Lentivirus-Mediated Disintegrin and Metalloproteinase 17 RNA Interference Reversed the Acquired Resistance to Gefitinib in Lung Adenocarcinoma Cells in *Vitro*

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Objective: *The aim of the study is to evaluate the effects of silencing a disintegrin and metalloproteinase 17 (ADAM17) gene expression by lentivirus-mediated RNA interference (RNAi) in the gefitinib-resistant lung adenocarcinoma cells, and then to explore whether the recombinant lentivirus mediated ADAM17 RNAi reversed the acquired resistance of lung adenocarcinoma to gefitinib in* vitro.

Methods: *The gefitinib-resistant RPC-9 cells were established and the mutations of EGFR were detected by gene sequencing. The ADAM17 shRNA expression vectors were constructed and packaged to recombinant lentivirus. The cell proliferation viability was detected by MTT, and cellular apotosis was analyzed by flow cytometry assay. The expression levels of ADAM17, EGFR and the phosphorylated EGFR were respectively detected by reverse transcription polymerase chain reaction and western blot. TGF-α production in the supernatant was detected by enzyme-linked immunosorbent assay.*

Results: *The gefitinib-resistant RPC-9 cells in which mutated EGFR (exon 20) carried 790T > T/M mutation were established. When the concentrations of gefitinib were less than 10μmol/L, there were no significant changes in the apoptosis and cellular proliferation of RPC-9 with the dose-escalation of gefitinib. The cell proliferation viability of RPC-9 was significantly decreased by lentivirus mediated ADAM17 RNAi ($P < 0.05$). Gefitinib did not inhibit ADAM17 expression in both the gefitinib-sensitive PC-9 and gefitinib-resistant RPC-9 cells ($P > 0.05$). Gefitinib had no significant effects on TGF alpha production in the supernatants ($P > 0.05$). Gefitinib did not inhibit EGFR expression in gefitinib-sensitive PC-9 and gefitinib-resistant RPC-9 cells ($P > 0.05$). The phosphorylation of EGFR in gefitinib-sensitive PC-9 cells was significantly inhibited by gefitinib ($P < 0.05$), but that in gefitinib-resistant RPC-9 could not be inhibited by gefitinib ($P > 0.05$). Lentivirus mediated ADAM17 RNAi significantly inhibited the mRNA and protein expression of ADAM17 in gefitinib-resistant RPC-9 cells ($P < 0.05$), as well as TGF alpha production in the supernatants ($P < 0.05$). Also, the phosphorylation of EGFR was significantly reduced in gefitinib-resistant RPC-9 cells by lentivirus mediated ADAM17 RNAi ($P < 0.05$); however, the mRNA and protein expression of EGFR could not be inhibited.*

Conclusion: *Lentivirus mediated ADAM17 RNAi may reverse the acquired resistance of lung adenocarcinoma to gefitinib via inhibiting the upstream of EGFR signal pathway, which may provide a new therapeutic target to solve the acquired resistance to EGFR tyrosine kinase inhibitors in lung adenocarcinoma. © 2017 The Authors Biotechnology Progress published by Wiley Periodicals, Inc. on behalf of American Institute of Chemical Engineers Biotechnol. Prog., 34:196–205, 2018*
Keywords: lung adenocarcinoma, epidermal growth factor receptor, drug resistance, disintegrin-metalloproteinase, RNA interference

Introduction

Lung cancer is one of the most common causes of cancer-related morbidity and mortality worldwide in males and females.[1] Non-small cell lung cancer (NSCLC) accounts for about 80% of lung cancer. Despite the improvements in therapeutic modalities, the 5-year survival rate of NSCLC patients is still around 15%. The ErbB family of receptor tyrosine kinases and their ligands are important regulators of tumor cell proliferation, angiogenesis, and metastasis. Epidermal growth factor receptor (EGFR), a member of the ErbB family, is a transmembrane tyrosine kinase receptor (RTK) consisting of an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. Activation of EGFR is achieved by the binding of a ligand, such as epidermal growth factor (EGF), transforming growth factor α (TGF-α) and neuregulins, to the extracellular portion. Binding with ligands leads to conformational changes in EGFR and homodimerization or heterodimerization with other human epidermal receptor (HER) family members.[2] Without a ligand bound to the receptor and the subsequent dimerisation there is no activity at the enzymatic site of the intracellular portion. First-generation EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib and erlotinib reversibly compete with adenosine triphosphate (ATP) binding at the tyrosine kinase domain of EGFR. This inhibits ligand-induced EGFR tyrosine phosphorylation, EGFR/HER1 activation, and subsequent activation of the downstream signaling networks.[3] Nowadays, it has been clearly established that the mutational status in the TK domain of *EGFR* is associated with responsiveness to EGFR-TKIs in

patients with NSCLC.[4] They have exhibited marked therapeutic effects against NSCLC with exon 19 deletions and L858R point mutations in EGFR.[5] However, acquired resistance to geifitinib and erlotinib will eventually develop in all patients after varying periods of time. Thus, the clinical efficacy of EGFR-TKIs in NSCLC is limited by the development of drug resistance mutations, which is a bottleneck problem in the targeted therapy of lung adenocarcinoma.

It is reported that two major mechanisms of acquired resistance to geifitinib are gatekeeper mutation (T790M) of EGFR and c-Met amplification in tumor cells.[6] To date, EGFR T790M mutation has been found in about 50% of tumors from patients that have developed acquired resistance to gefitinib or erlotinib. EGFR T790M mutation occurs in an analogous position to known resistance mutations to imatinib in other kinases.[7] The conserved threonine residue among these different kinases, located near the kinase active site, is often referred to as the gatekeeper mutation. The exact mechanism through which T790M causes gefitinib or erlotinib resistance is not completely understood. Lung adenocarcinoma that become resistant to the first-generation EGFR-TKIs through a secondary mutation are still likely to be dependent on the activated kinase for their growth and survival. Thus, alternative strategies of inhibiting EGFR T790M may be therapeutically efficacious. The second generation EGFR-TKI afatinib (BIBW-2992), designed to bind covalently with Cys-797 at the gatekeeper pocket, can potently and selectively block both wild-type and mutant forms of ErbB family receptors (EGFR, HER2, ErbB3 and ErbB4). In the clinical trial of LUX-lung 1,

the afatinib group experienced a prolonged progress-free survival (PFS) (3.3 months versus 1.1 months; HR 0.38, 95% CI 0.31–0.48; $P < 0.0001$), better confirmed objective response rate (ORR) (7% versus <1%), better disease control rate for ≥8 weeks (58% versus 18%) and improved life quality in the overall population than that of placebo. However, the primary endpoint overall survival (OS) showed no benefit from afatinib (afatinib versus placebo: 10.8 months versus 12.0 months; HR 1.08, 95% CI 0.86–1.35, $P = 0.74$).[8] The third-generation EGFR-TKIs including CO-1686 (Rociletinib) and AZD9291 exhibit potent inhibition of EGFR T790M.[9,10] In the phase I/II clinical trial, the CO-1686 group experienced a confirmed ORR of 80%, a PFS of over 6 months in T790M-positive NSCLC patients.[10]. Meanwhile, it also exhibited poor therapeutic effects in erlotinib -resistant NSCLC patients without T790M mutation in EGFR. Thus, it is still necessary to develop new strategies to overcome the resistance to EGFR-TKIs in EGFR-mutant lung adenocarcinoma.

EGFR signaling is regulated by proteolytic shedding of its membrane-tethered ligands.[11] Sheddases for EGFR-ligands such as TGF-α are the key signaling switches in the EGFR signaling pathway. Therefore, the therapies inhibiting ectodomain shedding of EGFR-ligands may be beneficial in individuals with EGFR-mediated responses. A disintegrin and metalloproteinases (ADAMs) are a family of transmembrane and secreted proteins with a variety of functions, including proteolytic cleavage of cell surface molecules, cell fusion, cell adhesion, and intracellular signaling.[12–14] ADAM17, a member of ADAM family, has been described as "a signaling scissor". The most well-known function of catalytically active ADAM17 is to cleave the ectodomains of various transmembrane proteins, such as growth factors, receptors and their ligands, cytokines, and cell adhesion molecules; therefore, ADAM17 is an important regulator of almost every cellular event.[12–14] It was reported that ADAM17 was overexpressed in NSCLC, which was required not only for heregulin-dependent HER3 signaling, but also for EGFR ligand-dependent signaling in NSCLC cell line.[15,16] The increased expression of ADAM17 in NSCLC was also associated significantly with aggressive progression and poor prognosis.[16] It was suggested that ADAM17 might be an attractive target for the treatment of NSCLC due to their role in shedding EGFR ligands. TGF-α expressions have been detected in 60%–80% of primary NSCLC, and they are also synthesized as membrane-anchored precursor forms that are later cleaved to generate soluble ligands in a metalloproteinase-dependent manner. However, it was still not clear whether ADAM17 and TGF-α were involved in the proliferation of the gefitinib-resistant lung adenocarcinoma cells and their potential values to overcome the acquired resistance to EGFR-TKIs in lung adenocarcinoma. Therefore, in the present study, we investigated the role of ADAM17/TGF-α/EGFR signaling pathway in the proliferation of lung adenocarcinoma cells with EGFR T790M-positive and then explored whether lentivirus-mediated ADAM17 RNA interference (RNAi) overcome the acquired resistance to gefitinib in lung adenocarcinoma cells in *vitro*.

Materials and Methods

Establishment of the Gefitinib-resistant lung adenocarcinoma cell line

The gefitinib-sensitive lung adenocarcinoma PC-9 cells that carried an in-frame deletion in EGFR exon 19 were obtained from American Type Culture Collection (ATCC,

Manassas, USA). The cells were cultured at a concentration of 1×10^9 cells/L in Dulbecco's modified eagle medium (DMEM, GIBCO, Carlsbad, CA, USA) containing 10% FBS in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C. The gefitinib-resistant lung adenocarcinoma cell lines were induced with the pulse dosage and dose-escalation of gefitinib in *vitro,* which was raised by Ogino and his colleagues.[17] Briefly, PC-9 cells were treated with gefitinib (AstraZeneca, Cambridge, UK) at the pulse dosage of 10 μmol/L for 48 h, when cells reached 80% fusion. Then the cells were cultured for about 8 months with gefitinib at a gradient of concentrations ranging from 0.01 to 10 μmol/L. Finally, the cells were able to grow in 10 μmol/L of gefitinib. So the gefitinib-resistant lung adenocarcinoma cell line, named RPC-9, was established successfully.

Lentivirus production and transduction

The human ADAM17 mRNA sequence (GenBank accession number: NM_003183) was used to determine suitable siRNA target sequences and CCTATGTCGATGCTGAACAAA was selected. The recombinant pLVTHM vectors were constructed by Sangon Biotech Co., Ltd. (Shanghai, China) as the previous study.[18] The orientation of the inserted shRNA cassettes was verified by restriction enzyme analysis and DNA sequencing. A negative control (NC) siRNA sequence (TTCTCCGAACGTGTCACGT) was used as a control for ADAM17 siRNA.The recombinant pLVTHM vectors and packaging helper plasmids were co-transfected into 293T cells (Shanghai Institute of Biology, Chinese Academy of Sciences, China) with calcium phosphate. The medium was replaced with fresh culture medium 12 h after transfection. The cultured supernatants were collected 48 h post-transfection and centrifuged at $800 \times g$ for 7 min at 4°C to remove cell debris. The supernatants were then filtered through a 0.45 μm pore filter prior to ultra-centrifugation at 50,000g for 90 min at 4°C. Viral particles were precipitated in ice-cold PBS re-suspension solution. Finally, the viral particles including in LV-ADAM17-shRNA and LV-NC-shRNA were stored at −80°C. The viral titer was detected via infection of 293T cells and subsequent flow cytometric assay.

Cell culture and treatment

The human lung adenocarcinoma PC-9 and RPC-9 cells were cultured in DMEM containing 10% FBS, 50 U/mL penicillin and 50μg/mL streptomycin in a humidified atmosphere of 5% carbon dioxide at 37°C. The PC-9 and RPC-9 cells were respectively treated for 48 h with 5 μmol/L of gefitinib (AstraZeneca, UK), and then the cells and supernatants were collected and stored at −80°C. The RPC-9 cells were respectively infected with LV-ADAM17-shRNA or LV-NC-shRNA at a multiplicity of infection of 15. At 96 h post-infection, the cells and supernatants were then harvested and stored at −80°C. The changes of cell apoptosis of them were respectively detected using a flow cytometer (Guava EasyCyte; Millipore, Billerica, MA, USA).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrozolium bromide (MTT) assay

The cellular proliferation was evaluated by MTT assay. Briefly, cells were seeded at a density of 1×10^4 cell/well in 96-well plates and incubated for 24 h, 20 μl of 5 mg/mL MTT was added to the 96 well plates and incubated for 4 h

at 37°C. At the end of the incubation, the purple formazan crystals were dissolved with the addition of 150μL DMSO. Then the absorbance values were determined at 540 nm wavelength using Bio-Tek Plate Reader (Model ELX 800, BioTek Instruments, Winooski, Vermont, USA). The proliferation viability of the treated cells relative to the control cells was determined.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells with RNAex reagent (Watson, Beijing, China) according to the manufacturer's instruction. First strand cDNA synthesis was performed with Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Promega, USA). The PCR reactions were performed in a 25 μL reaction volume, which included 2.5 μL first-strand cDNA, 2 μl sense primer (10 μmol/l), 2 μL antisense primer (10 μmol/l), 2.5 μL 10 × PCR buffer, 2 μL $MgCl_2$ (25 mmol/l), 1 μL dNTP (10 mmol/l) and 0.5 μL Taq-DNA polymerase (Tiangen Biotech, China). PCR parameters were as follows: 95°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec, and a final extension at 72°C for 5 min. β-Actin was used as an internal control. The primers were designed as follows (AuGT Biotech, China): ADAM17 (Genebank: NM_003183), 5'-GCTTGGCACACCTTTTCAC ATACC-3'(sense) and 5'-GTCCTCATTCGGGGCACATTC TG-3'(antisense); EGFR (GenBank: NM_005228), 5'-CCA GATGGATGTGAACCC-3' (sense) and 5'-GTAGCATTTA TGGAGAGTGAGTC-3' (antisense); β-actin, 5'-GGCACCA CACCTTCTACAATGA-3' (sense) and 5'-TCAGGAGGA GCAGCAATGATCTTG-3' (antisense). The expected sizes of the PCR products were 287 bp for ADAM17, 237 bp for EGFR and 745 bp for β-actin. PCR products were visualized by electrophoresis on ethidium bromide stained 1.5% agarose gels, and the band densities were analyzed with a UVP-GDS8000 gel analysis system (Ultra-Violet Products Ltd., UK). The expression level of ADAM17 or EGFR mRNA was evaluated by determining the ratio of ADAM17 or EGFR mRNA/β-actin mRNA.

Enzyme-linked immunosorbent assay (ELISA)

The amount of TGF-α in the supernatants was measured with ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, the supernatants were coated onto the 96-well plates and incubated overnight at 4°C. After blocking for 1 h at room temperature with blocking solution (1% FBS and 0.05% Tween-20 in PBS), monoclonal anti-human TGF-α was added to each well and incubated for 1 h at room temperature. After washing with 0.05% Tween-20 in PBS, peroxidase-conjugated immunopure goat anti-rabbit IgG (H + L) (Pierce, Rockford, IL, USA) was added and incubated for 1 h at room temperature. A color reaction was developed with tetramethylbenzidine for 10 min at room temperature. Following the addition of a stop solution, absorbance at 450 nm was measured by a microtiter plate reader.

Western blot assays

The total protein of treated cells was extracted using RIPA buffer with PMSF, and the concentration was measured with a protein assay kit (Micro BCA; Pierce, USA). Then proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted onto Hybond-ECL nitrocellulose membrane. The membranes were blocked with nonfat dry milk (Cell Signaling Technology, Danvers, Massachusetts, USA) for 1 h at room temperature, then incubated in primary antibodies for 1 h at room temperature, washed with Tris-buffered saline Tween (TBS-T), 5 times for 5 min each, and incubated with peroxidase-conjugated immunopure goat anti-rabbit IgG (H + L) (Pierce, USA) for 1 h at room temperature. After the membranes were again washed 5 times for 5 min each in TBS-T, the proteins were visualized with an enhanced chemiluminescence solution (Amersham Pharmacia biotech, Buckinghamshire, UK) and analyzed with a UVP-GDS8000 gel analysis system (Ultra-Violet Products Ltd., UK). The same membranes were stripped and blotted with anti-β-actin antibodies, which provided a loading control. The primary antibodies were obtained from the following sources: EGFR Antibody (Catalog# 2239) and phospho-EGFR (Tyr992) Antibody (Catalog# 2236) from Cell Signaling Technology (Danvers, Massachusetts, USA); ADAM17 antibody (Catalog# ab39163) from Abcam Biotechnology (Abcam, Cambridge, UK); β-actin (N-21) antibody (Catalog#sc-130656) from Santa Cruz Biotechnology (Santa Cruz, California, USA).

Statistical analyses

Data were analyzed for statistical significance via two-way analysis of variance (ANOVA), and multiple comparisons in ANOVA were analyzed via Sidak's test. Data were expressed as the mean ± SD. Values of $P < 0.05$ were considered statistically significant.

Results

Lung adenocarcinoma cells with EGFR T790M-positive emerged in the induction of gefitinib

To establish the gefitinib-resistant subline, the gefitinib-sensitive PC-9 cells that carried an in-frame deletion in EGFR exon 19 were cultured for 6 months in the induction of gefitinib as described in the methods (Figure 1). When the concentration of gefitinib escalated to 10 μmol/L, the gefitinib-resistant lung adenocarcinoma cells named as RPC-9 cells emerged. The persistence of the original 15 bp deletion (c.2235_2249delGGAATTAAGAGAAGC) was confirmed using gene sequencing (Figure 2). Moreover, a C-to-T base pair changed at nucleotide 2369 was also identified in EGFR exon 20 in the cells (Figure 2). Thus the gefitinib-resistant RPC-9 cells with EGFR T790M-positive were established successfully.

The different changes of the cell apoptosis induced by gefitinib in the lung adenocarcinoma cells

The cellular apoptosis levels of PC-9 were significantly increased with the dose-escalation of gefitinib ($P < 0.001$; Figure 3A–D). When the concentration of gefitinib was below 10μmol/L, there were no significant changes in the apoptosis of RPC-9 with the increase of gefitinib ($P > 0.05$; Figure 3E–H).

ADAM17 RNAi inhibited cellular proliferation of the gefitinib-resistant lung adenocarcinoma cells

The PC-9 and RPC-9 cells were respectively treated with gefitinb at the concentrations ranging from 0.01 to 10 μmol/L for 24h, and the cell proliferation viability was detected by
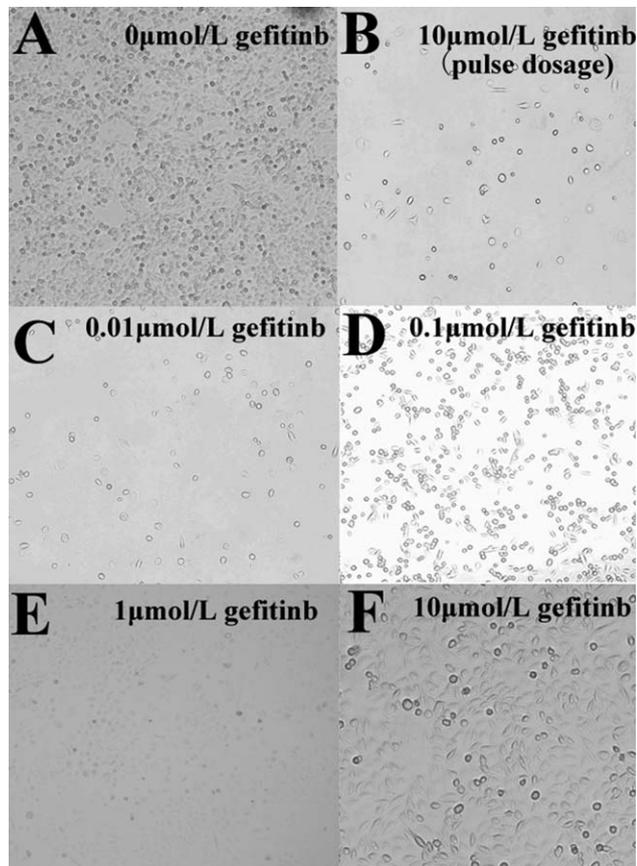
Figure 1. Induction of the gefitinib-resistant lung adenocarcinoma cells. A: The PC-9 cells; (B) The PC-9 cells were treated for 48 h with 10 μmol/L gefitinb; (C–F) the PC-9 cells were continuously subcultured respectively with gefitinb at the concentrations ranging from 0.01 to 10 μmol/L. 100$\times$.

MTT. The cellular proliferation of PC-9 was inhibited significantly when treated with dose-escalation of gefitinib ($P < 0.001$; Figure 4A), while there was no significant reduction in cell viability of RPC-9 ($P > 0.05$, Figure 4A). When the cells were treated for 0–48 h with 10 μmol/L gefitinib, the cell viability of PC-9 decreased markedly in a time-dependent manner ($P < 0.001$; Figure 4B), but gefitinib did not inhibit the cell viability of RPC-9 ($P > 0.05$; Figure 4B). When the gefitinib-resistant RPC-9 cells with EGFR T790M-positive were infected by LV-ADAM17-shRNA for 96 h, the cellular proliferation of RPC-9 was decreased significantly by lentivirus-mediated ADAM17 RNAi $P < 0.001$; Figure 5). When the gefitinib-resistant RPC-9 cells were infected by LV-ADAM17-shRNA for 96 h and then treated for 24 h with gefitinib at the concentrations ranging from 0.1 to 10μmol/L, it indicated there were no significant changes in the cellular proliferation of RPC-9 treated by gefitinib ($P > 0.05$; Figure 4C,D).

Lentivirus-mediated ADAM17 RNAi inhibited the expression of ADAM17 in lung adenocarcinoma cells

When the cells were treated for 48h with gefitinib at a concentration of 5 μmol/L, gefitinib could not inhibit the mRNA ($P > 0.05$) and protein ($P > 0.05$) expression of ADAM17 in the gefitinib-resistant RPC-9 cells (Figure 6). When the cells were treated for 96 h with LV-*ADAM17*-shRNA, lentivirus-mediated ADAM17 RNAi significantly

inhibited the mRNA ($P < 0.001$) and protein ($P < 0.01$) expression of ADAM17 in the gefitinib-resistant RPC-9 cells (Figure 6).

ADAM17 RNAi inhibited the release of TGF-α in the supernatants

When the cells were treated with gefitinib at a concentration of 5μmol/L for 48h, the release of TGF-α in the supernatants could not be inhibited by gefitinib ($P > 0.05$; Figure 7A). When the cells were treated for 96h with LV-NC-shRNA or LV-ADAM17-shRNA, lentivirus-mediated ADAM17 RNAi significantly inhibited the release of TGF-α in the supernatants of RPC-9 cells ($p < 0.001$; Figure 7B).

ADAM17 RNAi inhibited on phosphorylation of EGFR, but not EGFR expression in the lung adenocarcinoma cells

When the cells were treated for 48 h with gefitinib at a concentration of 5 μmol/L, gefitinib did not inhibit the mRNA and protein expression of EGFR in both the gefitinib-sensitive PC-9 cells and the gefitinib-resistant RPC-9 cells (Figure 8A–D). Gefitinib inhibited the phosphorylation of EGFR in the gefitinib-sensitive PC-9 cells ($P < 0.001$; Figure 8C,D), but not in the gefitinib-resistant RPC-9 cells ($P > 0.05$; Figure 8C,D). When the RPC-9 cells were treated for 96 h with LV-ADAM17-shRNA, lentivirus-mediated ADAM17 RNAi significantly inhibited the phosphorylation of EGFR in the gefitinib-resistant RPC-9 cells ($P < 0.001$; Figure 8C,D); however it could not inhibit the mRNA ($P > 0.05$) and protein ($P > 0.05$) expression of EGFR (Figure 8A–D).

Discussion

The acquired resistance to EGFR-TKIs is a key problem to be resolved in the targeted therapy of NSCLC. EGFR T790M mutation is considered to be one of the important mechanisms that cause the resistance to EGFR-TKIs in lung adenocarcinoma. The present study showed that the cell apoptosis levels in gefitinib-sensitive lung adenocarcinoma PC-9 cells that carried an in-frame deletion in EGFR exon 19 were significantly increased with the escalating concentration of gefitinib, and the survival rate of PC-9 cells was significantly decreased. The resistance to gefitinib in the lung adenocarcinoma cells emerged in the induction of gefitinib, and T790M mutation in EGFR exon 20 was also identified by gene sequencing. Thus, it indicated that the gefitinib-resistant lung adenocarcinoma cells with EGFR T790M-positive were established successfully. There are some strategies to reverse TKI resistance in lung adenocarcinoma, which include pulse-dose therapy of first-generation TKIs, chemotherapy combined with EGFR-TKI, third-generation EGFR-TKIs, such as AZD9291 and co-1686, and antiangiogenic agents such as bevacizumab.[19] Kuiper[20] described two patients with EGFR-mutated non-small cell lung cancer, were in both clinical and radiological response to high-dose, pulsatile erlotinib. Guan[21] reported that a dramatic and durable response to high-dose icotinib in a NSCLC patient who did not respond to a previous standard dose of erlotinib. The treatment extended the life of the patient for one additional year. However, the present study showed that there were no significant changes in the apoptosis of gefitinib-resistant RPC-9 cells with the dose-escalation of gefitinib, and the cellular proliferation of the RPC-9 cells could not be significantly inhibited
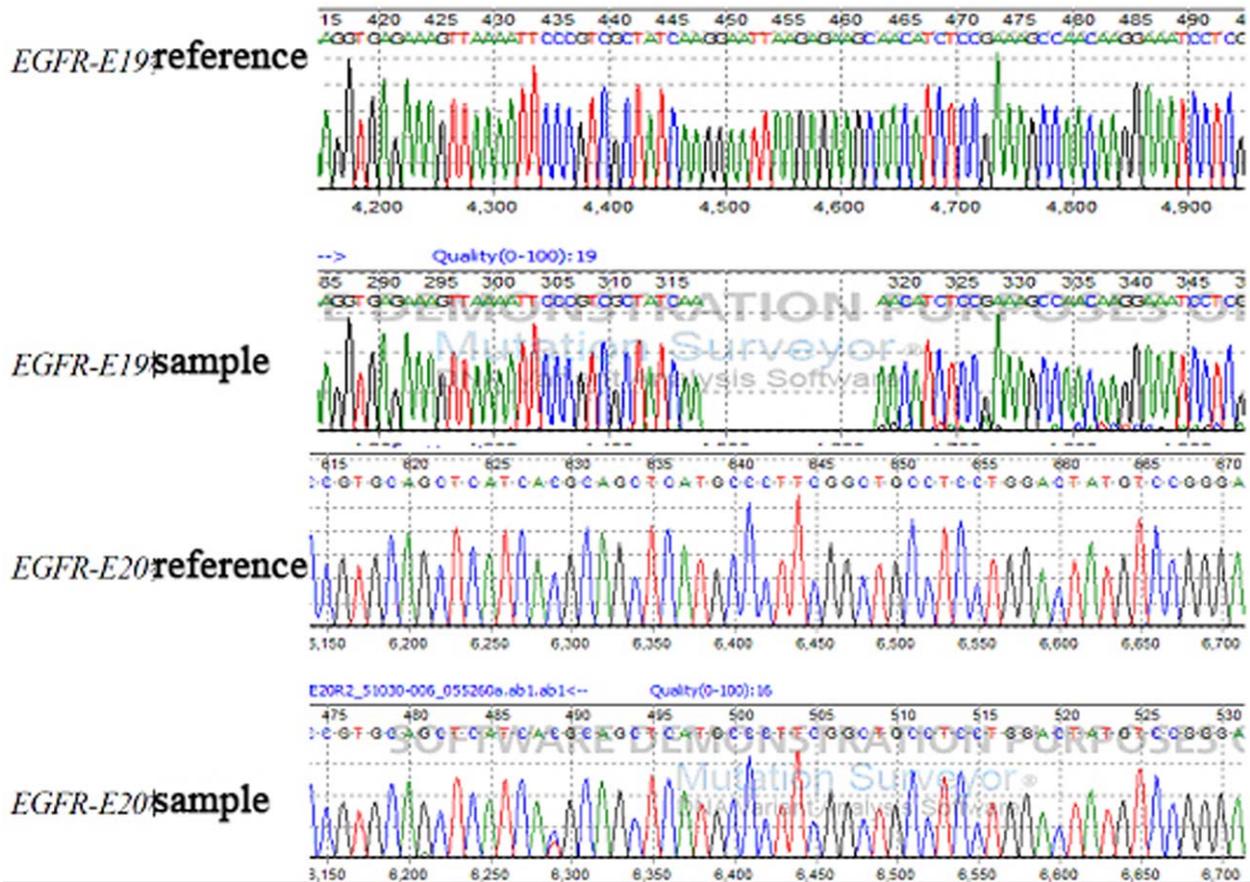
Figure 2. The mutation analyses of the EGFR gene in the gefitinib-resistant lung adenocarcinoma cells. A, C: the maps of *EGFR* exon 19 or 20 in reference standards; B, D: the maps of *EGFR* exon 19 or 20 in the RPC-9 cells.
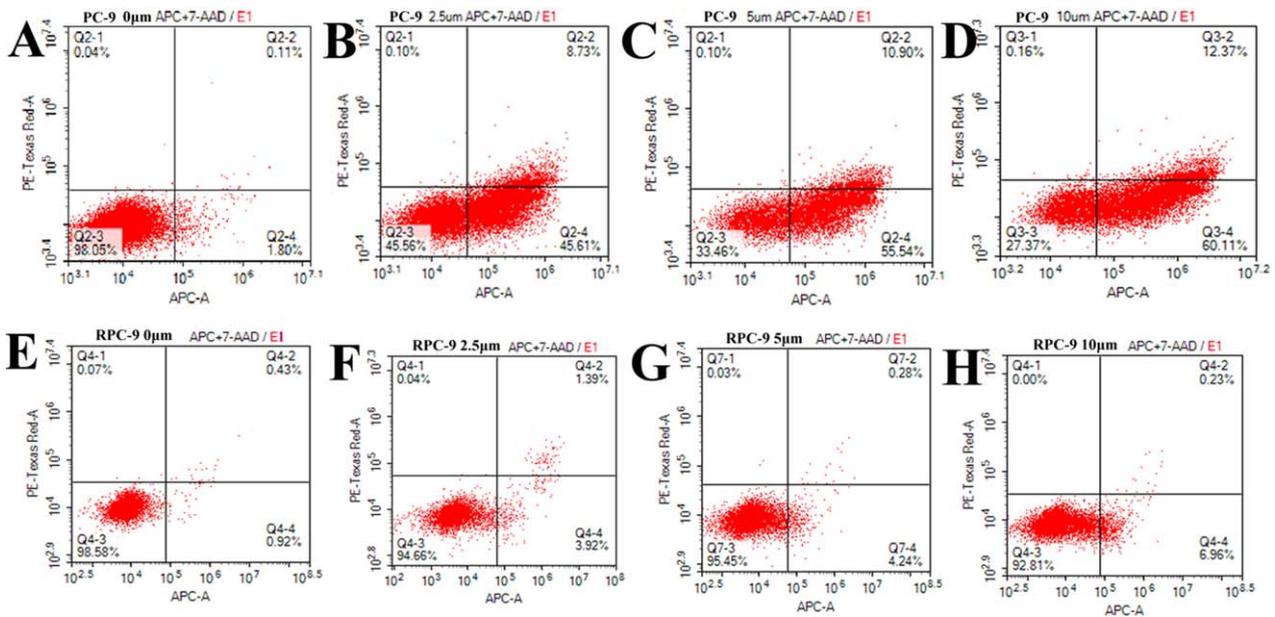

Figure 3. The changes of the apoptosis in PC-9 and RPC-9 cells induced by gefitinib. A–D: the apoptosis in PC-9 induced by gefitinib; (E–H) the apoptosis in RPC-9 cells induced by gefitinib. Data were analyzed for statistical significance via two-way analysis of variance followed by Sidak's multiple comparisons test, *$P < 0.05$ *vs* the group not treated with gefitinib.

by gefitinib in a dose-dependent manner, which accorded with Jackman' study.[22] Therefore, the responsiveness to EGFR-TKIs in lung adenocarcinoma may be related to the types of EGFR mutation and tumor heterogeneity. The IMPRESS III

clinical trial[23] indicated that continuation of gefitinib after radiological disease progression on first-line gefitinib did not prolong progression-free survival in patients who received platinum-based doublet chemotherapy as subsequent line of
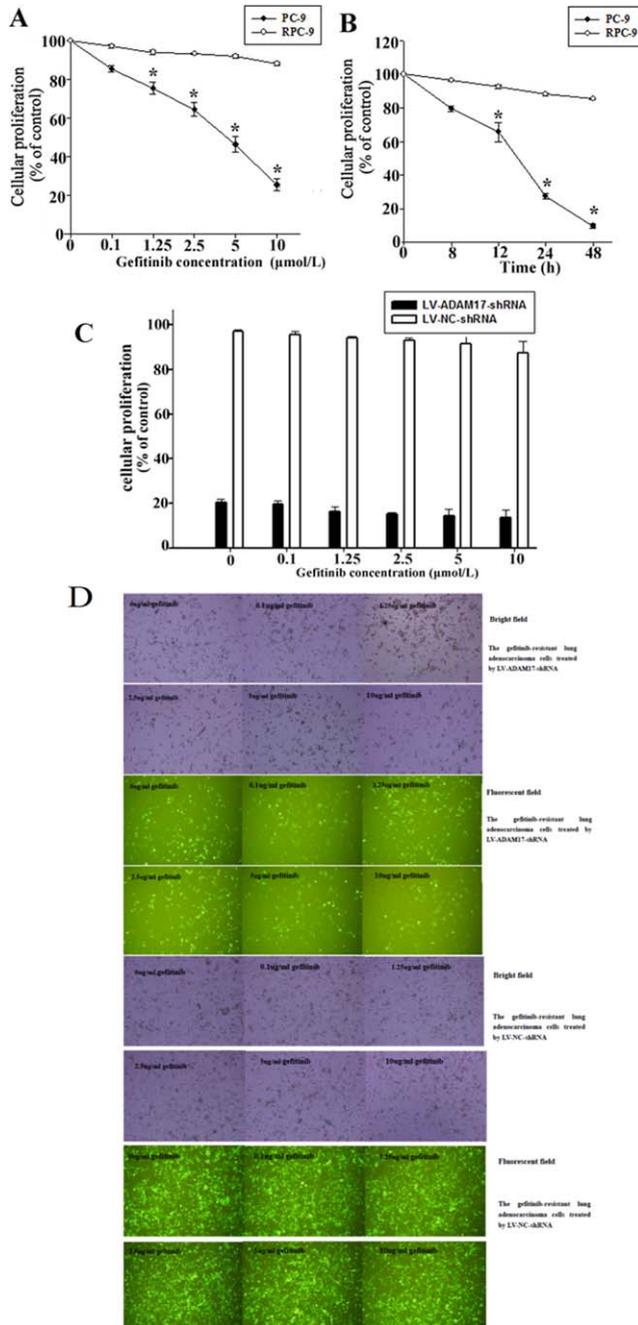
Figure 4. Effects of gefitinib on the proliferation viability of the lung adenocarcinoma cells. A: PC-9 and RPC-9 cells were treated with gefitinib at concentrations ranging from 0.1 to 10 μmol/L. B: PC-9 and RPC-9 cells were treated for different lengths of time (0–48 h) with gefitinib at a concentration of 10 μmol/L. C: RPC-9 cells were respectively infected with LV-ADAM17-shRNA or LV-NC-shRNA, and then treated for 24h with gefitinib at the concentrations ranging from 0.1 to 10μmol/L. D: Effects of gefitinib on the cellular proliferation of the gefitinib-resistant RPC-9 cells pretreated by LV-ADAM17-shRNA or LV-NC-shRNA. Data were analyzed for statistical significance via two-way analysis of variance followed by Sidak's multiple comparisons test, *$P < 0.05$ *vs* the group not treated with gefitinib.

Figure 5. Effects of lentivirus-mediated ADAM17 RNAi on the cellular proliferation of the gefitinib-resistant lung adenocarcinoma cells. A: PC-9 cells; (B) RPC-9 cells; (C) PC-9 cells treated by LV-NC-shRNA; (D) RPC-9 cells treated by LV-*ADAM17*-shRNA; (E) RPC-9 cells treated by LV-NC-shRNA; (F) RPC-9 cells treated by LV-*ADAM17*-shRNA. Data were analyzed for statistical significance via two-way analysis of variance followed by Sidak's multiple comparisons test, *$P < 0.05$ *vs* the group of RPC-9 cells without treatment, #$P < 0.05$ *vs* the group of PC-9 cells without treatment.

treatment. AZD9291, a third-generation EGFR-TKI, that is selective for EGFR tyrosine kinase inhibitor-sensitizing mutations and the T790M resistance mutation has shown to be not only highly active but also fairly tolerable in a large cohort of
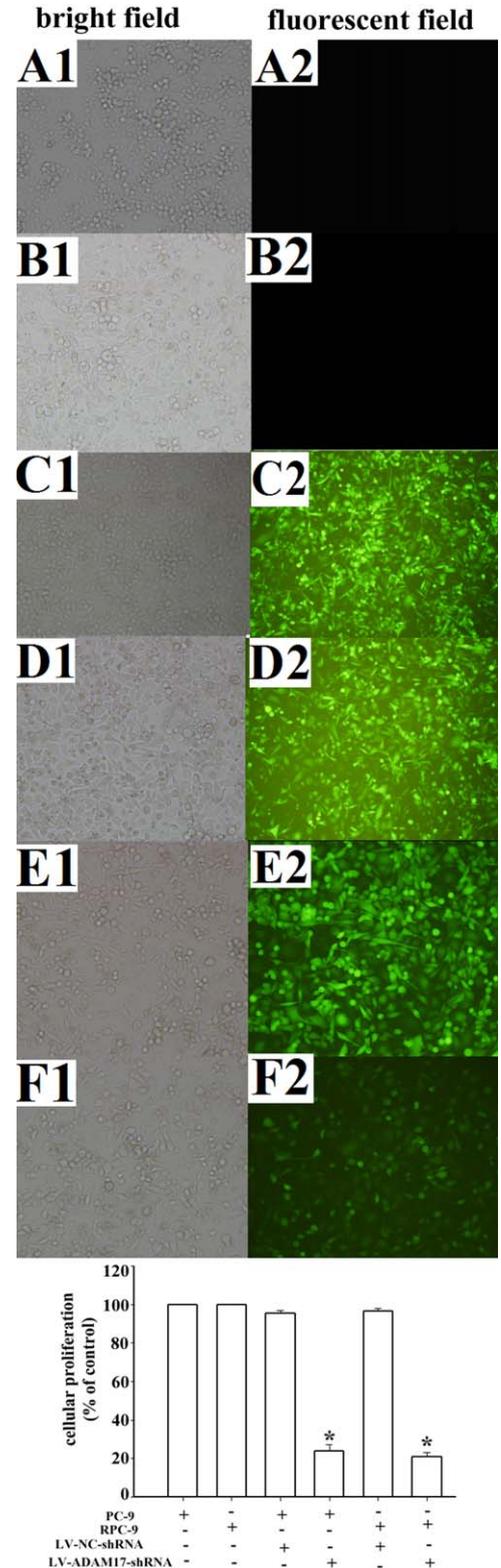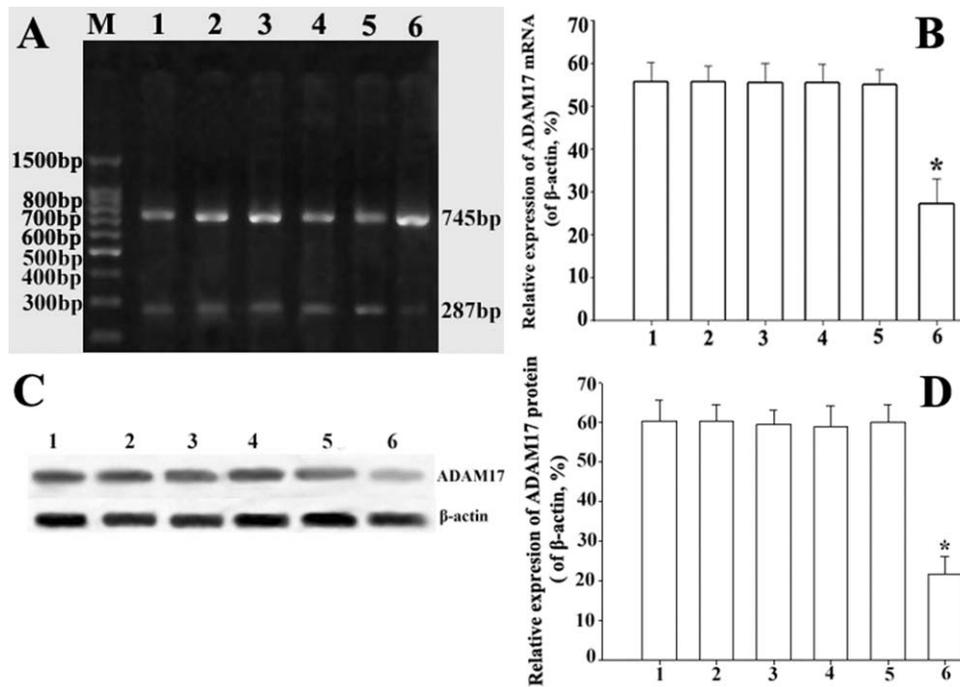
Figure 6. Effects of lentivirus-mediated ADAM17 RNAi on the expression of ADAM17 in lung adenocarcinoma cells. In A and B, the expression of ADAM17 mRNA in PC-9 and RPC-9 cells; In C and D, the expression of ADAM17 protein in PC-9 and RPC-9 cells.1, PC-9 cells; 2, RPC-9 cells; 3, PC-9 cells treated by gefitinib; 4, RPC-9 cells treated by gefitinib; 5, RPC-9 cells treated by LV-NC-shRNA; 6, RPC-9 cells treated by LV-ADAM17-shRNA. Data were analyzed for statistical significance viatwo-way analysis of variance followed by Sidak's multiple comparisons test, *$P < 0.05$ *vs* the group of RPC-9 cells without treatment,.
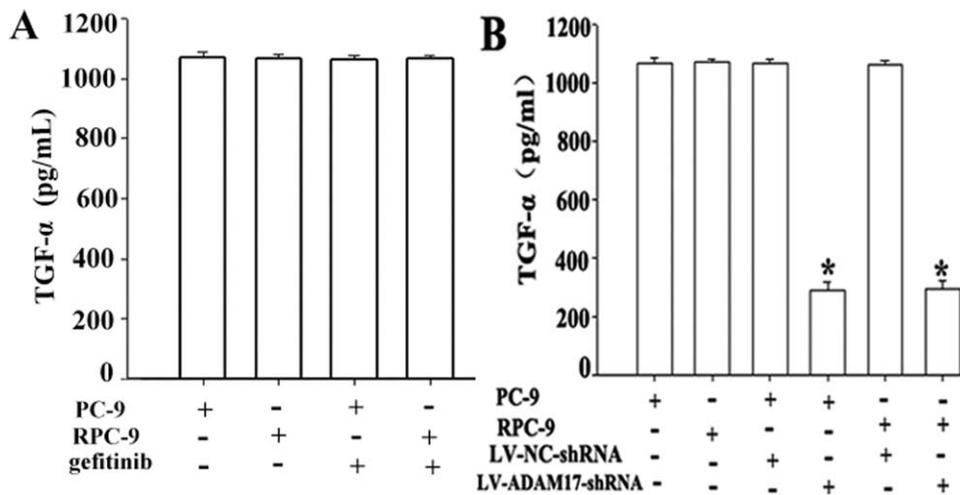

Figure 7. Effects of lentivirus-mediated ADAM17 RNAi on the release of TGF-α in the supernatants. The PC-9 and RPC-9 cells were respectively with 5μmol/L of gefitinib (A) and LV-ADAM17-shRNA (B). TGF-α production in the supernatant was detected by ELISA. Data were analyzed for statistical significance via two-way analysis of variance followed by Sidak's multiple comparisons test, *$P < 0.05$ *vs* the group of RPC-9 cells without treatment, #$P < 0.05$ *vs* the group of PC-9 cells without treatment.

patients.[24] However, acquired resistance to AZD9291 is also inevitable. The loss of EGFR-mutant clones plus alternative pathway activation or histologic transformation and EGFR ligand-dependent might result in the acquired resistance to AZD9291 in patients with EGFR-mutant NSCLC who failed in treatment with first-generation EGFR-TKIs.[25] Thus, it is still necessary to develop new strategies to reverse the acquired resistance to EGFR-TKIs.

The ectodomain shedding of EGFR ligands is critical for the activation of EGFR. The cleaved EGFR ligands binding

to EGFR causes receptor homodimerization or heterodimerization, which leads to recruiting, phosphorylating downstream molecules and initiating downstream signaling events. ADAM17, known as tumor necrosis factor (TNF)-α converting enzyme (TACE), is a multidomain, membrane-bound zinc metalloproteinase belonging to the ADAMs family.[13,14] ADAM17 can cleave ectodomains of various transmembrane proteins including TNF-α, TNF receptors, L-selectin, TGF-α, EGF, HB-EGF and amphiregulin,[14] and is an important regulator of almost every cellular event. It
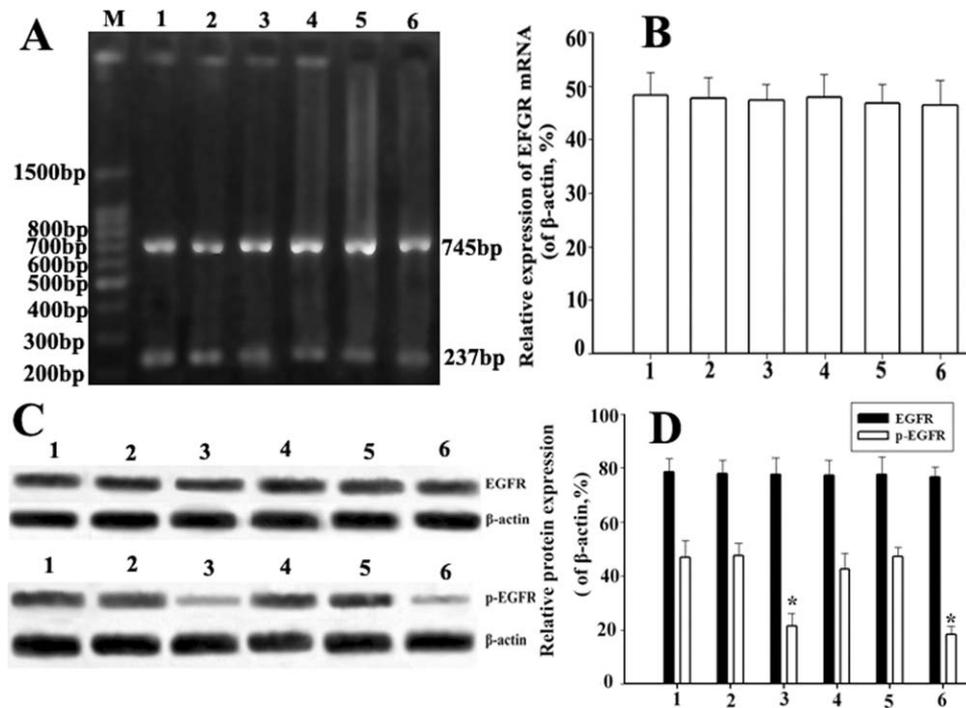
Figure 8. **Effects of lentivirus-mediated ADAM17 RNAi on EGFR expression and their phosphorylation in the lung adenocarcinoma cells. In A and B, the expression of EGFR mRNA in PC-9 and RPC-9 cells; In C and D, EGFR expression and their phosphorylation in PC-9 and RPC-9 cells.1, PC-9 cells; 2, RPC-9 cells; 3, PC-9 cells treated by gefitinib; 4, RPC-9 cells treated by gefitinib; 5, RPC-9 cells treated by LV-NC-shRNA; 6, RPC-9 cells treated by LV-ADAM17-shRNA. Data were analyzed for statistical significance viatwo-way analysis of variance followed by Sidak's multiple comparisons test, *$P < 0.05$ *vs* the group of RPC-9 cells without treatment, #$P < 0.05$ *vs* the group of PC-9 cells without treatment.**

can be expressed in epithelial cells, vascular smooth muscle cells, and macrophages in the lung. The previous study indicated that ADAM17 was expressed in type II alveolar epithelial cell, and ADAM17-EGFR signaling cascade mediated IL-8 production induced by LPS.[26] The present study showed that ADAM17 was overexpressed in PC-9 and RPC-9 lung adenocarcinoma cells, and lentivirus-mediated ADAM17 RNAi significantly inhibited the expression of ADAM17, and the proliferation viability of gefitinib-resistant RPC-9 cells was significantly inhibited, which was in accord with study of Cai et al.[27] Some studies indicated that both positivity of serum TGF-α and wild-type EGFR were independent poor prognostic factors in patients with non-squamous NSCLC, and the increases of TGF-α in serum also were an important predictor of the resistance to gefitinib in the patients with advanced NSCLC.[28,29] However, the effects of ADAM17 on the release of TGF-α and their roles in the therapy of lung cancer did not be explained in the studies. In the present study, it suggested that the release of TGF-α in the gefitinib-resistant RPC-9 cells was markedly inhibited by lentivirus-mediated ADAM17 RNAi. Lentivirus-mediated ADAM17 RNAi significantly inhibited the phosphorylation of EGFR in the gefitinib-resistant RPC-9 cells, instead of the inhibition of EGFR mRNA and protein expression, which might inhibit tumor progression and promote the cellular apoptosis in lung cancer. Thus, lentivirus-mediated ADAM17 RNAi may overcome the resistance to gefitinib by inhibiting release of TGF-α and the phosphorylation of EGFR in the lung adenocarcinoma cells with EGFR T790M-positive, which may provide potential drug targets to solve the acquired resistance to EGFR-TKIs.

REFERENCES

1. Torre LA, Siegel RL, Ward EM, et al . Global cancer incidence and mortality rates and trends–an update. *Cancer Epidemiol Biomarkers Prev.* 2016;25:16–27.
2. Goffin JR, Zbuk K. Epidermal growth factor receptor: pathway, therapies, and pipeline. *Clin Ther*. 2013;35:1282–1303.
3. Xu M, Xie Y, Ni S, et al. The latest therapeutic strategies after resistance to first generation epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) in patients with non-small cell lung cancer (NSCLC). *Ann Transl Med.* 2015;3:96.
4. Jorge SE, Kobayashi SS, Costa DB. Epidermal growth factor receptor (EGFR) mutations in lung cancer: preclinical and clinical data. *Braz J Med Biol Res.* 2014;47:929–939.
5. Cataldo VD, Gibbons DL, Perez-Soler R, et al. Treatment of non small-cell lung cancer with erlotinib or gefitinib. *N Engl J Med.* 2011;364:947–955.
6. Ahsan A. Mechanisms of resistance to EGFR tyrosine kinase inhibitors and therapeutic approaches: an update. *Adv Exp Med Biol.* 2016;893:137–153.
7. Takezawa K, Okamoto I, Tanizaki J, et al. Enhanced anticancer effect of the combination of BIBW2992 and thymidylate synthase-targeted agents in non-small cell lung cancer with the T790M mutation of epidermal growth factor receptor. *Mol Cancer Ther.* 2010;9:1647–1656.
8. Miller VA, Hirsh V, Cadranel J, et al. Afatinib versus placebo for patients with advanced, metastatic non-small-cell lung cancer after failure of erlotinib, gefitinib, or both, and one or two

lines of chemotherapy (LUX-Lung 1): a phase 2b/3 randomised trial. *Lancet Oncol.* 2012;13:528–538.

9. Cross DA, Ashton SE, Ghiorghiu S, et al. AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR inhibitors in lung cancer. *Cancer Discov.* 2014;4:1046–1061.

10. Sequist LV, Soria JC, Goldman JW, et al. Rociletinib in EGFR-mutated non-small-cell lung cancer. *N Engl J Med.* 2015;372:1700–1709.

11. Seshacharyulu P, Ponnusamy MP, Haridas D, et al. Targeting the EGFR signaling pathway in cancer therapy. *Expert Opin Ther Target* 2012;16:15–31.

12. Mullooly M, McGowan PM, Crown J, et al. The ADAMs family of proteases as targets for the treatment of cancer. *Cancer Biol Ther.* 2016;17:870–880.

13. Dreymueller D, Uhlig S, Ludwig A. ADAM-family metalloproteinases in lung inflammation: potential therapeutic targets. *Am J Physiol Lung Cell Mol Physiol.* 2015;308:L325–L343.

14. Rose-John S. ADAM17, shedding, TACE as therapeutic targets. *Pharmacol Res.* 2013;71:19–22.

15. Zhou BB, Peyton M, He B, et al. Targeting ADAM-mediated ligand cleavage to inhibit HER3 and EGFR pathways in non-small cell lung cancer. *Cancer Cell* 2006;10:39–50.

16. Ni SS, Zhang J, Zhao WL, et al. ADAM17 is overexpressed in non-small-cell lung cancer and its expression correlates with poor patient survival. *Tumour Biol.* 2013;34:1813–1818.

17. Ogino A, Kitao H, Hirano S, et al. Emergence of epidermal growth factor receptor T790M mutation during chronic exposure to gefitinib in a non small cell lung cancer cell line. Cancer *Res.* 2007;67:7807–7814.

18. Li YQ, Yan JP, Xu WL, et al. ADAM17 mediates MMP9 expression in lung epithelial cells. *PLoS One* 2013;8:e51701.

19. Santarpia M, Gil N, Rosell R. Strategies to overcome resistance to tyrosine kinase inhibitors in non-small-cell lung cancer. *Expert Rev Clin Pharmacol.* 2015;8:461–477.

20. Kuiper JL, Smit EF. High-dose, pulsatile erlotinib in two NSCLC patients with leptomeningeal Metastases–one with a remarkable thoracic response as well. *Lung Cancer* 2013;80:102–105.

21. Guan Y, Zhao H, Meng J, et al. Dramatic response to high-dose icotinib in a lung adenocarcinoma patient after erlotinib failure. *Lung Cancer* 2014;83:305–307.

22. Jackman DM, Holmes AJ, Lindeman N, et al. Response and resistance in a non-small-cell lung cancer patient with an epidermal growth factor receptor mutation and leptomeningeal metastases treated with high-dose gefitinib. *J Clin Oncol.* 2006;24:4517–4520.

23. Soria JC, Wu YL, Nakagawa K, et al. Gefitinib plus chemotherapy versus placebo plus chemotherapy in EGFR-mutation-positive non-small-cell lung cancer after progression on first-line gefitinib (IMPRESS): a phase 3 randomised trial. *Lancet Oncol.* 2015;16:990–998.

24. Gil-Bazo I, Rolfo C. AZD9291 in TKI EGFR resistance in non-small cell lung cancer and the new concept of phase I trials. *Transl Lung Cancer Res.* 2016;5:85–88.

25. Kim TM, Song A, Kim DW, et al. Mechanisms of acquired resistance to AZD9291: a mutation-selective, irreversible EGFR inhibitor. *J Thorac Oncol.* 2015;10:1736–1744.

26. Li Y, Yan J, Xu W, et al. Lentivirus-mediated ADAM17 RNA interference inhibited interleukin-8 expression via EGFR signaling in lung epithelial cells. *Inflammation* 2012;35:850–858.

27. Cai M, Wang Z, Zhang J, et al. Adam17, a target of Mir-326, promotes Emt-induced cells invasion in lung adenocarcinoma. *Cell Physiol Biochem.* 2015;36:1175–1185.

28. Masago K, Fujita S, Hatachi Y, et al. North-East Japan Study Group Clinical significance of pretreatment serum amphiregulin and transforming growth factor-alpha, and an epidermal growth factor receptor somatic mutation in patients with advanced non-squamous, non-small cell lung cancer. *Cancer Sci.* 2008;99:2295–2301.

29. Ishikawa N, Daigo Y, Takano A, *et al.* Increases of amphiregulin and transforming growth factor-alpha in serum as predictors of poor response to gefitinib among patients with advanced non-small cell lung cancers. *Cancer Res.* 2005;65:9176–9184.