modification can increase specificity and efficacy. Our findings revealed that the PSMA promoter is a good tool and double suicide gene strategies could be a potential option for future non-androgen signal-dependent prostate cancer therapy.

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