

Lentivirus-mediated RNAi knockdown of insulin-like growth factor-1 receptor inhibits growth, reduces invasion, and enhances radiosensitivity in human osteosarcoma cells

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Abstract The type 1 insulin-like growth factor receptor (IGF-1R) is essential for tumorigenicity, tumor proliferation, and protection from apoptosis. IGF-1R overexpression has been found in many human cancers including osteosarcoma. To explore its possibility as a therapeutic target for the treatment of osteosarcoma, lentivirus-mediated siRNA was employed to downregulate endogenous IGF-1R expression to study the function of IGF-1R in tumorigenesis and radioresistance of osteosarcoma cells. The IGF-1R expression was persistently and markedly reduced by lentivirus-mediated RNAi. Downregulation of IGF-1R expression in osteosarcoma cells significantly suppressed their growth rates *in vitro* and reduced the potential of tumorigenicity *in vivo*. Moreover, the specific downregulation arrested cells in G₀/G₁ phase of cell cycle and also induced apoptosis which correlated with the activation of

Caspase-3. Furthermore, we also observed that suppression of IGF-1R could reduce the invasiveness of osteosarcoma cells and enhance their radiosensitivity. Our study suggested that lentivirus-mediated RNAi silencing targeting IGF-1R could induce potent antitumor activity and radiosensitizing activity in human osteosarcomas.

Keywords Insulin-like growth factor-1 receptor · RNA interference · Growth · Invasion · Radiosensitivity · Osteosarcoma

Introduction

Osteosarcoma (also called osteogenic sarcoma) is the most common type of malignant bone cancer, which accounts for approximately 35% of primary bone malignancies [1]. For osteosarcoma, the most common pathologic subtype is conventional central osteosarcoma and the other subtypes are much less common, each occurring at a frequency of less than 5%. This kind of tumor is characterized by a relatively poorer prognosis, a high propensity for metastasis and resistance to chemotherapy or radiotherapy [2, 3]. Despite great advances made in surgery, radiotherapy, and chemotherapy, the long-term survival rates of patients with osteosarcoma have not significantly improved and many patients still died of this disease [4]. Therefore, a better understanding of the molecular mechanisms involved in osteosarcoma formation and development should be helpful to find novel therapeutic targets and develop new modalities of the treatment of human osteosarcoma.

The insulin-like growth factor-1 receptor (IGF-1R), a heterotetrameric plasma membrane glycoprotein, is composed of two α -subunits and two β -subunits linked by disulfide bonds [5]. Once ligand binds to the IGF-1R, it will

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induce activation of the intrinsic tyrosine kinase of the β -subunit, leading to phosphorylation of IGF-1R and activation of protein kinases [6]. Some studies have showed that the IGF-1R plays a fundamental role in cell growth control and tumorigenesis and is an important inhibitor of apoptosis [7, 8]. Highly upregulated IGF-1R expression was found in a variety of human malignancies such as non-small-cell lung cancer, gastrointestinal stromal tumors, cervical carcinoma, uveal melanoma, breast and prostate cancer [9–14]. Previously, Turner et al. showed that IGF-1R overexpression mediated cellular radioresistance of breast cancer which was associated with the activation of IGF-1R-initiated pathways. Moreover, we and other groups have demonstrated that the overexpression of IGF-1 receptor contributes to the malignant phenotype in human osteosarcoma, but the importance of IGF-1R overexpression with regard to tumor proliferation, survival, and response to radiotherapy in osteosarcoma is still unclear. In this study, we attempt to investigate whether IGF-1R downregulation affected phenotypes and enhanced radiosensitivity of osteosarcoma cells.

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism, which has demonstrated great prospects for human gene function, signal transduction research, and gene therapy [15]. Specific gene silencing can be achieved in a variety of cell systems using chemically synthesized small interference RNA (siRNA) or DNA vector-based shRNA. However, although small interfering RNA has been shown to be effective for short-term gene inhibition in mammalian cell lines, there is a clear problem in its use for stable transcript knockdown and a high efficiency of RNAi delivery. To overcome these obstacles, a lentiviral vector-based RNAi expression system was designed [16]. Lentivirus can efficiently infect not only dividing but also non-dividing cells and can be produced with high titers. Moreover, lentivirus is relatively easy to generate so that it can be applied for large-scale, high throughput RNAi assay for studying gene functions [17]. Additionally, lentiviral vectors show minimal immunogenicity [18]. Although lentivirus has been used as gene transfer vectors for many years, the use of lentiviral vector expressing shRNA as a therapeutic tool for osteosarcoma has not been clearly explored. In this report, for the first time, we explore the therapeutic potential of a lentivirus vector encoding short hairpin RNA (shRNA) targeting IGF-1R for the treatment of human osteosarcoma alone or combined with radiotherapy.

Materials and methods

Cell culture and tissue samples

Four human osteosarcoma cell lines (U2, MG63, LM-8, and Saos-2) were maintained in DMEM (Gibco, USA) and

supplemented with 10% FBS (Sigma, USA), 100 units/ml penicillin G, and 100 μ g/ml streptomycin (Gibco, USA) at 37°C in a humidified 5% CO₂/95% air atmosphere. The osteosarcoma tissues and accordingly normal bone tissues were collected from the Department of Orthopaedics, Chinese PLA 454 Hospital.

Construction of lentivirus vectors

Two DNA template oligonucleotides corresponding to IGF-1R gene (Genbank NM_000875) and a negative control oligonucleotide (NC) having no homology with human or mouse genomes were designed and synthesized as follows: IGF-1R-shRNA1, sense: 5'-CGCGTCCCCCTTCA GGTCCACC-CTCTCTTCAAGAGAGAGAGGGTGGACCTG AAGGTTTTGGAAAT-3'; IGF-1R-shRNA2: 5'-CGCG-TC CCCTTGCCATGAACTGTTGGATTCAAGAGATCCAACA GTTCATGGCAAATTTTTGGAAAT-3'; Negative control shRNA, sense: 5'-CGCGTCCCC CACCTTTCGGCACTC TCCCTTCAAGAGGGGA-GAGTGCCGAAAGGTGTTTT GGAAAT-3'. All the above sequences were inserted into the *mu*II and *cl*aI enzyme sites of pLVTHM vector, respectively. After recombination reaction using pCMV-dR8.74 and pMD2G3 vectors (Addgene, Cambridge, MA), the U6 RNAi cassette was cloned into the latter vector, and lentiviral vectors expressing shRNA were constructed. All the constructed plasmids were confirmed by DNA sequencing. Lentiviral vector DNAs and packaging vectors were then transfected into 293T cells. Supernatants containing lentiviruses were harvested 72 h later after transfection. Then, we performed subsequent purification using ultracentrifugation and the titer of lentiviruses was determined.

Transfection of lentivirus

The lentivirus stock was added to osteosarcoma cell line Saos-2. For stable silencing of IGF-1R, the transfected Saos-2 cell line was selected by 500 μ g/ml G418, followed by G418-resistant colonies picked, expanded, and analyzed separately. To avoid unintended differences related to gene insertion rather than to the expression of shRNA, we chose pooled clones to perform further study. Saos-2 cells transfected with lentivirus-mediated shRNA targeted IGF-1R were named Saos-2-s1 and Saos-2-s2 cells. Saos-2 cells transfected with lentivirus-mediated shRNA (NC) were named Saos-2-NC cells. Saos-2 cells transfected with parental lentivirus were named Saos-2-c cells.

Real-time reverse transcription-PCR

Approximately 1.0×10^6 Saos-2 cells (untransfected or stably transfected cells) were seeded into a six-well cell culture plate, respectively. Cells of each group were

harvested after culture for 72 h. Total RNA was extracted from cells using the RNeasy kit (Qiagen). The reverse transcription reaction was performed using high-capacity cDNA synthesis kit (Applied Biosystems). Expression of IGF-1R mRNA was detected by ABI 7700 Sequence Detection System (PE Applied-Biosystems) using specific primers, sense: 5'-TCTATCTTGGTTCCAC-3'; antisense: 5'-GGGAGCGA GCCGTCTG-3' (640 bp). β -actin as an internal, primers, sense: 5'-AGCAACCGGGAGCTGGTGG-3'; reverse: 5'-CAT TTCCGACTGAAGAGT-G-3' (358 bp). Relative gene expression was quantified.

Western blot analysis

The cells or tissues were lysed in 50- μ l lysis buffer (1 mM dithiothreitol, 0.125 mM EDTA, 5% glycerol, 1 mM phenylmethylsulfonylfluoride, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 μ g/ml aprotinin, 1% Triton X-100 in 12.5 mM Tris-HCl buffer, pH 7.0) on ice for 30 min, and the lysates were cleared by centrifugation. Proteins were separated by 10% SDS-PAGE and electroblotted onto nitrocellulose membrane, blocked by 5% skim milk, and probed with anti-IGF-1R- β (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Caspase-3 (BD Biosciences, USA), and anti- β -actin (Sigma, USA) antibody. Following incubation with horseradish peroxidase-conjugated goat antimouse secondary antibody (Amersham Pharmacia Biotech), immunoblots were visualized by chemiluminescence using a chemiluminescence kit (Invitrogen, USA) and the specific bands were recorded on X-ray film. Actin protein levels were used as a control to verify equal protein loading.

Measurement of cell proliferation by methylthiazolotetrazolium assay

The cell viability of four kinds of Saos-2 cells (untreated or stably transfected cells) was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma, USA). Above four kinds of Saos-2 cells, at 4.0×10^3 /well, were seeded into seven 96-well culture plates, with each plate having all three kinds of cells and each group consisted of 12 parallel wells. On each day for seven consecutive days, 200 μ l MTT (5 mg/ml) was added to each well, and the cells were incubated at 37°C for additional 4 h. Then, the reaction was stopped by lysing the cells with 150 μ l DMSO for 5 min. Optical densities were determined on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA) at 490 nm.

In vitro colony formation assay

Approximately, a total of 2.8×10^2 cells (Saos-2, Saos-2-c, Saos-2-NC, and Saos-2-s1) were plated in 10-cm culture

dishes, respectively. After 14 days, cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were manually counted.

In vitro invasion assay

Invasion assays were performed using two-chamber-Transwell (Corning, New York, NY) as described previously. The upper surface of a polycarbonate filter with 8 μ m pores was coated with 1 mg/ml Matrigel. Untransfected or stably transfected Saos-2 cells (1.0×10^6) were suspended in RPMI 1640 supplemented with 0.1% fetal bovine serum (FBS) and added to the upper chamber, and DMEM containing 10% FBS was placed in the lower chamber. Cells were incubated for 12 h at 37°C, 5% CO₂ incubator. At the end of incubation, the cells on the upper surface of the filter were completely removed by wiping with a cotton swab. Then, the filters were fixed in methanol and were stained with hematoxylin and eosin. Cells that had invaded the Matrigel and reached the lower surface of the filter were counted under a light microscope.

Flow cytometry analysis of cell cycle

The cells were harvested by trypsinization, fixed with cold 70% ethanal, and stored at 4°C until analyzed. The cells were resuspended in phosphate-buffered saline containing 20 μ g/ml propidium iodide (PI) and 10 μ g/ml RNase A for 30 min at room temperature, and DNA content was detected by flow cytometry (Coulter, Beckman). The relative proportions of cells in the G₁/G₀, S, and G₂/M phases of the cell cycle were determined from the flow cytometry data.

Detection of apoptosis by flow cytometry and fluorescence microscopy

Cells were stained with fluorescein isothiocyanate (FITC) labeled annexin-V, and simultaneously with PI stain, to discriminate intact cells (annexin-/PI-) from apoptotic cells (annexin+/PI-), and necrotic cells (annexin+/PI+). A total of 1.0×10^6 Saos-2 cells were washed twice with ice-cold PBS and incubated for 30 min in a binding buffer (1 μ g/ml PI and 1 μ g/ml FITC labeled annexin-V), respectively. FACS analysis for annexin-V and PI staining was performed by the same flow cytometer mentioned above. All experiments were performed in triplicate. For analysis of changes in nuclear morphology during apoptosis, DAPI (Sigma, Bornem, Belgium) was added to the culture medium by the procedures described previously. Fragmentation of the nucleus and chromatin condensation were examined by fluorescence microscopy.

Caspase-3 assay

The activity of Caspase-3 was measured using Caspase-3 Colorimetric Assay Kit (Nanjing Keygen Biotech. Co., Ltd) following the manufacturer's instruction. In brief, the untransfected or stably transfected Saos-2 cells were harvested, resuspended in 50 μ l of lysis buffer, and incubated on ice for 30 min, and cellular debris was pelleted. Lysates (50 μ l) were transferred to 96-well plates. The lysates were added to 50 μ l 2 \times reaction buffer along with 5 μ l Caspase-3 substrate and incubated for 4 h at 37°C, 5% CO₂ incubator. The activities were quantified spectrophotometrically at a wavelength of 405 nm. Caspase activity was calculated as the change in absorbance at 405 nm and divided by total protein concentration.

Murine xenograft model for tumorigenicity assay

The effect of IGF-1R on tumorigenicity was assessed by subcutaneous injection of untransfected or stably transfected Saos-2 cells into athymic nude mice. Each aliquot of 1.0×10^7 cells was injected into the back of BALB/c nude mice (Nu/Nu, female, 6–7 weeks old) which were maintained under pathogen-free conditions. The formation of subcutaneous tumors was monitored and measured with a digital caliper. The tumor volume formed was calculated by the following formula: $V = 0.4 \times D \times d^2$ (V , volume; D , longitudinal diameter; d , latitudinal diameter). At 35 days after inoculation, all mice were sacrificed, and s.c. tumors were resected and fixed in 10% PBS. Survival tests were performed using groups of mice ($n = 8$ /group) treated as above and monitored daily until all the mice died. For all the experiments, animal handling and experimental procedures were approved by the Animal Experimental Ethics Committee of Nanjing Medical University.

Clonogenic cell survival assay

A total of 1.0×10^5 untransfected or stably transfected Saos-2 cells were seeded in 24-well plates. After 24-h incubation, fresh medium was added to each well and incubation was continued for 24 h before further treatments. One day after the viral infection, cells were trypsinized, plated, and incubated for 24 h before irradiation. The time interval between viral infection and radiation treatment was 2 days. Following irradiation, duplicate cultures were incubated for 10–14 days for colony formation. Cultures were fixed with pure ethanol and stained with 1% crystal violet in ethanol, and colonies were counted. Surviving fraction was determined by normalizing to the plating efficiency of the untreated control cells. For dose fractionation, cells were irradiated with a high-dose rate ¹³⁷Cs unit (4.0 Gy/min) after the viral infections,

respectively. Irradiated cells were trypsinized and plated for survival analysis as described above.

Statistical analysis

Statistical analysis was performed using SPSS software (Release 11.0, SPSS Inc.). Data were expressed as the mean \pm SD. Results were considered significant, if $P < 0.05$ was obtained by an appropriate ANOVA procedure and Student's t -test.

Results

Downregulation of IGF-1R mRNA and protein expression in Saos-2 cells after lentivirus transduction

To exclude off-target silencing effect mediated by specific-shRNA, two different IGF-1R shRNAs (s1 and s2) were designed to silence the expression of IGF-1R gene in Saos-2 cell line, with non-specific control shRNA (NC). The mRNA and protein levels of IGF-1R gene expression were determined by semi-quantitative RT-PCR and Western blot. As shown in Fig. 1a, b, compared with untransfected Saos-2 cells, the levels of IGF-1R mRNA and IGF-1R β -subunit protein in Saos-2-s1 cells were significantly reduced by 57.5% and 48.9%, respectively ($P < 0.05$), but there were no obvious difference among Saos-2-s2, Saos-2-NC, and Saos-2-c cells. Finally, we chose Saos-2-s1 cells for further assays.

Effect of IGF-1R downregulation on in vitro cell growth and colony formation

To evaluate the potential effects of RNAi-mediated IGF-1R downregulation on cell growth and survival, we investigated osteosarcoma cell growth and the potential of colony formation in vitro. The results from MTT assays showed that the cell growth rate was significantly reduced in Saos-2-s1 cells and the highest inhibition rate was $33.3 \pm 2.4\%$ on day 5 ($P < 0.05$; Fig. 2a). Next, the effect of IGF-1R downregulation on the potential of colony formation was examined. As shown in Fig. 2b, Saos-2-s1 cells showed significantly lower colony numbers than other control cells ($P < 0.05$), whereas the growth rate at each time point and colony numbers showed no obvious difference among Saos-2-NC, Saos-2-c, and Saos-2 cells ($P > 0.05$).

Reducing the invasion activity of Saos-2 cells by lentivirus-mediated RNAi

We evaluated the effect of lentivirus-mediated RNAi targeted IGF-1R on the invasion activity of Saos-2 cells. As

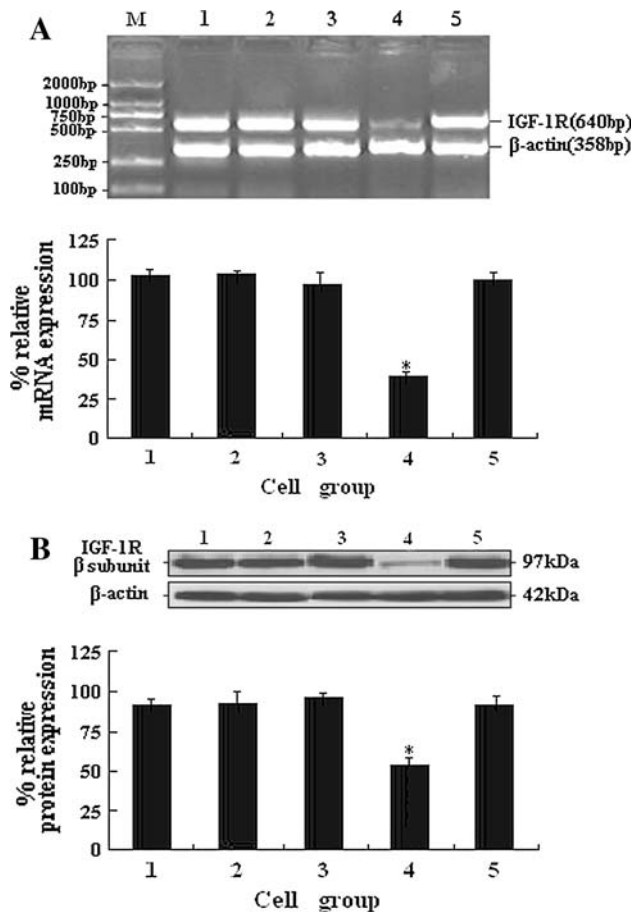


Fig. 1 Detection of IGF-1R mRNA and protein expression. **a** IGF-1R and β -actin mRNA levels were determined by RT-PCR. The PCR products were separated on a 1.5% agarose gel. Densitometric analysis was performed using the Labworks Image Acquisition. The level of IGF-1R mRNA expression in Saos-2-s1 cells was decreased by approximately 57.5%, but there were no obvious changes in Saos-2-s2 cells. **b** Membranes were probed with antibodies for target protein, and expression levels were normalized for loading by probing for β -actin. Densitometric analysis was performed using a chemiluminescence kit, and the level of IGF-1R protein expression in Saos-2-s1 cells was decreased by approximately 48.9%, but there were no obvious changes in Saos-2-s2 cells. All experiments were performed in triplicate ($n = 3$), $*P < 0.05$. *M* DGL2000 marker, *1* Saos-2, *2* Saos-2-c, *3* Saos-2-NC, *4* Saos-2-s1, *5* Saos-2-s2

shown in Fig. 3, Saos-2-s1 cells showed significantly lower invasion activities than other control cells (Saos-2, Saos-2-c, and Saos-2-NC), and the invasion activity of Saos-2-s1 cells was reduced by approximately 52.4% ($P < 0.05$). Thus, we concluded that the reduced invasion activity of Saos-2-s1 cells might be correlated with IGF-1R downregulation mediated by RNAi.

The changes of cell cycle induced by lentivirus-mediated RNAi

In the present study, the effect of IGF-1R downregulation on the cell cycle of Saos-2 cells was determined and each assay

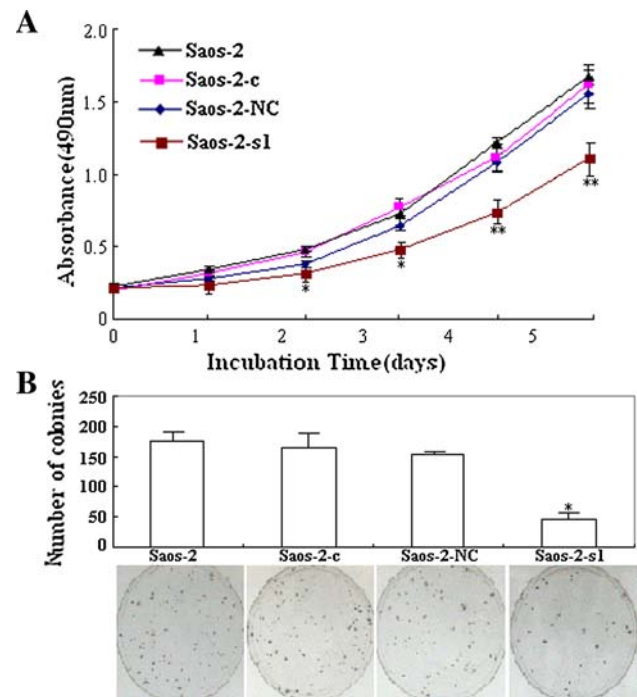


Fig. 2 Cell growth evaluated by MTT and colony formation assays. **a** The protracted cell growth curve and the results of the inhibitory rates of cell growth were applied to absorbance (**a**) at 490 nm. The viability of Saos-2-s1 cells was significantly decreased and the highest inhibitory rate was $33.3 \pm 2.4\%$ on day 5. Data shown are the mean results \pm SD of a representative experiment performed in triplicate ($n = 3$), $*P < 0.05$, $**P < 0.01$. **b** The Saos-2-s1 cells showed much less colonies than Saos-2-c, Saos-2-NC, and untransfected Saos-2 cells. These experiments were performed in triplicate ($n = 3$), $*P < 0.05$

was performed in triplicate. The FCM analysis revealed that most of Saos-2-s1 cells were arrested in the G_0/G_1 phase of the cell cycle ($65.2 \pm 3.7\%$), and the percentages of cells in the S ($20.9 \pm 2.2\%$) were decreased correspondingly ($P < 0.05$, Table 1). We concluded that the downregulation of IGF-1R expression led to G_0/G_1 arrest, which contributed to the inhibition of cell growth and proliferation.

Apoptosis enhancement by lentivirus-mediated RNAi

To further investigate the effects of siRNA on cell death, the rate of apoptosis was evaluated by flow cytometry analysis. As shown in Fig. 4, the results of FCM showed that the apoptosis rate of Saos-2-s1 significantly increased to 20.7% ($P < 0.05$), while there were no differences in cell apoptosis among Saos-2, Saos-2-c, and Saos-2-NC cells (5.2%, 6.6%, and 7.1%, respectively; $P > 0.05$). In addition, we examined the changes in nuclear morphology caused by the treatment of siRNA by staining of nuclear DNA with DAPI. Results indicated that a small proportion of cells with typical hallmarks of apoptosis, such as nuclear fragmentation and chromatin condensation, was obviously

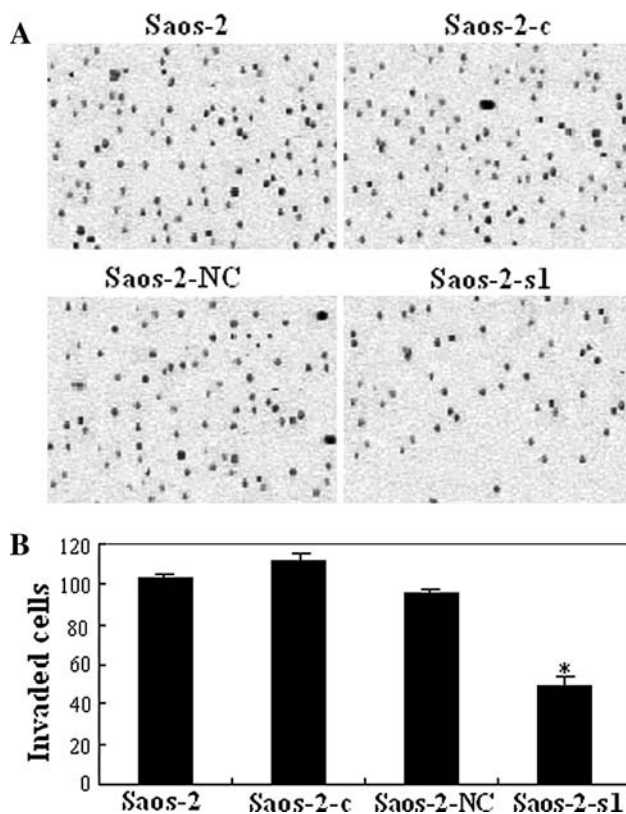


Fig. 3 Reducing invasion abilities of Saos-2 cells by lentivirus-mediated RNAi. **a** The cells that invaded through the Matrigel-coated inserts were counted and photographed under a light microscope (200 \times). **b** The invasion activity of Saos-2-s1 cells was obviously reduced by approximately 63.4% compared with Saos-2 cells. Experiments were performed in triplicate ($n = 3$), * $P < 0.05$

Table 1 Cell cycle phases detected by FCM

Cell	Cell cycle phases ($\bar{x} \pm SD$, %)		
	G ₀ /G ₁	S	G ₂ /M
Saos-2	44.5 \pm 4.2	40.7 \pm 5.1	14.8 \pm 2.6
Saos-2-c	43.1 \pm 2.4	43.8 \pm 1.9	13.1 \pm 1.8
Saos-2-NC	43.8 \pm 3.3	40.7 \pm 1.3	15.5 \pm 1.5
Saos-2-s1	65.2 \pm 3.7*	20.9 \pm 2.2*	13.9 \pm 1.2

Compared with Saos-2 cells, * $P < 0.05$ ($n = 3$)

detected in Saos-2-s1 cells but not in other control cells (Fig. 5). These data demonstrated that suppression of cell proliferation might be caused by apoptotic cell death resulting from lentivirus-mediated RNAi.

Detection of Caspase-3 activities

To understand the activation of the caspase cascade during RNAi-induced apoptosis in osteosarcoma cells, we investigated the changes of Caspase-3 activity in the IGF-1R-downregulated Saos-2 cells. As shown in Fig. 6a, b, the

activation of Caspase-3 could be observed and the Caspase-3 activity increased by 268% in Saos-2-s1 cells. In order to further confirm that the activation of caspases culminates in the apoptosis of Saos-2 cells, we employed the MTT assay to detect the cell viability of Saos-2-s1 cells or the cell viability of Saos-2-s1 treated with a pancaspase inhibitor. Results showed that a pancaspase inhibitor could partially restore the cell viability of Saos-2-s1 cells, confirming that the cell death of Saos-2 cells induced by RNAi was a caspase-dependent process (Fig. 6c).

Inhibiting in vivo tumor growth by lentivirus-mediated RNAi

Firstly, in order to confirm downregulation of IGF-1R expression by RNAi, tumor homogenates were subjected to Western blot analysis of IGF-1R protein expression. The IGF-1R protein expression in tumors formed from Saos-2-s1 cells was significantly downregulated than those in tumors formed from the control cells (Fig. 7a). The average tumor size of the xenografts formed from Saos-2-s1 cells at 35 days was significantly smaller than that of the xenografts formed from control cells ($P < 0.05$; Fig. 7b, c). Figure 7d showed the survival time of the mice. RNAi-mediated IGF-1R downregulation significantly prolonged the lifespan of mice bearing Saos-2 tumor cells. These results suggested that lentivirus-mediated RNAi targeted IGF-1R significantly inhibited tumor growth in vivo.

Enhancing radiosensitivity of Saos-2 cells by lentivirus-mediated RNAi

To determine the effects of IGF-1R expression on the radiosensitivity of human osteosarcoma cells, clonogenic cell viability assay was performed with the untransfected or stably transfected osteosarcoma cells (Saos-2, Saos-2-c, Saos-2-NC, and Saos-2-s1). The cell survival curve showed a significant decrease in D_0 and D_q which were 2.87 and 1.88 for Saos-2-s1 (Fig. 8; $P < 0.05$), and the radiation enhancement ratios were 2.45 (a ratio of D_0) and 1.65 (a ratio of D_q), respectively (Table 2). However, for Saos-2, Saos-2-c, and Saos-2-NC cells, their D_0 and D_q were almost similar, and the radiation enhancement ratios were close to 1.0. The further investigation about the exact mechanisms of radiosensitivity enhancement by IGF-1R downregulation is ongoing in our laboratory.

Discussion

Osteosarcoma is a neoplasm afflicting young adults, who in their prime years of life suffer debilitation if not death.

Fig. 4 Flow cytometry analysis of apoptosis. FCM data showed that the apoptotic rate of Hep-2-s1 significantly increased by 20.7% ($P < 0.05$), while there were no obvious differences in cell apoptosis among Saos-2, Saos-2-c, and Saos-2-s1 cells (5.2%, 6.6%, and 7.1%; $P > 0.05$). Experiments were performed in triplicate ($n = 3$)

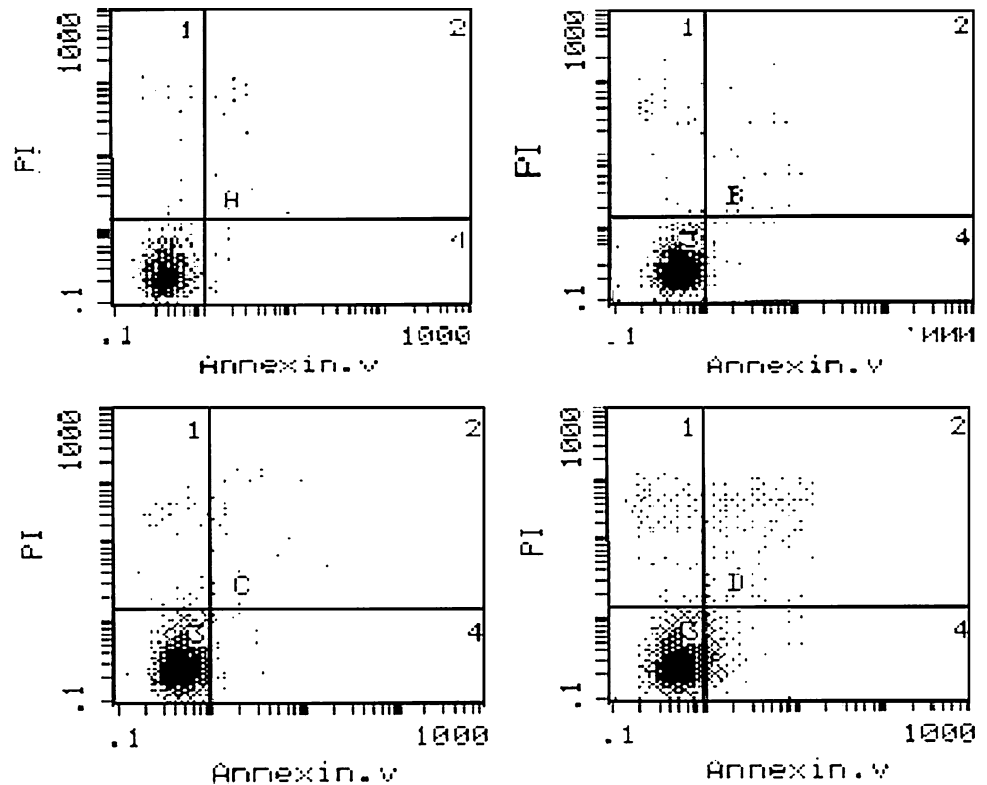
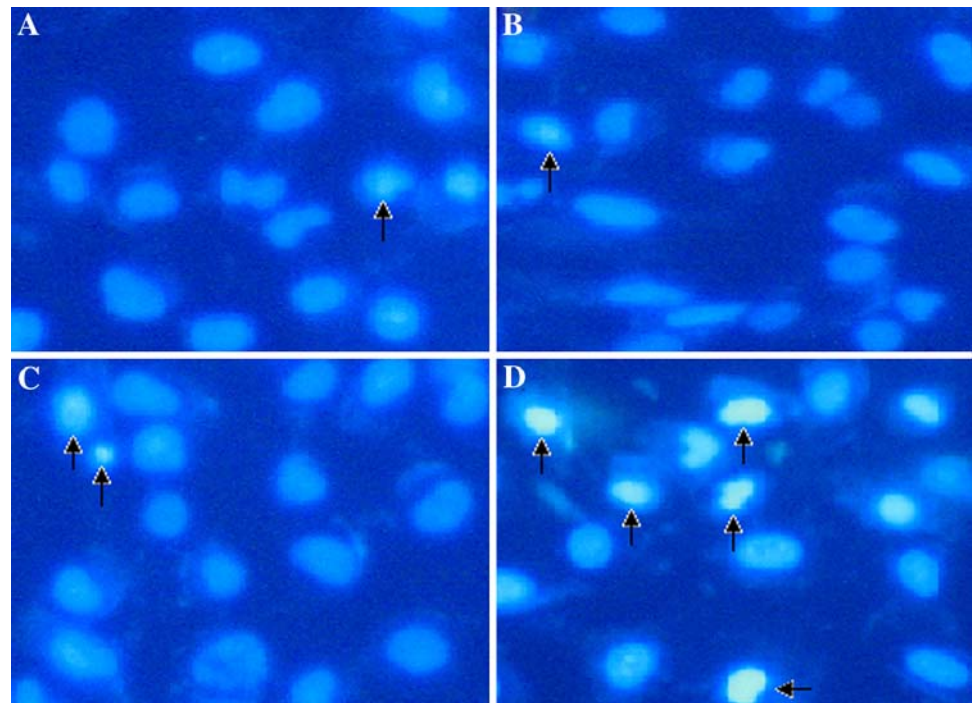


Fig. 5 DAPI staining of cells treated with lentivirus-mediated RNAi. Saos-2, Saos-2-c, Saos-2-NC, and Saos-2-s1 were cultured for 48 h; then, DAPI was added to the culture medium. Fragmentation of nucleus into oligonucleosomes and chromatin condensation were examined by fluorescence microscopy. Arrows indicate apoptotic cells. Experiments were performed in triplicate ($n = 3$)



Current osteosarcoma treatment strategies include surgery, radiation therapy, chemotherapy, or a combination of treatments, but treatment results of this disease are still unsatisfying [19, 20]. Thus, gene therapy, an applied form of biotechnology, has become a new and innovative approach to the treatment of human malignancies,

including osteosarcoma [21, 22]. Presently, more than 50% of all reported clinical trials for gene therapy are for cancers, though only a scant number for osteosarcoma. However, due to its frequent genetic mutations and accessibility for intra-tumoral administration, osteosarcoma might be a potential target for gene therapy.

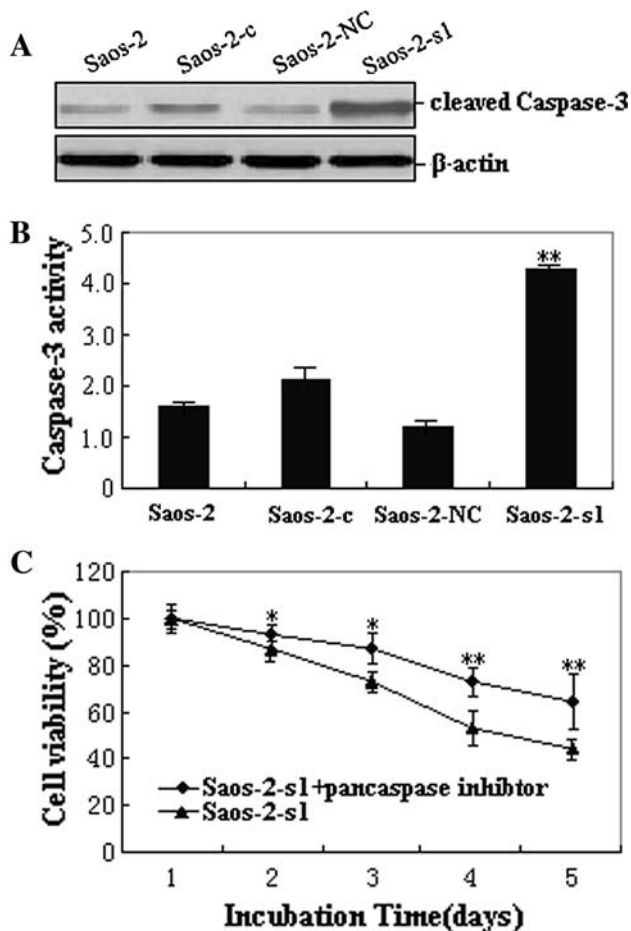


Fig. 6 Analysis of Caspase-3 activity. **a** Immunoblot of untransfected and stably transfected Saos-2 cells. Of total protein from cell lysates, 80 μ g was loaded per lane and blotted with anticleaved Caspase-3 antibodies. Equal loading was confirmed by showing equal β -actin levels. **b** Caspase-3 activity of Saos-2-s1 cells increased by 268%, ** $P < 0.01$. **c** The stably transfected Saos-2-s1 cells were treated with and without caspase inhibitor Z-VAD(OMe)-FMK. Cell viability was determined by the MTT assay. All experiments were performed in triplicate ($n = 3$), * $P < 0.05$, ** $P < 0.01$

The therapeutic efficiency of cancer gene therapy strategies strongly depends on the efficiency of gene transfer. A number of different viral systems are being developed for use as vectors for ex vivo and in vivo gene transfer such as retroviruses, adenoviruses, herpes-simplex viruses, and adeno-associated viruses [23]. Lentivirus, a genus of slow viruses of the Retroviridae family, was characterized by a long incubation period. Lentiviruses can deliver a significant amount of genetic information into the DNA of the host cell, so they are one of the most efficient methods of a gene delivery vector. A series of studies have showed that lentiviral vectors are safe when introduced into humans, as no adverse events have been reported [24, 25]. RNAi is a useful tool for functional analysis of genes and developing a potential therapeutic strategy for various diseases including cancers. Thus, the combination of

lentivirus and RNAi might provide a novel therapeutic strategy for cancer gene therapy. siRNAs are a small nucleic acid reagents which are unlikely to elicit an immune response, so these siRNAs can be easily manipulated and delivered by lentiviral vectors to target cells. The present study was conducted to determine whether lentivirus-mediated RNAi would be exploited for osteosarcoma gene therapy.

Next, we investigated the possibility of lentivirus-mediated shRNA exerting effects on the endogenous gene IGF-1R expression. IGF-1R plays critical roles in epithelial cancer cell development, proliferation, motility, and survival. IGF-1R has been found to be overexpressed in a variety of human malignant tumors, including osteosarcoma. Additionally, the overexpression of this gene is characterized clinically by a propensity for metastasis and resistance to conventional anticancer treatments including radiotherapy and chemotherapy. Many strategies have been employed to inhibit IGF-1R expression and function, including antisense oligonucleotides, antibodies to IGF-1R, or dominant negative IGF-1R mutants, which have been shown to inhibit in vitro or in vivo growth of these tumors, reverse of the transformed phenotype, and induce apoptosis [26–28]. In our study, we attempted to use lentivirus-mediated RNAi to silence the endogenous IGF-1R expression and explored the effects of IGF-1R downregulation on the phenotypes of osteosarcoma cells. Here, we have designed two shRNAs targeted at IGF-1R gene and successfully transfected them into a human osteosarcoma cell line (Saos-2) by lentivirus. IGF-1R-shRNA1 have been identified as IGF-1R-specific, because the stable transfectants show significantly decreased levels of IGF-1R mRNA and β -subunit protein, which showed that lentivirus-mediated IGF-1R-specific shRNA could silence the expression of IGF-1R effectively and specifically in Saos-2 cells. We observed that the downregulation of IGF-1R expression by RNAi significantly inhibited tumor proliferation both in vitro and in vivo, induced cell arrest in the G_0/G_1 phase, and significantly led to apoptosis induction in the Saos-2-s1 transfectants. Moreover, the IGF-1R-shRNA-induced apoptosis was proved to be correlated with enhancement of Caspase-3 activity, but the exact mechanism needs to be further clarified. In addition, the downregulation of IGF-1R also inhibited the invasiveness of Saos-2 cells, which might be correlated with the downregulation 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 previously reported [14, 29, 30]. Furthermore, to investigate whether the overexpression of IGF-1R affects the radiosensitivity of osteosarcoma cells, we compared the changes of radiosensitivity in vivo and in vitro before and after lentivirus transfection. Clonogenic survival assay showed that IGF-1R

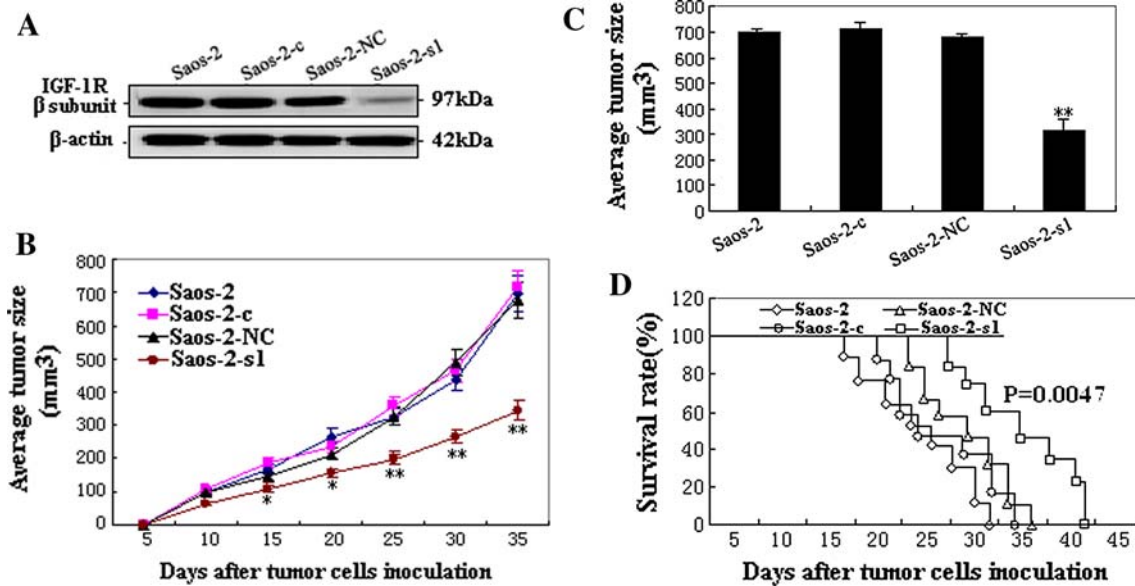


Fig. 7 The effect of IGF-1R downregulation on tumor proliferation in vivo. **a** Protein samples extracted from tumors were analyzed using Western blot analysis for IGF-1R expression levels. β -actin was included as a loading control. **b** Proliferation of tumors in the mice injected with Saos-2, Saos-2-c, Saos-2-NC, and Saos-2-s1 cells, * $P < 0.05$, ** $P < 0.01$. **c** Average tumor size at day 35 after the inoculation of Saos-2, Saos-2-c, Saos-2-NC, and Saos-2-s1 cells,

** $P < 0.01$. **d** Survival analysis of mice inoculated with above four Saos-2 cells. *Survival curves* were made by the Kaplan–Meier’s method, and statistical differences were evaluated by using the log-rank test. The differences between Saos-2-s1 group and other three control groups are statistically significant ($P = 0.0047$). All experiments were performed in triplicate ($n = 3$)

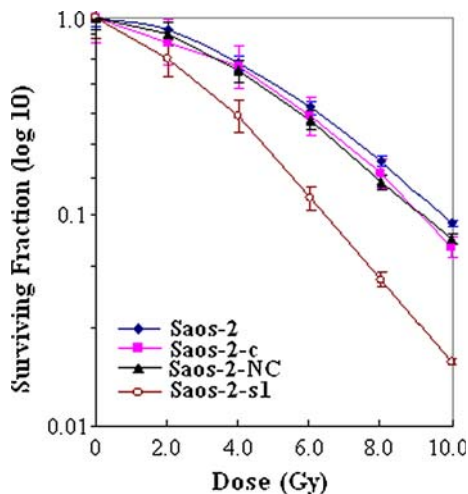


Fig. 8 Clonogenic survival after varying doses of irradiation exposure. The untransfected or stably transfected Saos-2 cells were irradiated followed by a further incubation for 24 h at 37°C before trypsinization and plating for clonogenic survival. After 10–14 days incubation, colonies were stained, and the surviving fraction were determined. The log survival was formed to the number of cells plated, after correcting for plating efficiency. There was a significant decrease in D_0 and D_q , which were 2.87 and 1.88, respectively ($P < 0.05$), and the radiation enhancement ratios were 2.45 (a ratio of D_0) and 1.65 (a ratio of D_q). *Error bars*, the mean \pm SD in three experiments ($n = 3$)

downregulation could significantly lead to radiosensitivity enhancement. The radiosensitization mechanism might be correlated with its effect on cell growth inhibition and

Table 2 Related parameters of cell survival curve standard model

Cell	D_0	D_q	N	Ratio of D_0	Ratio of D_q
Saos-2	7.03	3.12	6.36		
Saos-2-c	6.98	3.20	6.29	1.01	0.97
Saos-2-NC	7.13	3.21	6.16	0.99	0.97
Saos-2-s1	2.87*	1.88*	3.28	2.45	1.65

Compared with Saos-2 cells, * $P < 0.05$

apoptosis induction, but the accurate mechanism needs to be further investigated.

In summary, our study demonstrated that lentivirus-mediated RNAi targeted IGF-1R could significantly inhibit tumor proliferation, induce apoptosis, reduce invasion activity, and enhance radiosensitivity in osteosarcoma cells. This provides an attractive anticancer strategy and a means of enhancing sensitivity to conventional treatments for the treatment of human osteosarcomas.

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