Inhibition of epidermal growth factor receptor expression by RNA interference in A549 cells[1]

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KEY WORDS epidermal growth factor receptor; RNA interference; double-stranded RNA; small interfering RNA; non-small-cell lung carcinoma

ABSTRACT

AIM: To investigate the biological features of A549 cells in which epidermal growth factor (EGF) receptors expression were suppressed by RNA interference (RNAi). **METHODS:** A549 cells were transfected using short small interfering RNAs (siRNAs) formulated with Lipofectamine 2000. The EGF receptor numbers were determined by Western blotting and flowcytometry. The antiproliferative effects of sequence specific double stranded RNA (dsRNA) were assessed using cell count, colony assay and scratch assay. The chemosensitivity of transfected cells to cisplatin was measured by MTT. **RESULTS:** Sequence specific dsRNA-EGFR down-regulated EGF receptor expression dramatically. Compared with the control group, dsRNA-EGFR reduced the cell number by 85.0 %, decreased the colonies by 63.3 %, inhibited the migration by 87.2 %, and increased the sensitivity of A549 to cisplatin by four-fold. **CONCLUSION:** Sequence specific dsRNA-EGFR were capable of suppressing EGF receptor expression, hence significantly inhibiting cellular proliferation and motility, and enhancing chemosensitivity of A549 cells to cisplatin. The successful application of dsRNA-EGFR for inhibition of proliferation in EGF receptor overexpressing cells can help extend the list of available therapeutic modalities in the treatment of non-small-cell lung carcinoma (NSCLC).

INTRODUCTION

EGF receptor is a glycoprotein with a molecular weight of 170 000 to 180 000 with an intrinsic tyrosine-specific protein kinase, which is stimulated upon EGF binding. Activation of EGF receptor tyrosine kinase results in the generation of a number of intracellular signals that culminate in cell proliferation[1]. The known downstream effectors of EGF receptor include PI3-K, RAS-RAF-MAPK p44/p42, and protein kinase C signaling pathways. EGF receptor signaling involves in cell growth, angiogenesis and DNA repair, and has been recently assessed as an innovative target in cancer therapy and particularly in NSCLC[2].

RNAi is an evolutionarily conserved process in which recognition of dsRNA ultimately leads to post-transcriptional suppression of gene expression. This suppression is mediated by siRNA (21-23 nucleotides), which induces degradation of mRNA based on complementary base pairing. In several model systems, this natural response has been developed into a powerful

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tool for the investigation of gene function[3].

In mammalian cells, RNAi is initiated by the dsRNA-specific endonuclease III family, Dicer, which promotes cleavage of long dsRNA into siRNA between 21-23 nuleotides duplexes long. siRNA is incorporated into a protein complex (siRNA-induce-silencing-complex, RISC) that recognizes and cleaves target mRNAs. More recently it was discovered that introducing synthetic 21-nucleotide siRNA duplexes into mammalian cells could silence gene expression efficiently. RNAi, for its specificity, catalytic features, and effectiveness, offers a way to inactivate target genes and, thus, provides a powerful tool for the analysis of gene function[4].

Consideration of the importance of EGF receptor in human cancers and efficiency of RNAi led us to design siRNAs against EGF receptor. Here we demonstrated that RNAi could induce EGF receptor gene sequence-specific silencing in A549 cell line by introducing chemically synthesized siRNAs when formulated with lipophilic reagents, and hence suppressed the malignant phenotype of A549 cells. Therefore we can draw a conclusion that RNAi can be developed into a potential tool for gene therapy.

MATERIALS AND METHODS

Cell lines and cell culture A549 is a well-characterized human lung adenocarcinoma cell line, obtained from Shenergy Biocolor Biological Science & Technology Company. Cells were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM, Gibco,US) supplemented with 10 % fetal bovine serum (HyClone,US) in a well humidified atmosphere of 5 % CO_2 at 37 ºC.

siRNAs preparation siRNAs corresponding to EGF receptor gene were designed as recommended, with dTdT 3'-overhangs on each strand. They were chemically synthesized by Dharmacon Research[5]. The following EGF receptor gene-specific sequences were successfully obtained: siRNA-EGFR sense 5'-GGAGCUGCCCAUGAGAAAUdTdT-3' and antisense 5'-AUUUCUCAUGGGCAGCUCCdTdT-3'. The unrelated non-specific dsRNA were designed as follows: siRNA-unrelated sense 5'-GAACUUCAGGGUCAGCUUGCCUU-3' and antisense 5'-GGCAAGCUGACCCUGAAGUUCUU-3'. Annealing for duplex siRNAs formation was performed as described by the manufacture manual. The annealing dsRNAs were analyzed on 15 % PAGE gels electrophoresis.

Transfection experiments Transfection of siRNA-EGFR was conducted with Lipofectamine 2000 (Invitrogen,US) in 6-well plates according to the manufacturer's specifications. Briefly, the day before transfection, the cells were trypsinized, counted, and seeded at 1×10^5 cells per well into 6 well plates, so that they were 40 %-45 % confluence on the day of transfection. Lipofectamine 2000 diluted in serum-free DMEM was supplemented to the dsRNA mixture, and the formulation lasted for 25 min. 4 μg of 21-bp dsRNA formulated with 8 μL of Lipofectamine 2000 was applied to the final volume of 2 mL complete DMEM. The cells were incubated for another 48 h without the replacement of the medium. The cell number was determined after trypsinization using a hemocytometer before subsequent assays.

Western blotting The cells in a single well of a 6-well plate were lysed in SDS sample buffer, and the proteins were separated on 7 % polyacrylamide gels and transferred to nitrocellulose membranes. Mouse anti-EGFR monoclonal antibodies (MAb), a generous gift from Shanghai Institute for Cell Biology, Chinese Academy of Sciences, were used at 500-fold dilution, followed by peroxidase-conjugated goat anti-mouse immunoglobulin for immunoblotting. Enhanced chemiluminescence (Amersham, Germany) was adopted for detection.

Assessment of the EGF receptor numbers With the cells harvested by trypsinization, all the following steps were performed on ice. The cells were washed twice with 1×PBS, and then incubated with MAb for 1 h at 37 ºC. After that, they were washed twice again before they were stained with a FITC-conjugated rabbit anti-mouse antibody (Antibody Diagnostica Inc, Shanghai) for 45 min in the dark. Afterwards the cells received the same washing process and fixed in 0.5 mL of 4 % paraformaldehyde. The EGF receptor numbers were analyzed on a fluorescent microscopy or on a Becton Dickinson FACScan flowcytometry with excitation and emission settings of 488 and 530 nm respectively. To determine the change in EGF receptor numbers in A549 cells, we used positive cell percentage× mean intensity to evaluate the intensity of fluorescence.

Colony forming assay Survival after RNAi was defined as the ability of the cells to maintain their clonogenic capacity and form colonies. Briefly, after transfected with dsRNA/Lipofectamine 2000 mixture, cells were trypsinized, counted, and seeded for colony formation in 60 mm dishes at 300 cells/dish. After a

14-d incubation, the colonies were stained with crystal violet and manually counted. Colonies containing more than 50 cells were scored, and triplicates containing 10-150 colonies/dish were counted for each treatment.

Scratch assay Cells were seeded in triplicates in collagen IV coated 60-mm culture dishes at 1×10^5 cells/dish. A scratch through the central axis of the plate was gently made using a pipette tip when the cells were transfected for 4 h. Migration of the cells into the scratch was observed at time points of 24 h and 48 h.

Chemosensitivity assay The effects of dsRNA-EGFR on the chemosensitivity of A549 to cisplatin were evaluated using MTT assay as described previously. Briefly, exponentially growing cells were seeded into 96-well plates. Attached to the culture plates overnight, the cells were transfected with dsRNA/Lipofectamine 2000 mixture as described above. Four hours later, they were exposed to increasing concentrations of cisplatin ranging from 0 to 50 μg/L for another 48 h. MTT (1 g/L) 20 μL was added to each well and incubated for 4 h at 37 ºC to allow MTT to form formazan crystals by reacting with metabolically active cells. Subsequently the formazan crystals were solubilized by DMSO 150 μL. The absorbance of each well was measured in a microplate reader at 490 nm. The percentage of cell growth was calculated by comparison of the A_{490} readings from the treated versus the control.

Statistical analysis The effects of dsRNA-EGFR on EGF receptor gene expression, growth inhibition, colony formation and scratch assay were assessed by *t*-test. Differences were considered to be significant at $P<0.05$. SPSS10.0 software was used to perform statistical analysis. In chemosensitivity experiments, which involved multiple cisplastin doses, the linear quadratic model was fitted with Origin 6.0 software.

RESULTS

RNAi in A549 cells 21-nt sense and antisense single strand RNA (ssRNA) were synthesized separately, and the pairs were annealed to create the dsRNA with the characteristics of siRNA (Fig 1).

We observed the EGF receptor gene expression in A549 cell line on a fluorescent microscopy. In order to exclude the nonspecific binding, we termed A549 cells stained with FITC-conjugated rabbit anti-mouse antibody with or without MAb as positive control or negative control respectively. The results showed that EGF receptor was significantly inhibited by the corresponding dsRNA, but not by unrelated dsRNA in A549 cells (Fig 2).

The results obtained from Western blotting revealed that dsRNA-EGFR could inhibit EGFR expression markedly. The values of empty vector group, dsRNA-unrelated group and dsRNA-EGFR group were 0.85±0.30, 0.77±0.22 and 0.39±0.08, respectively. Compared with control, dsRNA-EGFR reduced EGF receptor expression by 61.0 % ($P<0.05$) (Fig 3A). The EGF receptor number assessed on a flowcytometry further conformed that dsRNA-EGFR dramatically reduced EGF receptor expression to the level which was 64.8 % lower than that observed in the control ($P<0.01$). Although the intensity of fluorescence decreased slightly in both empty vector and dsRNA-unrelated group, the difference was not significant ($P>0.05$) (Fig 3B & 3C).

Growth inhibiton We examined the antiproli-

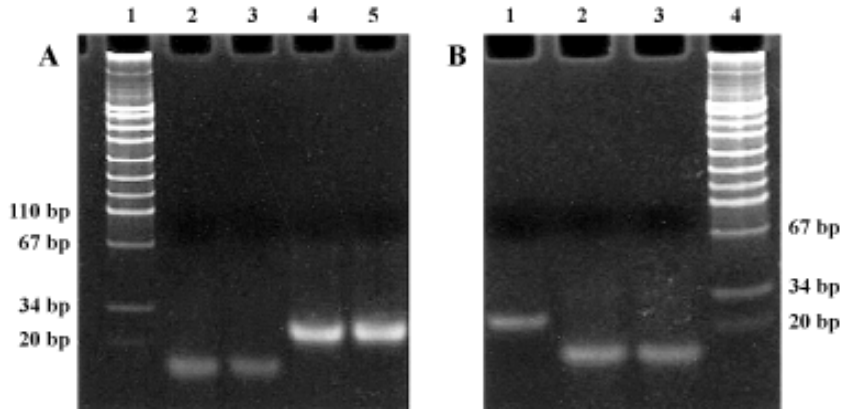

Fig 1. Results of siRNAs on 15 % PAGE gels. A) siRNA-EGFR on PAGE gels. Lane 1: Marker, Lane 2,3: sense and antisense strand of RNA-EGFR, Lane 4,5 dsRNA-EGFR; B) siRNA-unrelated on PAGE gels. Lane 1: dsRNA-unrelated, Lane 2,3: sense and antisense strand of RNA-unrelated, Lane 4: Marker.

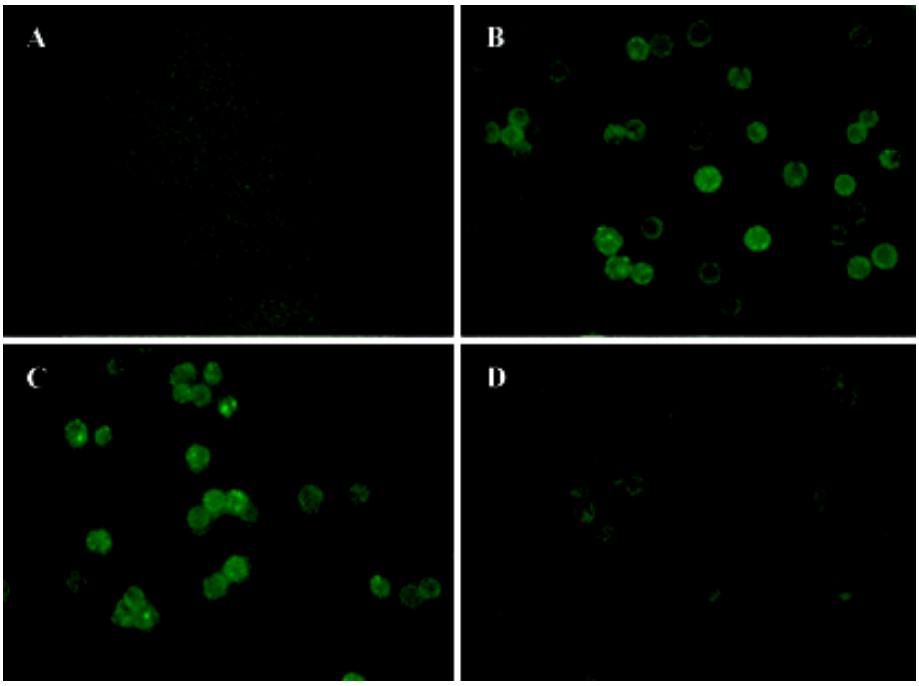

Fig 2. siRNA-mediated inhibition of the expression of EGFR gene in A549 cells. EGF receptor gene expression was observed on a fluorescent microscopy. A) Negative control; B) Positive control; C) Transfected with dsRNA-unrelated; D) Transfected with dsRNA-EGFR. ×200.

ferative effects of dsRNA-EGFR on A549 cells using cell count and colony assay. Our results demonstrated that cell numbers in the control, empty vector, dsRNA-unrelated and dsRNA-EGFR group were $(40\pm3)\times10^4$, $(31\pm3)\times10^4$, $(29\pm2)\times10^4$, $(6\pm1)\times10^4$, respectively. Compared with the control, dsRNA-EGFR reduced the cell number by 85.0 % ($P<0.001$). Empty vector and dsRNA-unrelated also inhibited the cell growth by 22.5 % and 27.5 %, respectively ($P<0.05$), but to a much lesser extent than dsRNA-EGFR. From the results that there existed no significant differences between the empty vector and dsRNA-unrelated group ($P>0.05$), we concluded that the antiproliferative effects induced by dsRNA were sequence specific and the cell growth inhibition in the empty vector and dsRNA-unrelated group originated from the cytotoxic effects of Lipofectamine 2000.

Colony assay achieved the similar results. dsRNA-EGFR decreased the colonies by 63.3 %, and thus, showed significantly growth inhibition ($P<0.01$). In comparison with the control, the empty vector and dsRNA-unrelated resulted in a little decrease in colony forming. The inhibitory rates were 14.8 % and 24.1 %, respectively, between which, however, there existed no difference (Fig 4).

Scratch assay The migration of A549 cells was quantitatively assessed at 24 h after the introduction of a scratch in monolayer cells grown on collagen IV. The results showed that cells transfected with dsRNA-EGFR demonstrated lower motility. Compared with the control, dsRNA-EGFR decreased the invasive ability of A549 cells by 87.2 % at 24 h ($P<0.01$). As shown in Fig 5, there was a negligible decrease in cell invasion in the plates treated with empty vector and dsRNA-unrelated, which reduced invading cells by 13.1 % and 22.7 % at 24 h respectively ($P>0.05$). By 48 h, the wound was barely visible in the control plates but was still clear in the treated ones, particularly in those transfected with dsRNA-EGFR. Therefore we concluded that dsRNA-EGFR had potential capacity to block invasive properties of A549 cells.

Cisplatin sensitivity The chemosensitivity of A549 cells transfected by dsRNA-EGFR to cisplatin was determined by MTT assay. Cisplatin inhibited cellular proliferation in a dose-dependent manner (Fig 6). This trend was supported by a significant interaction between growth inhibitory effect and the dose of cisplatin. Cells transfected with dsRNA-EGFR were more sensitive to cisplatin than the control. From the value of IC_{50} obtained by Origin 6.0 software, we concluded that dsRNA-EGFR increased the sensitivity of A549 to cisplatin by four-fold with an addictive or synergistic effect.

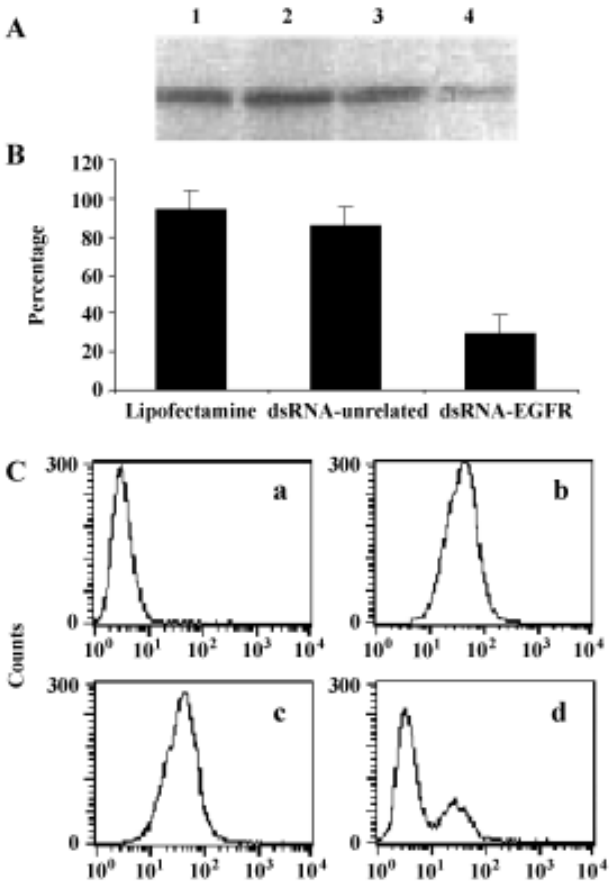

Fig 3. EGF receptor gene expression was quantified in both control and targeted cells by Western blotting and flowcytometry. A) Quantitative evaluation of inhibition of EGF receptor expression in A549 cells by Western blotting. 1, 2, 3, 4 indicated EGF receptor expression in control, empty vector, dsRNA-unrelated and dsRNA-EGFR group, respectively. B) EGF receptor gene expression was quantified by flowcytometry. Results were expressed as the percentage of intensity of fluorescence relative to controls. *n*=3. Mean±SD. C) EGF receptor gene expression was shown by the graph of flowcytometry. (a) Negative control; (b) Positive control; (c) Transfected with dsRNA-unrelated; (d) Transfected with dsRNA-EGFR.

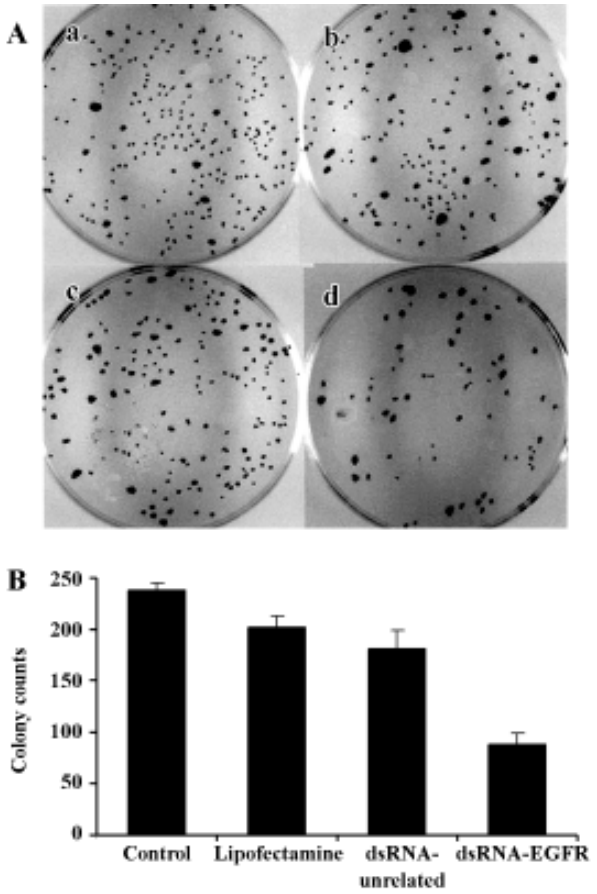

Fig 4. Effects of dsRNA-EGFR on the colony forming of A549 cells. A) Colonies were scanned by BIO-RAD Image Doc. Colonies of A549 cells in control group (a), empty vector group (b), dsRNA-unrelated group (c), dsRNA-EGFR group (d). B) Colonies containing more than 50 cells were scored, and triplicates containing 10-150 colonies/dish were counted for each treatment. *n*=3. Mean±SD.

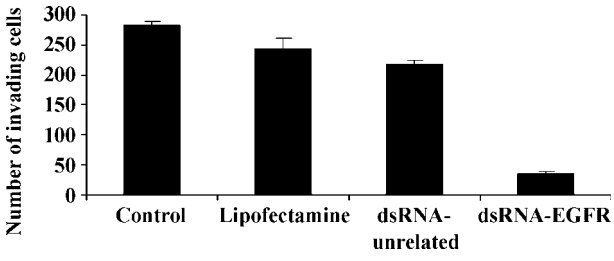

Fig 5. Effects of dsRNA-EGFR on the motility of A549 cells. *n*=3. Mean±SD.

DISCUSSION

Increased expression of the EGF receptor is common in cancer and correlates with neoplastic progression. Blockade of EGF receptor signaling pathway represents a new perspective on the development of novel and selective anticancer therapies. Although considerable progress has been made in the application of EGF receptor-targeted antibodies and small molecule tyrosine kinase inhibitors, none of these agents is curative[6]. In an effort to find new approaches to target EGF receptor overexpressing cancer cells, we set out to focus on the RNAi-induced gene suppression.

It had been reported previously that in mammalian cells RNAi should prove superior to antisense approaches in suppressing target gene expression. Furthermore dsRNA was more stable than antisense RNA[7]. Our current observation supported capped dsRNA was ac-

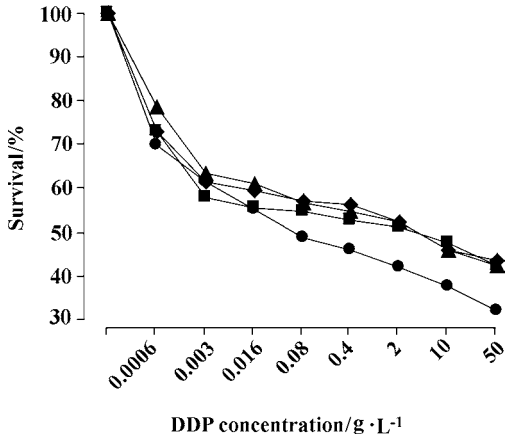

Fig 6. Effects of dsRNA-EGFR on the sensitivity of A549 cells to cisplatin. Cells were exposed to varying concentrations of cisplatin. ◆, ■, ▲, ●, represented the corresponding values in control, empty vector, dsRNA-unrelated and dsRNA-EGFR group, respectively.

tive and stable. It also indicated that Lipofectamine 2000 worked well in siRNAs transfections. We achieved a pronounced inhibition of EGF receptor expression in A549 cells, and the results proven quantitatively consistent from Western blotting and flowcytometry.

Previous literature has reported that the degree to which the expression of a given protein lent itself to inhibition by RNAi depended on the half-life and synthesis rate of the protein. Due to its much faster turnover, EGF receptor was considered to be more difficult to be knocked down using RNAi, because even if the biosynthesis of the protein was blocked, previously synthesized molecules persisted in the plasma membrane for a relatively long time[8]. Nevertheless the fact that we successfully inhibited the expression of EGF receptor in A549 cells provided the evidence for the high potency of the RNAi phenomenon.

Experimental and clinical data had indicated that EGF receptor overexpression correlated with various critical processes in the development, maintenance, and spread of malignant tumors[6]. The reduction of EGF receptor led to a failure in downstream signal cascades, and subsequently blocked the routes to gene activation or more direct modulators of mitogenesis and other cancer-promoting phenotypes. Although the expression of EGF receptor in A549 cells was reduced significantly by 64.8 %, the transfected cells still presented more than 30 % receptors. The fact that these cells displayed dramatic growth inhibition in cell counts and colony assay suggested an incomplete suppression of EGF receptor expression was sufficient to block growth factor-mediated signaling. This conclusion was consistent with the theory that the activation of tyrosine phosphorylation response might be completely switched off if the number of EGF receptor dropped below a given threshold[9]. In order to test whether the down-regulation of EGFR gene expression could also inhibit cell motility, we assessed the migration of A549 cells using a scratch-wound assay, which provided a qualitative picture of how rapidly cells migrated back to the disrupted region from a monolayer. We achieved affirmative results as expected.

Growing evidence indicates that activating the EGF receptor signal transduction pathway might induce chemoresistance in NSCLC cells[10]. One of the major causes involving in high levels of EGF receptor affecting the sensitivity of NSCLC cells to cisplatin, a commonly used DNA-damaging anticancer agent, was presumably by modification of DNA repair activity[11]. In previous studies, the degree of EGF-induced enhancement of sensitivity to cisplatin was only observed within the range of 2- to 4- fold. However this represented a clinically significant effect since acquired cisplatin resistance could be partially overcome by increasing cisplatin dose by 2-fold[12]. Our results revealed that reduction of EGF receptor resulted in enhanced chemosensitivity of A549 cells to cisplatin by four-fold probable due to this mechanism.

In summary, we demonstrated that dsRNA-EGFR had implications for further clinical studies to establish a radical therapy for NSCLC. Nevertheless, the experiments *in vivo* will be needed to determine the feasibility of this investigation.

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