

RGD Modification Improves the Transfection Efficiency of Adenovirus Vectors Carrying Double Objective Genes

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Abstract

The objective gene and transduction efficiency of a vector are the two key points in gene therapy. Recently, we carried out two-gene combination therapy by inserting two genes in one vector, which can significantly improve antitumor activity. However, bi-cistronic adenovirus may have a low efficiency of transfection and expression. Therefore, in this study, we aimed to construct an adenovirus vector modified with arginine- (R-) glycine-(G-) aspartate (D) (RGD) and to detect and compare the transfection efficiency of the vector carrying two genes: ING4 and PTEN. Based on our previous research results, we first constructed the transfer vector, pAdTrack-CMV-ING4-polyA-promoter-PTEN and pAdTrack-CMV-polyA-promoter-PTEN, and we then constructed the homologous recombinant adenovirus plasmids; finally, we obtained a homologous recombinant adenovirus. After PCR and sequence identification, different types of tumor cell lines were infected with these recombinant adenoviruses, and the expression of GFP was detected with fluorescence microscopy and flow cytometry. We successfully constructed adenovirus vectors with RGD modification carrying ING4 and PTEN genes. The GFP expression rate in cells infected with adenovirus containing RGD at 50 MOI was much greater than that of cells infected with adenovirus without RGD modification at 50 MOI. Modification with RGD could significantly improve the transfection efficiency of adenovirus vectors, even those carrying two objective genes, into most tumor cells.

Keywords: RGD; Adenovirus vector; Transfection efficiency; Double genes

Introduction

Tumors are a type of genetic disease; treatments include surgery, radiotherapy and chemotherapy. Currently, gene therapy is receiving increasing attention, and the therapeutic properties of the objective gene itself and the transduction efficiency of a vector towards the target cells are the two key points in gene therapy.

We have conducted gene therapy research for a long time [22,27], and we have cloned and obtained various anti-oncogenes, which have been applied in cancer gene therapy studies [20,26]. Subsequently, we carried out anti-cancer research with two-gene combination therapy, i.e., achieving the co-expression of two genes in a single vector. The results suggest that two-gene combination therapy can significantly improve antitumor activity [25]. ING4 as a member of inhibitor of growth (ING) tumor suppressor family has potent inhibitory effects on a variety of tumors. The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene located on human chromosome 10q23, was also a kind of anti-oncogene. The loss or inactivation of ING4 and PTEN occurs in a variety of tumors [7,24]; moreover, the inactivation of either PTEN or ING4 has a close relationship with the malignant progress of tumors. Therefore, theoretically, the anti-tumor effect might be improved by combining ING4 and PTEN in one adenoviral vector; however, there is another problem in that bicistronic adenoviruses may have a low efficiency of transfection and expression, which is another key factor in effective gene therapy. In fact,

optimization of the adenovirus vectors may be useful. There are various types of vectors for gene therapy, and among these, adenoviruses are one of the most promising vectors for cancer gene therapy [5,8]. The first step of adenovirus infection is combining the coxsackie virus and adenovirus receptor (CAR) on the surface of the target cells [4]. However, the absence or decrease of CAR on the surface of many types of tumor cells may lead to a low efficiency of adenovirus infection. Therefore, improvement of the infection efficiency is another difficulty to overcome.

The tripeptide sequence of arginine- (R-) glycine- (G-) aspartate (D) (RGD) exists in a variety of extracellular matrix proteins and can specifically combine with 11 types of integrins, resulting in effective adherence to integrin proteins on target cells. Fortunately, a type of RGD peptide that can specifically bind with integrin $\alpha v \beta 3$, has been screened out, and the sequence of this short peptide is CDCRGDCFC (abbreviated as RGD-4C). Dmitriev et al. inserted the RGD-4C sequence into the HI stalk loop of adenovirus flagellum zoon, so that the adenovirus could take advantage of integrin $\alpha v \beta 3$ as its receptor [6], which improved the infection capability of the adenovirus into CAR-deficient cells. Generally, tumor cells highly express integrin $\alpha v \beta 3$ [3,14,16], but is absent from the surface of many types of normal cells [13]. Accordingly, we can modify the adenovirus vectors by inducing the expression of RGD-4C in their capsid proteins to guide the specific adhesion between vectors and tumor cells and consequently to improve the transfection efficiency.

However, the modification of RGD in an adenovirus vector carrying two objective genes has not yet been reported; furthermore, whether the RGD modification may improve the infection efficiency

of an adenovirus vector carrying two or more objective genes has also rarely been reported. In this study, based on our previous research, we constructed an adenovirus vector co-expressing both ING4 and PTEN with double promoters by inserting a promoter sequence of polyA+, modified the plasmid frame with RGD-4C, and tested the problem of infection efficiency.

Materials and Methods

Vectors, cell lines, reagents

The adenoviral plasmid, containing green fluorescent protein (GFP), pAdTrack-CMV-PTEN, pAdTrack-CMV-PolyA-promoter, and pAdTrack-CMV-ING4-PolyA-promoter was constructed by our laboratory [19,21,23]. Recombinant adenovirus Ad-GFP, Ad-ING4, Ad-PTEN, Ad-ING4-PTEN were also constructed and kept by our laboratory. The backbone plasmid pAdEasy-1, BJ5183 bacteria and QBI-293A human embryonic kidney cell line were kindly provided by Dr. Jiang Zhong (Department of Microbiology, College of Life Science, Fudan University, Shanghai, China). The backbone plasmid pAd (RGD) was kindly provided by Prof. Albert Deisseroth (Sidney Kimmel Cancer Center, Canada). The K562, THP-1 and MEG-01 human leukemia cell lines, U87and U251 human glioma cell lines, A549 human lung adenocarcinoma cell line, and MCF-7 human breast cancer cell line were purchased from the American Type Culture Collection (Shanghai, China) and cultured in RPMI 1640 (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (FBS, Hyclone, Shanghai, China). The restriction enzymes NotI, XhoI, PmeI, PacI, KapI, SalI, T4DNA ligase, and DreamTaq™ Green PCR Master Mix were all bought from Fermentas Co.. Primers for PCR, synthesized by Sangon (Shanghai, China) are listed in table 1.

No. of primers	Sequences of primers
P1	5'-GCGCGGCCGCATGACAGCCATCATCAAAGAG-3'
P2	5'-CGCTCGAGTCAGACTTTTGTAAATTTGTGT-3'
P3	5'-tagagatctaccatggctgctgggatgtatttg-3'
P4	5'-accgtcgaccctattcttctccgttcttg-3'
P5	5'-ACCACAGTCCATGCCATCAC-3'
P6	5'-TCCACCACCCTGTTGCTGTA -3'

Table 1. the primers sequences for the PCR of different genes

Harvest of homologous recombinant adenoviruses

Based on the transfer vectors pAdTrack-CMV-PolyA-promoter and pAdTrack-CMV-ING4-PolyA-promoter (established by our laboratory), we inserted the wild-type PTEN gene sequence between the following multiple clone sites: Not I and XhoI, and got transfer vectors of pAdTrack-CMV-polyA-promoter-PTEN and pAdTrack-CMV-ING4-polyA-promoter-PTEN, both of which were identified by PCR and DNA sequencing. Then, plasmids of the transfer vector, including pAdTrack-CMV-PolyA-promoter pAdTrack-CMV-ING4-PolyA-promoter, pAdTrack-CMV-polyA-promoter-PTEN, and pAdTrack-CMV-ING4-polyA-promoter-PTEN, were first linearized by restriction endonuclease PmeI at 37 for 2 hours and were then co-transferred into competent E coli BJ5183 with the frame of the pAd-RGD adenovirus. After the screening from Kana (50 µg/ml), plasmids

extracted from positive clones were transferred into competent E coli DH5α for amplification. Next, we obtained four types of homologous recombinant adenovirus plasmids, i.e., pAd-RGD-pAdTrack-CMV-polyA-promoter (abbreviated as pAd.RGD-GFP), pAd-RGD-pAdTrack-CMV-ING4-polyA-promoter (pAd.RGD-ING4), pAd-RGD-pAdTrack-CMV-polyA-promoter-PTEN (pAd.RGD-PTEN), and pAd-RGD-pAdTrack-CMV-ING4-polyA-promoter-PTEN (pAd.RGD-ING4-PTEN). The four types of homologous recombinant adenovirus plasmids were linearized by restriction endonuclease PacI and were then analyzed by 1% agarose gel electrophoresis; the larger fragments were reclaimed and transfected into QBI-293A human embryo kidney cells for packaging. After transfection, the GFP fluorescence was observed intermittently under a fluorescence microscope. Then, 7-10 days after transfection, cells were collected and treated with repeated freeze-thaw cycles to harvest the homologous recombinant adenovirus supernatant of the first generation. After infection and amplification of these four types of virus in QBI-293A cells for another three generations, we obtained recombinant adenovirus at a high titer, i.e., Ad.RGD-GFP, Ad.RGD-ING4, Ad.RGD-PTEN, and Ad.RGD-ING4-PTEN (A schematic diagram were shown in Figure 1 to explain the process of harvest of homologous recombinant adenoviruses).

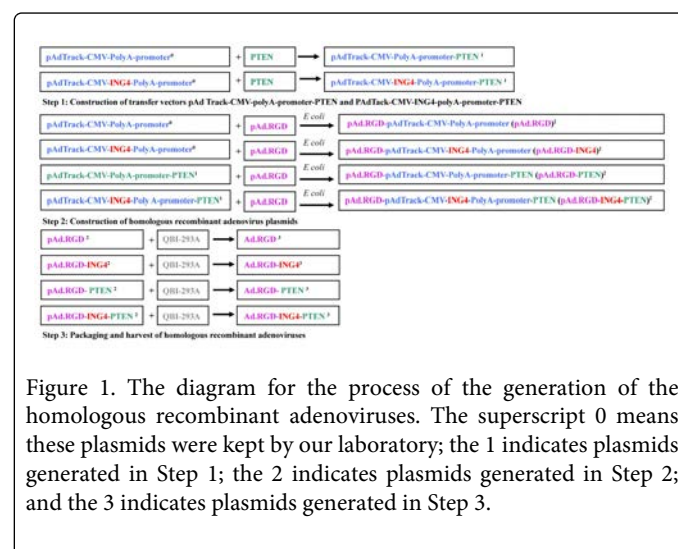


Figure 1. The diagram for the process of the generation of the homologous recombinant adenoviruses. The superscript 0 means these plasmids were kept by our laboratory; the 1 indicates plasmids generated in Step 1; the 2 indicates plasmids generated in Step 2; and the 3 indicates plasmids generated in Step 3.

RT-PCR

QBI-293A cells infected by Ad.RGD-GFP, Ad.RGD-ING4, Ad.RGD-PTEN, and Ad.RGD-ING4-PTEN were denoted as the corresponding experimental groups, and uninfected QBI-293A cells were denoted as the blank control group. Total RNA was extracted with TRIzol Reagent (Invitrogen). The first cDNA strands, reverse-transcribed from RNA, as well as plasmids pAdTrack-CMV-ING4-polyA-promoter-PTEN and pAdTrack-CMV-poly-A-promoter-PTEN were kept as templates. The PCR reaction was carried out using primers P1 and P2 for PTEN, P3 and P4 for ING4 (only for cDNA from QBI-293A cells), and P5 and P6 for GAPDH (only for cDNA from QBI-293A cells).

Potency detection for the recombinant adenovirus

QBI-293A cells in the logarithmic growth phase were trypsinized, and the cellular density was adjusted to 1×10⁷/ml; the cells were then seeded into a 96-well plate (100 µl cell suspension per well) for further

cultivation. Twenty-four hours later, 100 μ l recombinant adenovirus supernatant at different dilutions (e.g., 10^{-6} , 10^{-7} , 10^{-8} , and 10^{-9}) was added into the wells, and identical dilutions were repeated 3 times. After cultivation for another 18 hours, cells emitting green fluorescence (abbreviated as GFP cells) were counted under a fluorescence microscope, and we calculated the virus potency according to the formula (pfu/ml) = (mean number of GFP cells per well \times 10)/ dilution.

Qualitative detection of GFP expression by fluorescence microscopy

Cells of different tumor cell lines, including U87, U251, MCF-7, A549, K562, THP-1 and MEG-01, in the logarithmic growth phase were adjusted to a density of 1×10^5 /ml, seeded in 6-well plates, and cultured overnight under conventional conditions (culture medium containing 10% FCS, at 37 with 5% CO₂). The next day, recombinant adenovirus, including Ad-GFP, Ad-ING4, Ad-PTEN, Ad-ING4-PTEN, Ad.RGD-GFP, Ad.RGD-ING4, Ad.RGD-PTEN, and Ad.RGD-ING4-PTEN, at multiplicity of infection (MOI) 10, 50, 100 and 200 were added into the above-mentioned cell cultivation system. Then, 24 hours later, the expression of GFP in each type of cell was detected using a fluorescence microscope.

Quantitative detection of GFP-positive cells by flow cytometry

Cell of the U87, U251, MCF-7, A549, K562, THP-1 and MEG-01 lines that were infected, respectively, with 50 MOI Ad-GFP, Ad-ING4, Ad-PTEN, Ad-ING4-PTEN, Ad.RGD-GFP, Ad.RGD-ING4, Ad.RGD-PTEN, and Ad.RGD-ING4-PTEN were prepared as cell suspensions, and then the rate of cells expressing GFP fluorescence was detected by flow cytometry.

Statistical analysis

All data are presented as the mean \pm SD. Significant differences between two samples were evaluated by t-tests using SPSS 10.0 software. A value of $P < 0.05$ was considered statistically significant.

Results

Identification of the recombinant transfer vector

Plasmids pAdTrack-CMV-ING4-polyA-promoter-PTEN and pAdTrack-CMV-polyA-promoter-PTEN were kept as templates, and P1, P2 were used as primers for PTEN. The PCR results showed that the objective band was approximately 1,200 base pairs (bp) by agarose gel electrophoresis, which was consistent with the theoretical value of 1209 bp (Figure 2). The DNA sequencing results confirmed that the objective gene PTEN (wild-type) had been successfully inserted in the two recombinant transfer vectors: pAdTrack-CMV-ING4-polyA-promoter-PTEN and pAdTrack-CMV-polyA-promoter-PTEN (see supplementary).

Harvest of homologous recombinant adenovirus

Twelve hours after the transfection of the large fragments from the linearized homologous recombinant adenovirus plasmids (pAd.RGD-GFP, pAd.RGD-ING4, pAd.RGD-PTEN and pAd.RGD-ING4-PTEN) into QBI-293A incising cells, green fluorescence could be detected under a fluorescence microscope, moreover, the fluorescence intensity

gradually increased with time. Ten days after transfection, the viruses were harvested for second generation amplification by infecting QBI-293A incising cells, where the cytopathic effect (CPE) was obvious (Figure 3).

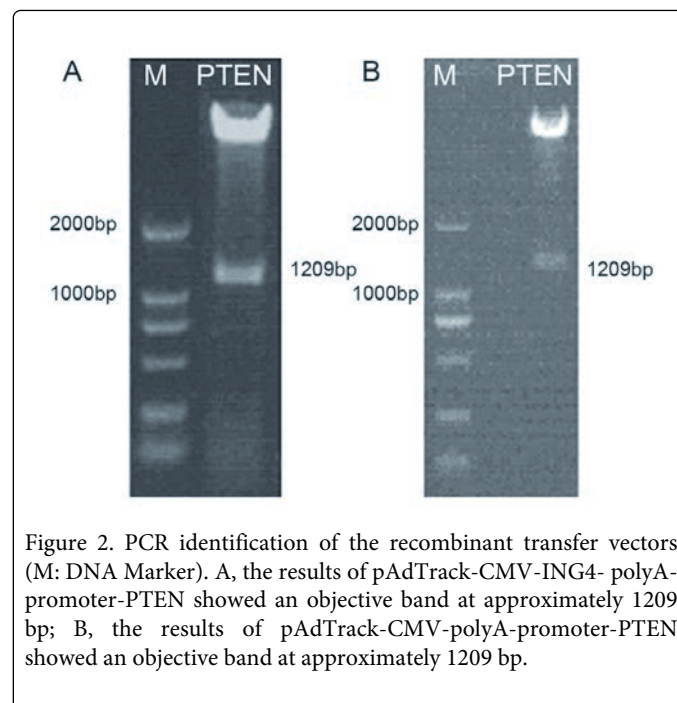


Figure 2. PCR identification of the recombinant transfer vectors (M: DNA Marker). A, the results of pAdTrack-CMV-ING4- polyA-promoter-PTEN showed an objective band at approximately 1209 bp; B, the results of pAdTrack-CMV-polyA-promoter-PTEN showed an objective band at approximately 1209 bp.

These results showed that the various viruses had been successfully packed in the QBI-293A cells. The virus supernatants from the third generation were kept for subsequent research.

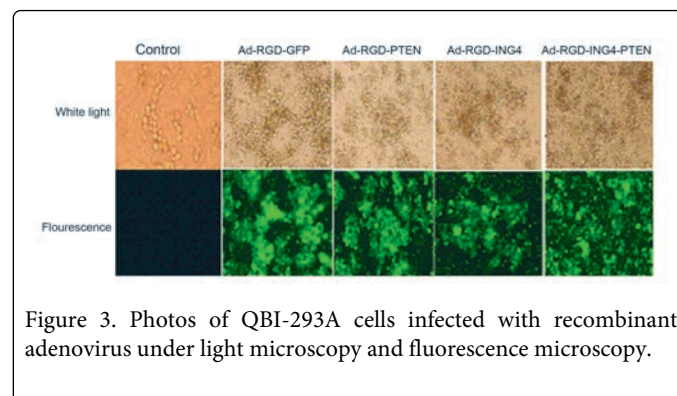


Figure 3. Photos of QBI-293A cells infected with recombinant adenovirus under light microscopy and fluorescence microscopy.

RT-PCR identification of the recombinant adenovirus

QBI-293A cells from the different groups were prepared for RT-PCR to detect the transcription of PTEN, ING4, and GAPDH. The results showed two objective bands at approximately 750 bp and 1209 bp for cells infected with Ad.RGD-ING4-PTEN; there was a band at approximately 750 bp for cells infected with Ad.RGD-ING4, but there was no band near 1209bp; and for the cells infected with Ad.RGD-PTEN, there was only an objective band at approximately 1209 bp with no band near 750 bp. There were no bands at either 1209 bp or 750 bp for cells infected with Ad.RGD-GFP or cells from the blank control group. Additionally, we detected bands for GAPDH in all groups (Figure 4).

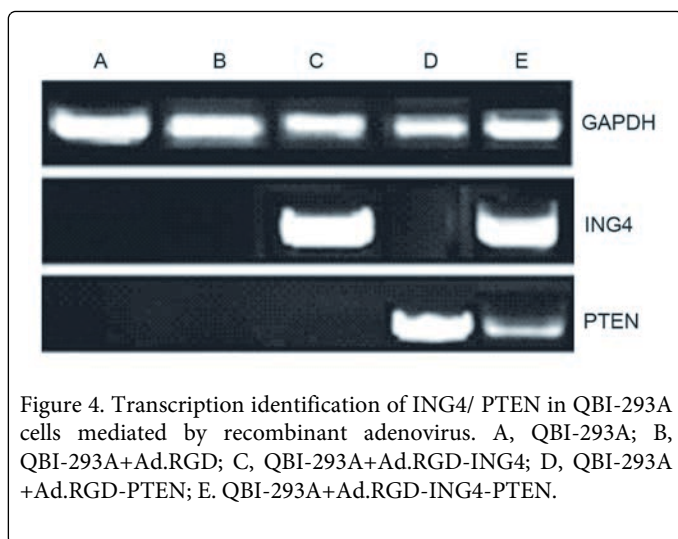


Figure 4. Transcription identification of ING4/ PTEN in QBI-293A cells mediated by recombinant adenovirus. A, QBI-293A; B, QBI-293A+Ad.RGD; C, QBI-293A+Ad.RGD-ING4; D, QBI-293A+Ad.RGD-PTEN; E. QBI-293A+Ad.RGD-ING4-PTEN.

Harvest of recombinant adenovirus with high potency

Adenovirus supernatants extracted from infected QBI-293A cells were used to infect QBI-293A cells again for several rounds for the amplification of the corresponding recombinant adenovirus. After three rounds of amplification, we obtained various adenoviruses in high titers, which were 5×10^9 , 3×10^9 , 5×10^9 , 2×10^9 , 2×10^{10} , 1×10^{10} , 2×10^{10} , 6×10^9 (pfu/mL), respectively, for Ad-GFP, Ad-ING4, Ad-PTEN, Ad-ING4-PTEN, Ad.RGD-GFP, Ad.RGD-ING4, Ad.RGD-PTEN, and Ad.RGD-ING4-PTEN. The results showed that the titers of adenovirus modified by RGD-4C were approximately 3-4 times higher than that of adenovirus without RGD-4C modification, which demonstrated that RGD could significantly enhance the adenovirus titers.

Enhanced infection efficiency by adenovirus modified with RGD compared with ordinary adenovirus

The U87, U251, MCF-7, A549, K562, MEG-01 and THP-1 cell lines were infected with various recombinant adenoviruses (Ad-GFP, Ad-ING4, Ad-PTEN, Ad-ING4-PTEN, Ad.RGD-GFP, Ad.RGD-ING4, Ad.RGD-PTEN, and Ad.RGD-ING4-PTEN) at different doses (10 MOI, 50 MOI, 100 MOI, and 200 MOI). Forty-eight hours later, the cells were observed under a fluorescence microscope. The results showed that all types of adenovirus without RGD modification could successfully infect U87, U251, MCF-7, and A549 cells at a titer of 50 MOI; however, K562, THP-1, and MEG-01 cells seldom expressed GFP. Moreover, cells infected with any type of adenovirus modified with RGD expressed GFP obviously and grew well (Figure 5).

These results demonstrated that modification with RGD could significantly improve the infection rate of adenovirus vectors, which was especially obvious for lymphoma cells, such as THP-1, K562, and MEG-01 cells. In addition, the dose of 50 MOI had low cytotoxicity, which suggested that 50 MOI would be a good titer for subsequent research. Cells expressing GFP were detected with flow cytometry, and the results showed that there were many more positive cells infected by adenovirus containing RGD at 50 MOI than there were cells infected by adenovirus without RGD modification at 50 MOI (Figure 6,7).

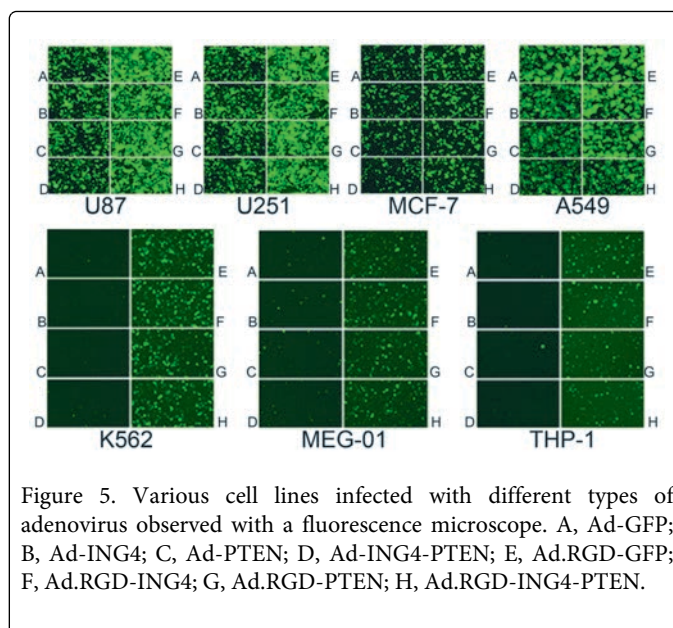


Figure 5. Various cell lines infected with different types of adenovirus observed with a fluorescence microscope. A, Ad-GFP; B, Ad-ING4; C, Ad-PTEN; D, Ad-ING4-PTEN; E, Ad.RGD-GFP; F, Ad.RGD-ING4; G, Ad.RGD-PTEN; H, Ad.RGD-ING4-PTEN.

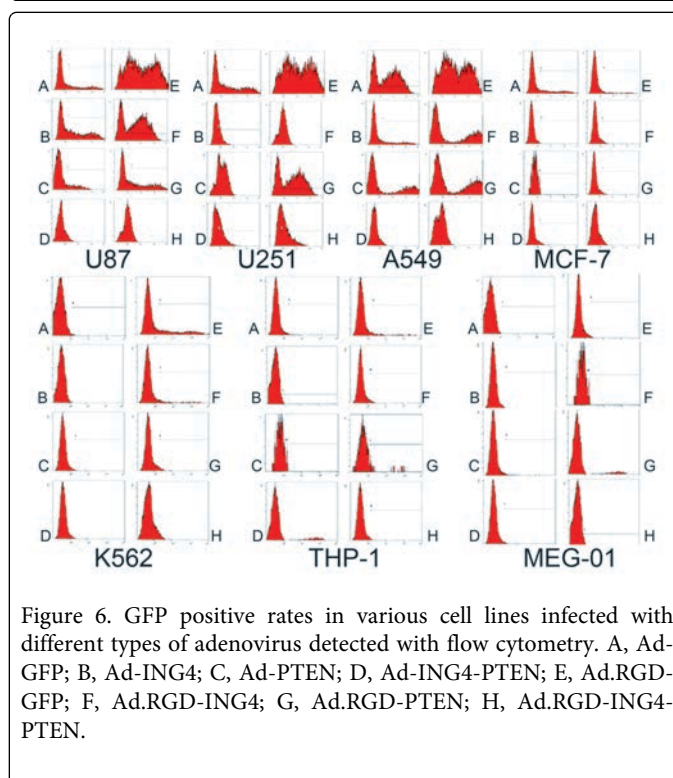


Figure 6. GFP positive rates in various cell lines infected with different types of adenovirus detected with flow cytometry. A, Ad-GFP; B, Ad-ING4; C, Ad-PTEN; D, Ad-ING4-PTEN; E, Ad.RGD-GFP; F, Ad.RGD-ING4; G, Ad.RGD-PTEN; H, Ad.RGD-ING4-PTEN.

Discussion

Adenovirus is a double-stranded DNA virus. In recent years, recombinant adenovirus has received increasing attention as a novel vector for gene therapy. One vector simultaneously expressing multiple exogenous genes has a wide range of applications in the field of biological therapies. In cancer gene therapy, constructing adenovirus vectors that express multiple exogenous genes with high transfection efficiency has particular significance by targeting multiple pathways [18].

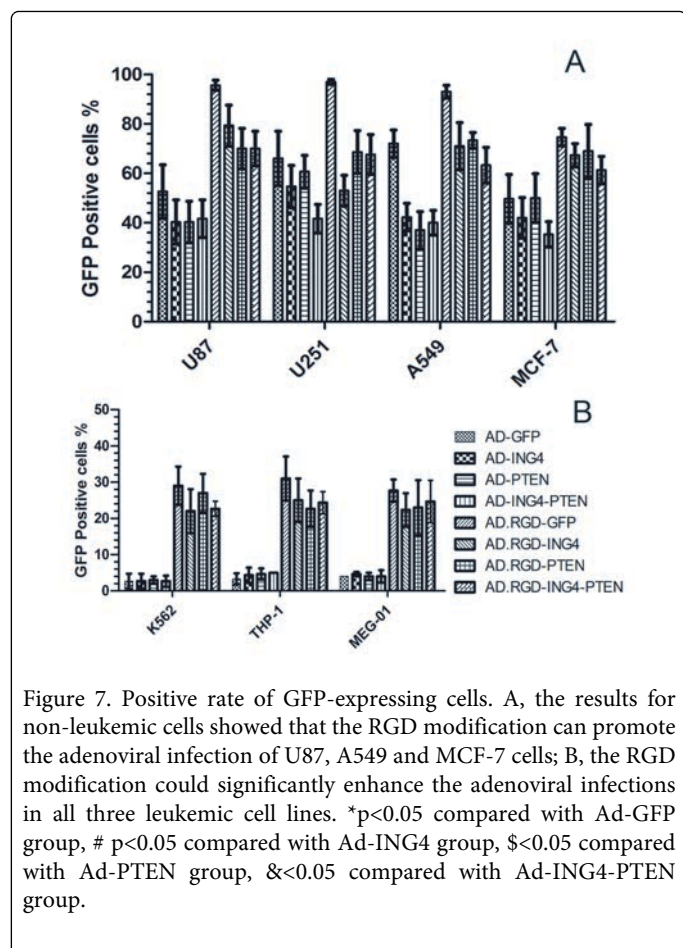


Figure 7. Positive rate of GFP-expressing cells. A, the results for non-leukemic cells showed that the RGD modification can promote the adenoviral infection of U87, A549 and MCF-7 cells; B, the RGD modification could significantly enhance the adenoviral infections in all three leukemic cell lines. * $p < 0.05$ compared with Ad-GFP group, # $p < 0.05$ compared with Ad-ING4 group, \$ $p < 0.05$ compared with Ad-PTEN group, & $p < 0.05$ compared with Ad-ING4-PTEN group.

At present, there are two main ways for multi-gene therapy: First, multiple independent vectors carrying different genes simultaneously transfect or infect target cells, and its advantage is that you can conveniently adjust the proportion of each expression-vector combination and coordination of time, the disadvantage is the efficiency of multi-gene co-expression is too low and onerous [11,15]. The second is to achieve the co-expression of multiple genes in one identical vector [10]. Comparing with a number of independent vectors carrying different genes to achieve co-expression, a multi-gene co-expression vector can increase the efficiency of transfection and expression. In this paper, we constructed such an adenovirus vector using conventional molecular biology methods. Our vectors have some obvious advantages [8], including: 1) simple operation - multiple gene fragments can be inserted in the transfer plasmid; 2) high efficiency of homologous recombination and rapid multiplication of bacteria; 3) vectors containing the GFP reporter gene, which facilitates the detection for viral packaging, infection efficiency and virus titer.

Successful infection of a target cell by an adenovirus requires an interaction with $\alpha v \beta 3$ or $\alpha v \beta 5$ integrin on the cell surface [1,2,17]. The integrin family is a class of cell surface receptor proteins that mediate adhesion between cells or between cells and the extracellular matrix [9,12]. The RGD peptide is composed of arginine, glycine, and aspartic acid (Arg-Gly-Asp), and the attachment proteins in blood or the extracellular matrix are the most common human RGD-containing proteins.

The homologous recombination of pAd-RGD backbone plasmids and adenovirus shuttle plasmids results in the specific expression of an RGD-4C short peptide on the intact adenovirus capsids. Then, through RGD, they can directly recognize and combine with integrin proteins on the surface of the target cell, which greatly improves the potency and infection capability of the adenovirus. In our study, compared with adenovirus without RGD modification and at the same MOI, Ad-RGD had much higher infection efficiency in tumor cell lines including the K562, Thp-1, MEG-01, U87, A549, and MCF-7 cell lines, but not in U251 cells. Therefore, the successful construction of Ad-RGD may result in more effective expression of the objective genes and lower adenovirus toxicity in cancer gene therapy, especially for cancer cells from lymphocytes, which lack CAR.

The loss or inactivation of ING4 and PTEN occurs in a variety of tumors; in this study, we constructed a recombinant adenovirus vector containing both ING4 and PTEN with RGD modification, which will be a very promising research tool for future research on cancer gene therapy. However, our results suggest that because the RGD modification could not significantly improve the infection efficiency in U251 glioma cells, our vectors still need further refinement.

Acknowledgments

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