

Research article

**SILENCING OF THE TYPE 1 INSULIN-LIKE GROWTH FACTOR RECEPTOR INCREASES THE SENSITIVITY TO APOPTOSIS AND INHIBITS INVASION IN HUMAN LUNG ADENOCARCINOMA A549 CELLS**

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**Abstract:** The type 1 insulin-like growth factor receptor (IGF-1R), which is over-expressed or activated in many human cancers, including lung cancer, mediates cancer cell proliferation and metastasis. Several studies indicate that blocking IGF-1R expression can inhibit tumor cell proliferation and metastasis. In this study, inhibition of the endogenous IGF-1R by recombinant adenoviruses encoding short hairpin RNAs against IGF-1R was found to significantly suppress IGF-1R expression, arrest the cell cycle, enhance the apoptotic response, and inhibit proliferation, adhesion, invasion and migration in A549 cells. Moreover, silencing IGF-1R decreases the expression of invasive-related genes including matrix metalloproteinase-2 (MMP-2), MMP-9, and urokinase-plasminogen activator (u-PA), and the phosphorylation of Akt and ERK1/2. These results suggest that the silencing of IGF-1R has the potential to be an effective cancer gene therapy strategy for human lung cancer.

**Key words:** IGF-1R, Lung cancer, Short hairpin RNA, MMPs, Apoptosis, Metastasis

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Abbreviations used: ECM – extracellular matrix; HRP – horseradish peroxidase; IGF-1R – type 1 insulin-like growth factor receptor; McAb – monoclonal antibody; MMP – matrix metalloproteinase; NSCLC – non-small cell lung cancer; PcAb – polyclonal antibody; shRNA – short hairpin RNA; u-PA – urokinase-plasminogen activator

## INTRODUCTION

Lung cancer accounts for 32% of cancer deaths in men and 24% in women, and represents the leading cause of cancer death worldwide, independently of gender. Approximately 75-80% of lung cancers are of the non-small-cell (NSCLC) histology. The overall 5-year survival rate for NSCLC is only 8-14% when diagnosed, and this rate increases to 40% after complete surgical resection [1, 2]. Cancer mortality is related to the ability of tumor cell to proliferate, invade, and metastasize. Novel strategies for anticancer therapy to target these processes are the focus of much medical research.

The type 1 insulin-like growth factor (IGF-1R, GeneBank accession No.: NM\_000875 ) and its ligands IGF-I and IGF-II have been shown to play a central role in the regulation of the cell cycle, protection from apoptosis, transformation, tumor invasion, and metastasis. IGF-1R is a heterotetramer with tyrosine kinase activity consisting of two extracellular  $\alpha$  subunits and two transmembrane  $\beta$  subunits linked by disulfide bonds [3]. Binding of the ligands to IGF-1R leads to the activation of the intrinsic tyrosine kinase activity, which subsequently phosphorylates several intracellular substrates, including members of the insulin receptor substrate family and Shc, and triggers multiple signaling pathways, such as the MAPK (also called ERK) pathway, which is implicated in receptor-mediated mitogenesis and transformation, and the PI-3K/Akt (protein kinase B) pathway, implicated in the transmission of the cell survival signal [4, 5]. Recent studies have shown that IGF-1R is overexpressed in various human cancers, including lung cancer [6], and that a high level of expression of IGF-1R and IGF-I can act in an autocrine/paracrine manner [7]. Furthermore, overexpression or constitutive activation of IGF-1R causes ligand-dependent transformation of fibroblasts [8], plays a key role in the survival of transformed colonocytes [9], and leads to the development of tumors in nude mice [10]. Besides its role in tumorigenesis, IGF-1R protects tumor cells from apoptosis induced by chemotherapy, radiotherapy, or cytokine [11-13]. Moreover, not only are elevated levels of IGF-I associated with an increased risk in several cancers [14-16], including lung cancer, but IGF-I enhances tumor cell migration through interaction with integrins, modulates cell-cell attachment via crosstalk between the IGF axis and cell adhesion molecules such as E-cadherin [17, 18], and increases metastatic potential by constitutively activating IGF-1R [19]. The invasion of a tumor cell is a complex process requiring the proteolytic degradation of the extracellular matrix (ECM) barriers, which are lysed by specific proteinases. Of the involved proteinases, matrix metalloproteinase-2 (MMP-2), MMP-9 and urokinase-plasminogen activator (u-PA) are the most vital for the degradation of the base membrane [20-22]. In addition, the blockade of IGF-1R by monoclonal antibodies [23], dominant-negative mutants [3] or antisense oligonucleotides [24] against IGF-1R has been reported to suppress tumor cell growth, inhibit tumor cell metastasis, and induce the regression of

established tumors. Therefore, IGF-1R targeting is a potential therapy for human cancer treatment.

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing process in which double-strand RNA (dsRNA) induces potent and specific inhibition of eukaryotic gene expression via the degradation of complementary messenger RNA [25]. This novel approach has opened new avenues in cancer gene therapy. In this study, we developed recombinant adenoviral vectors encoding short hairpin RNAs (shRNA) that are then processed into small interfering RNAs (siRNA) by the Dicer enzyme against IGF-1R, and investigated their efficacy with regard to the suppression of IGF-1R expression in A549 cells and consequent anti-tumor potential.

## MATERIALS AND METHODS

### Materials and reagents

IGF-1R McAb (MS-645-P0) was purchased from LAB VISION. Caspase-3 PcAb (sc-1225), t-ERK McAb (sc-94), p-ERK McAb (sc-7383), t-Akt PcAb (sc-1619), p-Akt PcAb (sc-7985-R), MMP-2 PcAb (sc-1359), MMP-9 McAb (sc-6840) and human Actin PcAb (sc-1616), and horseradish peroxidase-conjugated goat anti-mouse (sc-2060) and goat anti-rabbit IgG-HRP IgG (sc-2004) antibodies were purchased from Santa Cruz Biotechnology (USA). u-PA McAb was purchased from Sigma (USA). The BLOCK-iT<sup>TM</sup> Adenoviral RNAi Expression System was from Invitrogen (USA).

### shRNA preparation and recombinant adenovirus construction

The siRNA-1-targeting IGF-1R cDNA at nucleotide (nt) 365~385 was found using BLOCK-IT<sup>TM</sup> RNAi Designer (at <https://rnaidesigner.invitrogen.com/rnaexpress/>). siRNA-2 (nt612~630) was derived from siRNA R2 [26], which was reported to effectively suppress IGF-1R expression. siRNA-luc (nt4413~4433) against the *Photinus pyralis* luciferase gene (GeneBank accession no.: X65324) was used as the control. As shown in Tab. 1, all of the shRNAs were generated using the chemical synthesis method of Sangon Inc. (Shanghai, China), and cloned into the pENTR<sup>TM</sup>/U6 vector under the control of the human U6 promoter (BLOCK-iT<sup>TM</sup> U6 RNAi Entry Vector Kit, Invitrogen) according to the manufacturer's instructions. The positive entry clones were confirmed by DNA sequencing, and the resulting pENTR<sup>TM</sup>/U6 vector and the E1- and E3-deleted pAd/BLOCK-iT<sup>TM</sup>-DEST expression vector underwent a homologous recombination reaction to generate pAd/BLOCK-iT<sup>TM</sup> expression constructs as per the manufacturer's protocol. The positive expression constructs were confirmed by sequencing, and transfected into 293A cells by lipofection using Lipofectamine2000 reagent (Invitrogen) to produce recombinant adenoviruses containing the U6 RNAi cassette of the corresponding target (pAd-GW/U6-IGF-1R-1, pAd-GW/U6-IGF-1R-2, and pAd-GW/U6-luc). Viral titers were determined by plaque assays.

Tab. 1. Oligonucleotide sequences contained in the pAd/BLOCK-iT™ expression constructs.

Name	shRNA sequence	Corresponding siRNA sequence
pAd-GW/ U6-IGF-1R-1	5'-CACCGCGCTGGAAACTCTTCTA CACGAATGTAGAAGAGTTTCCAGCC GC-3'	siRNA-1(nt365~385): 5'-GCGGCTGGAAACTCTTCTACA-3'
pAd-GW/ U6-IGF-1R-2	5'-CACCGCCGATGTGTGAGAAGACC TTCAAGAGAGGTCTTCTCACACATC GGC-3'	siRNA-2(nt612~630): 5'-GCCGATGTGTGAGAAGACC-3'
pAd-GW/ U6-luc	5'-CACCGCTCACCGGCTCCAGATTT ATCGAAATAAATCTGGAGCCGGTGA GC-3'	siRNA-luc(nt4413~4433): 5'-GCTCACCGGCTCCAGATTTAT-3'

### Cell culture and transduction

The human lung adenocarcinoma A549 cells, which are one of the types of non-small cell lung cancer cell, were a gift from Dr. Xy-Lu. They were maintained in RPMI 1640 medium supplemented with 10% newborn bovine serum (NBS), at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. For all the infection experiments, A549 cells were plated in a 6-well plate at a concentration of  $6 \times 10^5$  cells/well for 16 hours, and then transduced with each adenoviral construct at an MOI of 10. These were incubated in complete medium at 37°C overnight. The medium containing the virus was replaced with fresh RPMI 1640 medium with 10% NBS, and cells were harvested for assay after 72 hours of transduction.

### Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from the cells with TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and stored at -80°C until use. Four micrograms of total RNA were reverse-transcribed to the first strand of cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, USA). The primers for PCR amplification were: IGF-1R, forward 5'-AAATGTGCCCGAGCGTGTG-3' and reverse 5'-TGCCCTTGAAGATGGTGCATC-3'; MMP-2, forward 5'-CACTTTCCTGGGCAACAAAT-3' and reverse 5'-TGATGTTCATCCTGGGACAGA-3'; MMP-9, forward 5'-CTGGGCTTAGATCATTCCTCA-3' and reverse 5'-AGTACTTCCCATCCTTGAACAAATA-3'; u-PA, forward 5'-TTGCGCCATCTACAGGAG-3' and reverse 5'-ACTGGGGATCGTTATACATC-3'; and human  $\beta$ -actin (internal control), forward 5'-TTCCAGCCTTCCTTCTGGG-3' and reverse 5'-TTGCGCTCAGGAGGAGCATT-3'. The PCR conditions were: 94°C for 3 min; denaturation at 94°C for 30 s; annealing at 53.5°C for 30 s for IGF-1R and  $\beta$ -actin, for 50 s at 55°C for MMP-2, for 30 s at 55°C for MMP-9, and for 60 s at 56°C for u-PA; extension for 60 s at 72°C, for 30 s for MMP-9, with over 25 cycles for  $\beta$ -actin, 30 cycles for MMP-9, 35 cycles for IGF-1R and MMP-2, and 40 cycles for u-PA; and a final extension at 72°C for 10 min. The PCR products were visualized with ethidium bromide on 2% agarose gels.

**Western blot analysis**

At 72 hours post transduction, A549 cells were lysed on ice for 10 min in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1.2% Triton-X114, 1 mM NaF, 200  $\mu$ M NaVO<sub>4</sub>) that contained 1 complete Mini Protease Inhibitor Cocktail Tablet (Roche). The protein concentrations were determined using the BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). One hundred micrograms of total cellular proteins (30  $\mu$ g for caspase-3 and  $\beta$ -actin) were separated on 10% SDS-polyacrylamide gels, and then transferred to polyvinylidene difluoride (PVDF) membranes (Hybond-P, Amersham, USA). After the non-specific binding sites were blocked by incubating the membranes in TBS-0.1% Tween20 with 5% skimmed milk (PBS-1% Tween-20 without skimmed milk for IGF-1R) for 0.5 hours at room temperature, the membranes were probed with primary antibodies (1:200 dilutions for IGF-1R, MMP-2, MMP-9 and u-PA; 1:400 dilution for caspase-3; 1:1000 dilution for  $\beta$ -actin) for 2 hours (3 hours for IGF-1R) at room temperature, and then washed three times with TBS-T (PBS-T for IGF-1R). The membranes were then incubated with HRP-conjugated goat anti-mouse (or goat anti-rabbit) IgG (1:2500 dilution) for 1 hour at room temperature, and washed three times with TBS-T (PBS-T for IGF-1R). The blots were developed according to the Chemiluminescence protocol (Roche), and analyzed using Scion Image Software.  $\beta$ -actin was used as an internal control.

The effects of pAd-GW/U6-IGF-1R-1 on Akt and ERK phosphorylation were analyzed as described previously, with some modification [27]. Briefly, A549 cells were transduced with pAd-GW/U6-IGF-1R-1 and pAd-GW/U6-luc. After 48 hours, the cells were starved in serum-free medium for 24 hours and stimulated by serum for 30 min at 37°C. Cell extracts were separated on 10% SDS-polyacrylamide gels and immunoblotted with t-Akt, p-Akt, t-ERK or p-ERK McAbs (1:200 dilution) for 2 hours at room temperature. Horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG antibodies (1:2500 dilution) were used as the secondary antibody for an hour at room temperature, visualized by ECL, and analyzed using Scion Image Software.

**DNA laddering assay**

A549 cells were seeded into 6-well plates for 16 hours, infected with pAd-GW/U6-IGF-1R-1 and pAd-GW/U6-luc, and after 72 hours of transduction, apoptosis was induced by 3% ethanol for 6 hours. Total DNA was extracted from each well using the apoptotic DNA ladder kit (AxyPrep Multisource Genomic DNA Miniprep Kit, Axygen Biosciences) according to the manufacturer's instructions, and the extracted DNA was separated by 2% (w/v) agarose gel electrophoresis in order to analyze the internucleosomal DNA cleavage.

**Cell cycle analysis**

To analyze the cell cycle, cells were removed by trypsinization 72 hours after adenovirus infection, washed in PBS, and fixed in 70% ice-cold ethanol for 2 hours. The fixed samples were centrifuged, treated with 1 mg/ml RNase solution (Sigma) for 30 min at 37°C, and resuspended in 0.1 mg/ml propidium iodide (PI) solution (Sigma) at 4°C for 1 hour. PI-stained cells were analyzed with a flow cytometer (Beckman Coulter).

**Cell proliferation, measured using the MTT assay**

The MTT assay was performed to evaluate the effects of pAd-GW/U6-IGF-1R-1 on cell proliferation. A549 cells were seeded in a 96-well plate at a concentration of  $4 \times 10^3$  cells/well for 16 hours, and then infected with pAd-GW/U6-IGF-1R-1 and pAd-GW/U6-luc. Thereafter, 20  $\mu$ l/well MTT solution (5 mg/ml; Sigma, USA) was added at 0, 24, 48, and 72 hours post transduction at 37°C for 4 hours. The media and MTT were removed, dimethyl sulfoxide was added for 10 minutes, and absorbance was measured at 570 nm. All the experiments were performed in triplicate.

**Cell adhesion, invasion, and migration assays**

An *in vitro* cell adhesion assay was performed according to the method of Zhang *et al.* [28] with some modification. Briefly, A549 cells were transduced with pAd-GW/U6-IGF-1R-1 and pAd-GW/U6-luc. After 72 hours, the cells were harvested by trypsinization and added to flat bottom 96-well plates that had been precoated with Matrigel (30  $\mu$ g/well) at a concentration of  $1 \times 10^5$  cells/100  $\mu$ l and incubated for 2 hours. Non-adherent cells were carefully removed by PBS washes, and adherent cells were fixed in 100% methanol, stained, and counted under a light microscope (Olympus, Japan).

The *in vitro* invasive ability of cells was tested in the transwell system (Corning Inc., USA). Polycarbonate membrane filters with 8.0  $\mu$ m pores and Matrigel coating served as the extracellular matrix, and were dried in a laminar hood overnight. Before performing the invasive assay, the inserts were rehydrated with 100  $\mu$ l of PBS at 37°C for 2 hours. Afterwards, infectant cells were seeded in the upper compartment at a density of  $1 \times 10^5$  cells/well in 50  $\mu$ l of SFM. The lower compartments were filled with 400  $\mu$ l conditioned medium derived from NIH3T3 cells. After 24 hours incubation at 37°C in a humidified atmosphere of 5% carbon dioxide, the cells that had invaded the lower surface of the membrane were fixed with 100% methanol for 10 min and stained with 0.2% Crystal Violet solution for 5 min. The number of invaded cells were counted in random fields under a microscope.

To quantify cell migration, cells were seeded into inserts on membrane filters which were not coated with Matrigel. The migration of infectant cells was calculated in the same way as for the invasion assay. All the experiments were performed in triplicate.

### Statistical analysis

All the quantitative data is presented as means  $\pm$  SD. The statistical significance of differences was determined using Student's two-tailed t test in two groups, and one-way ANOVA in multiple groups. A P value  $< 0.05$  was considered statistically significant. All the data was analyzed with the SPSS13.0 software.

## RESULTS

### Transduction of shRNA against IGF-1R inhibits IGF-1R expression in A549 cells

To suppress IGF-1R expression, we constructed two recombinant adenoviruses targeting IGF-1R, and the control adenovirus against the luciferase gene, and transduced these separately into A549 cells. 72 hours later, we estimated the expression of IGF-1R in the transduced cells. RT-PCR analysis (Fig. 1A and B) demonstrated that the IGF-1R mRNA expression in cells infected with pAd-GW/U6-IGF-1R-1 and pAd-GW/U6-IGF-1R-2 was respectively  $11.1 \pm 1.6\%$  and  $63.8 \pm 3.2\%$  of that for those transduced with pAd-GW/U6-luc ( $P < 0.05$ ). Similarly, the immunoblot analysis (Fig. 1C) showed that the expression of IGF-1R protein in A549 cells transduced with shRNA expressing adenoviruses targeting IGF-1R was strongly inhibited. Densitometric analysis (Fig. 1D) revealed that the amounts of IGF-1R protein remaining in cells transduced with

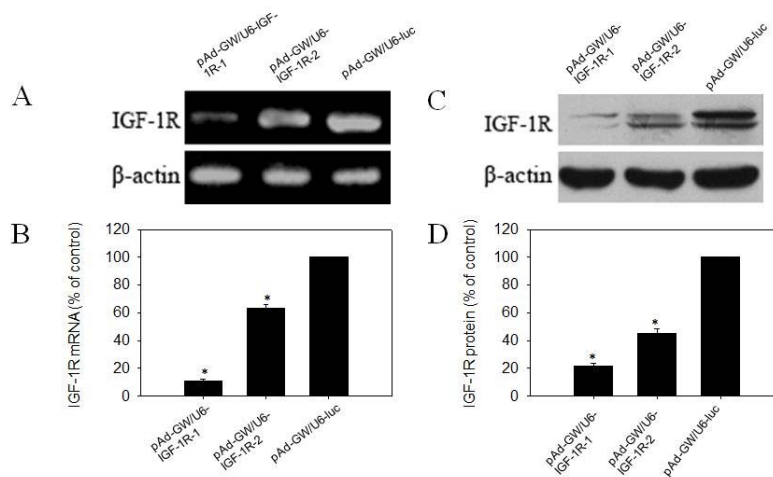


Fig. 1. The effects of adenovirus-based shRNA against IGF-1R on the expression of IGF-1R in human lung cancer A549 cells. A549 cells infected with adenovirus-based shRNA were subjected to semi-quantitative RT-PCR (A) and Western blot (C) with  $\beta$ -actin as a loading control. The respective expressions of IGF-1R in cells transduced with pAd-GW/U6-IGF-1R-1 and pAd-GW/U6-IGF-1R-2 were  $11.1 \pm 1.6\%$  and  $63.8 \pm 3.2\%$  at the mRNA level (B), and  $20.1 \pm 2.4\%$  and  $44.8 \pm 3.4\%$  at the protein level (D) compared with that of cells infected with pENTR/U6-shRNA-luc. The data is the means  $\pm$  SD from three independent experiments with the level of IGF-1R expression in the control set to 100% (\* $P < 0.05$ ).

pAd-GW/U6-IGF-1R-1 and pAd-GW/U6-IGF-1R-2 were respectively  $20.1 \pm 2.4\%$  and  $44.8 \pm 3.4\%$  of that found in cells infected with the control virus pAd-GW/U6-luc ( $P < 0.05$ ). These results indicate that the recombinant adenovirus expressing shRNA against IGF-1R can effectively suppress the IGF-1R expression. pAd-GW/U6-IGF-1R-1 proved to be more potent than pAd-GW/U6-IGF-1R-2 in the above analyses, so we used pAd-GW/U6-IGF-1R-1 in the subsequent experiments.

#### Down-regulation of IGF-1R blocks A549 cells in $G_0/G_1$

To determine the apoptosis-inducing potential of pAd-GW/U6-IGF-1R-1 in A549 cells, flow cytometric analysis of propidium iodide-stained cells was performed. The percentage of cells in the  $G_0/G_1$  population after infection with pAd-GW/U6-IGF-1R-1 ( $78.5 \pm 1.5\%$ ) was much higher than that observed after infection with pAd-GW/U6-luc ( $46.9 \pm 1.2\%$ ) (Fig. 2A,  $P < 0.05$ ).

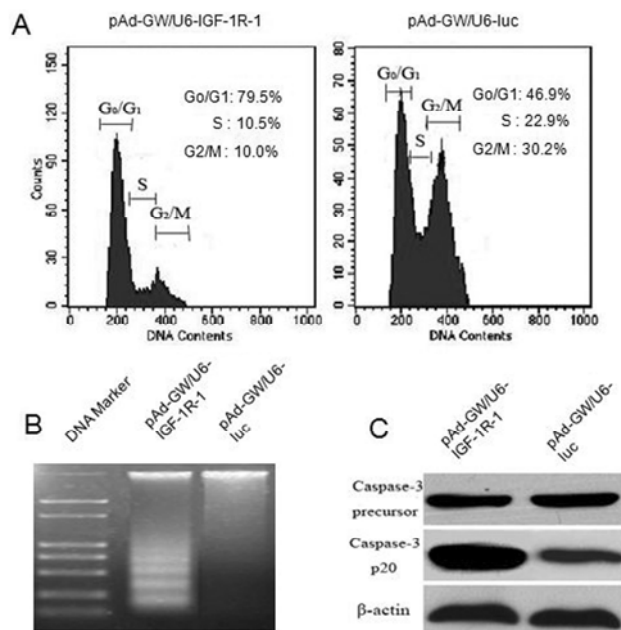


Fig. 2. The effects of the RNAi knockdown of IGF-1R expression on the cell cycle and apoptosis in A549 cells. A – Flow cytometric analysis of propidium iodide-stained cells. The percentage of cells in the  $G_0/G_1$  population after infection with pAd-GW/U6-IGF-1R-1 ( $78.5 \pm 1.5\%$ ) was much higher than that observed after infection with pAd-GW/U6-luc ( $46.9 \pm 1.2\%$ ). The experiments were performed in triplicate, and the representative data is shown. B – The DNA fragmentation assay showed that ethanol-induced apoptosis was much more apparent in cells after infection with pAd-GW/U6-IGF-1R-1 than in the control. C – The immunoblot analysis for caspase-3 in IGF-1R knockdown A549 cells detected caspase-3 activation in the cells after the transduction with pAd-GW/U6-IGF-1R-1.  $\beta$ -actin was used as a loading control. Representative immunoblots are shown.



### IGF-1R gene silencing increases ethanol-induced apoptosis and up-regulates the cleavage of caspase-3 in A549 cells

The DNA fragmentation analysis yielded evidence of apoptotic induction. As shown in Fig. 2B, after infection with pAd-GW/U6-IGF-1R-1, A549 cells stressed by 3% ethanol showed enhanced DNA laddering, a typical hallmark of apoptosis, on agarose gel electrophoresis, in comparison to pAd-GW/U6-luc transduced cells. This data suggests that inhibiting IGF-1R expression elicits apoptosis in A549 cells. In addition, an immunoblot analysis was carried out for caspase-3, which is an executioner caspase, in order to confirm that the apoptosis induced by pAd-GW/U6-IGF-1R-1 was via the activation of caspase-3. The analysis revealed that the cleaved fragment of caspase-3 (p20) in the cells infected with pAd-GW/U6-IGF-1R-1 was up-regulated compared with the control, as would be expected upon the activation of caspase-3 (Fig. 2C).

### Blockage of IGF-1R expression inhibits the proliferation, adhesion, invasion and migration of A549 cells

To investigate whether the above-mentioned apoptosis-inducing potential of pAd-GW/U6-IGF-1R-1 might result in the suppression of tumor cell growth *in vitro*, MTT analysis was performed at 0, 24, 48, and 72 hours post transduction (Fig. 3A). It was revealed that cells infected with pAd-GW/U6-IGF-1R-1 exhibited a markedly reduced proliferation capacity *in vitro*, whereas infection with pAd-GW/U6-luc yielded a minimal change in terms of cell growth. The percentages of viable cells at 48 and 72 hours after infection were respectively  $212 \pm 6.5\%$  and

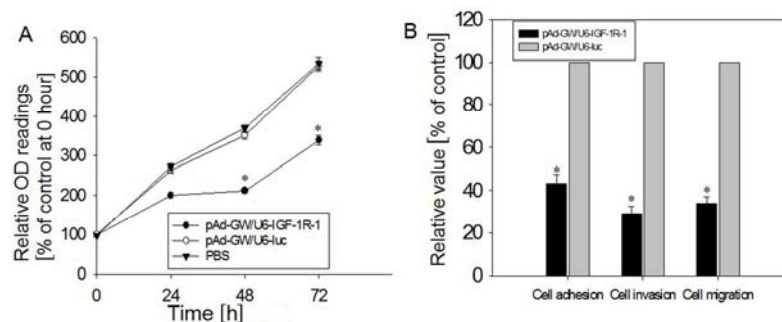


Fig. 3. The inhibitory effects of IGF-1R gene knockdown on the proliferation, adhesion, invasion and migration of A549 cells. A – The proliferation of A549 cells transduced with either the shRNA adenovirus targeting IGF-1R or the control was evaluated via the MTT assay at 0, 24, 48, and 72 hours post transduction. pAd-GW/U6-IGF-1R-1 significantly reduced *in vitro* growth in A549 cells. B – At 72 hours post transduction, cells infected with either the shRNA adenovirus targeting IGF-1R or the control were then subjected to analyses for the adhesion, invasion and migration of A549 cells as described in the Materials and methods section. The adhesion, invasion and migration of A549 cells infected with pAd-GW/U6-IGF-1R-1 were respectively reduced to  $43 \pm 5\%$ ,  $29 \pm 4\%$ , and  $34 \pm 3\%$  of the control. The data is the means  $\pm$  SD from three independent experiments with the control set as 100% (\* $P < 0.05$ ).

$339 \pm 11.7\%$ , as compared with the control cells ( $351 \pm 8.3\%$  and  $529 \pm 13.4\%$ ) ( $P < 0.05$ ), when the percentage of control cells was set as 100% at 0 hours.

We also analyzed the effects of pAd-GW/U6-IGF-1R-1 transduction on the adhesion, invasion and migration of A549 cells. When compared with the cells infected with pAd-GW/U6-luc, the pAd-GW/U6-IGF-1R-1-transduced cells showed a substantial reduction in cell adhesion, invasion and migration (Fig. 3B). The adhesion, invasion and migration of A549 cells infected with pAd-GW/U6-IGF-1R-1 were respectively reduced to  $43 \pm 5\%$ ,  $29 \pm 4\%$ , and  $34 \pm 3\%$  of the control ( $P < 0.05$ ). These results indicate that IGF-1R expression is required for A549 cell proliferation as well as invasion, and that adenovirus-mediated silencing of IGF-1R expression can suppress the proliferation, adhesion, invasion and migration of A549 cells.

#### **The adenovirus encoding shRNA against IGF-1R decreases MMP-2, MMP-9 and u-PA gene expression**

In the cell migration and invasion processes, ECM degradation is substantial. To clarify whether invasive-related genes, including MMP-2, MMP-9 and u-PA, were involved in the inhibition of the adhesion, invasion and migration of A549 cells after infection with the adenovirus encoding shRNA against IGF-1R, RT-PCR and western blot were performed at 72 hours post transduction. As shown in Fig. 4A, the analysis of the expression of MMP-2, MMP-9 and u-PA in cells infected with pAd-GW/U6-IGF-1R-1 via RT-PCR demonstrated a specific reduction in mRNA levels for each gene relative to the control, respectively  $17.5 \pm 2.1\%$ ,  $14.3 \pm 1.5\%$ , and  $23.4 \pm 2.6\%$  of the control (Fig. 4B,  $P < 0.05$ ). A similar trend was observed using the immunoblot assay for protein levels (Fig. 4C). Densitometric analysis (Fig. 4D) showed that the amounts of MMP-2, MMP-9 and u-PA protein remaining in the cells transduced with pAd-GW/U6-IGF-1R-1 were respectively  $45.4 \pm 2.4\%$ ,  $48.9 \pm 2.8\%$ , and  $43.4 \pm 1.9\%$  of those for cells infected with the control virus pAd-GW/U6-luc ( $P < 0.05$ ). These results indicate that MMP-2, MMP-9 and u-PA are involved in the inhibition of the adhesion, invasion and migration of A549 cells, and that their levels are decreased by the down-regulation of IGF-1R expression.

#### **Suppression of IGF-1R expression by the adenovirus expressing shRNA against IGF-1R blocks its downstream signaling through Akt and ERK1/2**

The activation of IGF-1R results in the activation of the phosphatidylinositol 3-kinase target Akt and the phosphorylation of ERK1/2 of the mitogen-activated protein kinase pathway. To further explore the effects of IGF-1R gene silencing on its downstream signaling pathways, immunoblot assays for total and phosphorylated Akt and Erk1/2 were performed. As shown in Fig. 5, the analysis revealed that the levels of phosphorylated Akt and ERK1/2 in serum-starved cells infected with pAd-GW/U6-IGF-1R-1 were significantly decreased after the cells were treated with serum, whereas the total Akt and ERK1/2 levels were similar, compared with the control. This data suggests that IGF-1R gene silencing can block downstream signaling via the deactivation of Akt and ERK1/2.

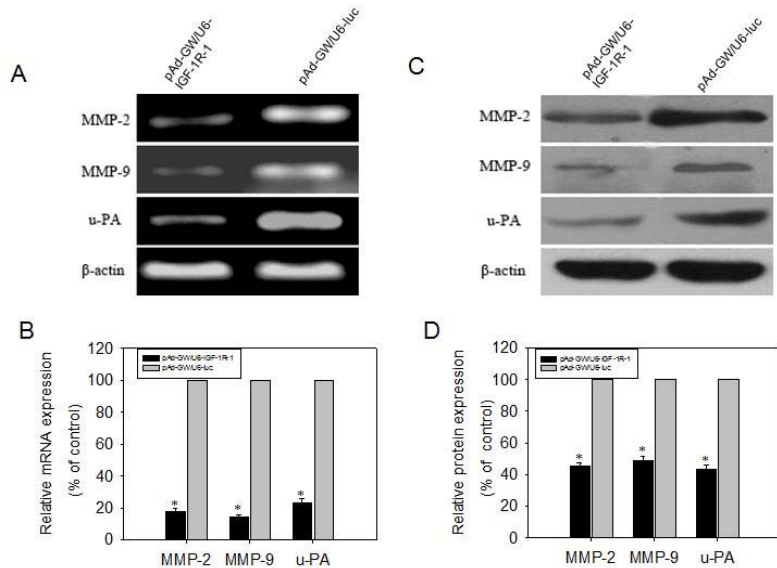


Fig. 4. Down-regulation of IGF-1R expression by RNAi reduces MMP-2, MMP-9 and u-PA gene expression. A549 cells infected with pAd-GW/U6-IGF-1R-1 were subjected to semi-quantitative RT-PCR (A) and Western blot (C) for MMP-2, MMP-9 and u-PA gene expression at 72 hours post transduction, with  $\beta$ -actin as a loading control. The respective expressions of MMP-2, MMP-9 and u-PA in cells transduced with pAd-GW/U6-IGF-1R-1 were  $17.5 \pm 2.1\%$ ,  $14.3 \pm 1.5\%$ , and  $23.4 \pm 2.6\%$  at the mRNA level (B), and  $45.4 \pm 2.4\%$ ,  $48.9 \pm 2.8\%$ , and  $43.4 \pm 1.9\%$  at the protein level (D) compared with cells infected with pENTR/U6-shRNA-luc. The data is the means  $\pm$  SD from three independent experiments with the control set as 100% (\* $P < 0.05$ ).

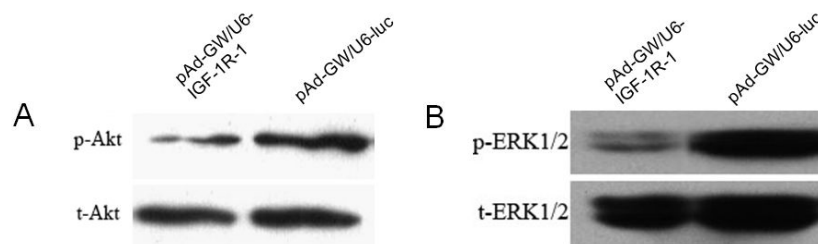


Fig. 5. The suppression of IGF-1R expression effectively blocks serum-induced activation of Akt (A) and ERK1/2 (B). Immunoblot assays for total and phosphorylated Akt and ERK1/2 revealed that the levels of phosphorylated Akt and ERK1/2 in serum-starved cells infected with pAd-GW/U6-IGF-1R-1 were significantly decreased after the cells were treated with serum, whereas the total Akt and ERK1/2 levels were similar, compared with the control. The results are representative of triplicate experiments.

## DISCUSSION

In this study, we constructed recombinant adenoviral vectors encoding shRNA against IGF-1R, which can significantly inhibit the IGF-1R expression in A549 cells. The blockade of IGF-1R expression by IGF-1R gene silencing not only arrests A549 cells in G<sub>0</sub>/G<sub>1</sub> and enhances the apoptotic response, but also inhibits proliferation, adhesion, invasion and migration in A549 cells. Moreover, silencing IGF-1R decreases the expression of invasive-related genes including MMP-2, MMP-9 and u-PA, and the phosphorylation of Akt and ERK1/2.

A number of experimental strategies have been employed to blunt IGF-1R function or expression. For instance, using  $\alpha$ -IR3, a monoclonal antibody to IGF-1R, to block IGF-1R signaling significantly inhibited Ewing's sarcoma cells *in vitro*, and induced the regression of established tumors [29]. Genetic blockage can be accomplished using an antisense oligonucleotide [24], and vectors [30] expressing antisense IGF-1R have been shown to inhibit cell growth, suppress tumorigenesis, alter the metastatic potential, and prolong survival *in vivo*. Another approach is to utilize dominant-negative mutants to inhibit the function rather than the expression of the naturally expressed receptor. Using mutant receptors for IGF-1R that contain a portion of the molecule including only the extracellular domain or the extracellular domain with a mutant or deleted intracellular tyrosine kinase domain resulted in the induction of differentiation and the inhibition of adhesion, invasion and metastasis [3, 27, 31]. However, the stable induction and delivery efficacy of antisense, dominant-negative plasmids or the use of antisense oligonucleotides seem to be critical limiting factors for practical cancer gene therapy. RNAi has been shown to be superior to the antisense approach, in that only a few molecules of dsRNA per cell can trigger gene silencing [32], and the siRNA duplex is more stable in cells than the cognate single-stranded sense or antisense RNA with transcription under the control of an identical promoter [33]. Recently, siRNAs expressed by vectors against IGF-1R have been employed by our group. They effectively inhibit the growth of human lung cancer cell line A549 *in vitro* and in nude mice [34]. Adenoviral vectors are considered to be a powerful tool in cancer gene therapy because they have been shown to transduce genes efficiently into many types of cancer cells. In this study, we demonstrated that adenoviral vectors encoding shRNA against IGF-1R significantly suppress IGF-1R expression, the inhibitory efficacy of which is up to nearly 80-90%, elicit cell apoptosis, and inhibit proliferation, adhesion, invasion and migration in A549 cells. Our results, consistent with previous studies, suggest that this strategy is promising for the treatment of human lung cancer.

It has been reported that at least two distinct signaling pathways are involved in the proliferative and anti-apoptotic activities of IGF-1R and its ligand. Both Akt and MAPKs (ERK1/2, and p38) are highly activated in several cancers [4, 35]. We showed that silencing of IGF-1R expression significantly inhibits serum-stimulated activation of Akt and ERK1/2. This result is in agreement with

previous observations that antisense oligodeoxynucleotides to IGF-1R inhibited breast cancer growth via the inactivation of the PI-3K/Akt and p42/p44 MPAK signaling pathways [36]. By contrast, another study demonstrated that the dominant-negative receptors of IGF-1R blocked the phosphorylation of Akt-1 and p38, but not of ERK1/2, indicating that IGF derived growth signals through the PI-3K/Akt-1 pathway [4]. In addition, recent evidence showed that the adhesion to fibronectin and migration of IGF-1-induced human multiple myeloma cells are achieved only when PI-3K/Akt is activated [37]. A truncated receptor to inhibit the activation of endogenous IGF-1R by IGF-1 inhibits the IGF-1-stimulated phosphorylation of Akt in the metastatic process of human cancer cells, whereas the ERK1/2 of the mitogen-activated protein kinase pathway are constitutively active, suggesting that the MAPK pathway is not involved in metastatic processes regulated by IGF-1 [19]. This data indicates that the PI-3K/Akt and MAPK pathways are independent, and that the PI-3K/Akt pathway is involved in the anti-apoptotic and metastatic activities of IGF-1R and its ligand, and that the role of the MAPK pathway in the anti-apoptotic and metastatic activities of IGF-1R needs to be investigated further.

In many types of neoplasm, including lung cancer, a high level of expression of MMP-2, MMP-9 and u-PA has been found to correlate with the migration of cancer cells through the digested extracellular matrix and to contribute to tumor cell invasion and metastasis, and may give prognostic information independent of stage [38-40]. However, suppression of MMP-9 and u-PA impairs tumor cell migration, invasion, survival and tumorigenicity [41, 42]. Our results showed that silencing IGF-1R decreases the expression of invasive-related genes such as MMP-2, MMP-9 and u-PA. A recent study also showed that blocking IGF-1R has an inhibitory effect on MMP-2 transcripts and protein synthesis in androgen-independent PC-3 cells, suggesting that IGF-1R can regulate the expression of MMP-2 [43]. Another paper reported that IGF-1R can dually regulate MMP-2 expression via the PI-3K/Akt and MAPK/ERK signaling pathways [5]. This means that in addition to its growth modulating effects, the IGF/IGF-1R system can also affect the invasive/metastatic potential via the collagenolytic activity of cancer cells.

In summary, our results provide further evidence that IGF-1R targeting is a potential therapy for human lung cancer, and that RNAi is a powerful alternative to genetic tools such as antisense oligonucleotides, dominant-negative mutants, and ribozyme technologies to reduce target gene expression. Our results support the therapeutic potential of RNAi as a method for lung cancer gene therapy.

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