EUR. J. ONCOL.; Vol. 19, n. 1, pp. 13-20, 2014

Enhanced anti-tumor activity by adenovirus mediated LIF and IL-24 co-expression on glioma cells and its mechanism

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Summary. *Objective:* Gene therapy on the glioma has been studied for many years, but most studies still remain at the level of single gene therapy. However, the glioma is characterized by a multistep process of genetic and molecular changes to oncogenes and tumor suppressor genes, which limits the efficacy of single gene-mediated therapy due to the difficulty of finding a pivotal gene conferring its occurrence in glioma gene therapy. In this paper, we try to study the anti-tumor effects and mechanism of a recombinant adenoviral vector co-expressing leukemia inhibitory factor (LIF) and interleukin-24 (IL-24) on glioma cells. *Materials and Methods:* LIF/IL-24 bicistronic adenovirus (Ad-LIF-IL-24) was constructed and preserved by our laboratory. The enhanced growth-suppressing and apoptosis-inducing effect of Ad-LIF-IL-24 on the U251 glioma cells *in vitro* was assessed, and the expressions of the apoptosis-related genes (bcl-2, bax, and ICE) were determined. *Results:* Ad-LIF-IL-24 could induce much more cellular apoptosis of U251 glioma cells than either Ad-LIF or Ad-IL-24 alone at the same virus titer. Molecularly, Ad-LIF-IL-24 could significantly enhance the expressions of bax and ICE though it inhibits the expression of bcl-2. *Conclusion:* Cancer gene therapy combining LIF and IL-24 may constitute a novel and more effective therapeutic strategy for gliomas, with good potential prospects of clinical application.

Key words: gene therapy, bicistronic adenovirus, LIF, IL-24, glioma

«Aumento dell'attività anti-tumorale tramite adenovirus mediato da co-espressione LIF e IL-24 su cellule di glioma e suo meccanismo»

Riassunto. *Oggetto:* La terapia genica su glioma è stata studiata per anni, ma tuttora la maggior parte degli studi resta a livello di terapia di gene singolo. Tuttavia il glioma è caratterizzato da un processo multifase di cambiamenti molecolari e genetici, che coinvolgono oncogeni e geni soppressori che limitano l'efficacia di una terapia mediata da singolo gene a causa della difficoltà di trovare un gene decisivo il cui utilizzo possa trasformarsi in terapia genica per il glioma. In questo studio abbiamo cercato di approfondire l'effetto anti-tumorale di un vettore ricombinante adenovirale co-espressivo del fattore di inibizione leucemico (LIF) e interleuchina-24 (IL-24) sulle cellule e il metabolismo del glioma. *Materiali e metodi:* Il complesso LIF/IL-24 adenovirus bicistronico (ad-LIF-IL-24) è stato composto e mantenuto presso il nostro laboratorio. L'effetto sul miglioramento sulla soppressione della crescita e sull'induzione dell'apoptosi da parte del complesso Ad-LIF-IL-24 sulle cellule di glioma è stato accertato, e l'espressione dei geni relativi all'apoptosi (bcl-2, bax, e ICE) è stata determinata.

The first 2 authors contributed equally to this article.

The current research was supported by National Natural Science Foundation of China (No. 81001016; 81101909); Health Bureau Foundation of Shanghai (No. 2010133).

Risultato: il complesso Ad-LIF-IL-24 può provocare una maggiore apoptosi cellulare sulle cellule di glioma U251 rispetto ai complessi Ad-LIF o Ad-IL-24 da soli o combinati con lo stesso virus. A livello molecolare, il complesso Ad-LIF-IL-24 può migliorare in modo significativo l'espressione di bax ed ICE e inibire l'espressione di bcl-2. *Conclusioni:* La terapia genetica sul cancro combinante LIF e IL-24 può costituire una nuova e più efficace strategia terapeutica per il glioma con buone potenziali prospettive per l'applicazione clinica.

Parole chiave: terapia genica, adenovirus bicistronico, LIF, IL-24, glioma

Introduction

Gliomas are the most common primary brain tumor and are among the most challenging tumors for neurosurgeons. So far, an effective glioma therapy has still not been devised, even combining surgery, radiotherapy and chemotherapy, with median survival times being less than 15 months from time of diagnosis (1). Thus, there is increasing interest in finding new agents for glioma treatment. Cancer gene therapy represents a promising new therapeutic modality for cancers, and gene therapy was ranked as one of the Top Ten scientific discoveries in 2009 by SCIENCE (2). Adenovirus is one of the most promising vectors for cancer gene therapy. Adenoviral vectors harboring therapeutic genes have been used successfully for gene transfer *in vitro* and *in vivo*.

Leukemia inhibitory factor (LIF) is named for its function since it can inhibit the proliferation of myeloid leukemia cells in mice (3). LIF has a variety of biological functions. It can induce differentiation and inhibit proliferation of tumors of the hematological system; it also can induce cell apoptosis or proliferation in solid tumors by activating different genes (4): e.g. LIF may have a tumor suppressive potential specific to medullary thyroid carcinoma (5), but promote the proliferation of pancreas carcinoma cells (6).

The other tumor suppressor, interleukin-24 (IL-24), displays ubiquitous antitumor properties and tumor specific killing activity in a broad spectrum of cancer cells but not in normal cells (7). IL-24 can also inhibit tumor angiogenesis through directly suppressing vascular endothelial cell differentiation and migration (8, 9), repressing tumor cell invasion and migration via downregulation of phosphatidylinositol 3-kinase, focal adhesion kinase and matrix metalloproteinase-2 (10). Our previous study showed that exogenous IL-24 mediated by adenovirus can inhibit the growth of U251 glioma cells (11). Thus, LIF and IL-24 are promising tumor suppressors which negatively modulate tumor growth via multiple pathways.

After Nobel Prize winner Shinya Yamanaka successfully converted mature cells into induced pluripotent stem cells (iPS) by simultaneously transfecting multiple transcription factors, instead of one transcription factor per experiment, more and more geneticists have transferred their research focus to genic modification based on the expression of multiple genes. At present, there are two main paths for multi-gene therapy. First, multiple independent vectors carrying different genes simultaneously transfect or infect target cells, the advantage of which is that you can conveniently adjust the proportion of each expression-vector combination and coordination in time, the disadvantage being that the efficiency of multi-gene co-expression is too low and onerous (12, 13). The second is to achieve co-expression of multiple genes in one identical vector (14). Compared with various independent vectors carrying different genes to achieve co-expression, a multigene co-expression vector can increase the efficiency of transfection and expression. Because the low efficiency of gene transfer is the bottleneck in gene therapy, we constructed the LIF/IL-24 bicistronic adenovirus-mediated gene co-transfection vector (Ad-LIF-IL-24).

The therapeutic potential of combining LIF and IL-24 for cancers has not been reported in the glioma. To enhance the therapeutic efficacy and develop a novel combined therapeutic modality for gliomas, based on the antitumor features of LIF and IL-24, we hypothesized that combination treatment of LIF and IL-24 tumor suppressors would enhance antitumor efficacy. Hence, in this study, we investigated the potential combined effect of LIF and IL-24 double tumor suppressor genes (Ad-LIF-IL-24) against U251 hu-

man glioma cells, and also tried to elucidate the underlying molecular mechanism.

Material and methods

Vectors, cell lines, and reagents

Ad-LIF, Ad-IL-24, Ad-LIF-IL-24 and adenovirus (Ad-GFP) replication-incompetent adenoviral vectors were constructed and preserved by our laboratory. The U251 human glioma cell line was purchased from the American Type Culture Collection (Shanghai, China) and cultured in RPMI-1640 (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (Hyclone, Shanghai, China). The Reverse transcription (RT)-PCR detection kit was purchased from Invitrogen (Shanghai, China). The MTT kit was purchased from Sigma (Shanghai, China). The Annexin V-PE/7-AAD apoptosis detection kit was purchased from BD Biosciences (Shanghai, China).

Control groups and experimental groups

All experiments were processed in the following 5 groups: a normal control group treated only with phosphate buffered saline (PBS), a negative control group treated with blank adenovirus (Ad-GFP), an LIF group treated with recombinant adenovirus containing human LIF (Ad-LIF), an IL-24 group treated with recombinant adenovirus containing human IL-24 (Ad-IL-24) and a bicistronic adenovirus group containing both LIF and IL-24 (Ad-LIF-IL-24). Cells in all groups were observed under an inverted microscope and an inverted fluorescent microscope after culture for 48 hours.

MTT assay

The *in vitro* cytotoxic effect of Ad-LIF-IL-24 on U251 human glioma cells was evaluated by MTT assay. Briefly, U251 tumor cells were dispensed into 96-well culture plates at 1×10^4 cells/well. After 24 h incubation at 37°, the U251 tumor cells were infected with Ad-LIF, Ad-IL-24, Ad-LIF-IL-24 or Ad-GFP used as a blank adenovirus control at 100 MOI or without adenovirus (PBS control), and cultured for the time periods indicated (0-4 days). Before treatment and at different time points after treatment, the viability of U251 tumor cells was analyzed using an MTT kit according to the company's protocol. Inhibition ratios were calculated as the following formula: inhibition ratio= (OD_{570} of control group- OD_{570} of experimental group)/OD_{570} of control group.

Flow cytometric analysis of apoptosis

U251 human glioma cells (1×10^6) were cultured with Ad-LIF, Ad-IL-24, Ad-LIF-IL-24 or Ad at 100 MOI or without adenovirus (PBS control), respectively. Forty-eight hours later, the treated and untreated U251 tumor cells were harvested, washed in cold PBS, and apoptosis was assessed by flow cytometric analysis using an Annexin V-PE/7-AAD apoptosis detection kit following the manufacturer's instructions. Briefly, the treated and untreated U251 tumor cells (1×10^6) were incubated with 5 μl Annexin V-PE (early apoptotic marker) and 5 μl 7-AAD (late apoptotic marker) in 100 μl of 1× Annexin V binding buffer at room temperature. After 15 min incubation, 400 μl of 1× binding buffer was added and the apoptotic cells were then analyzed by flow cytometry.

Reverse transcription (RT)-PCR analysis

The apoptosis related genes Bcl-2, Bax and interleukin-1 β converting enzyme (ICE) were determined by RT-PCR analysis in U251 human glioma cells. Briefly, the U251 cells (5×10^6) were infected with Ad-ING4, Ad-IL-24, Ad-ING4-IL-24 or Ad used as a blank adenovirus control at 100 MOI, or without adenovirus PBS control, respectively. After 48 h treatment, the infected and uninfected MDA-MB-231 tumor cells were collected, and the total cellular RNA was extracted with Trizol for RT-PCR. PCR was carried out using cDNA as templates and primers as follows: 5'-TGT GGC CTT TCT TTG AGT TCG-3'; and 5'-CTA CCC AGC CTC CGT TAT CC-3' for Bcl-2; 5'-GGA TGC GTC CAC CAA GAA-3'; and 5'-GCA CTC CCG CCA CAA AGA-3' for Bax; 5'-ACA TCC TCA GGC TCA GAA GG -3'; and 5'-TGC TGT CAG AGG TCT TGT GC-3' for ICE;

and 5'-TGC GTG ACA TTA AGG AGA AG-3'; 5'-CTG CAT CCT GTC GGC AAT G-3' for human β-actin. The RT-PCR products were then analyzed by 1% agarose gel electrophoresis.

Statistical analysis

All data are presented as means ± standard deviation (SD). Significant differences between two samples were evaluated by the t-test using SPSS 10.0 software. A value of $p<0.05$ was considered statistically significant.

Results

Adenovirus-mediated LIF and IL-24 expression

U251 human glioma cells were treated with Ad-GFP, Ad-LIF, Ad-IL-24, Ad-LIF-IL-24 and PBS. The results of RT-PCR yielded bright bands, indicating transcription of LIF and IL-24 in the Ad-LIF and Ad-IL-24 and Ad-LIF-IL-24 treatment groups but not in the other groups (Figure 1).

Enhanced tumor suppression by LIF and IL-24 co-expression

U251 human glioma cells cultivated in the Ad-GFP, Ad-LIF, Ad-IL-24 and Ad-LIF-IL-24 groups expressed green fluorescence protein (GFP), which did not appear in the PBS group (Figure 2A). However, fewer cells were observed in the Ad-LIF, Ad-IL-24 and Ad-LIF-IL-24 groups, but more in the Ad-GFP and PBS groups. Moreover, there were even fewer cells in the Ad-LIF-IL-24 group than in the Ad-LIF and Ad-IL-24 groups (Figure 2A). For a quantitative purposes, the cell viability of each group was examined daily *in vitro* before and after treatment using MTT assays. As shown in Figure 2B, compared with the Ad-GFP and PBS control groups, adenovirus-mediated LIF and/or IL-24 expression significantly suppressed *in vitro* U251 human glioma cell growth in a time-dependent manner with peak inhibition at day 4 after infection ($p<0.05$). Interestingly, the results of combination treatment with LIF and IL-24 co-expression showed greater inhibition (though not significantly so) on the growth of U251 glioma cells compared with the Ad-LIF- and Ad-IL-24-treated groups where the inhibition ratios were 32%, 49% and 68% respectively in groups Ad-LIF, Ad-IL-24, Ad-LIF-IL-24 on day 4 ($p<0.05$).

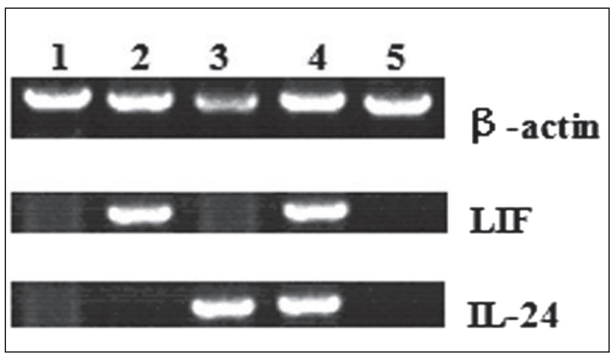
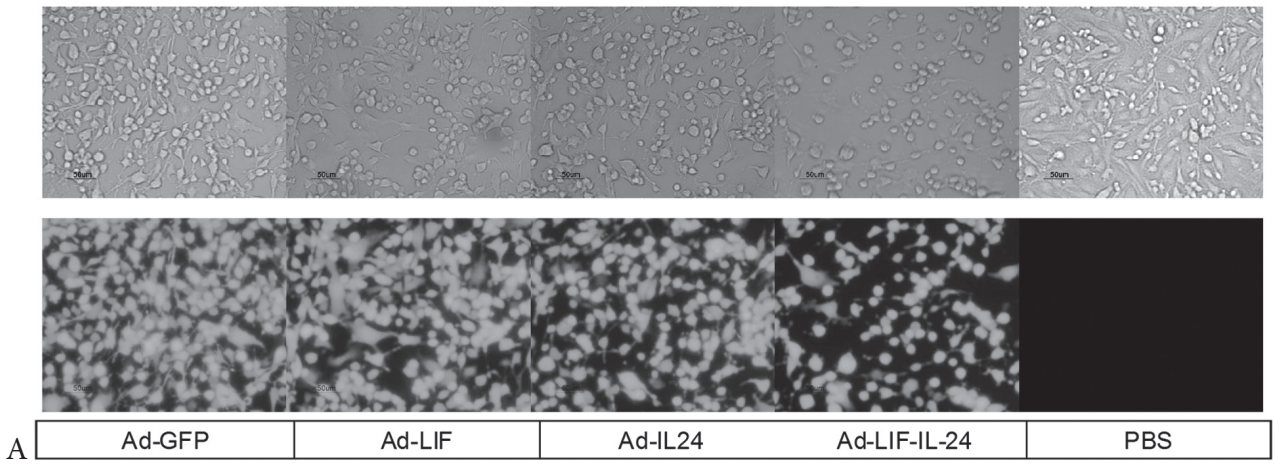


Figure 1. Transcription of LIF and IL-24 in vitro. Numbers 1-5 refer to Ad-GFP, Ad-LIF, Ad-IL-24, Ad-LIF-IL-24, and PBS, respectively. RT-PCR demonstrated the successful transcription of LIF and IL-24 in corresponding group.

Enhanced apoptosis by LIF and IL-24 co-expression

To explore the mechanism by which Ad-LIF-IL-24 synergistically inhibits U251 glioma cell growth, the apoptosis of U251 glioma cells treated with Ad-LIF, Ad-IL-24, Ad-LIF-IL-24, Ad (100 MOI) or PBS for 48 h was analyzed using Annexin V-PE (early apoptotic marker) and 7-AAD (late apoptotic marker) double staining by flow cytometry. As shown in Figures 3A and B, Ad-LIF-IL-24 treatment resulted in 59.4±3.6 % of U251 glioma cell apoptosis, whereas there was 1.6±0.3 %, 2.85±0.5 %, 35.4±3.0 % and 39.8±2.1 % apoptosis of U251 glioma cells when grown in the medium with PBS, Ad-GFP, Ad-LIF and Ad-IL-24, respectively. Compared with the Ad-GFP and PBS control groups, adenovirus-mediated LIF and/or IL-24 expression significantly induced the apoptosis of U251 human glioma cells ($p<0.05$). Moreover, the apoptosis rate of the Ad-LIF-IL-24 group was also significantly higher than that of the Ad-LIF and Ad-IL-24 groups ($p<0.05$) (Figure 3).



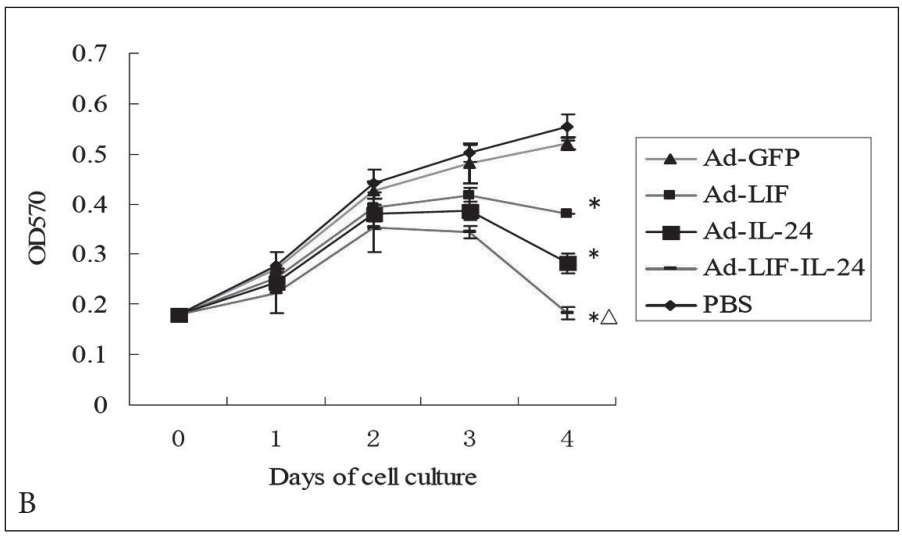


Figure 2. The in vitro cytotoxic effect of Ad-LIF-IL-24 on U251 human glioma cells. (A) U251 human glioma cells were treated with Ad-GFP, Ad-LIF, Ad-IL-24, and Ad-LIF-IL-24 and PBS, and were observed with differential interference contrast and fluorescence microscopy (×100) 48h later. (B) The survival cells were evaluated at days 0, 1, 2, 3 and 4 after treatment by MTT assay. *P<0.05 compared with the PBS and Ad-GFP groups; DP<0.05 compared with the Ad-LIF and Ad-IL-24 groups, two-way repeated measures ANOVA and multiple comparisons, n=4 replicates/condition.

Ad-LIF-IL-24 cooperatively regulates apoptotic pathways

To further address the underlying molecular mechanism responsible for Ad-LIF-IL-24- promoting apoptosis, the transcriptions of apoptosis-related genes including Bax, Bcl-2, and ICE in Ad-GFP, Ad-LIF-, Ad-IL-24-, Ad-LIF-IL-24- treated and untreated (PBS) U251 glioma cells were analyzed by RT-PCR. The transcriptions of Bax and ICE in the Ad-LIF, Ad-IL-24 and Ad-LIF-IL-24 groups were significantly increased; especially the transcriptions of ICE in the Ad-LIF-IL-24 group were significantly higher than in both Ad-LIF and Ad-IL-24 groups. Again, the transcriptions of Bcl-2 in the Ad- IL-24 and Ad-LIF-IL-24 groups were decreased (Figure 4A and B).

Discussion

Multigene-based combination therapy represents an effective practice in cancer gene therapy, which can achieve greater therapeutic benefit by targeting multiple pathways (15). Recent studies have reported that LIF as a tumor suppressor plays an important role in many cancer-related cellular processes including oncogenesis, apoptosis, and tumor angiogenesis, implying that it is a potent tumor suppressor for cancer therapy. Expanding studies have demonstrated that another tumor suppressor, IL-24 as a cytokine-tumor suppressor, can discriminate between normal and tumor cells, induce apoptosis, stimulate immune responses, promote bystander antitumor activity, and synergize with anticancer drugs and radiation, suggesting

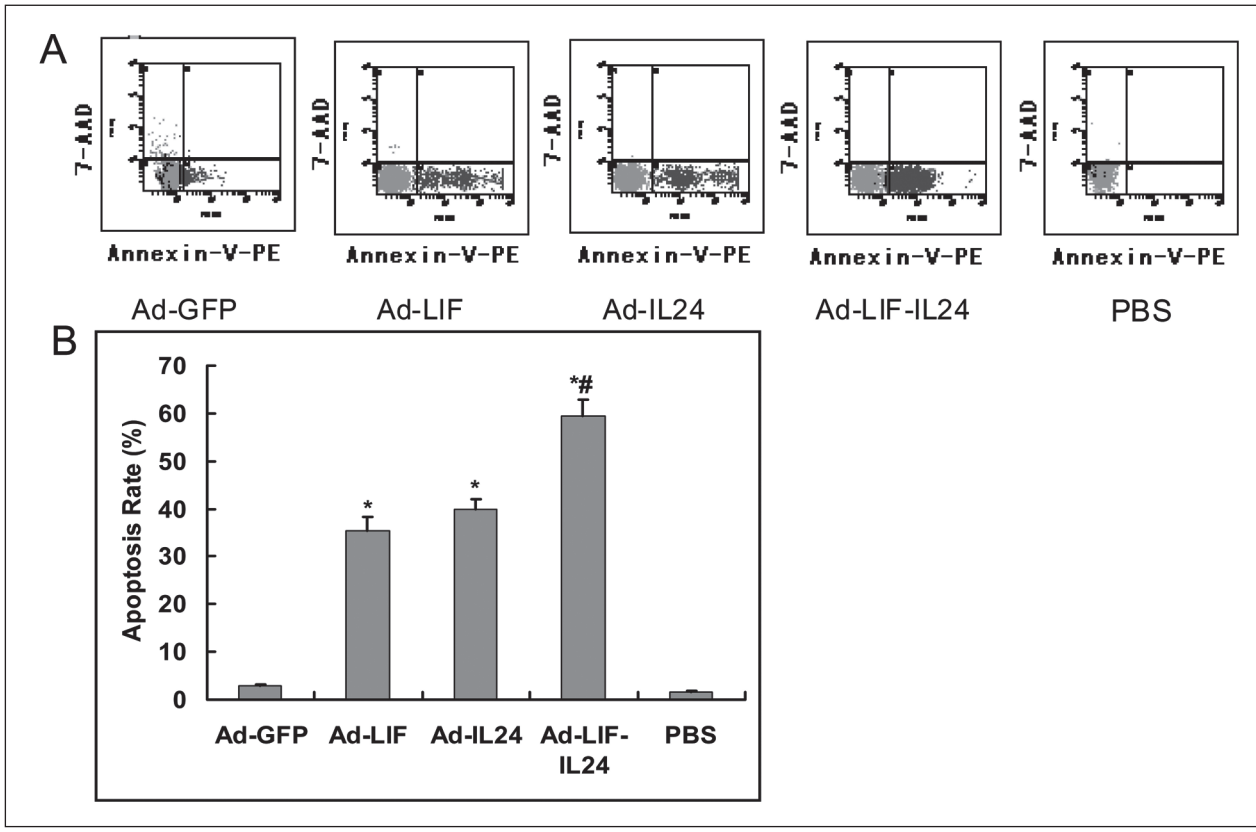


Figure 3. Enhanced induction of apoptosis in U251 glioma cells by Ad-LIF-IL-24. (A) The U251 glioma cells were treated with Ad-LIF, Ad-IL-24, Ad-LIF-IL-24 or Ad-GFP used as the Ad control at the optimal MOI of 100 or PBS as the control for 48 h, and apoptosis was then analyzed using Annexin V-PE/7-AAD double staining by flow cytometry. The Annexin V single-positive cells in the total cell population represented the apoptotic cells. (B) *P<0.05 compared with the PBS and Ad-GFP groups; #P<0.05 compared with the Ad-LIF and Ad-IL-24 groups, one-way repeated measures ANOVA and multiple comparisons, n=3 replicates/condition.

that it is also an effective agent for cancer treatment. However, no therapeutic effect of combination glioma treatment with LIF and IL-24 for cancer has yet been reported. Based upon the antitumor properties of LIF and IL-24, in this study we constructed an LIF/IL-24 biscistronic adenovirus harboring LIF and IL-24 double tumor suppressor genes (Ad-LIF-IL-24) and evaluated its combined therapeutic effect on U251 human glioma cells *in vitro*. Our results showed that combination treatment of Ad-mediated LIF and IL-24 co-expression induced in vitro synergistic growth suppression and apoptosis in U251 glioma cells.

Genes regulating apoptosis could be divided into apoptosis-related oncogenes and anti-oncogenes, one to promote apoptosis, such as Bax and ICE, and the other being anti-apoptotic, such as Bcl-2. The ratio between Bcl-2/Bax heterodimers and Bax/Bax homodimers appears to be pivotal in deciding the life or death of a cell (16). Bcl-2/Bax constitutes a rheostat that sets the threshold of susceptibility to apoptosis (17). Meanwhile, ICE appears to be involved in the regulation of apoptosis. Overexpression of ICE induces cell death (18, 19).

To elucidate the underlying mechanism involved in Ad-LIF-IL-24-mediated synergistic antitumor activity, the *in vitro* transcription of apoptosis-related proteins such as Bcl-2, Bax, and ICE in U251 human glioma cells was assessed by RT-PCR. Our evidence demonstrated that adenovirus-mediated LIF and IL-24 co-expression caused a cooperative and overlapping effect on upregulation of the apoptosis-promoting genes Bax and ICE, and downregulation of anti-apo-

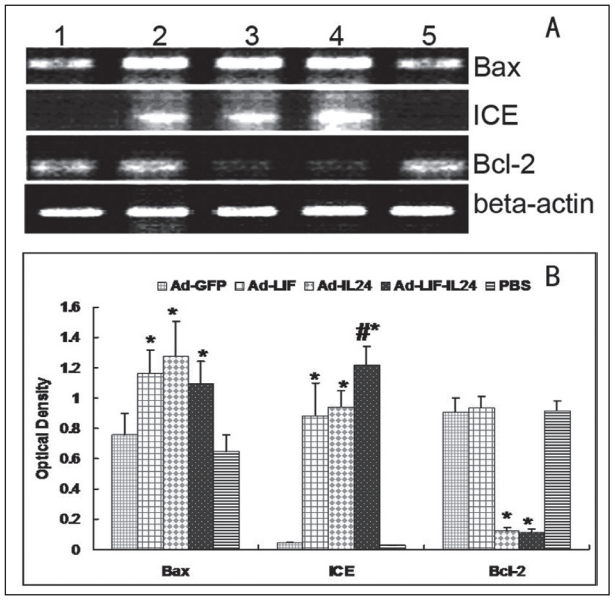


Figure 4. Ad-LIF-IL-24 modulates apoptosis-related molecules. (A) The transcription of Bax, ICE and Bcl-2 in U251 glioma cells was detected by RT-PCR. Lane 1: Ad-GFP; lane 2: Ad-LIF; lane 3: Ad-IL-24; lane 4: Ad-LIF-IL-24; lane 5: PBS. (B) Semiquantitative analysis of these gene transcriptions, *P<0.05 compared with the PBS and Ad-GFP groups; #P<0.05 compared with the Ad-LIF and Ad-IL-24 groups, one-way repeated measures ANOVA and multiple comparisons, n=3 replicates/condition.

ptotic gene Bcl-2 in U251 human glioma cells. These results may closely account for the Ad-LIF-IL-24-induced synergistic growth inhibition and apoptosis in U251 tumor cells.

Our research may provide a new idea for, and potential method of, gene therapy for gliomas by the combination of two anti-oncogenes. However, before any real application in patients, a lot of work, such as *in vivo* studies, and clinical research, still needs to be done. Meanwhile, the malignant progression of the glioma is the result of multiple gene changes, and if we can change three or more genes at a time, it may be possible to get an even better result.

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Received: 13.11.2013
Accepted: 17.1.2014
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