

Amplified interleukin-15 expression vectors for cancer immunogene therapy

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Abstract. Interleukin-15 (IL-15) is a pleiotropic cytokine that plays a key role in the regulation of both innate and adaptive immune responses. It promotes the survival, proliferation, activation and maintenance of natural killer and CD8⁺ T cells. It also stimulates the function of neutrophils, macrophages and dendritic cells, and could therefore be a potential cytokine for cancer immune therapy. The therapeutic effects of cytokines are related to their expression levels. In this study, we investigated an amplified IL-15 expression plasmid vector, pHi2-spIL15-CMV-tat, and a carcinoembryonic antigen (CEA)-positive tumor-specific amplified IL-15 expression plasmid vector, pHi2-spIL15-CEA-tat. In pHi2-spIL15-CMV-tat, IL-15 expression was driven by HIV2 LTR, which was transactivated by CMV promoter-controlled expression of HIV *tat*. In addition, the native IL-15 signal peptide was replaced by the IL-2 signal peptide to enhance its secretion. A significant amount of IL-15 expression was achieved when transfected into tumor cells *in vitro*. In order to target IL-15 expression in CEA-positive cells, the CEA promoter positively-controlled IL-15 expression plasmid vector pHi2-spIL15-CEA-tat was constructed by replacing the CMV with the CEA promoter. Efficient and targeted IL-15 expression was achieved in CEA-positive tumor cells by pHi2-spIL15-CEA-tat.

Introduction

Cancer is one of the leading causes of death in the world. In dissecting the molecular mechanism of carcinogenesis, gene therapy has been developed as a promising approach to cancer treatment. In fact, more than two-thirds of gene therapy clinical trials are designed to control cancer (1). Gene therapy with cytokine genes provides a strategy to enhance immune responses to cancer, simultaneously avoiding systemic adverse

effects. Many cytokines have been investigated in preclinical settings and phase I/II gene therapy clinical trials, including granulocyte-macrophage colony-stimulating factor, tumor necrosis factor- α , IFN- γ and the interleukins (2). Cytokine-based gene therapy was demonstrated to eliminate solid tumors in preclinical models, but only weak results were achieved in clinical trials with the currently available vectors. Low efficiency of transgene expression was associated with non-viral vectors, and safety concerns regarding the clinical implementation of viral vectors hampered the whole gene therapy field. However, advances in gene delivery and improvements in vector design are generating new momentum for gene therapy-based cancer treatment.

Interleukin-15 (IL-15) is a cytokine which acts on both innate and adaptive immune cells and promotes the survival, proliferation, activation and maintenance of natural killer and CD8⁺ T cells, as well as stimulating the function of neutrophils, macrophages and dendritic cells (3,4). In this study, we aimed to construct an efficient IL-15 expression vector and target IL-15 expression to carcinoembryonic antigen (CEA)-positive tumor cells. Amplified IL-15 expression plasmid vector pHi2-spIL15-CMV-tat and CEA-positive tumor-specific amplified IL-15 expression plasmid vector pHi2-spIL15-CEA-tat were constructed. In these vectors, the HIV2 promoter directed IL-15 expression, while its efficacy was enhanced by the CMV or CEA promoter driving transcription transactivator *tat* expression. Our data show that a significant amount of IL-15 was expressed, and that the CEA-specific positively-controlled IL-15 expression plasmid increased IL-15 efficiency in CEA-positive human colon carcinoma SW480 cells. This research lays a foundation for further study to develop IL-15-based immunogene therapies for cancer *in vivo*.

Materials and methods

Cell lines. Human colon carcinoma SW480 cells and human breast carcinoma MCF-7 cells were obtained from American Type Culture Collection (Manassas, VA). All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Beijing, P.R. China), 2 mM glutamine, 1 mM pyruvate, 50 μ M 2-mercaptoethanol, penicillin (200 units/ml) and streptomycin (200 μ g/ml) at 37°C in a 5% CO₂/95% air atmosphere. For gene-modified cells, Geneticin (600 μ g/ml) (Invitrogen) was added to the medium.

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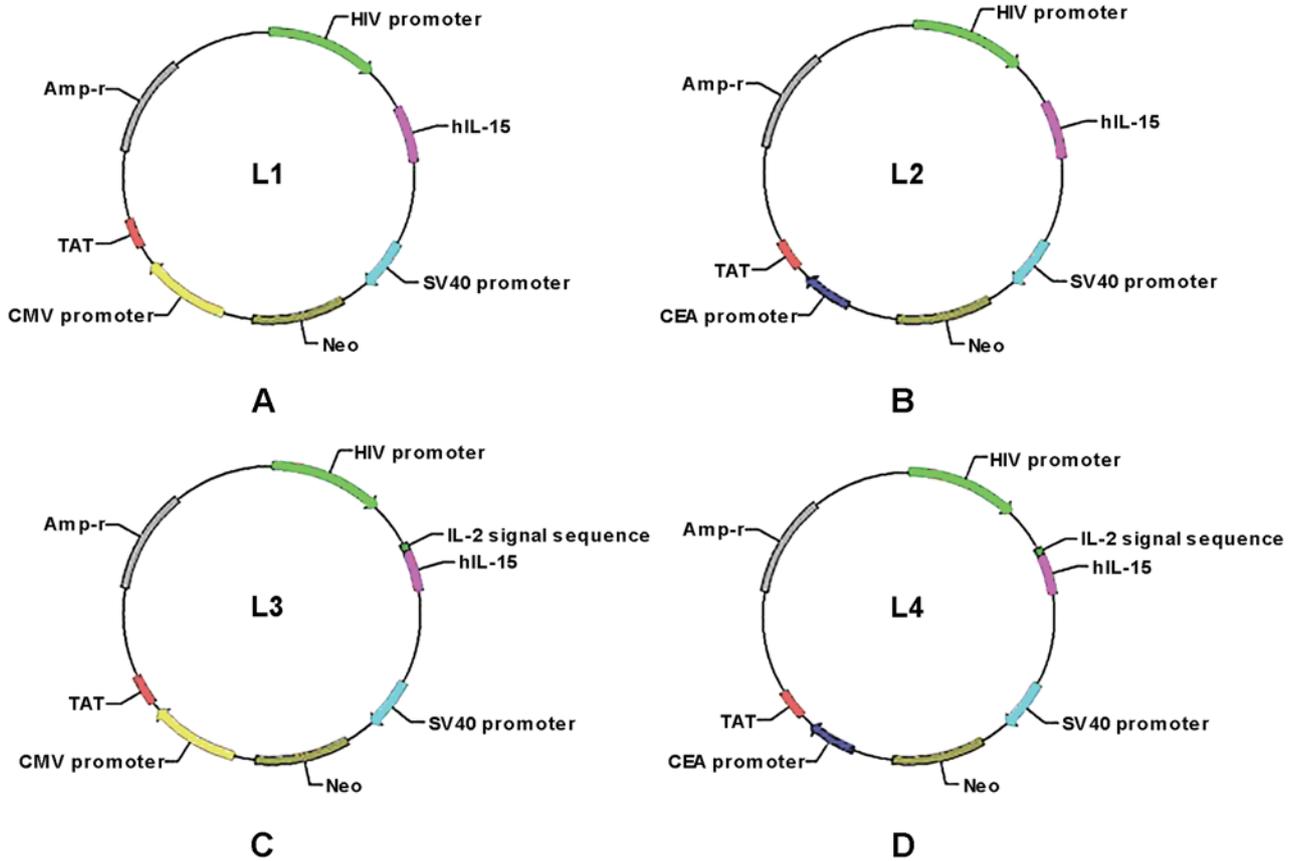


Figure 1. IL-15 expression genetic constructs. (A) pHi2-IL15-CMV-tat: the HIV2 promoter drives human IL-15 gene expression and the CMV promoter drives HIV transcriptional transactivator *tat* gene expression. (B) pHi2-IL15-CEA-tat: the HIV2 promoter drives human IL-15 gene expression and the CEA promoter drives *tat* gene expression. (C) pHi2-spIL15-CMV-tat: the HIV2 promoter drives human IL-15 gene expression, the signal sequence of IL-15 is replaced by the human IL-2 signal sequence, and the CMV promoter drives *tat* gene expression. (D) pHi2-spIL15-CMV-tat: the HIV2 promoter drives human IL-15 gene expression, the IL-15 signal sequence is replaced by the human IL-2 signal sequence, and the CEA promoter drives *tat* gene expression.

Genetic constructs. All restriction enzymes and their buffers were purchased from New England Biolabs (Beijing, P.R. China). The following plasmids were constructed.

pHi2-IL15-CMV-tat (L1). pHi2-IL15-CMV-tat was constructed by inserting the IL-15 gene fragment into the *EcoRI* and *MuII* sites behind the HIV2 promoter of the backbone plasmid pHi2-MCS-CMV-tat (M7) (kindly provided by Dr Harris, University of Arizona). Briefly, the IL-15 gene fragment was amplified from pORF-IL15 (Invivogen, San Diego, CA) using the following primers: upstream primer containing the *EcoRI* restriction site, 5'-CCGGCCGAATTCCGAAGGAGGGCC ACCATG-3'; and downstream primer containing the *MuII* site, 5'-CCGGCCACGCGTTCAATTGCAATCAAGAAGT-3'. The PCR product was 538 bp. After digestion with *EcoRI* and *MuII*, the IL-15 fragment was ligated into the same restriction sites in the plasmid pHi2-MCS-CMV-tat, resulting in the plasmid pHi2-IL15-CMV-tat (Fig. 1A).

pHi2-IL15-CEA-tat (L2). pHi2-IL15-CEA-tat was constructed by replacing the CMV promoter in pHi2-IL15-CMV-tat with the CEA promoter. Briefly, CEA promoter was amplified from pDRIVE-hCEA (Invivogen) using the following primers: upstream primer containing the *SpeI* site, 5'-GGCCGGACT AGTGCCCTGGAGAGC-3'; and downstream primer containing the *SacI* site, 5'-CCGGCCGAGCTCGGTCTCTGC

TGTCTGCTC-3'. The PCR product was 452 bp. After digestion with *SpeI* and *SacI*, the CEA promoter sequence was inserted into the same restriction sites of the plasmid pHi2-MCS-CMV-tat, resulting in pHi2-MCS-CEA-tat. Then, the IL-15 gene fragment was inserted into the *EcoRI* and *MuII* sites as described above, resulting in the plasmid pHi2-IL15-CEA-tat (Fig. 1B).

pHi2-spIL15-CMV-tat (L3). pHi2-spIL15-CMV-tat was constructed by replacing the IL-15 signal sequence with the IL-2 signal sequence by site-directed mutagenesis (Fig. 1C). Briefly, upstream and downstream primers were synthesized whose 5' ends were nucleotides corresponding exactly to the sequences upstream and downstream of the IL-15 signal sequence in the plasmid pHi2-IL15-CMV-tat. Their 3' ends were nucleotides corresponding exactly to the 5' and 3' end sequence of the IL-2 signal sequence in the plasmid pHi2-IL2-CMV-tat (kindly provided by Dr Harris, University of Arizona). After PCR amplification using the pHi2-IL2-CMV-tat as a template, the PCR product was harvested. According to the protocol described by the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), 1 μ l dNTP and 2.5 μ l PfuTurbo DNA polymerase were added to 250 ng of the PCR product and 50 ng of the plasmid pHi2-IL15-CMV-tat for a final volume of 50 μ l. The PCR reaction was denatured at 95°C for 30 sec, followed by 18 cycles for

SPANDIDOS PUBLICATIONS 95°C, 1 min at 55°C and 8 min at 68°C. At the end of the ligation reaction, 10 U *DpnI* was added to the reaction tube and incubated at 37°C for 1 h to remove wild-type original methylated template DNA. The PCR product was then used to transform XL1-Blue Supercompetent cells. Clones were tested to verify the replacement by DNA sequence analysis (performed by Takara, Dalian, P.R. China).

pHi2-spIL15-CEA-tat (L4). To construct pHi2-spIL15-CEA-tat (Fig. 1D), the plasmid pHi2-spIL15-CMV-tat was digested with *EcoRI* and *MuII* and the resulting fragment was inserted into the same sites in the plasmid pHi2-MCS-CEA-tat.

pHi2-EGFP-CEA-tat (L5) and pHi2-EGFP-CMV-tat (L6). In order to evaluate transfection efficiency, a gene-encoding enhanced green fluorescence protein (EGFP) was cloned into the same backbone plasmid behind the HIV2 promoter, resulting in the EGFP-expressing plasmids pHi2-EGFP-CEA-tat and pHi2-EGFP-CMV-tat.

Cell transfection. Tumor cells were transfected with plasmid DNA using the cationic lipid Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 24 h before transfection, cells were plated at a concentration of 5×10^5 cells/well in 2 ml of medium on 6-well plates and grown to 70-80% confluency. DNA/lipid complexes were prepared 30 min before transfection. Plasmid DNA (1 μ g) and 3 μ l DMRIE-C were individually diluted in 250 μ l of OptiMEM medium (Gibco, Rockville, MD), mixed together, incubated at room temperature for 20 min, then added to the well. Cells were incubated with lipid/DNA complexes for 4 h. The medium was then replaced with fresh culture medium. Transfection efficiency was determined by simultaneous transfection of an EGFP expression plasmid. IL-15 expression was tested by ELISA.

ELISA for IL-15 production. At 24 and 48 h after transfection, the culture supernatants were collected and tested for IL-15 expression using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (R&D Systems, USA). Briefly, a 96-well plate was coated with anti-mouse IL-5 antibody and blocked with bovine serum albumin-containing buffer. After washing, a standardized IL-15 solution and the cell culture supernatants were added to the wells. Following 2 h of incubation, the plate was washed, then biotin-labeled detection antibody and avidin-horseradish peroxidase were added. After another 1-h incubation and washing, the substrate solution was added and the plate was read at 450 nm.

Statistical analysis. Values were represented as the means \pm standard deviation (SD). Results were compared using the Student's t-test. p-values <0.05 were considered to be statistically significant.

Results

pHi2-IL15-CMV-tat gave rise to a significant amount of IL-15 expression in human tumor cells. The bioactivity of cytokines is related to their expression levels. In order to achieve a high level of IL-15 expression, we cloned the IL-15 gene into the

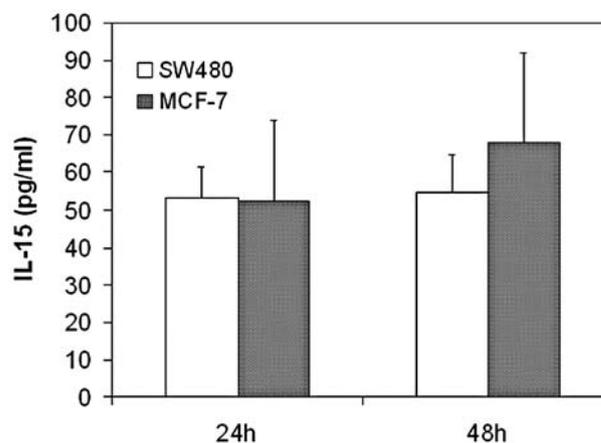
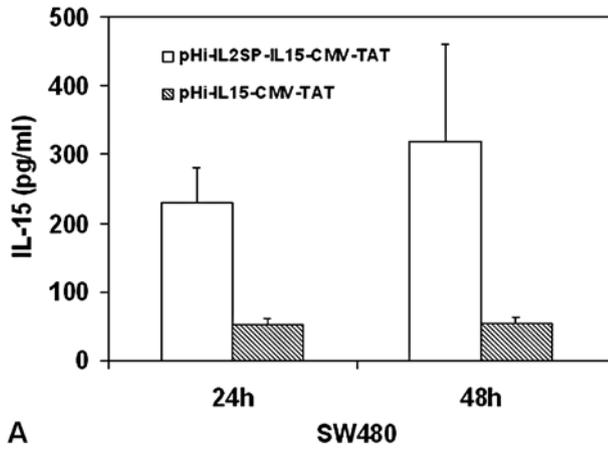


Figure 2. IL-15 expression by pHi2-IL15-CMV-tat-transfected cells. SW480 and MCF-7 cells were plated at a concentration of 5×10^5 cells/well in 2 ml of medium on 6-well plates. After 24 h, cells were transfected with IL-15 expression plasmid pHi2-IL15-CMV-tat using Lipofectamine 2000. Culture supernatants were collected at 24 and 48 h after transfection, and IL-15 expression was detected by ELISA. The mean \pm SD of three triplicate experiments are shown.

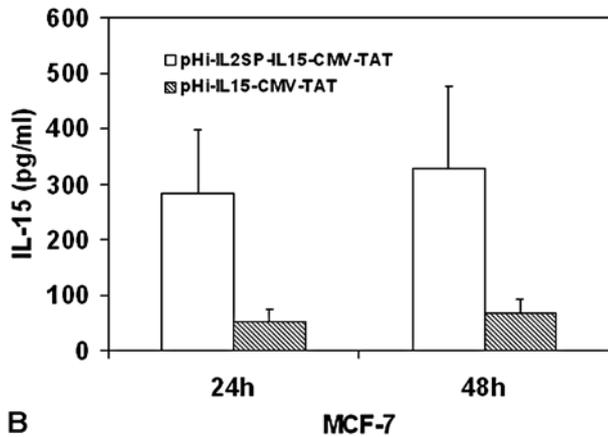
amplified gene expression vector pHi2-MCS-CMV-tat, which resulted in the plasmid pHi2-IL15-CMV-tat. In pHi2-IL15-CMV-tat, the HIV2 promoter drives human IL-15 gene expression and CMV promoter HIV transcriptional activator *tat* gene expression. The expression of Tat protein enhances the transcription initiated by the HIV promoter. After transfection into human tumor MCF-7 and SW480 cells, a significant amount of IL-15 expression was achieved (Fig. 2). The transfection efficiency was ~21% for both cell lines, determined by transfected EGFP-expressing plasmid pHi2-EGFP-CMV-tat under the same conditions (data not shown).

Replacement of the IL-15 with the IL-2 signal sequence significantly enhanced IL-15 secretion of transfected cells. In order to further increase IL-15 expression, we replaced the IL-15 signal sequence with the human IL-2 signal sequence. In the plasmid pHi2-spIL15-CMV-tat, the IL-15 signal sequence was replaced by the IL-2 signal sequence using site-directed mutagenesis. Compared to pHi2-IL15-CMV-tat, pHi2-spIL15-CMV-tat increased IL-15 expression 4.3-/5.9-fold and 5.4-/4.8-fold (24/48 h) after being transfected into SW480 and MCF-7 cells, respectively (Fig. 3). These results indicate that the IL-2 signal peptide can more efficiently direct IL-15 secretion.

pHi2-spIL15-CEA-tat targeted IL-15 expression in CEA-positive tumor cells. To target IL-15 expression to tumor tissue we constructed the plasmid pHi2-spIL15-CEA-tat, the tumor-specific CEA promoter of which controls *tat* expression. HIV2 promoter is weak without Tat. Through this modification, IL-15 expression was low in CEA-negative tumors, but high in CEA-positive tumors. After transfection, the plasmid pHi2-spIL15-CEA-tat produced 7.0- and 9.8-fold increases in the level of IL-15 at 24 and 48 h, respectively, after transfection into SW480 cells compared with transfection into MCF-7 cells (Fig. 4). These results indicate that the CEA promoter can efficiently drive *tat* expression and amplify IL-15 expression in turn.



A SW480



B MCF-7

Figure 3. Increased IL-15 expression by the replacement of the IL-15 with IL-2 signal sequence. SW480 (A) and MCF-7 (B) cells were plated at a concentration of 5×10^5 cells/well in 2 ml of medium on 6-well plates. After 24 h, cells were separately transfected with the plasmids pHi2-spIL15-CMV-tat and pHi2-IL15-CMV-tat using Lipofectamine 2000. Culture supernatants were collected at 24 and 48 h after transfection, and IL-15 expression was detected by ELISA. The mean \pm SD of three triplicate experiments are shown.

Comparable levels of IL-15 expression were achieved by CEA-specific IL-15 expression plasmid in CEA-positive cells. To compare the IL-15 expression of pHi2-spIL15-CEA-tat with that of pHi2-spIL15-CMV-tat in CEA-positive tumor cells, we transfected both plasmids into SW480 cells. The supernatants of transfected cells were harvested at 24 and 48 h after transfection, and the results indicate that comparable levels of IL-15 were expressed by both plasmids in SW480 cells. At 24 h after transfection, expression by pHi2-spIL15-CEA-tat was 332.49 ± 151.32 pg/ml, whereas that of pHi2-spIL15-CMV-tat was 229.69 ± 52.37 pg/ml ($p=0.361$). At 48 h, expression by pHi2-spIL15-CEA-tat was 341.12 ± 44.78 pg/ml, whereas that of pHi2-spIL15-CMV-tat was 320.73 ± 139.00 pg/ml ($p=0.998$) (Fig. 5).

Discussion

With the dissection of the molecular mechanism of carcinogenesis and tumor progression, great progress in gene therapy for cancer has been made. In fact, over two-thirds of gene therapy clinical trials currently target cancer. Of the approaches to cancer gene therapy, immunotherapy predominates. Gene transfer is being used to overcome the low immunogenicity

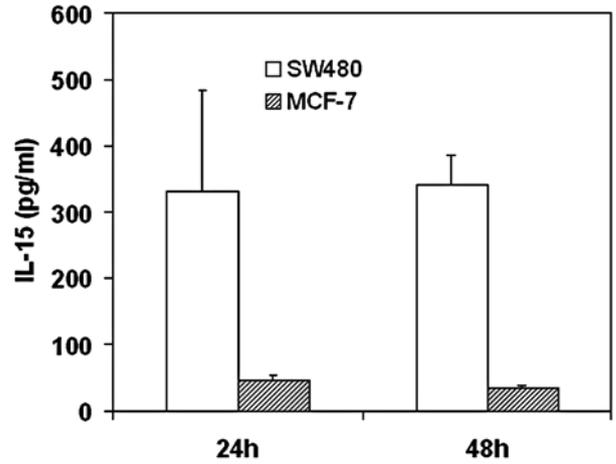


Figure 4. CEA-specific IL-15 expression by pHi2-spIL15-CEA-tat. SW480 and MCF-7 cells were plated at a concentration of 5×10^5 cells/well in 2 ml of medium on 6-well plates. After 24 h, cells were transfected with pHi2-spIL15-CEA-tat using Lipofectamine 2000. Culture supernatants were collected at 24 and 48 h after transfection, and IL-15 expression was detected by ELISA. The mean \pm SD of three triplicate experiments are shown.

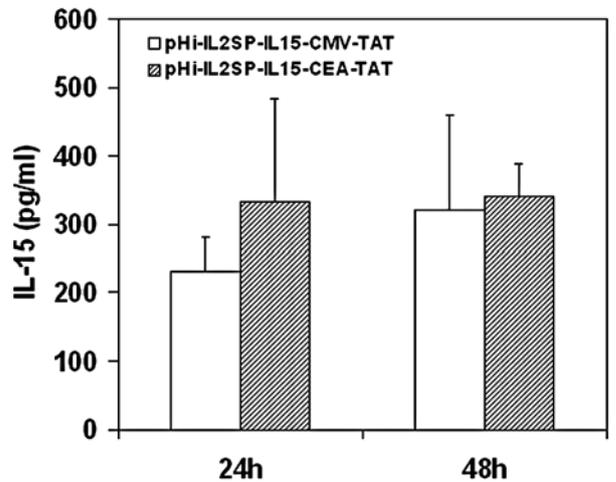


Figure 5. IL-15 expression of SW480 cells transfected with pHi2-spIL15-CEA-tat and pHi2-spIL15-CMV-tat. SW480 cells were plated at a concentration of 5×10^5 cells/well in 2 ml of medium on 6-well plates. After 24 h, cells were separately transfected with pHi2-spIL15-CEA-tat and pHi2-spIL15-CMV-tat using Lipofectamine 2000. Culture supernatants were collected at 24 and 48 h after transfection, and IL-15 expression was detected by ELISA. The mean \pm SD of three triplicate experiments are shown.

and immune evasion of malignant cells. Cytokines are a group of proteins or peptides which play an important role in the regulation of immune responses. Recombinant cytokines, such as IL-2 and IFN- γ , have been used in clinical settings. However, their application is impeded by their systemic toxicity in high therapeutic doses (5). The transfer of genes encoding immunostimulatory cytokines *in vivo* and *ex vivo* to tumor cells consequently becomes an attractive approach. Cytokines locally expressed by tumor cells can augment tumor antigen presentation, as well as recruit tumor-specific immune cells. Many cytokines have been investigated in preclinical studies, including granulocyte-macrophage colony-stimulating factor, tumor necrosis factor- α , IFN- γ and the interleukins (6,7).

SPANDIDOS is a pleiotropic cytokine that plays a key role in the of both innate and adaptive immune responses. First isolated from a simian kidney epithelial cell line in 1994 by Grabstein *et al* (8), it promotes the survival, proliferation, activation and maintenance of natural killer and CD8⁺ T cells, as well as stimulating the function of neutrophils, macrophages and dendritic cells. Increased anti-tumor activity was reported following IL-15 administration to tumor-bearing mice in 1995 (9). However, although the cytokine has been known in the field of cancer gene therapy since shortly after its discovery (10), few preclinical studies have analyzed the effects of IL-15 gene transfer. Yajima *et al* demonstrated that overexpression of IL-15 *in vivo* enhanced anti-tumor activity against malignant melanoma through augmented NK activity and cytotoxic T-cell response (11). Araki *et al* reported that weakly immunogenic murine colon 26 cells could be completely rejected when expressing a high level of IL-15 (12). Vera *et al* showed that dendritic cells transferred with IL-15 have an enhanced capability to induce curative anti-tumor immunity when injected into malignant lesions (13). In this study, we constructed amplified IL-15 expression plasmid vector pHi2-spIL15-CMV-tat and CEA-specific amplified IL-15 expression vector plasmid pHi2-spIL15-CEA-tat to achieve a high level of IL-15 expression for cancer gene therapy.

Efficient expression of the therapeutic gene in target tissue is the prerequisite for gene therapy. Cytokine genes can be delivered through the use of viral or non-viral vectors. The main problems encountered when using viral vector systems are toxicity and immunogenicity, as well as a possible insertion mutation for retroviral vectors. Plasmid-based non-viral vectors hold great promise for future gene therapy due to their safety, stability, low cost and, most importantly, the progress of physical methods in gene transfer (14-16). High levels of transgene expression have been achieved by modified plasmid vectors. Tsang *et al* constructed an amplified plasmid vector, pHi2-IL2-CMV-tat, which gave rise to a 28-fold increase in IL-2 expression compared to the CMV promoter-driven IL-2 expression plasmid (17). In this study, based on a sample backbone, we constructed the IL-15 expression plasmid pHi2-IL15-CMV-tat, among others. In these plasmids, the CMV promoter controlled HIV *tat* and, upon expression, the Tat protein mediated the transcriptional transactivation of HIV2 LTR, which directed IL-15 expression. Bamford *et al* reported that replacing the IL-15 with the IL-2 signal peptide increased IL-15 expression up to 20-fold (18). Therefore, in order to further increase the IL-15 expression of our vector, we successfully replaced the IL-15 with the IL-2 signal sequence by site-directed mutagenesis. Our data show a 5.9-fold increase in IL-15 expression after replacement. Consequently, by cloning the IL-15 gene into the amplified vector and replacing the IL-15 signal sequence with the IL-2 signal sequence, efficient IL-15 expression was achieved.

Targeting therapeutic genes to tumor cells could improve the effect of gene therapy and could lower systemic side effects. Strategies using tumor-specific promoters in conjunction with therapeutic genes have been attempted. CEA is a membrane oncofetal glycoprotein expressed on most adenocarcinomas of the gastrointestinal tract and on lung and breast cancer (19). Utilization of the CEA promoter to target therapeutic genes to CEA-positive tumors has been investigated

(20,21). However, the low efficiency of the CEA promoter limits its application. In the present study, we constructed the IL-15 expression plasmid vector pHi2-spIL15-CEA-tat. In this plasmid, the CEA promoter was used to direct HIV *tat* expression, while HIV2 LTR drove IL-15 expression. Expression of Tat controlled by the CEA promoter transactivated HIV2 LTR, achieving a high level of IL-15 expression in CEA-positive tumor cells. Our data show that pHi2-spIL15-CEA-tat gave rise to a very basic level of IL-15 expression in CEA-negative cells because the HIV2 promoter was inefficient in directing transgene expression without Tat, whereas it increased IL-15 expression ~10-fold in CEA-positive cells. In addition, the expression level of IL-15 was comparable to CMV promoter-directed *tat* expression plasmid pHi2-spIL15-CMV-tat in CEA-positive cells. The underlying mechanism could be that the cumulative activity of Tat on the HIV promoter renders the need for a strong promoter to drive expression unnecessary. The pHi2-spIL15-CEA-tat expression vector may provide a feasible strategy for the locally-inducible, and hence safer, high-level expression of IL-15 for anti-cancer gene therapy.

We constructed an amplified IL-15 expression plasmid vector, pHi2-spIL15-CMV-tat, and a CEA promoter-controlled amplified IL-15 expression plasmid vector, pHi2-spIL15-CEA-tat. A high level of IL-15 and targeted expression were achieved after transfection into tumor cells. Our results confirm that incorporating amplifier strategy into a single vector expression cassette significantly enhances transgene expression. Furthermore, an inefficient low activity tumor-specific promoter can be used in the amplified vector to achieve targeted high-level gene expression.

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