Inhibition of EGFR signaling in prostate cancer treated with EGFR siRNA and Gefitinib

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Abstract: To validate the therapeutic effects and modification of EGFR-induced signaling proteins of gefitinib and a small interfering RNA targeting human EGFR (EGFR siRNA) on prostate cancer cell lines PC-3. MTT assay and tumor inhibitory rate were used to evaluate the antitumor activity of EGFR siRNA and/or gefitinib on PC-3 cells *in vitro* and *in vivo*. Real-time PCR was used to measure expression of EGFR mRNA; Western blot assay was applied to evaluate the level of EGFR and its downstream signalling proteins Akt (protein kinase B), MAPK (mitogen-activated protein kinase) and PKC (protein kinase C). Gefitinib inhibited PC-3 cells proliferation in a dose-dependent manner with significant decreased level of EGFR protein and phosphorylation of only Akt, but not either MAPK or PKC; on the other hand, knockdown of EGFR mRNA by siRNA led to lower proliferation of PC-3 cells with decreased phosphorylation of Akt and MAPK, but not PKC. Combination of both had more inhibitory effects on cells than gefitinib and EGFR alone with decreased level of Akt, MAPK and PKC phosphorylation ($P < 0.05$). In *in vivo* models, compared with control group, siRNA could significantly inhibited tumor growth at the rate of 34.83% ($P<0.05$) which is lower than 53.95% in gefitinb group and 59.28% in combined group ($P<0.05$), but no differences in the latter both groups ($P>0.05$). Gefitinib and EGFR siRNA could effectively inhibit the proliferation and tumor growth of prostate cancer, probably via inhibiting the activation of EGFR and the phosphorylation of Akt and MAPK.

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1. Introduction

Prostate adenocarcinoma is one of the most common cancers in aged men (Shteynshlyuger and Andriole, 2010). Most of the patients with prostate cancer died from the metastasis to the bone and lymph nodes of cancer cells that conventional androgen-deprivation therapy ultimately fails so that new therapeutic strategies are needed for its treatment and prevention. Prostate cancer commonly overexpresses several growth factors and their receptors, including epidermal growth factor (EGF) and its receptor (EGFR). EGFR plays a critical role in tumor growth and the prostate tissue becomes more susceptible to the growth-promoting action of EGF family growth factors during androgen withdrawal (Traish et al., 2009). Therefore, inhibiting the activation of EGFR and EGFR-induced tyrosine kinase signaling pathways provides therapeutic advantage especially against prostate cancer metastasis (Kim et al., 2006).

EGFR is a membrane glycoprotein composed of an extracellularbinding domain, a transmembrane domain containing a single hydrophobic anchor sequence and an intracellular domain containing tyrosine kinase activity. Activation of EGFR involved in the recruitment and activation of downstream intracellular-signaling cascades, mainly including the mitogen-activated protein kinase (MAPK) pathway, protein kinase C and phosphatidylinositol-3-kinase (PI-3K) /Akt pathways (Hynes and MacDonald, 2009). These signaling cascades can promote proliferation, angiogenesis and invasion and inhibit apoptosis, key mechanisms underlying tumor growth and progression (Iain et al., 2006).

Considering the overexpression of EGFR in prostate cancer, silencing of EGFR expression appears to be a rational strategy for targeting prostate caner. Small interfering RNA (siRNA) would specifically target cells expressing EGFR, providing significant cell specificity for this strategy. siRNA molecules can be efficiently introduced into cells in a permanent manner through expression systems such as lentiviruses that are capable of integration into the cellular genome. This approach for permanent delivery of siRNA has the potential of being translated into important clinical applications (Naldini, 1999). In this study, we have used siRNA-expression lentivirus targeting human EGFR to silence EGFR expression in prostate cancer.

Gefitinib (Iressa, ZD1839) is a quinazoline derivative and an orally active EGFR tyrosine kinase inhibitor (TKI) that competitively binds ATP of EGFR and blocks signal transduction processes implicated in the proliferation, metastasis and

angiogenesis of cancer cells. Gefitinib has been approved as a single drug therapy for lung cancer following very encouraging data obtained in clinical trials (Cohen et al., 2004). It has also shown antiproliferative and anti-invasive effects in other human cancers with amplified or transfected EGFR. Angelucci et al (2006) have shown that gefitinib was effectively inhibiting EGFR-dependent growth in prostate cancer primary cultures and cells lines, independently of their sensitivity to androgen.

In the present study, we undertook not only to investigate and compare the antitumor activity of gefitinib and/or EGFR siRNA against PC-3 cells *in vitro* and *in vivo*, but also to examine the inhibitory effect on EGFR expression and EGFR-induced downstream survival signaling proteins phosphorylation, such as Akt, MAPK and PKC.

2.Materials and Methods

Chemicals and antibodies: Gefitinib was purchased from AstraZeneca Pharmaceutical Co. (Macclesfied, UK). The primary antibodies included rabbit monoclonal antibody against phosphorylated (p)-Akt purchased from Cell Signaling (Beverly, MA, USA), and EGFR, p-PKC and p-MAPK purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibody were peroxidase-labeled antibodies against rabbit (Santa Cruz, CA, USA). Anti-β-actin antibody was purchased from Sigma-Aldrich Company Ltd. (Allentown, PA, USA). Drugs were dissolved in dimethyl sulfoxide (DMSO) for *in vitro* and stored at –20°C. The final concentration of DMSO in culture medium for all treatments was not greater than 0.1%.

Cell culture and transfection of siRNA: PC-3 cell was purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China) and cultured in dulbecco's modified Eagle medium (DMEM, Gibco, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 mg/ml) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. A lentiviruse expressing siRNA targeting human EGFR (EGFR siRNA), marked with green fluorescent protein (GFP), was purchased from Shanghai Benefit Biothechnology Company (Shanghai, China). It's virus titer is 1.7×10^5 ifu/μl. The sequences of EGFR siRNA duplex are: sense strand, 5'-GCAGAGGAATTATGATCTT-3'; antisense strand, 5'-TAATCGTCGTAGACGGTTG-3'. It is homologous to 456–474 nt of human EGFR transcript. PC-3 cells were incubated with EGFR siRNA solution (final concentration of 150 nM) at 37°C. 72 h after initial transfection, cell infection rate was measured by counting green fluorescence and EGFR siRNA stably transfected PC-3 cells (si-PC-3 cells) were obtained. si-PC-3 cells were seeded into 96-well plates (for cell proliferation assays) or 6-well plates (for RT-PCR of immunoblot analysis).

MTT assay: The MTT ((3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, Sigma, St. Louis, MO, USA) assay was performed to determine cell proliferation. Briefly, prostate cancer cells were plated in 96-well plates at a density of 3×10^3 per well in 100 μl of medium. After attachment overnight, cells were divided into different groups including PC-3 or si-PC-3 cells treated with DMSO (0.2%) as a control, PC-3 cells treated with gefitinib at varying final concentrations (0.5, 1, 2.5, 5 and 10 mg/mL) or si-PC-3 cells treated with gefitinib at a final concentrations of 5 mg/ml for various time periods (24 h, 48 h, 72h, 96h and 120h respectively). After treatment, 20 μL of MTT solution (5 mg/mL) was added to each well. After 4 h incubation, medium was removed and then dissolved by adding 150ul of DMSO to the plates. Color intensity of the solubilized formazan was measured at 570 nm and 630nm with an enzyme linked immunosorbent assay (ELISA) plate reader (Varioskan flash, American Thermo Instruments). This procedure was repeated four times. The growth inhibition was calculated according to the following formula: growth inhibition (%) = (mean OD of the control wells - mean OD of the treated wells)/mean OD of the control wells×100%. The 50% inhibitory concentration (IC50) was calculated from the linear equation, which was deduced using concentration versus growth inhibition regression curve.

Fluorescent realtime PCR: Following the provider's protocol, Trizol reagent kit (Promega, Shanghai, China) was used to isolated total RNA from PC-3 cells or si-PC-3 cells, treated or untreated with gefitinib (5mg/L) for 72 h. The purity of DNA was verified by the ratio A260/A280 = 1.80 – 1.93. The RNAs were frozen at -80℃ until analyzed. cDNA was synthesized from mRNA samples and subsequently used as template for fluorescent real-time PCR assays. Amplicons were visualized on a 1% agarose gel containing 0.2 μg / μL ethidium bromide. A 100-bp ladder (promega) was used as a size standard. A set of primers was designed for real-time PCR (sequences 5'→3') as follows: *EGFR* forward (F): GTGGGGCCGACAGCTATGAGAT, *EGFR* reverse (R): ACCGGCAGGATGTGGAGAT (190bp amplicon); β-actin (as an internal control) F: CCTGTACGCCAACACAGTGC, β-actin R: ATACTCCTGCTTGCTGATCC (211 bp amplicon). For fluorescent realtime PCR, the Qiagen Master Mix kit was used according to the vendor guidelines. A total reaction volume of 25 uL containing 12.5 uL Master Mix, 2 uL 25 mM MgCl2, and 0.25 uL 25x SyBr Green (BioWhittaker Molecular Applications,

Rockland, ME). For EGFR, 5 uL cDNA was used, and 3 uL was used for β-actin control. Samples were processed using the Cepheid Smart Cycler software (Cepheid Systems, Sunnyvale, CA) following 40 amplification cycles (15 seconds of denaturizing at 94℃; 20 seconds of annealing at 60℃; and 20 seconds of extension at 72℃). Melt curve analysis of each sample was supplemented with agarose gel electrophoresis of randomly selected samples to confirm the success of reactions. Fluorescence spectra were recorded during the annealing phase of the reaction. Second derivative analysis of the amplification curves was performed to determine the threshold cycles for each sample. The EGFR mRNA level for each sample was measured by using UVIDOC software (Topac, Cohasset, MA) in order to calculate the values of EGFR / β-actin.

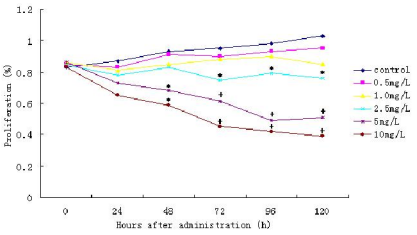
Western blotting: PC-3 or si-PC-3 cells were treated with DMSO (0.2%) as a control or gefitinib at a final concentration of 5 mg/mL for 72 hours. After treatment, cells were harvested and lysed on ice with lysis buffer. Protein concentrations of the supernatants were measured by using the Bradford assay (Bio Rad, Hercules, CA, USA). Protein (20 mg) was separated on 8% or 10% SDS-polyacrylamide gel by electrophoresis and transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). Membranes were blocked overnight at 4°C in blocking buffer and then immunoblotted with primary antibodies (all at a 1:1000 dilution) overnight at 4°C. The blots were then incubated with the appropriate secondary antibodies (at a 1:2000 dilution) conjugated with horseradish peroxidase for 2 h at room temperature. The proteins were visualized with the Super Signal Chemiluminescent Substrate (Pierce, USA). The intensity of visualized bands was measured with Quantity One software (Ver 4.4.0, Bio-Rad, Hercules, CA).

Tumor xenograft model: Female nude mice (6 weeks old, weighing 20-25 g, Balb/c) were purchased from Suzhou University Animal Center (Suzhou, China). The certificate number is SYXK (Jiangsu) 2007-0035. Mice were introduced to establish xenograft tumor models of PC-3 cells and si-PC-3 cells respectively. All *in vivo* animal studies described here were carried out in compliance with the standards for use of laboratory animals. The axilla of the upper limb of nude mice were injected subcutaneously with exponentially growing 1×10^7 PC-3 cells or si-PC-3 cells suspended in 0.1 ml physiological saline respectively. When the diameter of the tumors reached over 0.5 cm in diameter after 2 weeks of implantation, selective 24 mice were randomized in average into 4 groups and then drug treatment was initiated. Four groups included: control group (treated with saline only, n = 6), gefitinib group (n = 6), si-PC-3 group (treated with saline only, n = 6) and si-PC-3 cells treated with Gefitinib group (combined treatment, n = 6). Gefitinib (50 $mg\cdot kg^{-1}\cdot d^{-1}$) were orally administrated for 6 days every week for 2 weeks. When all the mice were sacrificed 10 days after the last treatment, the tumors were removed and tumor volume (length × $width^2$ /2) was measured and weighed. The relative tumor volume at day n (RTVn) versus day 0 was expressed according to the following formula: RTVn = TVn/TV0. Tumor regression (T/C (%)) in treated versus control mice was calculated using: T/C (%) = (mean RTV of treated group)/(mean RTV of control group) × 100%.

Statistical analysis: Data are expressed as the mean ± standard deviation (SD). Pictures of the Western blot assay were analyzed using software Image J. Data of the representatives were analyzed for statistical significance using analysis of variance (ANOVA). All statistical analyses were performed with SPSS12.0. *P* values <0.05 was considered statistically significant.

3.Results

Inhibitory effect of gefitinib and/or EGFR siRNA on the proliferation of PC-3 cells: To determine the effects of gefitinib, EGFR siRNA and their combination on cellular proliferation, the MTT assay was performed on PC-3 cells at different time points. PC-3 cells were incubated with various concentrations of gefitinib for various time periods. Gefitinib inhibited cellular proliferation in a dose-dependent manner. The percentage of growth inhibition was lower from 30% to 80% at concentrations of 0.5, 1, 2.5, 5 and 10 mg/mL of gefitinib than control (Fig. 1A). The values of IC_{50} of gefitinib were (5.12 ± 0.41) mg / mL. GFP labeled EGFR siRNA (EGFR-GFP) was used to test the efficiency of EGFR siRNA expressing lentivirus transfecting PC-3 cells. When PC-3 cells were tranfected by EGFR-GFP, the intensity fluorescence from GFP was measured to calculate transfection efficiency under fluorescence microscope. A PC-3 cell line that stably expresses EGFR-GFP was generated 72 h after initial treatment. The transfection efficiency of lentivurus to PC-3 cells was about 85%. Compared with control, the viability of the PC-3 cells stably transfected with EGFR siRNA (si-PC-3 cells) maintained a lower rate at 40% - 50% ($P < 0.01$) and did not rebound (Fig. 1B and 1C). The combination treatment had a more potent inhibitory effect on cell viability than gefitinib or EGFR siRNA alone, with viability at 35%, 61% of gefitinib and 46% of EGFR siRNA at 72 h, respectively (P<0.05, Fig. 1B).



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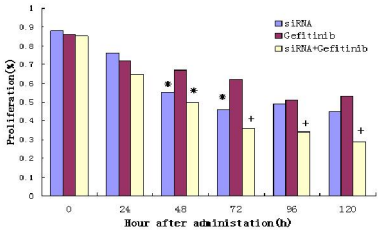

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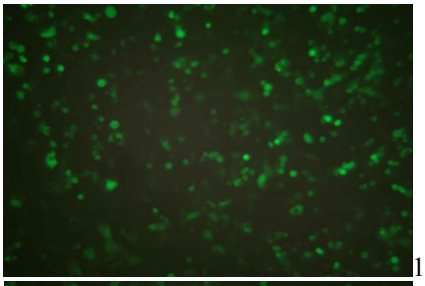

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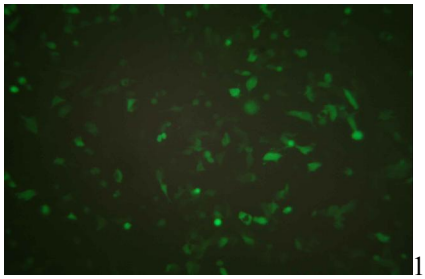

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Fig. 1 Inhibitory effect of gefitinib and/or EGFR siRNA on PC-3 cells measured by MTT. **1A.** Inhibitory effect of gefitinib on PC-3 cells at different doses for various periods. Gefitinib inhibited PC-3 cell proliferation in a dose-dependant manner. **P* < 0.05 versus control; +*P* < 0.05 versus gefitnib (2.5 mg/L). 1B. Combined effects of gefitinib (5 mg/L) and EGFR siRNA (150 nM) on PC-3 cell proliferation. Cell viability of PC-3 cells was measured by MTT assay after treatment with gefitinib, EGFR siRNA or a combination of both for various periods. **P* <0.05 compared with gefitinib; +*P* <0.05 compared with EGFR siRNA. 1C. PC-3 cells were transfected by EGFR siRNA expressing lentivirus labeled with GFR. The transfect efficiency of lentivirus for PC-3 cells was 85% (green, Fig. 1c1, × 100). After 72 h of initial tranfection, proliferation of PC-3 decreased dramatically (Fig. 1c2). The experiment was performed three times and each time triplicate cell cultures were examined. Data are shown as mean ±SD.

Gefitinib and/or EGFR siRNA decreased expression of EGFR: Overexpression or amplification of EGFR plays a key role in prostate cancer progression. To determine the effects of gefitinib (5 mg/mL), EGFR siRNA (150 nM) and their combination on cells, EGFR expression was measured on PC-3 cells at two selected time points (0 and 72 h) to determine the role of EGFR signaling in prostate cancer. Our study showed that the expression level of EGFR protein was high at 48% in PC-3 cells (Fig. 2B). Therefore, we measured EGFR expression by realtime PCR and Western blot respectively to determine the role of EGFR signaling in gefitinib-induced growth inhibition in PC-3 cells. Gefitinib decreased total EGFR protein by 78.3% after 72 h of exposure (Fig. 2B), but the level of EGFR mRNA unchanged compared with control (Fig. 2A). Such a discrepancy between the level of EGFR mRNA and protein strongly suggested that gefitinib, as a TKI would inhibit EGFR expression by regulating translation from EGFR, which would be elucidated by further study. Testing the efficiency and specificity of the individual EGFR siRNA in knocking down EGFR was necessary to confirm the results of the screen. Therefore, we examined EGFR siRNA effectiveness using real-time PCR and Western blot respectively. The result revealed that EGFR mRNA levels were reduced up to 92.5% after treatment with EGFR siRNA (Fig. 2A) consistent with the similar effect on EGFR protein expression (88.9%, Fig. 2B). Thus, EGFR siRNA, when added to PC-3 cells, demonstrated a near complete knockdown of EGFR expression. This experiment suggested that treatment of PC-3 cells with EGFR siRNA lead to an efficient depletion of EGFR mRNA and protein. As shown in Fig. 1B, proliferation of EGFR knockdown cells (si-PC-3 cells) was still inhibited by gefitinib despite that there was no big difference on EGFR expression between EGFR siRNA alone treatment and combination of both. It suggests that gefitinib may have other targets beside EGFR. It was reported that gefitinib induced apoptosis of cancer cells via VEGF and IL-8. However, Our above results showed downregulation of EGFR expression along with cell proliferation suppressed significantly, indicating that EGFR plays an important role in proliferation of PC-3 cells.

Gefinib and/or EGFR inhibited the phosphorylation of EGFR-induced downstream signaling proteins, such as p-Akt, p-MAPK and p-PKC: The PI3K/Akt, ras/MAPK and PKC pathway were recognized as playing critical regulatory roles in the cell survival/death decision.

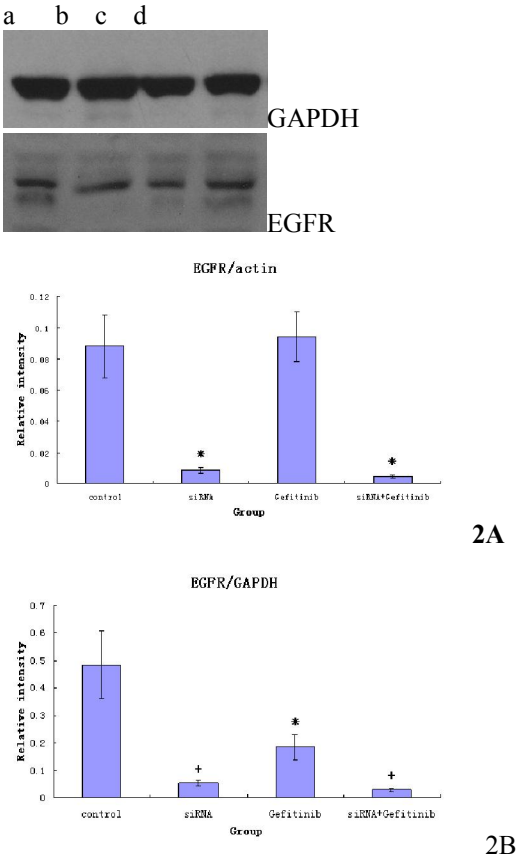

Fig. 2 Effects of gefitinib (5 mg/L), EGFR siRNA (150 nM) or combination of both on the EGFR expression after 72-hour exposure. 2A. Inhibition of EGFR mRNA expression in PC-3 cells treated by gefitinib, EGFR siRNA or combination of both. For evaluation of the EGFR mRNA, realtime RT-PCR was used. Actin was used as an equal loading control. Relative intensity of EGFR mRNA was measured by densitometry analysis. *P <0.05, compared with control and gefitnib. 2B. PC-3 cells were pretreated with compounds for 72 h. For evaluation of the EGFR protein, western blotting was used. GAPDH was used as an equal loading control. Relative intensity of EGFR was measured by densitometry analysis. *P <0.05 compared with control; +P <0.05 compared with gefitinib. a, control; b, gefitinib; c, EGFR siRNA; d, combination of gefitinib and EGFR siRNA.

To analyze those downstream signaling events of the EGFR pathway in our study, we next examined EGFR-induced phosphorylation of Akt, MAPK and PKCl in PC-3 cells treated with gefitinib, EGFR siRNA or their combination 72 h after initial treatment by using Western blot assay (Fig. 3). Our study showed that level of p-MAPK was higer than that of p-Akt and p-PKC in PC-3 cells. Gefitinib could efficiently inhibit level of p-Akt, but not levels of p-MAPK or p-PKC.

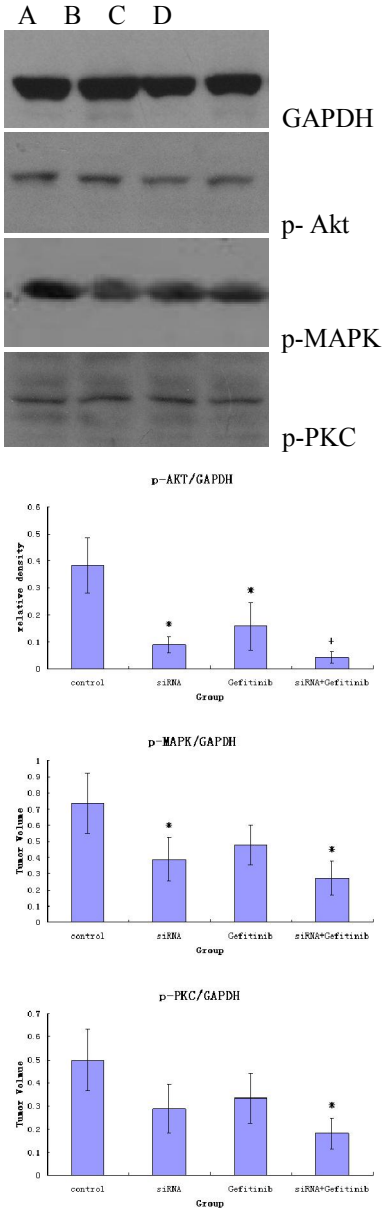

Fig. 3. Inhibition of EGFR-induced downstream signaling proteins phosphorylation in PC-3 cells by combination of gefitinib and EGFR siRNA. PC-3 cells were treated with compounds (gefitinib 5 mg/mL; EGFR siRNA 150 nM; or their combination) for 72 hours. Protein extracts were used for Western blot analysis of EGFR-induced downstream signaling proteins phosphorylation by using phospho-specific antibodies. Phosphorylation of Akt, MAPK or PKC was measured using phospho-specific antibodies by western blotting. Relative value of grey scale of p-Akt, p-MAPK, and p-PKC compared with GAPDH was calculated by software, respectively. *P<0.05 compared with control; +P<0.05 compared with gefitinib. A, control; B, gefitinib; C, EGFR siRNA; D, combination of gefitinib and EGFR siRNA.

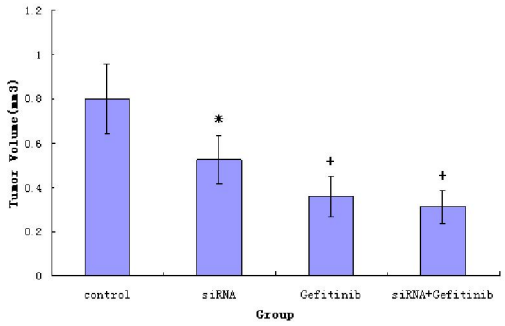

Fig. 4. Gefitinib and/or EGFR siRNA inhibited growth of PC-3 xenograft in nude mice. Luteolin inhibits growth of PC-3 xenograft in nude mice. Twenty-four mice were randomly divided into four groups (n=6 mice/each group). The mice of the treatment group were administered I.P. with gefitinib at 50 mg/kg. The mice of control group received solvent at equal volume. Treatment was begun 3 days after implantation and continued daily throughout the study. *P<0.05 compared with control; +P<0.05 compared with EGFR siRNA. For details, please see Materials and methods. After treatment, the tumors were harvested and measured to calculate volume. The data represent mean ± SD (n=6).

Densitometry analysis showed that p-Akt protein level was decreased by gefitinib by 59.01%, P<0.05; level of p-MAPK and p-PKC were decreased by 34.83% and 33.40%, respectively, P>0.05, as compared with control. However, EGFR siRNA decreased p-Akt and p-MAPK protein levels by 76.49% and 47.15%, respectively, P<0.05; and p-PKC protein level unchanged significantly by 42.2%, P>0.05, as compared with control. Importantly, the combination treatment of gefitinib and EGFR siRNA showed much more significant inhibition of the Akt, MAPK and PKC phosphorylation than the single treatment, especially with respect to the p-PKC protein. Densitometry analysis showed that p-PKC protein levels was inhibited by 33.4%, 44.2% and 63.8% when the cells were treated with gefitinib, EGFR siRNA and the combination, respectively. These data suggest that gefitinib, EGFR siRNA or their combination could selectively inhibit the phosphorylation of EGFR-mediated cell survival signals such as Akt, MAPK and PKC. Furthermore, the combination of gefitinib and EGFR siRNA demonstrated additive inhibitory effects on these critical cell survival signaling proteins and, importantly, in cell survival.

In vivo antitumor activity of gefitinib and/or EGFR siRNA on PC-3 tumor: Finally, the antitumor effect of gefitinib and/or EGFR siRNA *in vivo* was evaluated using the model of nude mice bearing PC-3 tumor xenografts. There was no evidence of systemic toxicity to the mice as evidenced by normal food intake and body weight (data not shown). The average size of tumors in the right flank (control group) reached 0.803±0.157 mm^3 at the end of experiment. Compared with the control group, EGFR siRNA showed a stronger tumor inhibitory effect with a percent tumor regression (T/C) of 34.83% (P <0.05). Gefitinib (50 mg /·kg) inhibited tumors growth more significantly than either EGFR siRNA or control treated with a T/C of 53.95% (P <0.05). But combination of gefitinib and EGFR siRNA did not show significant inhibitory effect against tumor growth than gefintinb alone with a T/C of 59.28% (Fig. 4), P > 0.05.

4.Discussion

Amplification or overexpression of EGFR was significantly associated with high grade, advanced stage, and high risk for prostate-specific antigen (PSA) recurrence in prostate cancer progression. EGFR-targeted drugs could be of therapeutic relevance in prostate cancer (Schlomm et al., 2007). There is increasing interest in the combined use of low doses of therapeutic agents with differing modes of action, rather than the administration of a single agent at a higher dose, as a means of obtaining increased efficacy and minimized toxicity. This approach is extremely important when a promising therapeutic agent demonstrates significant efficacy but may produce some untoward side effects at higher effective doses. Gefitinib is a molecular TKI that has showed significant inhibitory effects of many cancers. To date, small-molecule TKIs and siRNAs targeting EGFR are used frequently to treat prostate cancer (Festuccia, et al., 2009; Addepalli et al., 2010). It is interesting to evaluate the therapeutic effects of gefitinib, EGFR siRNA or combination of both on cells as well as their intracellular mechanisms useful to guide how to use these agents efficiently. In this study, we compared the inhibitory effects of gefitinib, EGFR siRNA and combined treatment on PC-3 cells along with investigating EGFR-induced downstream signaling proteins phosphorylation.

The overexpression of EGFR in prostate cancer has been implicated in the stimulation of EGFR signaling pathways that drive the progression of neoplastic events (de Muga, et al., 2010). In our *in vitro* study, overexpression of EGFR mRNA and protein on PC-3 cell was candidated again by PT-PCR and western blot assay. Gefitinib and EGFR siRNA could effectively inhibited PC-3 cells prolification with deceased expression of EGFR (Fig. 2). Gefitinib blocked the growth of PC-3 cells in a dose-dependent manner; however, PC-3 cells showed a lower growth

rate at 40%~50% for knockdown of EGFR 72 hours after stably trasfected by EGFR siRNA expressing lentivirus. siRNA molecules can be efficiently introduced into cells in a permanent manner through expression systems such as lentiviruses that are capable of integration into the cellular genome. Lentiviruses are considered as powerful integrative vector systems and are highly efficient for *in vivo* application (Naldini, 1999). The latest generation of lentiviral vectors is one of the safest and most efficient tools for stable gene transfer, even eliminating the risk of vector mobilization, due to infection with a wild-type HIV-1 (Montini, et al., 2006). The anti-EGFR lentivirus was efficiently capable of silencing EGFR, which resulted in a complete suppression at 120 h post-infection. Interestingly, in our study gefitnib still significantly inhibited the growth si-PC-3 cells with EGFR knockdown by EGFR siRNA. Therefore, combination of gefitinib and EGFR siRNA block PC-3 cells growths via other pathways, for example, directly blocking p-PKC that could not be effectively inhibited by gefitinib or EGFR siRNA alone. Protein kinase C activation can, in turn, result in MAPK and c-Jun NH2-terminal kinase activation (McClellan et al., 1999). One study investigated whether the antitumor effect of gefitinib was partly attributable to antiangiogenic activity that could be mediated directly by blocking EGF-induced neovascularization and also indirectly by inhibition of vascular endothelial growth factor (VEGF) or interleukin-8 production (Hirata et al., 2002). Our results was inconsistent with Bates et al (2005) who noted that successful therapy by such EGFR-targeting drugs could be expected for patients whose EGFR family members are amplified or overexpressed in cancer cells.

With regard to EGFR-induced downstream signaling proteins in PC-3 cells in our study, EGFR siRNA efficiently inhibited levels of p-Akt and p-MAPK, but not p-PKC; however, gefitinib significantly downregulated level of p-Akt and show no effects on the levels of p-MAPK and p-PKC. Activation of EGFR leads to the phosphorylation of key tyrosine residues within the COOH-terminal portion of EGFR and, as a result, provides specific docking sites for cytoplasmic proteins containing Src and phosphotyrosine-binding domains (Yarden et al., 2001). Akt, PKC and MAPK belong to cell-survival kinases. MAPK is a critically important protein in Ras/Raf pathway to regulate cell proliferation and survival. Activated MAPKs are imported into the nucleus where they phosphorylate specific transcription factors involved in cell proliferation (Liebmann et al., 2006). Both Ras-MAPK and PI3K-Akt signaling have been implicated in modulating androgen receptor (AR) activity in androgen-independent prostate cancer cells. Cross-talk between PI3K-Akt and Ras-MAPK pathways may also be important for androgen-independent prostate cancer cells to maintain their growth in an androgen-depleted state. Wu et al (2008) examined cross-talk between the Ras–MAPK and PI3K–Akt signaling pathways in androgen-independent C4-2 CaP cells. They found that PTEN expression in C4-2 cells made cells hypersensitive to EGF or serum stimulation as indicated by increased pERK levels. This hypersensitivity of MAPK signaling was due to the PTEN inhibition of PI3K–Akt pathway. PKC activation can result in MAPK and c-Jun NH2-terminal kinase activation. In PC-3 cells, combination of EGFR siRNA and gefitinib could more dramatically reduced level of p-PKC than either EGFR siRNA or gefitinib, but showed no significant reduction on the levels of p-Akt or p-MAPK compared with EGFR siRNA. Therefore, EGFR siRNA could inhibit PC-3 cells growth via efficiently downregulating expression of EGFR and the phosphorylation of EGFR-induced downstream signaling protein Akt and MAPK; however, gefitinib did so only selective inhibition of Akt activation, and its inhibitory effects on PC-3 cells could be enhanced by EGFR siRNA via further reducing p-PKC level.

Gefitinib is the first EGFR-targeting drug to be registered for advanced NSCLC, but might possesses slightly different pharmacologic characteristics on prostate cancer. In our study, PC-3 cells treated with gefitinib, p-Akt levels were downregulated by 59.01% lower than reduced levels of p-MAPK or p-PKC by 34.83% or 33.4%. Akt is another important regulator of cell survival and cell proliferation that significantly contributes to tumor growth and progression by promoting cell invasiveness and angiogenesis. Akt is phosphorylated on EGFR activation, transmitting signals for cell survival. It is reported that patients with phosphor-Akt positive tumours or EGFR-dependent Akt activation had a better response rate, disease control rate, and time to progression by gefitinib treatment (Cappuzzo et al., 2004; Ono et al., 2004). The Akt signaling pathway activated by EGFR harboring activating mutations or gene gain is rather more specifically involved in enhanced drug sensitivity and therapeutic efficacy than the ERK1/2 pathway, suggesting phosphorylated AKT as one of the molecular determinants of response to EGFR-targeting drugs. However, loss of PTEN gene in cancer cells such as PC-3 cells leads to constitutive activation of the PI3K/Akt signal transduction pathway. Gefitinib is unable to downregulate Akt activity in PTEN-negative cells while pharmacologic downregulation of constitutive PI3K/Akt pathway signaling using the PI3K inhibitor LY294002 restores EGFR-stimulated Akt signaling and sensitizes cells to

gefitinib. These results suggested that sensitivity to gefitinib requires intact growth factor receptor-stimulated Akt signaling activity. Reconstitution of PTEN in these cells re-established EGFR-driven Akt signaling and thereby restored gefitinib sensitivity. The factors that determine gefitinib sensitivity have long been an enigma (Uramoto and Mitsudomi, 2007). However, further study is required to determine how phosphorylated AKT expression can be applied to determination of the clinical therapeutic efficacy of gefitinib.

At last, we tested the effects of gefitinib and/or EGFR siRNA on prostate cancer growth by in vivo experiments. Gefitinib inhibited more significantly PC-3 xenografts growth in nude mice than EGFR siRNA. However, no significant difference in the tumor growth inhibition was observed between gefitinib group and combination group. This may be due to, (1) transfection of siRNA by lentivirus to PC-3 cells is insufficient, (2) the treatment time is not long enough or (3) the sample size is not big enough. This needs further investigation in our future study. In this study we have identified high levels of EGFR-induced p-Akt, p-MAPK and p-PKC as an essential mediator of growth factor-activated cell proliferation in PC-3 cells in vitro. Gefitinib and EGFR siRNA could inhibit the growth of PC-3 cells mainly via decreasing levels of p-Akt and p-MAPK. Considering great complexity and redundancy of EGFR pathway, it is natural to assume that one cannot expect a sole determinant of clinical benefit of EGFR-TKIs (Tsao et al., 2005). Combining EGFR-targeting drugs with anticancer agents could modify the characteristics of drug sensitivity in ways that might be unique for each drug type. Cooperative growth inhibition is often observed following a combination of EGFR-targeting drugs against various cancer cell types (Jimeno et al., 2005).

In summary, we have demonstrated that gefitinib in combination with EGFR siRNA mainly inhibits the activation of EGFR-induced downstream protein p-Akt and p-MAPK in PC-3 cells. EGFR/Akt or EGFR/MARP signaling pathways are critical for maintaining cell survival. These mechanisms may be exploited for the prevention and/or treatment of human prostate cancer. Our results clearly show that combination treatment of PC-3 cells with gefitinib and EGFR siRNA had a very potent inhibitory effect on the phosphorylation and activation of EGFR and Akt, and on the NF-kB pathway, when compared with single compound treatment. The concerted inhibitory effect of the combination on EGFR and its downscream proteins led to enhanced apoptotic cell death when compared with the effects of the individual compounds. The combination may thus offer therapeutic advantages in the treatment and prevention of human prostate cancer.

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References

1. Shteynshlyuger A, Andriole GL. Prostate cancer: to screen or not to screen? Urol Clin North Am. 2010; 37: 1-9.
2. Traish AM, Morgentaler A. Epidermal growth factor receptor expression escapes androgen regulation in prostate cancer: a potential molecular switch for tumour growth. Br J Cancer. 2009; 101:1949-56
3. Kim JH, Xu C, Keum YS, Reddy B, Conney A, Kong AN. Inhibition of EGFR signaling in human prostate cancer PC-3 cells by combination treatment with b-phenylethyl isothiocyanate and curcumin. Carcinogenesis, 2006, 27:475–482.
4. Hynes NE, MacDonald G. ErbB receptors and signaling pathways in cancer. Curr Opin Cell Biol. 2009; 21:177-84.
5. Iain RH, Janice MK, Helen EJ, et al. Inductive mechanisms limiting response to anti-epidermal growth factor receptor therapy. Endocrine-Related Cancer, 2006,13:S89–S97.
6. Naldini L. In vivo gene delivery by lentiviral vectors. Thromb Haemost 1999; 82:552–554.
7. Cohen MH, Williams GA, Sridhara R, et al. United States Food and Drug Administration Drug Approval summary: gefitinib (ZD1839; Iressa) tablets. Clinical Cancer Research. 2004,10:1212-1218.
8. Angelucci A, Gravina GL, Rucci N, Millimaggi D, Festuccia C, Muzi P, Teti A, Vicentini C, Bologna M. Suppression of EGF-R signaling reduces the incidence of prostate cancer metastasis in nude mice. Endocrine-related Cancer, 2006,13:197-210.
9. Schlomm T, Kirstein P, Iwers L, Daniel B, Steuber T, Walz J, Clinical significance of epidermal growth factor receptor protein overexpression and gene copy number gains in prostate cancer. Clin Cancer Res. 2007; 13:6579-84.
10. Festuccia C, Gravina GL, Biordi L, et al. Effects of EGFR tyrosine kinase inhibitor erlotinib in prostate cancer cells in vitro. Prostate. 2009; 69:1529-37.
11. Addepalli MK, Ray KB, Kumar B, et al. RNAi-mediated knockdown of AURKB and EGFR shows enhanced therapeutic efficacy in prostate tumor regression. Gene Ther. 2010;

17:352-9.
12. de Muga S, Hernández S, Agell L, et al. Molecular alterations of EGFR and PTEN in prostate cancer: association with high-grade and advanced-stage carcinomas.Mod Pathol. 2010; 23:703-12.
13. Naldini L. In vivo gene delivery by lentiviral vectors. Thromb Haemost 1999; 82:552–4.
14. Montini E, Cesana D, Schmidt M, Sanvito F, Ponzoni M, Bartholomae C, Sergi Sergi L, Benedicenti F, Ambrosi A, Di Serio C, Doglioni C, von Kalle C, Naldini L. Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat Biotechnol 2006; 24:687–96.
15. McClellan M, Kievit P, Auersperg N, Rodland K. Regulation of proliferation and apoptosis by epidermal growth factor and protein kinase C in human ovarian surface epithelial cells. Exp Cell Res 1999;246:471- 9.
16. Hirata A, Ogawa S, Kometani T, Kuwano T, Naito S, Kuwano M, Ono M. ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. Cancer Res 2002; 62:2554-60.
17. Bates SE, FojoT. Epidermal growth factor receptor inhibitors: a moving target? Clin Cancer Res 2005; 11:7203 - 5.
18. Yarden Y, Sliwkowski M. Untangling the ErbB signaling network. Nat RevMol Cell Biol 2001; 2:127-37.
19. Liebmann C. Regulation of MAP kinase activity by peptide receptor signaling pathway: paradigms of multiplicity. Cell Signal 2001; 13:777- 85. GaestelM.MAPKAP kinasesIMKsItwo's company, three's a crowd. Nat Rev Mol Cell Biol 2006; 7:120 - 30.
20. Wu Z, Gioeli D, Conaway M, et al. Restoration of PTEN expression alters the sensitivity of prostate cancer cells to EGFR inhibitors. Prostate. 2008; 68:935-44.
21. Cappuzzo F, Magrini E, Ceresoli GL, et al. Akt phosphorylation and gefitinib efficacy in patients with advanced nonsmall-cell lung cancer. J Natl Cancer Inst, 2004,96: 1133– 1141.
22. Ono M, Hirata A, Kometani T, Miyagawa M, Ueda S, Kinoshita H, Fujii T, Kuwano M. Sensitivity to gefitinib (Iressa, ZD1839) in non-small cell lung cancer cell linesco rrelates with dependence on the epidermal growth factor (EGF) receptor/extracellular signal-regulated kinase 1/2 and EGF receptor/Akt pathway for proliferation. Mol Cancer Ther 2004; 3: 465-72.
23. Uramoto H and Mitsudomi T. Which biomarker predicts benefit from EGFR-TKI treatment for patients with lung cancer? Br J Cancer. 2007,96, 857 – 863.
24. Tsao MS, Sakurada A, Cutz JC, Zhu CQ, Kamel-Reid S, Squire J, Lorimer I, Zhang T, Liu N, Daneshmand M, Marrano P, da Cunha Santos G, Lagarde A, Richardson F, Seymour L, Whitehead M, Ding K, Pater J, Shepherd FA. Erlotinib in lung cancer-molecular and clinical predictors of outcome. N Engl J Med 2005; 353:133-44.
25. Jimeno A, Rubio-Viqueira B, Amador ML, Oppenheimer D, Bouraoud N, Kulesza P, Sebastiani V, Maitra A, Hidalgo M. Epidermal growth factor receptor dynamics influences response to epidermal growth factor receptor targeted agents. Cancer Res 2005; 65:3003-10.

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