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Clinical significance of proliferation, apoptosis and senescence of nasopharyngeal cells by the simultaneously blocking EGF, IGF-1 receptors and Bcl-xl genes

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ABSTRACT

Background: In previous work, we constructed short hairpin RNA (shRNA) expression plasmids that targeted human EGF and IGF-1 receptors messenger RNA, respectively, and demonstrated that these vectors could induce apoptosis of human nasopharyngeal cell lines (CNE2) and inhibit ligand-induced pAkt and pErk activation.
Method: We have constructed multiple shRNA expression vectors of targeting EGFR, IGF1R and Bcl-xl, which were transfected to the CNE2 cells. The mRNA expression was assessed by RT-PCR. The growth of the cells, cell cycle progression, apoptosis of the cells, senescent tumor cells and the proteins of EGFR, IGF1R and Bcl-xl were analyzed by MTT, flow cytometry, cytochemical therapy or Western blot.
Results: In group of simultaneously blocking EGFR, IGF1R and Bcl-xl genes, the mRNA of EGFR, IGF1R and Bcl-xl expression was decreased by (66.66 ± 3.42)%, (73.97 ± 2.83)% and (64.79 ± 2.83)%, and the protein expressions was diminished to (67.69 ± 4.02)%, (74.32 ± 2.30)%, and (60.00 ± 3.34)%, respectively. Meanwhile, the cell apoptosis increased by 65.32 ± 0.18%, 65.16 ± 0.25% and 55.47 ± 0.45%, and senescent cells increased by 1.42 ± 0.15%, 2.26 ± 0.15% and 3.22 ± 0.15% in the second, third and fourth day cultures, respectively.
Conclusions: Simultaneously blocking EGFR, IGF1R and Bcl-xl genes is capable of altering the balance between proliferating versus apoptotic and senescent cells in the favor of both of apoptosis and senescence and, therefore, the tumor cells regression.

1. Introduction

Nasopharyngeal carcinoma (NPC) is highly prevalent in China, in which the incidence is approximately 30–80 cases per 100,000 people per year [1,2]. It usually affects a relatively young population in comparison to other head and neck carcinomas. Most NPCs are undifferentiated or poorly differentiated squamous carcinomas with a fast growth rate and a high metastatic potential [3]. 70–80% of new cases present with lymphnode metastasis in the neck, and 4.2% of those present with distant metastasis. NPC is a multistep process with morphological progression involving multiple genetic events [4]. Many of the characteristics of the malignant phenotype are mediated by overexpression and/or activation of receptor tyrosine kinases and several anti-apoptotic proteins, making these molecules attractive anti-cancer treatment targets.

The EGF and IGF receptor tyrosine kinase (RTK) families are well-recognized mediators of tumor cell proliferation and survival [5]. Bcl-xl is a transmembrane molecule in the mitochondria. It is involved in the signal transduction pathway of the FAS-L. It is one of several anti-apoptotic proteins which are members of the Bcl-2 family of proteins.

RNAi has emerged as one of the most important discoveries in the field of molecular biology in the last several years. Due to its high efficacy and specificity in down-regulating gene expression, RNAi was considered to be a potential therapeutic strategy against human cancer. In previous study, EGF and IGF-1 receptors individual gene has been targeted by RNAi technology in nasopharyngeal tumor cells (CNE2), leading to successful silencing of the protein, subsequently, cancer impairment [6–9]. However, this study has selected a single gene rather than multiple genes. It is now generally accepted that there are many genes abnormally expressing in malignant tumors. In most cancers, silencing a single gene may be insufficient to therapeutically treat cancer cells. Therefore, simultaneously blocking multiple genes that are abnormally expressed may be more effective in treating cancer cells than in

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silencing a single gene. Numerous studies have suggested that the growth of factor receptors and anti-apoptosis genes is essential to maintain tumor proliferating and progression, and it is therefore plausible that simultaneously blocking these genes: EGFR, IGF1R and Bcl-xl genes, is capable of breaking the balance between proliferating verus apoptosis. Therefore the aim of this study was to investigate whether simultaneously blocking EGFR, IGF1R and Bcl-xl genes is capable of triggering tumor cell to enter quiescence, either apoptosis or senescence or both.

2. Materials and methods

2.1. Materials

Anti-Bcl-xl (sc-7382,CA, USA), rabbit polyclonal anti-IGF1Rβ (C20; Santa Cruz Biotechnology, Inc, Santa Cruz, California; 1:1000) chain and goat anti-human primary polyclonal antibody of EGFR (Santa Cruz Biotechnology, Inc), anti-rabbit and antigoat secondary antibody IgG (Pierce, Rockford, Illinois), nitrocellulose membrane (Pall Corporation, East Hills, New York), Oligo (dT) primer (Invitrogen Life Technologies, Carlsbad, California), Trizol reagent (Gibco, New York), dNTP (Invitrogen Life Technologies), RNasein (Invitrogen Life Technologies), RTase (Gibco), and Taq DNA polymerase (Promega, Madison, Wisconsin), extraction buffer and lysed in Nonidet P-40 (Sigma–Aldrich Corp, St Louis, Mo) isotonic lysis buffer and ECL Test Kit and an enhanced chemiluminescent agent (Pierce Biotech Inc., Rockford, IL, USA), Cell Death Detection ELISA Plus kit and β-Galactosidase Antibody Staining Kit were commercially obtained. The NPC cell lines CNE2 were purchased from Wuhan University. The primer and probe were designed by Primer 5.0 software and synthesized by Invitrogen. RPMI 1640 (SH30197.01; Hyclone; Logan, UT), fetal bovine serum (Gibco-BRL, Carlsbad, CA, USA) were commercially obtained. The plasmids were constructed by Wuhan Genesil Biotechnology Co., Ltd. (Wuhan, China).

2.2. Methods

2.2.1. Construction of shRNA segments expression vectors

The recombinant plasmids pU6-EGFR-shEGFR-shIGF1R-shBcl-xl was constructed according to the complementary DNA sequence of EGFR, IGF1R and Bcl-xl genes. All of these plasmids could simultaneously express different shRNAs targeting EEGF, IGF1R and Bcl-xl mRNA. The specific base sequences of the targeted mRNA sites are as follows:

EGFR (341–361 bp), 5′-GGCACGAGTAACAAGCTCA-3′; IGF1R ((444–464 bp), 5′-CCTGAGGAACATTACTCGG-3′; Bcl-xl (605–625 bp), 5′-GGA GAT GCA GGT ATT GGT GAG-3′. For each targeted sequence, a pair of sense and anti-sense strands were designed. A control plasmid, pU6-EGFR -shHK, which did not target any specific human genes, was also constructed. The sequence of shHK was: 5′-GAC TTC ATA AGG CGC ATGC-3′. The shRNAs were subcloned into the pEGFP vector (containing the enhanced green fluoresce in protein [EGFP] gene and kanamycin marker) with a human U6 promoter between the BamHI and HindIII restriction sites. All of the constructs used in this study were verified by DNA sequencing. The structure of multiple shRNAs and their vectors were as described previously.

2.2.2. CNE2 cell culture and transfection

These CNE2 cells were cultured in RPMI 1640 medium supplemented with 10 FCS, 100 μ penicillin and 100 mg/ml streptomycin, and humidified with 5% CO_2 at 37 °C. 12 h before transfection, cells were seeded onto 24-well plates at a density of 5×10^4 cells per well. The confluence reached approximately 70–80% at the time of transfection. Cells were washed twice using RPMI 1640 and then a further 0.2 ml of RPMI 1640 without penicillin, streptomycin and 10% FCS. Complexes of plasmid vector and transfection reagent were prepared by gently mixing either 2 μg pU6-EGFR-shEGFR-shIGF1R-shBcl-xl plasmid vector (Multiple group), 2 μg pU6-EGFR-shHK plasmid vector (HK group) or equal amount of saline (Saline group), respectively, in 100 μl of RPMI (pH 7.4), with 10 μl transfection reagent in 100 μl of 5 RPMI 1640 (pH 7.4), respectively. The three solutions were incubated 15 min at room temperature and then they were added to the cells, three wells each group. After incubation for 8 h, their medium were removed and new RPMI 1640 medium supplemented with 10% FCS, 100 μ penicillin and 100 mg/ml streptomycin were added to each well in humidified with 5% CO_2 at 37 °C for 48 h.

2.2.3. Analysis of CNE2 cells growth and cell cycle progression

2.2.3.1. CNE2 cells growth. CNE2 cells of three groups (multiple, HK, Saline) and normal CNE2 cells (NC group) were plated into 6-well plastic plates at a seeding density of 3×10^3 cells/well, and cultured in medium for 3 days in the existence of 10% FCS, respectively. On the indicated day, triplicate wells from CNE2 cells of four groups were trypsinized and resuspended in 10 ml isotonic saline solution and were counted with a haemocytometer under a phase-contrast microscope, respectively. The cells were cultured in a 96-well plate (10^4 cells/well) in medium for 3 days in the existence of 10% FCS. After being washed, the cells were incubated in the medium containing 0.5% MTT for 4 h at 37 °C followed by a reaction with 150 μl DMSO for 10 min. The assays above were repeated in three culture plates. The cultures were analyzed on a plate reader at 492 nm. Data were expressed as means of absorbance from 5 wells at each group CNE2 cells.

2.2.3.2. Cell cycle progression. Each group CNE2 cells were seeded into 6-well plastic plates at 3×10^3 cells/well and incubated for 3 days in the medium with 10% FCS, and then were collected by centrifugation and washing, and fixed in 70% cold ethanol at 4 °C for 10 min. The cells were washed twice with ice cold PBS buffer, and incubated with RNase (100 mg/l) and DNA intercalating dye propidium iodide (50 mg/l) for 30 min in a 37 °C aqueous bath before analysis. The cell cycle phases were analyzed using a FACScab Licbur flow cytometer and CXP software (Beckman Coulter, Mountain View, CA, USA). Triplicate samples were assessed for each group CNE2 cells. The proliferative index (PI) was calculated with the following formula: PI (%) = (S + G2/M)/(G0/G1 + S + G2/M) × 100%.

2.2.4. Detections of apoptosis

The apoptotic rate represented by the percentage of sub-G1 peak in flow cytometry histogram with propidium iodide stain was used to estimate the number of apoptotic cells. To retrieve the discrepancy of the above assay in discriminating the apoptotic cell and corpuscle fragment, Cell Death Detection ELISA Plus kit was used to measure histone-bound DNA fragments (nucleosome) in an ELISA format. The CNE2 Cells from each group were prepared according to the protocol provided by the manufacturer and analyzed on a microplate spectrophotometer at 405 nm. Data was expressed by means of absorbance from triplicate experiments performed in each groups CNE2 cells.

2.2.5. Protein assays for IGF1R and EGFR

The cells were scraped off the dish and transferred into centrifuge tubes. After centrifuging at 2000 rpm for 5 min at 4 °C, the pellets were lysed in ice-cold lysing buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.02% Sodium Azide, 1% NP-40, 100 μg/mL PMSF, 1 μg/mL aprotinin) for 20 min in ice. After the removal of cell debris by centrifugation (12,000 *g*, 5 min), cell protein (80 μg

per lane) was separated in 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) by electrophoresis and transferred onto nitrocellulose membrane (Pall Corporation, East Hills, NY), then was blocked by 5% skimmed milk in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) at 4 °C overnight. The membranes were then incubated with either rabbit polyclonal anti-IGF1Rβ or goat anti-human primary polyclonal antibody of EGFR antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) at 1:200 in blocking solution at 37 °C in a water bath which were shaken for 2 h, respectively, then they were incubated with alkaline phosphatase-labeled anti-goat or anti-rabbit secondary antibody IgG at 1:600 for 1 h, respectively. Anti-β-actin antibody was used as an internal standard for protein concentration and integrity. Quantitative analysis for all the pixels in each band was carried out with GeneTools software (Syngene, Cambridge, UK). The relative expression levels of the proteins were expressed as ratio of EGFR or IGF1R raw volumes (integrated intensity of all the pixels in each band) divided by the corresponding β-actin value.

2.2.6. Protein assays for Bcl-xl

Three group cells were collected and lysed in ice-cold RIPA lysis buffer (Beyotime, Shanghai, China). Following centrifugation at 12,000 *g* for 5 min at 4 °C, the supernatants were collected and stored at −70 °C until use. Equal amounts of protein extracts (20 mg/lane) were subjected to SDS–PAGE on a 10% separating gel and electrophoretically transferred onto PVDF membrane. After being blocked with 50 g/l skim milk powder and 1 g/l Tween-20 in TBS buffer for 1 h, the membranes were then incubated with either anti-Bcl-xl antibody for 10 h at 4 °C, followed by the corresponding horseradish peroxidase-conjugated second antibody for another 1 h. Anti-β-actin antibody was used as an internal standard for protein concentration and integrity. The proteins were detected by using the ECL Test Kit and an enhanced chemiluminescent agent and exposed to an X-ray film. Quantitative analysis for all the pixels in each band was carried out with GeneTools software (Syngene, Cambridge, UK). The relative expression levels of the proteins were expressed as ratio of Bcl-xl raw volumes (integrated intensity of all the pixels in each band) divided by the corresponding β-actin value.

2.2.7. RT-PCR for IGF1R, EGFR and Bcl-xl mRNA expression

There were 1×10^5 cells collected using Trizol Reagent (Gibco) to extract total RNA. cDNA was synthesized from 1 μg total RNA using Oligo (dT) primer (Invitrogen, Carlsbad, CA) 1.0 μl (50 μg/mL), then the mixture was heated at 70 °C for 5 min. The tube was chilled on ice for 2 min. The mixture was then added in 5× buffer 4.0 μl, 10 Mm dNTP (Invitrogen) 2.0 μl, 20 units of RNasein (Invitrogen), and 200 units of RTase (Gibco) and incubated at 37 °C for 60 min. It was then heated at 95 °C for 5 min and stored at −20 °C. In the PCR reaction, 1 μl of cDNA production was amplified with gene specific primers, in a total volume of 50 μl. The mixture was: cDNA 1 μl, 10× buffer 5 μl L, Mgcl2 (25 mM) 7 μl, dNTP (10 mM) 1 μl, either EGFR, IGF1R, Bcl-xl or β-actin upstream primer (20 pmol/μl) 0.8 μl, EGFR or β-actin downstream primer (20 pmol/μl) 0.8 μl, Taq DNA polymerase (5 U/μl) (Promega, Madison, WI) 0.5 μl, with H_2O_2 finally added to 50 μl. The primers of EGFR, IGF1R, Bcl-xl or β-actin were designed by Primer 5.0 software and synthesized by Invitrogen. RT-negative tubes served as the negative control. The standard curves for each mRNA were constructed using serial dilutions of a known standard cDNA. The targeted gene expression was normalized by using the reference gene β-actin. The ratio between the copy numbers of the target gene (EGFR or IGF1R or Bcl-xl) and that of β-actin represented the relative value of the expression of the targeted gene for each sample; each experiment was repeated 3 times. Negative contrast excluding cDNA was used. The experiment was repeated three times. Finally, 50 cycles of PCR were performed with primers specific for the human EGFR, IGF1R, Bcl-xl and β-actin mRNA sequence and the resulting PCR products analyzed by agarose gel electrophoresis. The relative expression levels of the proteins were expressed as ratio of EGFR, IGF1R and Bcl-xl raw volumes (integrated intensity of all the pixels in each band) divided by the corresponding β-actin value.

2.2.8. Senescence β-galactosidase staining

CNE2 Cells from four groups were washed in PBS, fixed for 3–5 min (room temperature) in 2% formaldehyde/0.2% glutaraldehyde (or 3% formaldehyde), washed, and incubated at 37 °C (no CO_2) with fresh senescence associated β-Gal (SA-β-Gal) stain solution: 1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) per ml (stock = 20 mg of dimethylformamide per ml)/40 mM citric acid/sodiumphosphate, pH 6.0/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM $MgCl_2$. Staining was evident in 2–4 h and maximal in 12–16 h. The CNE2 Cells from each group were prepared according to the protocol provided by the manufacturer and analyzed on.

2.2.9. Statistical analysis

All values are expressed as the mean ± SD. Statistical analyses were carried out by Student's *t*-test performed by using the SPSS statistical software (version 11.5; SPSS Inc., Chicago, IL, USA). Probability values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Four groups CNE2 cells growth

As is shown in Figs. 1 and 2, the average cell numbers and the average absorbance by the MTT assay of the NC, HK and saline group cells were very similar, while the average cell numbers and the average absorbance in multiple group were significantly lower than those in NC cultures. By Day 3, the NC, HK and saline groups became confluent. The result indicates that simultaneously blocking EGFR, IGF1R and Bcl-xl genes had a transient growth-inhibiting effect on the CNE2 cells.

3.2. The effect of simultaneously blocking EGFR, IGF1R and Bcl-xl genes on CNE2 cell cycle progression

Cell cycle analysis (Table 1) shows that the proportions of multiple group at the G0/G1 phase significantly increased, while those

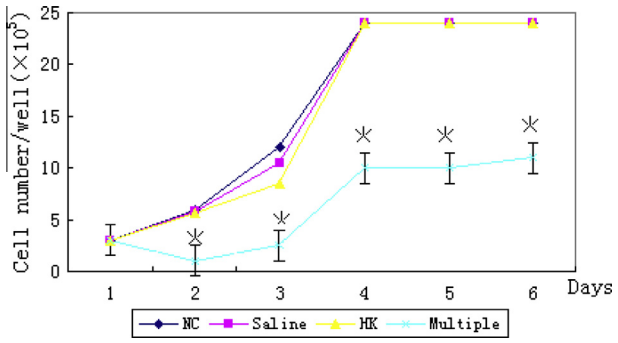

Fig. 1. Cell count for growth of four groups CNE2 cells. *$P < 0.05$, compared with NC, Saline and HK groups. Multiple: transfected with pU6-EGFR-shEGFR-shIGF1R-shBcl-xl plasmid vector CNE2; HK: transfected with pU6-EGFR-shHK plasmid vector CNE2; Saline: saline-treated with equal amount of saline CNE2; NC: without treated CNE2.

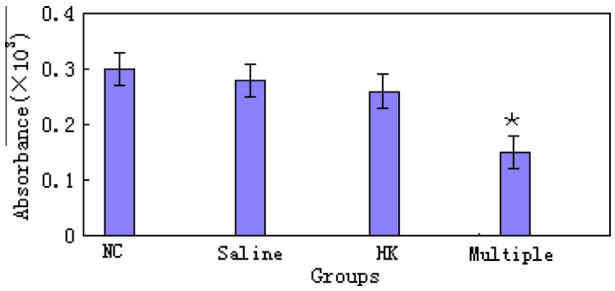

Fig. 2. MTT assay for growth of CNE2 cells from four groups. The cells were analyzed at 492 nm and data were expressed as average values of absorbance from 5 wells at each group. Multiple: transfected with pU6-EGFR-shEGFR-shIGF1R-shBcl-xl plasmid vector CNE2; HK: transfected with pU6-EGFR-shHK plasmid vector CNE2; Saline: saline-treated with equal amount of saline CNE2; NC: without treated CNE2.

Table 1
Cell cycle progression of different group CNE2 cells (mean ± SD).

Groups	G0/G1	S	G2/M	RI%
Multiple	84.00 ± 0.18*	10.72 ± 0.28*	5.38 ± 0.06*	16.05 ± 0.38*
HK	47.91 ± 0.65	42.71 ± 0.11	9.38 ± 0.14	52.09 ± 0.08
saline	44.64 ± 0.28	45.70 ± 0.15	9.65 ± 0.01	55.35 ± 0.08

Multiple: pU6-EGFR-shEGFR-shIGF1R-shBcl-xl plasmid vector–transfected CNE2; HK: pU6-EGFR-shHK plasmid vector- transfected CNE2; Saline: saline-treated CNE2.
* $P < 0.01$, compared with the NC, Saline and HK groups.

at the S and G2/M phase decreased, which resulted in a significant decrease of proliferative index. It indicates that simultaneously blocking EGFR, IGF1R and Bcl-xl genes inhibits the growth of CNE2 cells by decreasing their cell cycle progression from the G1 to the S and G2 phases. The sub-G1 population appeared and increased in multiple group (Table 1). Cycle of tumor cells was arrested in G0/G1 phase, rather than progression.

3.3. The effect of simultaneously blocking EGFR, IGF1R and Bcl-xl genes on apoptosis

FCM analysis on the sub-G1 rate reveals that the absorbance of CNE2 cells in the multiple group was 65.52 ± 0.15 and 64.34 ± 0.96 on Day 2–3, respectively. The CNE2 cells of simultaneously blocking EGFR, IGF1R and Bcl-xl genes presented significant differences to the HK group (1.09 ± 0.43) and Saline group (1.01 ± 0.36), $P < 0.01$. The other two groups did not demonstrate significant differences compared to the NC group. Results indicate that the

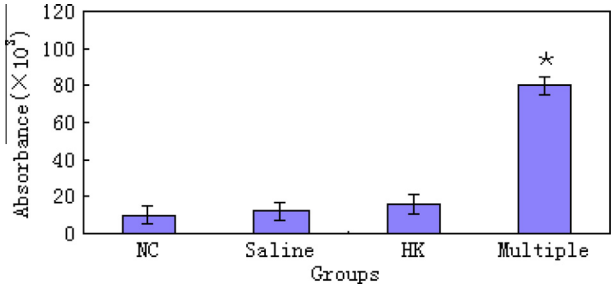

Fig. 3. Analysis for the nucleosomes from four groups CNE2 cells by ELISA. The relative levels of nucleosomes are expressed as average values of absorbance at 405 nm. *$P < 0.05$, compared with the NC, Saline and HK groups. Multiple: transfected with pU6-EGFR-shEGFR-shIGF1R-shBcl-xl plasmid vector CNE2; HK: transfected with pU6-EGFR-shHK plasmid vector CNE2; Saline: saline-treated with equal amount of saline CNE2; NC: without treated CNE2.

apoptotic rates rose significantly in cells of multiple group. This result was compatible with the ELISA data (as is shown in Fig. 3).

3.4. The effects of simultaneously blocking EGFR, IGF1R and Bcl-xl genes on expressions of EGFR, IGF1R and Bcl-xl mRNA and proteins

Western blotting and RT-PCR analysis showed that of Multiple group, the levels of EGFR, IGF1R and Bcl-xlmRNA and proteins showed obvious down-regulates in comparison with the NC, Saline and HK groups cells: the expression of the EGFR mRNA and protein declined to 66.67% and 65.00% ($P < 0.05$); the expression of the IGF1R mRNA and protein decreased to 73.97% and 74.32% ($P < 0.05$) and the expression of the Bcl-xl mRNA and protein was reduced to 64.79% and 59.22% ($P < 0.05$), respectively. EGFR, IGF1R and Bcl-xl expression remained unchanged in groups of NC (A), Saline (B) and HK (C) cells (Fig. 4).

3.5. The effects of simultaneously blocking EGFR, IGF1R and bcl-xl genes on cellular senescence in CNE2 cells

Senescence-associated beta-galactosidase activity, which is detected at pH 6.0, was absent in the majority of cells in CNE2 cells of NC, Saline and HK groups cultures (<2 cumulative population doublings), but was present in a proportion of cells (up to 25–28%) in Multiple shRNA group (for third and second day); in intermediate passage cultures (15–28 cumulative population doublings) it was found in fewer than 15% of the cells. The increase in the percentage of senescence-associated beta-galactosidase-positive cells correlated with a decrease in the cell density at confluence and with a marked increase in cell size (Table 2).

4. Discussion

Many of the characteristics of the malignant phenotype are mediated by overexpression and/or activation of receptor tyrosine kinases, making these molecules attractive anti-cancer treatment targets. Inhibitors of the EGFR/ErbB1 and Her2 (ErbB2), a 170 Kd surface receptor with intrinsic tyrosine kinase activity, have already shown clinical activity in cancers of the lung and breast [10]. The IGF1R is a heterotetrameric receptor tyrosine kinase with close homology to the insulin receptor (IR). The IGF1R is synthesised as a proreceptor, which undergoes cleavage into alpha and beta subunits before assembly into a 2β2 tetramers and insertion into the plasma membrane [11]. The IGF1R is increasingly recognized as an attractive anti-cancer treatment target: it is frequently overexpressed in tumors, often as a result of tumor suppressor gene loss, and mediates proliferation, survival, and properties required for invasion and metastasis [12]. These functions are predominantly mediated through IGF-induced activation of the phosphoinositide 3-kinase (PI3K)-AKT, RASRAF-MAPK and p38 MAPK signaling cascades [13].

On the other hand, a decrease in the rate of apoptosis is the main characteristic of malignant tumor cells. Apoptosis plays an essential role as a protective mechanism against carcinogenesis by eliminating genetically damaged cells. There are some anti-apoptotic genes in cancer cells that contribute to chemotherapy or radiotherapy resistance and can serve as RNAi targets. Bcl-xl, the long splice variant of Bcl-2, is a potent antagonist of apoptosis [14]. In our previous work, we found that shRNA targeted to EGFR or IGF1R mRNA can inhibit, respectively, the growth of nasopharyngeal cancer cells CNE2 by 50.05% and 53.50% in vitro. However, malignant tumors result from a multi-step process of accumulated genetic alterations, in this study, we further have observed that EGFR and IGF1RmRNA and proteins were more down-regulated, growth for CNE2 cells more inhibited, cell apoptosis more

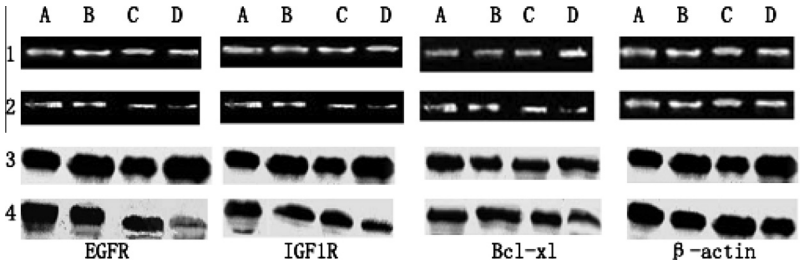

Fig. 4. Representative graphs of Western blotting and RT-PCR analysis for EGFR, IGF1R and Bcl-xl mRNA (Lane 1 and 2) and proteins (Lane 3 and 4) from different groups CNE2 cells. Lane 1 and 3 showed that the cells were transfected before and Lane 2 and 4 transfected after, respectively. The equal loading of the samples was confirmed by β-actin as an internal control. $^{*}P < 0.05$, compared with the NC, Saline and HK groups. $\Delta P < 0.05$,compared with the transfected before. Letters A, B, C and D represent NC, Saline, HK and Multiple groups, respectively. (A) without treated CNE2 (NC). (B) saline-treated with equal amount of saline CNE2 (Saline); (C) transfected with pU6-EGFR-shHK plasmid vector CNE2 (HK); (D) transfected with pU6-EGFR-shEGFR-shIGF1R-shBcl-xl plasmid vector CNE2 (multiple).

Table 2
Senescence-associated beta-galactosidase-positive cells of different group CNE2 cells (%, mean ± SD).

Groups	Second day	Third day	Fourth day
Multiple	1.42 ± 0.15*	25.26 ± 0.15*	28.22 ± 0.15*
HK	0.19 ± 0.44	0.36 ± 0.23	0.49 ± 0.33
saline	0.21 ± 0.36	0.50 ± 0.26	0.22 ± 0.04

Multiple: pU6-EGFR-shEGFR-shIGF1R-shBcl-xl plasmid vector-transfected CNE2; HK: pU6-EGFR-shHK plasmid vector- transfected CNE2; Saline: saline-treated CNE2.
* $P < 0.05$, compared with saline and HK groups.

increased by blocking EGFR, IGF1R and bcl-xl genes simultaneously than by blocking single gene that were studied before.

This indicates that simultaneous blocking multiple genes has accumulating and enlarging effect on apoptosis for homeostasis of alteration of cell cycle. Here, the present study further showed that the action of simultaneous blocking multiple genes was pointed at the G1 phase – decreasing the transition of the CNE2 cells from G1 to S phases by FCM detection. An unexpected finding in cell cycle analysis is that the decreased S and G2/M phase cell rate in the simultaneous blocking multiple gene group was much more than that of blocking single gene .It is known that in general, apoptosis and proliferation are two closely related, but mutual exclusive progressions. Why does simultaneous blocking multiple genes have the multiple effects on the CNE2? A possible mechanism, as revealed in the present study, is that the simultaneous blocking multiple genes not only inhibits much more cell proliferation by decreasing G1–S phase transition by down-regulating EGFR and IGF1R but also induces much more apoptosis of the cells by down-regulating Bcl-xl, compared with blocking single gene. In our experiment, the simultaneous blocking EGFR, IGF1R and Bcl-xl genes did affect the expression of Bcl-xl, so that resulted in a down-regulation of Bcl-xl, leading to an increased ratio of Bax/Bcl-2 – a crucial factor to trigger apoptosis.

Another interesting phenomenon in the present study is that the actions of simultaneous blocking multiple genes showed that senescence-associated beta-galactosidase activity was absent in the cells of NC, HK and saline groups, but was present in a proportion of cells (up to 25–28%) in simultaneous blocking multiple genes group in the third and fourth day culture. The increase in the percentage of senescence-associated beta-galactosidase-positive cells correlated with a decrease in the cell density at confluence and with a marked increase in cell size. This indicates that simultaneous blocking EGFR, IGF1R and Bcl-xl genes triggering CNE2 cells to enter apoptosis and senescence process. The apoptosis and senescence are cellular failsafe programmes that counteract excessive mitogenic signalling from activated oncogenes. Cancellation of apoptosis or senescence is therefore a prerequisite for tumor formation, and the ability of the cancer cell to disrupt these processes can be considered its 'lifeline'. Ironically, the efficacy of anticancer agents also depends on the activation of apoptosis or an acutely inducible form of cellular senescence. Understanding how the 'lifelines' of the cancer cell interfere with treatment sensitivity is of crucial importance for developing safer and more effective treatment strategies [15]. It is indicated that Cellular senescence refers to the arrest in the G1 phase of the cell cycle of continuously proliferating cells, which was in correspondence with the present study, in response to simultaneous blocking EGFR, IGF1R and Bcl-xl genes that puts them at safety of malignant transformation. When growth inhibition plus anti-apoptosis accumulates irreversibly, the CNE2 cells from multiple blocking genes group rely on dual mechanisms to avoid replication so that CNE2 from Multiple blocking genes may be permanently arrest the cell cycle (cellular senescence) or trigger cell death programs. Apoptosis (self-killing) is the best-described form of programmed cell death, but autophagy (self-eating), which is a lysosomal degradation pathway essential for homeostasis, reportedly contributes to cell death as well [16].

Senescence and apoptosis are two key mechanisms that protect against cancer development. Many cell cycle regulators are important in G1 cell cycle arrest and oncogene-induced senescence. The Bcl-xl protein is one of the key components that control apoptosis. There are two basic cellular mechanisms by which cells are prevented from cancer development: apoptosis and senescence. Senescence is a form of growth arrested state where metabolically active cells undergo stable cell cycle arrest at the G1 phase [17–21].

Recently, several studies have developed siRNA-expression vectors containing multiple tandem sequences [22]. In order to silence multiple genes simultaneously. Ter Brake used multiple shRNAs against conserved HIV-1 regions, and demonstrated that they inhibit HIV-1 production much more strongly compared to a single shRNA [23]. Hong blocked c-myc and stat3 by *Escherichia coli*-expressed and enzyme-digested siRNA in mouse melanoma, and revealed that the mixed treatment of esiC-MYC and esiSTAT3 had a better inhibitory effect (81%) than the individual treatment of esiC-MYC (62%) or esi STAT3 (51%) on mouse B16 melanoma cells [24–25]. Therefore, we suggest that the simultaneous blockage of multiple genes have a better inhibitory effect on cancer cells than the blockage of a single gene.

In summary, our results demonstrate that the application of vector-based RNAi technology involving multiple targets will be a promising therapeutic modality in the gene therapy of cancers. The data strongly suggests that simultaneously blocking multiple genes in human cancers using an RNAi approach should be considered in cancer therapy. On the other hand, the most suitable

targeted genes which mutual combination together get more effect should be investigated in further.

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