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• GASTRIC CANCER •

Construction and identification of recombinant vectors carrying herpes simplex virus thymidine kinase and cytokine genes expressed in gastric carcinoma cell line SGC7901

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Abstract

AIM: To construct and identify the recombinant vectors carrying herpes simplex virus thymidine kinase (HSV-TK) and tumor necrosis factor alpha (TNF-α) or interleukin-2 (IL-2) genes expressed in gastric carcinoma cell line SGC7901.

METHODS: The fragments of HSV-TK, internal ribosome entry sites (IRES) and TNF-α or IL-2 genes were inserted in a TK-IRES-TNF-α or TK-IRES-IL-2 order into pEGFP-N_3 and pLXSN to generate the therapeutic vectors pEGFP-TT, pEGFP-TI, pL(TT)SN and pL(TI)SN respectively, which were structurally confirmed by the digestion analysis of restriction endonuclease. The former two plasmids were used for the transient expression of recombinant proteins in the target cells while pL(TT)SN and pL(TI)SN were transfected into SGC7901 cells by lipofectamine for the stable expression of objective genes through G418 selection. The protein products expressed transiently and stably in SGC7901 cells by the constructed vectors were confirmed by fluorescent microscopy and Western blot respectively.

RESULTS: The inserted fragments in all constructed plasmids were structurally confirmed to be consistent with that of the published data. In the transient expression, both pEGFP-TT and pEGFP-TI were shown expressed in nearly 50% of the transfected SGC7901 cells. Similarly, the G418 selected vectors PL(TT)SN and PL(TI)SN were confirmed to be successful in the stable expression of the objective proteins in the target cells.

CONCLUSION: The constructed recombinant vectors in the present study that can express the suicide gene TK in combination with cytokines genes may serve as the potential tools to perform more effective investigations in future for the gene therapy of gastric carcinoma.

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INTRODUCTION

Gastric cancer is one of the most common malignancies both in China and abroad[1-7]. Despite the improvements in surgical techniques, radiation and chemotherapeutic regimens, the disease remains a great challenge. Most patients still die finally of their disease, even after apparent "curative resection". In recent years, the promising conception of gene therapy has been advocated in a hope to deal with the malignant diseases more effectively. One of the landmark discoveries for this therapeutic strategy is the transfer of suicide genes, such as HSV-TK, into the tumor cells, which has been shown to exert antitumor efficacy on a variety of cancer cells[8-11]. The expressed HSV-TK/ganciclovir (GCV) system can not only inhibit the DNA synthesis of the target cells but also produce a bystander effect against tumors[12-16]. However, these effects have been found to be unstable in some of the transfected cells, which may result in a decreased efficiency in the treatment of malignancies. The use of tissue-specific vectors to deliver genes and combination of TK with cytokine genes may improve the efficacy of the antitumor effects[17-20]. In the present study, therefore, we tried to construct and identify the recombinant vectors containing HSV-TK, IRES in combination with cytokine genes, TNF-α or IL-2, in an attempt to establish more effective recombinants for the gene therapy of gastric malignancies.

MATERIALS AND METHODS

Materials

Reagents Restriction enzymes (*EcoR* I, *Hpa* I, *Xho* I and *BamH*I), T_4 DNA ligase and *Taq* DNA polymerase were purchased from Gibco Co., USA. Lipofectamine was supplied by Boeringer Mannheim Co., Mannheim, Germany. Monoclonal antibodies of mouse anti-IL-2 and anti-TNF-α proteins and horseradish peroxidate-conjugated antimouse immunoglobulin were purchased from Zhongshan Co., Shanghai, China.

PCR primers The primer sequences used in the study were designed according to that of the individual genes (Table 1) with the modification of adding the recognition site sequences for the corresponding restriction enzymes (*Eco*R I, *Hpa* I, *Xho* I and *Bam*H I), and synthesized by Shanghai GeneCore Bio Technologies Co.

Plasmids and cell lines Plasmid pBluescript-TK was provided by Shanghai Institute of Biochemistry. pIRZA1neo with 585 bp IRES sequence[21,22] was purchased from Invitrogen Co., USA. The plasmid PT_7-TNF-α, pEGFP-N_3 carrying green fluorescent protein (GFP) gene, pGEM-T-Easy vector and *E.coli* JM109 were provided by Orthopedic Oncology Institute of PLA,

Table 1 Primer sequences of amplified genes

Genes	Primer sequences	Fragment length
TK	Forward: 5'-GC *GAA TTC* ATG GCT TCG TAC CCC TGC CAT C-3'	1 128 bp
	Reverse: 5'-GC *GTT AAC* TTA AGC CTC CCC CAT CTC CCG G-3	
IL-2	Forward: 5'-GC *CTC GAG* ATG TAC AGG ATG CAA CTC CTG-3'	456 bp
	Reverse: 5'-GC *GGA TTC* TTA AGT CAG TGT TGA GAT GAT GC-3'	
TNF-α	Forward: 5'-GC *CTC GAG* ATG GTC AGA TCA TCT TCT CGA AC -3'	474 bp
	Reverse: 5'-GC *GGA TCC* TTA CAG GGC AAT GAT CCC AAA -G-3'	
IRES	Forward: 5'-GC *GTT AAC* AAT TCC GCC CCT CTC CCT CCC CC-3'	585 bp
	Reverse: 5'-GC *CTC GAG* AAT AGT AGC ACA AAA AGT TTC C-3'	

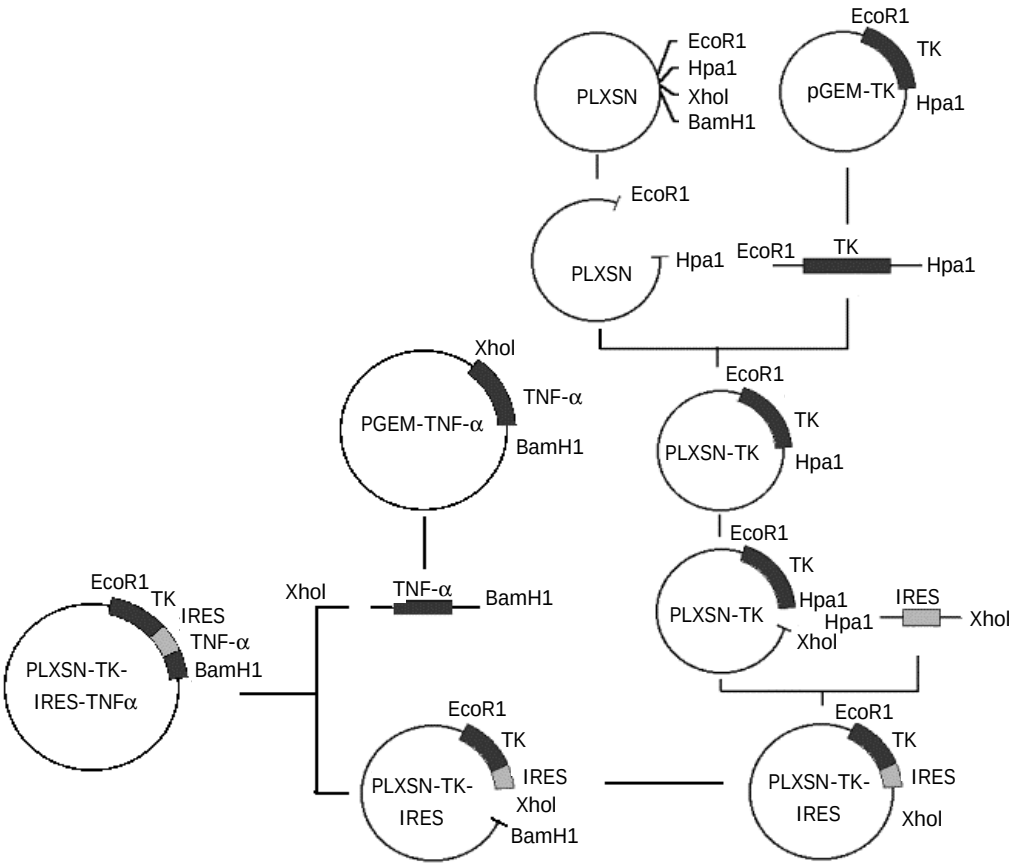

Figure 1 Diagram of the construction of the expression vector PL(TT)SN.

Xi' an, China. The retroviral expressing vector pLXSN was provided by Dr. Yu Bing from Fourth Military Medical University, Xi' an, China. IL-2 cDNA was made from human peripheral blood by RT-PCR.

The gastric carcinoma cell line SGC7901 was provided by Shanghai Institute of Biochemistry. Virus packaging cell PA317 and NIH3T3 cell lines were provided by Dr. Yu Bing. Cells were maintained in RPMI 1640 medium supplemented with 10% FBS (Hangzhou, Sijiqing Biotech Company), 2 mM L-glutamine, 100 units /ml penicillin and 100 μg/ml streptomycin. The PA317 was used as the packaging cell and the NIH 3T3 cells were used to assay the virus titre. The cell cultures were maintained at 37 ℃ in a humidified atmosphere with 5% CO_2.

Methods

Construction of the recombinant vectors The recombinant vectors were constructed with routine molecular cloning techniques[23-25]. The DNA fragments of HSV-TK, IRES, TNF-α and IL-2 were obtained by PCR amplification with specific primers from their corresponding templates. For the transient expression of the recombinant genes, the fragments of HSV-TK, IRES, TNF-α and the fragments of HSV-TK, IRES, IL-2 were cloned into pEGFP-N_3 to generate pEGFP-TT and pEGFP-TI respectively. Similarly, those fragments were separately inserted into pLXSN to generate plasmid PL (TT) SN and PL (TI) SN as shown in Figure 1 for the selection of the stable expression vectors. The structure of all these constructed vectors was confirmed by the digestion analysis of restriction endonuclease.

Transient expression The transient expression of recombinants was performed according to the literature[26]. The constructed vectors pEGFP-TT and pEGFP-TI, and the control plasmid were transfected into SGC7901 cells with a routine protocol by lipofectamine. Twenty hours after the transfection, cells were harvested and the expressed marker protein GFP fused with the objective genes were detected under a fluorescent microscope.

Stable expression The plasmids pL(TT)SN, pL(TI)SN and pL(TK)SN were transfected respectively into PA317 cells with lipofectamine (Gibco) according to the manufacturer's

instruction. After 48 h of transfection, G418 (Promega) was added to the culture media at a concentration of 500 mg· L^{-1} to select G418-resistant colonies. After 2-weeks' culture with the changing of the G418-containing media every 3 days, the supernatant of G418-resistant colony was collected and diluted to different concentrations to infect NIH3T3 cells, which was further undergone the G418 selection for 2w when the G418-resistant NIH3T3 colonies were counted for the determination of viral titre. The viral titer of pL(TT)SN, pL(TI)SN, pL(TK)SN and empty plasmid pLXSN were 5×10^8 CFU/L, 6×10^8 CFU/L, 1×10^9 CFU/L and 1×10^9 CFU/L respectively.

For the stable expression of recombinants, a total number of 5×10^5 SGC7901 cells were incubated in a 6-well plate for 24 h, then rinsed with serum-free RPMI 1640 medium twice and incubated with 100 μl supermatant of G418-resistant PA317 colony for 3 h. After 4-weeks' cultivation, the G418-resistant colonies designated as SGC/TK-TNF-α, SGC/TK-IL-2, SGC/TK and SGC/0 respectively were used to confirm the objective gene expression by Western blot analysis.

Western blotting analysis The SGC/TT and SGC/TI cells were incubated respectively in the six-well plates at a density of 2.5×10^5 cells/well for 24 h, followed by a further cultivation of 48 h with the culture medium replaced with 1 ml of serum-free RPMI 1640. Then the serum-free medium was totally collected, concentrated in a microconcentrator to 20 μl and subjected to electrophoresis on a 120 g· L^{-1} SDS/PAGE gel. Proteins were transferred to a nitrocellulose membrane and incubated overnight in 50 ml· L^{-1} fat free milk in PBS at 4 ℃. After washed in 10 ml· L^{-1} fat free milk, the membrane was incubated with monoclonal antibody of mouse anti-rhIL-2 or anti-TNF-α, followed by incubation with horseradish peroxidate-conjugated antimouse immunoglobulin. Proteins were detected by using the ECL kit according to the manufacturer's protocol (Amersham).

RESULTS

Identification of constructed vectors

The segment analysis by restriction endonuclease digestion confirmed that the inserted gene sequences in all of the constructed plasmids were structurally consistent with that of the published data. The inserted fragments in pEGFP-TI and pEGFP-TT were identified as shown in Figure 2.

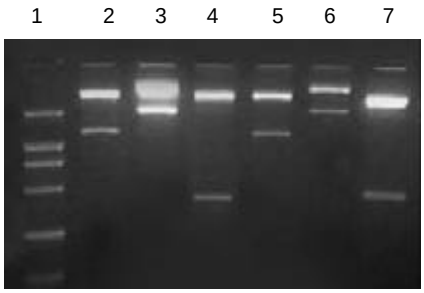

Figure 2 Segment analyses of pEGFP-TI and pEGFP-TT by restriction endonuclease digestion. Lane 1: DNA marker (from top to bottom: 1 543, 994, 697, 515 and 337 bp); Lane 2: pEGFP-TI/*Eco*R I and *Hpa* I (TK gene 1 128 bp); Lane 3: pEGFP-TI/*Eco*RI and *Xho*I (TK+IRES 1.71kb); Lane 4: pEGFP-TI/*Xho*I and *Bam*H I (IL-2 gene 456 bp); Lane 5: pEGFP-TT/*Eco*RI and *Hpa*I (TK gene 1 128 bp); Lane 6: pEGFP-TT/*Eco*RI and *Xho*I (TK+IRES 1.71 kb); Lane 7: pEGFP-TT/*Xho*I and *Bam*HI (TNF-α gene 474 bp).

Transient expressions of GFP-TT and GFP-TI protein

Twenty hours after the transfection, the GFP fluorescence was detected under fluorescent microscope in nearly 50% of the total cells transfected with pEGFP-TT or pEGFP-TI. The fluorescence was gradually increased with time and peaked at 72 h, which indicated that the TK-IRES-IL-2 and TK-IRES-TNF-α were transiently expressed in SGC7901 cells (Figure 3 A and B).

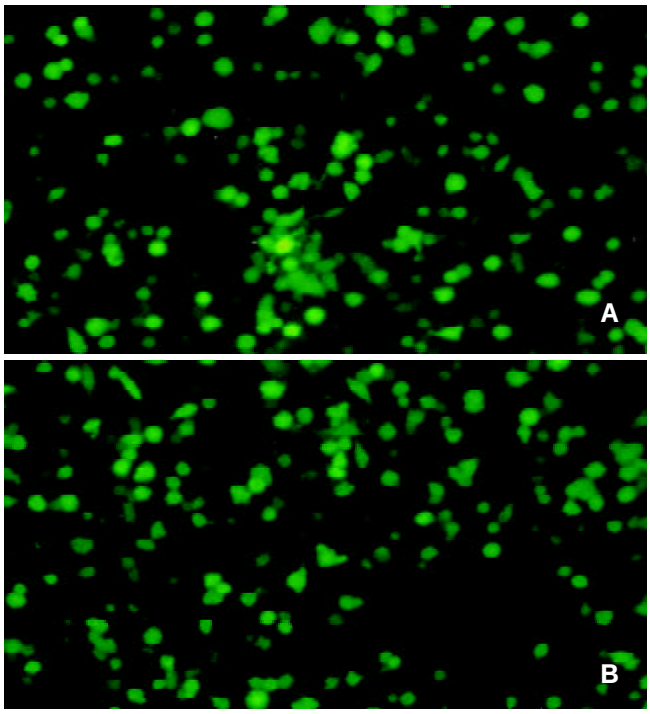

Figure 3 Transient expression of the recombinant genes in SGC7901 cells. A: Transient expression of pEGFP-TT in SGC7901 cells×100. B: Transient expression of pEGFP-TI in SGC7901 cells ×100.

Expressions of stable transfectants

The stable expression of recombinant proteins in SGC7901 cells was confirmed by Western blotting, in which two distinct bands of 15ku and 17ku were observed on the nitrocellulose membrane, corresponding to the fragment sizes of IL-2 and TNF-α respectively (Figure 4).

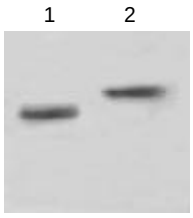

Figure 4 Stably expressed recombinant protein in transduced SGC7901 cells confirmed by Western blot. Lane 1: IL-2 (15ku) expressed in SGC/TI cells; Lane 2: TNF-α (17ku) expressed in SGC/TT cells.

DISCUSSION

Gene therapy has become a promising strategy for the treatment of gastric cancer[27-31], in which the transfer of suicide genes into tumor cells has emerged as an attractive modality for the selective elimination of cancer cells[14-16,30,31]. The suicide genes encode non-mammalian enzymes that can convert nontoxic prodrugs into cellular toxic metabolites. The most widely used suicide gene is the HSV-TK/ganciclovir (GCV) system that can convert prodrug GCV into GCV monophosphate. The latter is further phosphorated by cellular kinase to form GCV

triphosphate, a toxic substance that can inhibit cellular DNA synthesis and lead to cell death. Besides, the "bystander effect" induced by TK gene can also enhance the tumor-killing capacity of the HSV-TK/GCV system[16-20,32]. Because of the antitumor properties exerted by certain cytokines such as IL-2 and TNF-α, it is believed that the gene therapy combining cytokine with TK suicide gene would be more beneficial and effective for the treatment of cancers, which has been strongly supported by some of the recently published literatures[33]. However, there are fewer reports of this therapeutic strategy applied to the antitumor study of gastric malignancy.

Construction of a bicistronic retroviral vector with an internal ribosome entry site (IRES)[34,35] allows the simultaneous expression of two genes from a single transcript, which has been demonstrated to be efficient. However, the expressive levels of the objective protein are found lower in some circumstance. Five different genes, including human IL-2, IL-4, granulocyte macrophage stimulating factor, HSV-TK and hepatitis C virus core gene, have been tested using the modified vector for the gene transfer[36,37]. The new bicistronic vector, modified by abolishing the functional viral gag initiation codon and keeping it before the 5' end to the first initiation codon of the transduced gene, has made the protein expression greatly increased compared with the original vector. As the RNA levels and splicing patterns of these two vectors remain similar, the improvement was most likely at the translation level. Thus, the incorporation of the internal ribosome entry site sequence into a proper location of the retroviral vector for gene therapy represents a promising strategy to facilitate the simultaneous and efficient expression of several genes from the same promoter[21, 22].

In the present study, we employed the strategy to construct our expression vectors, in which the IRES gene was cloned in TK-IRES-TNF-α or TK-IRES-IL-2 order and the recombinants were constructed in combination with TK and cytokine genes. In the transient expression, the constructed vectors pEGFP-TT and pEGFP-TI were shown to be effectively expressed *in vitro* as demonstrated by the appearance of the fusing fluorescent protein GFP[26,38] in nearly 50% of the transfected SGC7901 cells. Similarly, in the experiment of stable expression, the G418 selected vectors PL(TT)SN and PL(TI)SN were also confirmed to be successful in both of the transfection into the target cells and the expression of the objective proteins. All of these results indicate that the expressive vectors constructed in the present study may serve as the potential tools to perform more effective investigations for the gene therapy of gastric carcinoma in future. Further studies are therefore needed to elucidate and characterize the antitumor effects of these constructed vectors on the transfected SGC7901 cells.

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