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Original Research

Stable silencing of IGF1R using Lentiviral-mediated shRNA in HEK293T cells

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Abstract: Insulin-like growth factors are among the peptide mitogens that regulate cell proliferation and differentiation as well as mediator of antiapoptotic signals. The imbalance between the expression and activities of these molecules may lead to malignancy in cells. Evidences have suggested the insulin-like growth factor 1 receptor (IGF-1R) signaling pathway as a therapeutic target in the management and treatment of cancer. In this present study, we have generated silencing stable clones of HEK cells using six different pGIPZ (lentiviral vector) shRNAs targeted to human IGF-1R gene and a pGIPZ non-silencing shRNAmir lentiviral vector (as negative control). The recombinant lentiviral vectors were separately transduced into human embryonic kidney 293 T (HEK293T) cell lines. The knockdown of IGF-1R was confirmed by reverse transcription polymerase chain reaction (RT-PCR) and the relative IGF-1R mRNA levels were expressed as a ratio of IGF-1R to β-actin by REST software. The results showed significant reduction in the expression of IGF-1R mRNAs in cells transduced with all six pGIPZ-IGF-1R recombinant lentivirals compared to non-silencing negative control. No significant difference was observed among the six cassettes. Results indicated that recombinant lentiviral vectors provided an efficient and stable knockdown of IGF-1R providing useful tool for IGF-1R pathway studies.

Key words: IGF-1R; Knockdown; Transduction; HEK293T cells.

Introduction

Insulin-like growth factor 1 receptor (IGF-1R) is a protein responsible for the mediation of the effects of insulin-like growth factors (IGFs). As potent mitogens, IGFs are involved in the regulation of cell proliferation, differentiation, and apoptosis (1). A balance between the production and use of these molecules is required to ensure normal physiological conditions and functioning. However, certain changes, such as the overexpression of one factor, disturb this balance and initiate a series of molecular events that may eventually cause malignancy.

Due to its potent anti-apoptotic activity, IGF-1R plays a major role in determining cell and body size. Owing to this property, transformed cells will be able to develop into macroscopic tumors and to detach from their parent cells (as an initial stage of metastasis) (2). The cross-talk between IGF-1R pathway and other signaling pathways increases resistance to therapeutic agents targeting (3). The existing evidences suggest the IGF-1R signaling pathway as a potential therapeutic target in cancer management. Therefore, anti-IGF-1R-targeting therapeutic agents not only reverse resistance but also decrease the efficacy of cancer treatments (1-3).

RNA interference (RNAi), a major strategy in gene silencing, has turned into a valuable tool for drug discovery and drug target validation processes in cell culture studies (4). siRNA (21-23 base pairs in length) are being subsequently processed by the cellular machinery by one of three pathways: RNAi, plasmid and viral vector systems that express short hairpin RNAs (shRNA) (5-7). However, methods of efficient silencing provides useful tools in research as well as gene therapy. In this regards, the lentiviral vectors, in addition to their wide local applications, are commonly used for *ex-vivo* gene therapy. Lentiviral shRNA vectors are used by the Lenti-X shRNA Expression System to introduce the constructs of short hairpin RNA expression into cells. This process mainly aims to suppress specific genes via RNAi. By stable integration of their genomes into the host cell DNA, these lentiviral vectors facilitate the long-term expression of the introduced therapeutic genes (8).

Considering the key role of IGF-1R in the growth of cancer cells, we hypothesized that the knockdown of IGF-1R via a stable transgene using a lentiviral vector may serve as a unique and promising strategy for *ex-vivo* gene therapy. In present study, we assessed six different lentiviral shRNA to find the best model for IGF-1R knockdown in HEK293T cell line.

Materials and Methods

Cell cultures

HEK293T (human embryonic kidney, ATCC CRL-3216) was obtained from Mede Bioeconomy Company, Iran and cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Carlsbad, USA). It was then incubated at 37 ºC in a hu-

midified 5% CO_2 atmosphere.

Cell viability was evaluated by the trypan blue dye exclusion assay and only cell suspensions with > 95% viability were used.

Lentiviral vectors preparation

Six pGIPZ lentiviral vectors containing shRNAs against human IGF-1R gene (Genbank accession NM_ 000875.4) and pGIPZ non-silencing shRNAmir lentiviral vector (as the negative control) were purchased from Dharmacon Company. A scrambled sequence, i.e. 22mer: ATCTCGCTTGGGCGAGAGTAAG which has no significant homology to human gene sequences, was used as the negative control. presents the characteristics of the six selected shRNA sequences "Table 1".

Virus production

The seven lentiviral vectors were transfected with three plasmids, i.e. GIPZ-shRNA IGF-1R - turbo green fluorescent protein (tGFP), psPAX2, and pMD2.G, in HEK-293T cells using the standard calcium phosphate precipitation method according to Tronolab protocol with some modifications (9, 10). On day one, 5×10^6 HEK-293T cells were seeded in a 10 cm plate in DMEM (Gibco, USA) with 10% FBS (Gibco, USA). On the second day, 21 µg of transfer vector GIPZ, 21 µg of psPAX2 vector, and 15 µg of pMD2.G vector were mixed with transfection buffer and added dropwise to the cells. The transfection medium was replaced with fresh medium within 14 hours after transfection. Green fluorescent protein (GFP) expression was observed by fluorescent microscopy after 24 hours. The packaged recombinant lentiviruses were harvested from the supernatant of cell cultures 48 hours after transfection, centrifuged at 2000 rpm and 4 °C for 5 minutes, and filtered through a 0.22 µm filter. The recombinant viruses were stored at -70 °C for subsequent experiments. Viral titers were determined with counting the number of GFP-positive cells by flow cytometry.

Transduction of HEK 293T cells

A total of 1.5×10^5 HEK 293T cells were prepared in a six-well plate. On the next day, the cells were transduced with recombinant lentivirus at a high multiplicity of infection (MOI = 7). After 24 hours, the transduction media was replaced with fresh DMEM plus 10% FBS. The transduced cells were passaged every three days. The fluorescence properties of tGFP were examined under a LABOMED® Model T121100 fluorescent microscope (Labo America Inc., USA) and the obtained images were evaluated by Image J software (11).

Quantitative reverse transcription Polymerase Chain Reaction (RT-qPCR)

After infection with lentiviruses for four days, the total RNA from each group of cells was extracted by RNeasy ® Plus Mini Kit (Alameda, CA, United States). DNA contamination (cDNA) was prevented by a QIAGEN kit (Germany) according to the manufacture's instructions. It was quantified by an ultraviolet spectrophotometer at a wavelength of 260 nm. The reverse transcription (RT) reaction was performed with a RevertAid First Strand cDNA Synthesis kit (Fermentas, Lithuania) according to the manufacturer's instructions.

In order to perform the RT-qPCR for IGF-1R, the reaction was repeated for 40 cycles. Each cycle consisted of denaturing at 95 ºC for 5 seconds, annealing at 60 ºC for 34 seconds, and at 78 ºC for 5 seconds. The RT-qPCR for β-actin was performed for 40 cycles, each cycle consisted of denaturing at 95 ºC for 5 seconds, annealing at 60 ºC for 34 seconds, and extension at 80 ºC for 5 seconds. The primer sequences used to amplify IGF-1R and β-Actin are listed in "Table 2".

The cutoff point (Ct) for each sample was plotted on the standard curve and the mRNA copy numbers were calculated. The β-actin gene was used as an endogenous control. The relative IGF-1R mRNA levels were expressed as a ratio of IGF-1R to β-actin by REST software.

Statistical analysis

All the experiments were carried out in triplicate and IGF-1R to β-actin ratios were expressed as mean percent ± standard deviation (SD). Statistical differences between groups were compared by one-way analysis of variance (ANOVA) using OpenEpi 3.03 free statistical software. P values less than 0.05 were considered statistically significant.

Table 1. DNA sequences of insertion fragments for shRNAs.

Name	shRNA anti IGF1R sequences	Target site
V2LHS-131070	5'-TAACTGAGAAGAGGAGTTC -3'	3006-3024
V2LHS-131071	5'-TAGAAATGACAGTTCTCTC- 3'	3399-3417
V2LHS-131072	5'-TTGACTGTGAAATCTTCGG- 3'	4474-4492
V2LHS-20148	5'-AAAGAATCCAGAGTATATC- 3'	12090-12108
V3LHS-377848	5'-TGCATGACATCTCTCCGCT- 3'	3256-3274
V3LHS-377851	5'-TGACTGTGAAATCTTCGGC- 3'	4473-4491

Table 2. Primer sequences for RT-qPCR.

Target gene	Primer sequences (5'→3')	TM	Amplification length (bp)
IGF-1R-F	TCCTGTGTTCTTCTATGTCC	49	141
IGF-1R-R	GCTGTTATTTCTCTTTCTATGG	48.7	
β-actin-F	CTGACGGCCAGGTCATCAC	64.9	174
β-actin-R	CTGACGGCCAGGTCATCAC	60.5	

Results

Lentiviral vector preparation and cassettes confirmation

After preparation of GIPZ lentiviral vectors (11774bp, Dharmacon Company), the quality of pGIPZ lentiviral vectors was checked by their digestion with SalI enzyme (data not shown).

Packaging and titration of the recombinant Lentiviral

Fluorescence microscopy was used to verify the packaging of the recombinant lentiviruses based on the expression of tGFP at 24, 48, and 72 hours after transfection. As shown in "Figure 1", more than 90% of HEK293T cells were transfected by pGIPZ lentiviruses. The lentiviruses' titer was determined based on the expression of tGFP in a cell-based assay. The titer of the recombinant viruses was approximately 1.5×10^6 IU/mL in all groups.

Recombinant Lentiviral assessment based on tGFP expression

The transduction efficiency of the recombinant lentiviral vectors was investigated by analyzing tGFP expression in HEK293T cells. The number of tGFP-expressing cells and tGFP intensity were assessed three days after transduction. A significant increase in the percentage of cells expressing tGFP was detected through fluorescence intensity. Typically, at an MOI of 7, more than 80% of the transduced cells were found to be tGFP-positive using fluorescence-activated cell sorting analysis "Figure 2". In addition, since the GIPZ lentiviral vectors' structure has a drug resistant marker (Puromycin), 100% of the cells would be transduced after a few days.

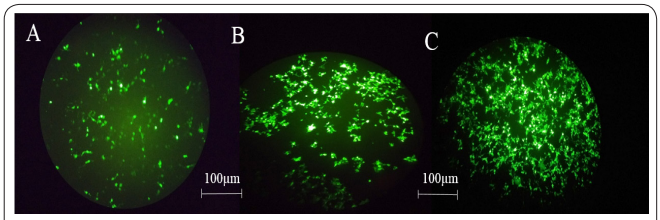

Figure 1. Microscopy fluorescent images show transfection of HEK-293T for achieving viral particles by pGIPZ lentiviral IGF1R shRNAmir vector. **A**: HEK- 293T at 24 hours after transfection, Panel **B**: HEK- 293T at 48 hours after transfection and **C**: HEK-293T at 72 hours after transfection.

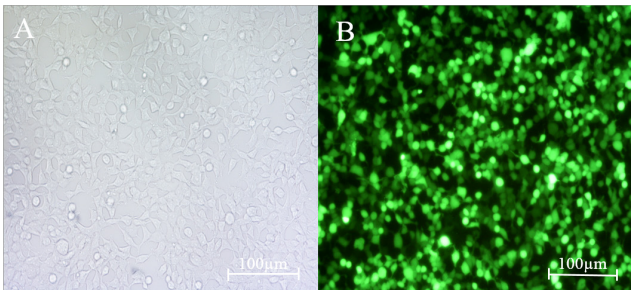

Figure 2. Transduction of HEK-293T cells by lentiviral particles. A: HEK-293T cells prior to transduction and B: transducted HEK-293T cells by pGIPZ lentiviral IGF1R shRNA vector.

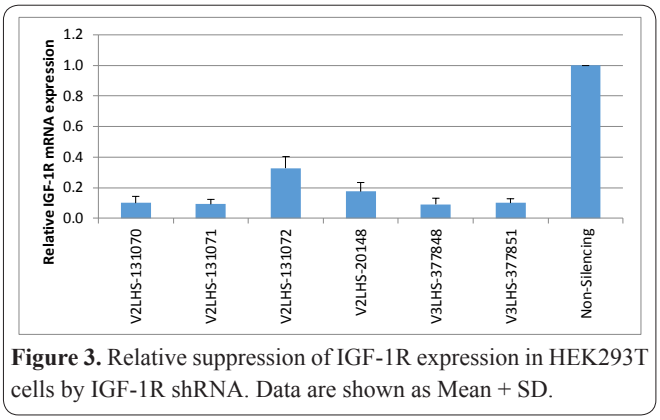

Figure 3. Relative suppression of IGF-1R expression in HEK293T cells by IGF-1R shRNA. Data are shown as Mean + SD.

Recombinant Lentiviral vector assessment based on RT-qPCR analysis

The mRNA expression IGF-1R was investigated using RT-qPCR. IGF-1R gene expression was normalized based on β-actin gene expression (as the internal standard gene). Based on the results obtained from REST software, the expression of the IGF-1R mRNAs was significantly lower in cells transduced with pGIPZ-IGF-1R recombinant construct than in cells treated with the non-silencing negative control ($P < 0.01$). In all six cassettes, IGF-1R gene expression was significantly down regulated (70-90%) compared to the negative control "Figure 3". However, the cassettes targeted to 3000-3400 target sites were more efficient in silencing (90%) compared to other distant cassettes (~70%). Comparing all cassettes, the ANOVA showed no statistically significant difference between the six intervention groups in terms of expression reduction. Therefore, all Six pGIPZ lentiviral vectors containing shRNAs against human IGF-1R gene decreased the expression of IGF-1R gene in HEK293T cells.

Discussion

The IGF-1R signaling pathway has a critical role in not only normal cell growth, differentiation, and transformation, but also anti-apoptotic signaling. The overexpression of IGF-1R has been detected in several human cancers and is believed to cause a variety of functional consequences including the blockage of apoptosis resulting from various agents or adverse tumor microenvironments (2, 3).

Different methods, including plasmids and viral-based methods, have been developed for gene delivery (4, 8). Adenoviral-mediated gene expression and plasmid-mediated gene knockdown cannot be maintained for a very long periods of time. Meanwhile, adenovirus vectors may induce an immune response in the host (12).

Due to their ability to integrate into host genomes with high efficiency, lentiviral vectors are efficient tools for gene delivery (12). The use of a lentiviral vector has important advantages such as prolonged expression of foreign genes, large capacity (up to 7.5 kb of DNA), efficient infection of many kinds of cells, high titer, and low immunogenicity (less immunogenic than adenoviral vectors) (6, 13). Recent studies have demonstrated the high efficiency of lentiviral transgenesis in various animal species and cell lines (12). Lentiviral GIPZ is an shRNA lentiviral vector system based on the main

structure of microRNA (miR30). This design has been shown to greatly increase gene silencing efficiency. Adding the miR-30 loop and 125 nucleotides (nt) of miR-30 flanking sequence on either side of the hairpin results in greater than 10-fold increase in shRNA production and greater potency for expressed hairpins. (14)

ShRNA is the focus of molecular biology studies and can be used to block the expression of specific genes. The shRNA expression vector is considered to treat diseases and to have advantages over other strategies because of its high selectivity and potency (3, 13, 15).

ShRNA exhibiting higher silencing levels can be obtained via this carrier. As mentioned earlier, the pGIPZ shRNAmir lentiviral vector contains GFP (visual marking) in the expressing cells and Puromycin (drug resistance marker) for stable cell lines selection. These vectors are usable for in vitro and in vivo applications (13, 14, 16).

We used six lentivirus-mediated shRNA-targeting cassettes to knockdown IGF-1R in HEK 293T cells in six different positions. Some studies proposed different algorithms of shRNA to find the best cassette to knockdown the IGF-1R (17-22). Up to our best knowledge, no study has performed before using lentiviral mediated multiple shRNA targeting IGF1R on HEK cell lines; Chen (23) and Wang (24) used similar method targeting IGF1R on breast cancer and osteosarcoma cell lines respectively. The efficiency of shRNA molecules and the infection rate of target cells are two major determinants of success when inhibition of certain genes is involved. In fact, only highly efficient shRNA molecules (knockdown efficiency > 85%) are suitable for the production of lentiviral transfer vectors and complex lentiviruses (25). Four cassettes"Figure 3" in this study showed this ability. Although our results showed no significant differences in gene expression reduction between different cassettes, it seems that V2LHS-131072 cassette may not be suitable for IGF-1R knockdown.

We assayed the effects of recombinant lentiviral vector on the expression of IGF-1R and found that recombinant lentiviral decreased the expression of mRNA IGF-1R. In this study, we successfully transfected HEK293T cells with the recombinant vector and observed packaged viral particles with a high titer. Recombinant viral particles were able to infect HEK293T cells and were a suitable model cell type for biological studies.

According to the result of this study and considering the important role of IGF1 gene in the development of many cancers (e.g. prostate, colorectal, and premenopausal breast carcinomas as well as lung, endometrial, and bladder cancers) (2), it is recommended to apply shRNA IGF-1R cassettes in GIPZ lentiviral vectors for future *in vivo* (animal models) and *in vitro* (cancer cell lines such as Raji and Mantel) studies on cancer treatment with or without other chemotherapy medications.

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