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Potential role of short hairpin RNA targeting epidermal growth factor receptor in growth and sensitivity to drugs of human lung adenocarcinoma cells

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ABSTRACT

Upregulation of expression and activation of epidermal growth factor receptor (EGFR) is involved in the development and progression of a wide range of human cancers. The present study aims at determining gene-silencing effects of vector-based short hairpin RNA (shRNA) targeting EGFR on receptor expression and cell growth and evaluating its modulation of responsiveness to drugs in human lung adenocarcinoma cells (HLAC). A vector-based polymerase 3-promotor system was used to express shRNA targeting EGFR in HLAC lines (A549 and SPC-A1). EGFR was detected by immunofluorescence staining and quantified by Western blot. The effect of shRNA targeting EGFR on tumor cell growth was assessed by colony formation assay, cell cycle and apoptosis by flow cytometry, and the responsiveness of HLAC lines to cytotoxic drugs by 3-[4,5-dimethylthiozol-2yl]-2,5-diphenyltetrazolium bromide [MTT] assay. Vectors expressing shRNA against EGFR significantly downregulated receptor expression by 74 and 85% and the colony number by 63 and 69% in A549 and SPC-A1, respectively. Vector-based shRNA against EGFR caused G1 arrest, induced apoptosis, and subsequently increased the sensitivity to cisplatin, doxorubicin and paclitaxel by about four- to seven-fold in both HLAC lines. Our data suggest that vector-based shRNA could be considered as an alternative to effectively inhibit EGFR expression in HLACs, probably with the higher efficacy in combination therapies with conventional chemotherapeutic drugs.

1. Introduction

The epidermal growth factor receptor (EGFR) is highly expressed in a range of solid tumors including head and neck, non-small cell lung, breast and prostate cancers [1]. The reported frequency of EGFR overexpression in non-small cell lung carcinoma (NSCLC) is 40–80%, associated with advanced tumor stage, poor prognosis, and resistance to radiochemotherapy [2]. EGFR has been identified as an important target in anticancer therapy by blocking EGFR function with the detailed understanding of the biology of EGFR protein and its downstream signal pathway [3,4], e.g. anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors (Fig. 1).

Other potentials indicated by experimental studies were the feasibility of interfering with EGFR expression by antisense oligonucleotides and synthesized small interference RNA (siRNA) [5–7]. The complexity of blocking EGFR-mediated pathway by modulating EGFR expression is that the receptor persisted in the cell membrane may function for a relatively long time, even though the gene is blocked. It is important to achieve long-term downregulation of EGFR gene expression to inhibit EGFR function more effectively. Experimental evidence

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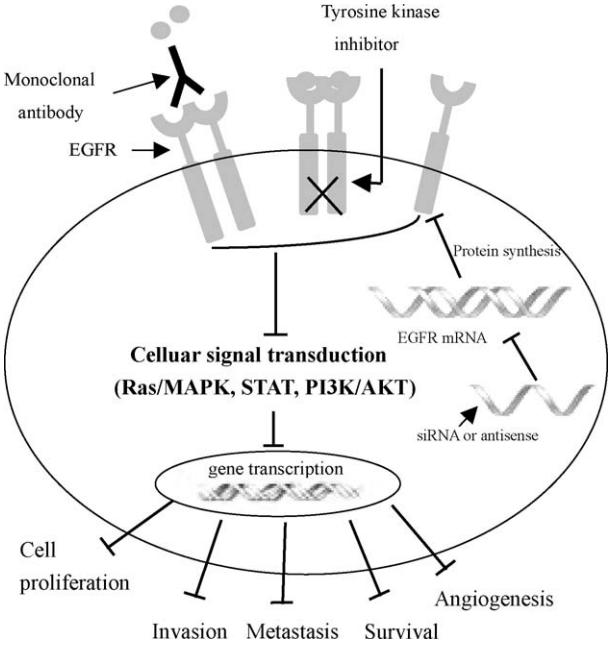

Fig. 1 – EGFR-mediated signal pathway and strategies targeting EGFR in cancer.

showed that expression of small hairpin (shRNA) under control of an RNA polymerase III promotor could result in a longer silencing of targeting gene in mammalian cells, overcoming the limitations of transient and non-renewable nature of antisense oligonucleotides and synthesized siRNA [8].

The present study aims at investigating inhibitory effects of vector-based shRNA on HLAC growth by downregulating EGFR expression effectively and potential influence of shRNA targeting EGFR in sensitivity to drugs, in order to understand whether vector-based shRNA targeting EGFR could be an attractive treatment strategy alone or in combination with other drugs.

2. Materials and methods

2.1. Construction of plasmid with DNA template

The siRNA sequence targeting EGFR corresponded to the coding region 133570–133588 of EGFR (Genbank accession number NM_288738), which showed no homology to any other sequences by a blast search. The shRNA template oligonucleotides were designed by entering the siRNA target sequence into the web-based converter at the address (http://ambion.com/techlib/misc/psilencer_converter.html) as follows: sense, GATCCGGAGCTGCCCATGAGAAATTTCAAGAGAATTTCTCATGGGCAGCTCCTTTTTTTGGAAA and antisense, AGCTTTTCCAAAAAAGGAGCTGCCCATGAGAAATTCTCTTGAAATTTCTCATGGGCAGCTCCG. The synthesized oligonucleotides were annealed and ligated into *p*Silencer 2.1-U6 siRNA expression vector (Ambion, Inc., Austin, TX, USA) according to manufacturer's protocol. The resulting shRNA expression vector (*p*ShEGFR) was utilized to silence the EGFR gene. The control vector (*p*ShNEG) served as the negative control provided by the kit. Plasmid DNA was extracted from *Escherichia coli* transormants using Wizard Plus Minipreps (Promega, San Luis Obispo, CA, USA). The constructs of the vectors (*p*ShEGFR) were confirmed by DNA sequencing (Sangon, Shanghai, China).

2.2. Cell lines and culture

Human lung adenocarcinoma cell (HLAC) lines, SPC-A1, and A549, were obtained from Shanghai Cell and Biology Institute. Cells were grown in RPMI 1640 medium (HyClone, Logan, Utah, USA) supplemented with 10% bovine serum (Alphabetical Gibco, Lenexa, Kansas, USA), 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacture's protocol. Briefly, cells at 70–80% confluence in 6-well plate were incubated for 6 h with the mixture of 4 μg *p*ShEGFR or *p*ShNEG and 10 μl of lipofectomine 2000 in serum-free Dulbecco's modified Eagle's medium (HyClone). The transfection medium was then replaced with RPMI 1640 medium. Cells were harvested at indicated time points for experiments.

2.3. EGFR measurements

Expression and location of EGFR were investigated by immunofluorescence. Cells in coverslips were fixed in 4% paraformaldehyde for 20 min, then incubated with mouse anti-EGFR monoclonal antibody (1:50, a generous gift from Shanghai Cell and Biology Institute) in humid chamber at 37 °C for 1 h. Cells were washed with PBS thrice, and then incubated with goat anti-mouse IgG antibody conjugated to Cy3 at 37 °C for 1 h. After then, cells were washed again, dehydrated, and visualized using Zeiss microscope (Carl Zeiss, Germany).

The amount of expressed EGFR was measured by western blot. Cells were harvested and resuspended in PBS. Total protein was extracted using cell lysis buffer (0.01 M Tris–HCl pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS pH 7.4), and separated on a 7% polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was incubated with anti-EGFR primary antibody (1:500) at 37 °C for 1 h, and then with peroxidase-conjugated goat anti-mouse IgG (Santa Cruz biotechnology, Inc., Santa Cruz, CA, USA) at room temperature for 1 h. GAPDH was used as the internal control. Protein was measured by using enhanced chemiluminescence (Amersham, Freiburg, Germany).

2.4. Measurements of cell growth and death

Cell growth and survival ability was determined by the colony-formation assay [20]. On Day 2 after transfection, cells were seeded at a density of 500 cells/dish in 6-cm dishes and incubated for another 2 weeks. Then they were washed with PBS and fixed with 95% ethanol. Finally, cells were stained with crystal violet and colonies containing more than 50 cells were counted. Each experiment was done in triplicates per cell lines. Cell cycles and apoptosis were analyzed with flow cytometry (Becton-Dickinson, USA) and the data were consequently evaluated by CellQuest and ModFit software (BD Bioscience, USA). Cells were trypsinized

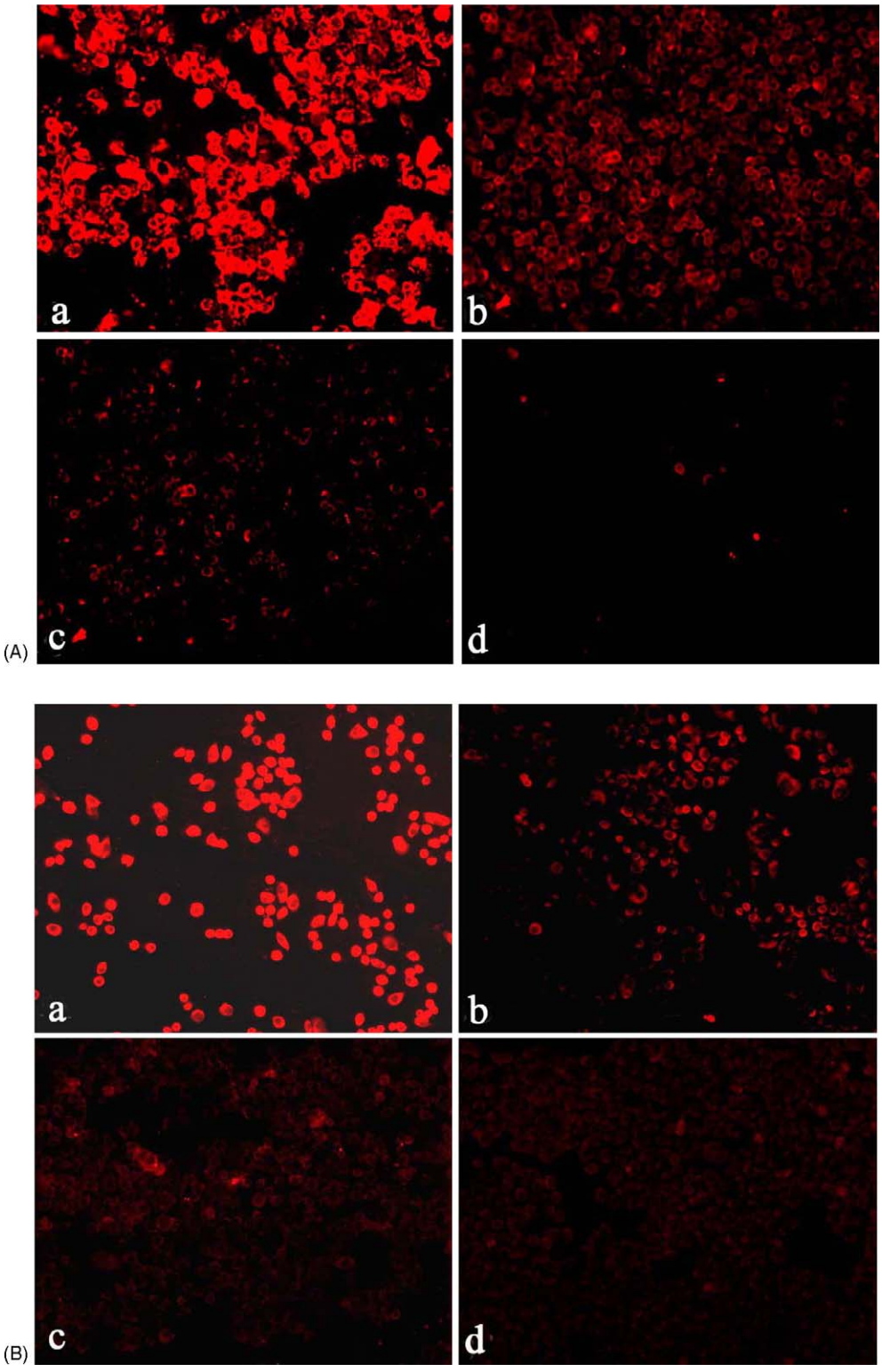

Fig. 2 – Inhibitory effects of vector-based shRNA on EGFR expression of A549 (A) and SPC-A1 (B), transfected with *p*ShNEG as controls (a) and *p*ShEGFR on Days 2 (b), 4 (c), and 6 (d), respectively, detected by immunofluorescence (×200).

and washed with PBS and fixed in 70% ethanol at 4 °C overnight. After being washed twice with cold PBS, cells were resuspended in PBS containing 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A followed with incubation at 4 °C for 30 min.

2.5. *Effects of drug combination*

The effects of *p*ShEGFR on sensitivity of cells to other drugs were determined by 3-[4,5-dimethylthiozol-2yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were transfected with

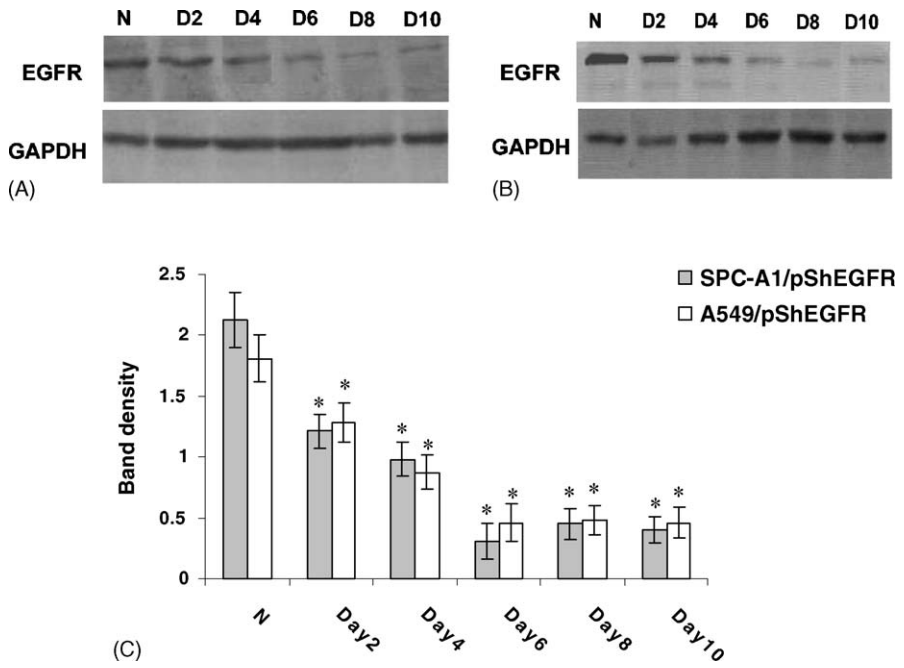

Fig. 3 – Inhibitory effects of vector-based shRNA on EGFR protein expression by Western blot. EGFR expression was suppressed after transfection with *p*ShEGFR in A549 cells (A) and SPC-A1 cells (B), as compared with cells treated with *p*ShNEG (N). EGFR expression was measured 2, 4, 6, 8, and 10 days after transfection with *p*ShEGFR (Day 2–Day 10). The amount of expressed EGFR was quantified from band intensities in A549 and SPC-A1 cells (C). Data are shown as the mean ± S.D. of three independent for each cell lines. (*) stands for the *P* value less than 0.01, as compared with cells with *p*ShNEG.

*p*ShEGFR or *p*ShNEG as described above. After 48 h of transfection, cells were passaged into 96-well plates at the density of 5×10^3 per well and incubated for 96 h with cisplatin, doxorubicin, or paclitaxel at various concentrations. Twenty microliters of MTT (5 g/l) was added to each well and incubated for 4 h to form formazan crystals in active cells. Subsequent DMSO 150 μl was added to each well and absorbance of each well was measured at 595 nm using a microplate reader (Bio-Rad, USA). The survival rate was calculated by the ratio of drug-treated cells to the control.

2.6. *Statistics*

Statistics were conducted by SPSS software. The results were presented with mean ± standard deviation. Student's *t*-test analysis was used to compare mean values after ANOVA analysis. A probability value of $P < 0.05$ was defined as statistical significance. The concentration of inhibiting cell growth at 50% (IC_{50}) was determined by nonlinear regression analysis.

3. Results

HLAC that express EGFR were transiently transfected with *p*ShEGFR, leading to the synthesis of shRNA specifically targeting EGFR. After transfection, EGFR protein level was determined by immunofluorescence and Western blot, respectively, at the indicated time point. The inhibition of EGFR expression was observed in A549 and SPC-A1 cells by *p*ShEGFR, respectively. EGFR expression on A549 cells transfected with *p*ShEGFR decreased from Day 2 and almost disappeared 6 days after transfection, as compared with that on *p*ShNEG-transfected cells (Fig. 2A). Similar results were also seen in SPC-A1 cells (Fig. 2B). The inhibition rate of EGFR amount on harvested cellular member was from 29 to 74% in A549 cells and from 43 to 85% in SPC-A1 cells, respectively, from Day 2 to Day 6 after *p*ShEGFR transfection, remaining at the low level until 10 days (Fig. 3). The *p*ShNEG transfectants did not show suppressive effects on EGFR protein expression.

With the evidence of inhibitory effects of *p*ShEGFR on EGFR expression, we furthermore evaluated its effect on cell growth

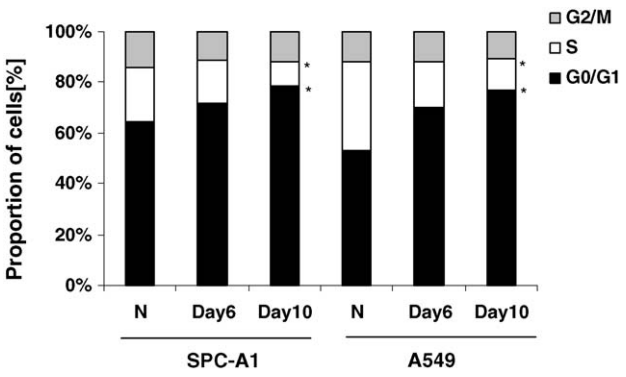

Fig. 4 – Induction of cell cycle arrest in the G0/G1 phase of cells treated with *p*ShEGFR or *p*ShNEG (N). Data are shown as the mean of three independent for each cell lines. (*) stands for the *P* value less than 0.01, as compared with cells with *p*ShNEG.

by colony-formation assay. The colony numbers decreased by 63 and 69% in *p*ShEGFR-transfected A549 and SPC-A1 cells, respectively. Fig. 4 demonstrated that *p*ShEGFR transfection could arrest the proliferation of both A549 and SPC-A1 cells in G0/G1 phase of the cycle, with a time-related response, measured by flow cytometry. It was accompanied by a corresponding decrease in S phase, whereas the proportion of cells in the G2/M phase was almost unaffected in both cell lines (Fig. 4). Apoptotic cells were noted in 26 and 22% of A549 and SPC-A1 cells 7 days after transfection with *p*ShEGFR, while in 2.2 and 0.5% of the control cells.

We furthermore evaluated whether vector-based siRNA targeting for EGFR could alter the sensitivity of A549 and SPC-A1 cells to cisplatin, doxorubicin and paclitaxel, which are commonly used in NSCLC chemotherapy. There was a significant difference in the drug response curves between *p*ShEGFR- and *p*ShNEG-transfected cells (Fig. 5). IC_{50} values of the different drugs are summarized in Table 1. The sensitivity to cytotoxic drugs by shRNA targeting EGFR increased about four- to seven-fold, an independent pattern of action of the chemotherapeutic agents and cell types.

4. Discussion

A growing number of experimental and clinical data demonstrates that the blockade of the EGFR-activated mitogenic pathway is a promising novel therapeutic strategy in the control of human cancer [1]. In addition to approaches targeting the expressed EGFR proteins directly such as EGFR-specific tyrosine kinase inhibitor and anti-EGFR blocking MAbs, other strategies based on modulating biosynthesis of EGFR have been developed, e.g. antisense oligonucleotides specific for EGFR [7]. However, efficiency, specificity and stability of the oligonucleotides limit the widespread use of the approach [9]. Recently double-stranded RNA-mediated interference has recently emerged as a powerful reverse genetic tool to silence gene expression, and might be exploited for gene therapy [10]. There are two main methods for RNAi including chemically synthesized siRNA and vector-based shRNA [11]. In this study, siRNA expression vector silencing technology was used to downregulate the expression of EGFR. We found inhibition of EGFR protein appeared from 2 days and on, lasting at low levels until 11 days after transfection, similar to previous findings when downregulating the expression of other proteins by vector-based shRNA [12,13]. In our previous study, the silencing effect using chemical synthesized RNA targeting EGFR mRNA was found short-lived, and the synthesis of EGFR protein could recover to the original level 6 days after transfection [5]. These results suggested that vector-based shRNA seem to suppress the expression of target gene more durably than chemical synthesized siRNA. Potential explanation on the different inhibitory rate between SPC-A1 (85%) and A549 (74%) cells may be due to various levels of EGFR existed, transfection efficiency and ability to process hairpin RNA into smaller cleavage products.

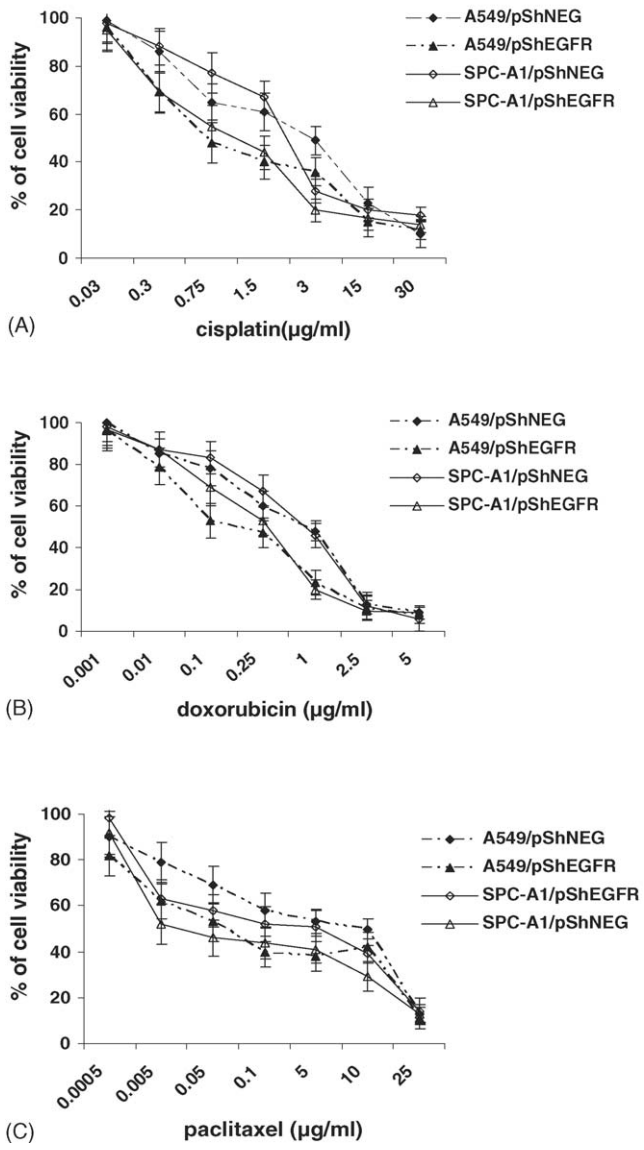

Fig. 5 – Inhibitory effects of *p*ShEGFR on cell growth in combination with cisplatin (A), doxorubicin (B) or paclitaxel (C) by MTT assay. (*) stands for the *P* value less than 0.01, as compared with cells with *p*ShNEG.

Table 1 – IC_{50} values of different drugs in A549 and SPC-A1 cells in combination with vector-based shRNA targeting EGFR (μg/ml)

	A549/*p*ShEGFR	A549/*p*ShNEG	SPC-A1/*p*ShEGFR	SPC-A1/*p*ShNEG
Cisplatin	0.71	4.73	0.82	4.91
Doxorubicin	0.12	0.66	0.23	1.08
Paclitaxel	0.59	3.89	0.51	3.27

Downregulation of EGFR expression by the vector-based shRNA significantly inhibited NSCLC cell growth in both cell lines and led to a time-dependent arrest of cells in the G0/G1 phrase, decreasing the proportion of cells in S phase. Our data suggested that RNAi-downregulated EGFR expression could decrease cell proliferation and growth through apoptosis, rather than cytostatic effect, evidenced by findings observed in other tumor cells after inhibiting EGFR function by either anti-EGFR blocking MAbs or an EGFR-specific tyrosine kinase inhibitor [14].

Effects of gene-silencing targeting EGFR on cell cycle arrest in G0/G1 phrase seemed to be more pronounced in SPC-A1 cells, suggesting these cells more sensitive to RNAi or various ability to process dsRNA or hairpin RNA into smaller cleavage products. Further investigation is necessary to clarify the relationship of the protein level and gene-silencing by RNAi in these cell types. Although 70–80% decrease in EGFR expression by RNAi, our results indicate that such amount of inhibitory effects by *p*ShEGFR is sufficient to abolish EGFR-mediated signaling and cell growth. Partial inhibition of EGFR expression by siRNA could also switch off receptor-dependent tyrosine phosphorylation response in A431 cells [6]. These results supported the hypothesis that EGFR-mediated signal pathway might be completely blocked when the number of EGFR dropped below a given threshold [15]. It may provide feasibility for developing the RNAi technique in modulating expression in anti-cancer therapy targeting EGFR because it is difficult to achieve complete gene knock-out in cancer patients.

The antiproliferative effect of RNAi targeting EGFR may have important clinical implication for anticancer therapy. We combined vector-based shRNA for EGFR with drugs with diverse mechanisms of action and found that the procedure of vector-based shRNA targeting EGFR increased the sensitivity of both SPC-A1 and A549 cells to cisplatin, doxorubicin and paclitaxel. The changes of sensitivity to drugs by RNAi targeting EGFR was four- to seven-fold higher, with an independent mode of action of the chemotherapeutic agents, although we did not know such effects resulted from additive or synergistic mechanisms. These results are consistent with the previous reports showing enhanced antitumor activity of drugs by modulating EGFR biosynthesis with antisense oligonucleotide or approaches targeting the expressed protein such as anti-EGFR monoantibodies and EGFR-TK small molecular inhibitors [7,16]. Blockade of EGFR mitogenic signaling in combination with cytotoxic drugs could cause irreparable cancer cell damage [17]. In addition to the application of RNAi for cancer therapy, it may be also useful for treating other diseases, such as organ dysfunction, a systemic consequence of acute and chronic diseases [21].

In conclusion, we demonstrated that vector-based shRNA could effectively reverse EGFR expression of HLAC and showed the higher efficacy in combination therapies with conventional chemotherapeutic regimens. These findings indicate that combination treatment with vector-based shRNA targeting EGFR may be an attractive strategy in anti-cancer gene therapy. Further investigations on potential therapeutic effects of shRNA on tumor growth in the in vivo system are needed by using different delivery systems, e.g. adenovirus vector [18] or lenti-virus vector [19].

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