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Original Article

Synergistic anti-tumor effects of lenalidomide and gefitinib by upregulating ADRB2 and inactivating the mTOR/PI3K/AKT signaling pathway in lung adenocarcinoma

Shuangshuang Zhou[1,2], Meiyi Deng[1], Xuyu Bian[1], Jindong Shi[3], Rongrui Liang[1,#,*], Min Tao[1,4,#,*]

[1] *Department of Oncology, Dushu Lake Hospital Affiliated to Soochow University, Suzhou, Jiangsu Province, 215123, China*
[2] *Department of Oncology, Shanghai Fifth People's Hospital, Fudan University, Shanghai 200240, China*
[3] *Department of Pulmonary and Critical Care Medicine, Shanghai Fifth People's Hospital, Fudan University, Shanghai, 200240, China*
[4] *Department of Oncology, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu Province, 215006, China*

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Abstract

Gefitinib is commonly used to be the first-line therapy for advanced non-small cell lung cancer (NSCLC). Therapeutic effect of gefitinib is reduced due to acquired resistance, and combined treatment is recommended. In this research, we planned to explore the impacts of combined treatment of lenalidomide and gefitinib on gefitinib-sensitive or -resistant NSCLC cells. The co-treatment results demonstrated that enhanced antitumor impact on NSCLC cell growth, migration, invasion, cell cycle process and apoptosis. The tumor-bearing mouse models were established using PC9/GR cells. In vivo assays also showed that lenalidomide and gefitinib synergistically inhibited mouse tumor growth along increased the survival of mice. ADRB2 was identified as a lowly expressed gene in PC9/GR cells and LUAD tumor tissues. LUAD patients with high ADRB2 expression were indicated with favorable survival outcomes. Moreover, ADRB2 was upregulated in lenalidomide and/or gefitinib-treated PC9/GR cells. ADRB2 deficiency partially offsets the suppressive impacts of lenalidomide and gefitinib co-treatment on the viability and proliferation of PC9/GR cells. Additionally, lenalidomide and gefitinib cotreatment significantly inactivated the mTOR/PI3K/AKT signaling pathway compared with each treatment alone. Rescue assays were performed to explore whether lenalidomide and gefitinib synergistically inhibited the growth of PC9/GR cells via the PI3K/AKT pathway. PI3K activator SC79 significantly restored reduced cell proliferation, migration and invasion along with elevated cell cycle arrest and apoptosis caused by lenalidomide and gefitinib cotreatment. In conclusion, lenalidomide and gefitinib synergistically suppressed LUAD progression and attenuated gefitinib resistance by upregulating ADRB2 and inactivating the mTOR/PI3K/AKT signaling pathway in lung adenocarcinoma.

Keywords: ADRB2, Gefitinib, Lenalidomide, NSCLC, PI3K/AKT, Resistance

1. Introduction

Lung cancer belongs to the second most frequently diagnosed malignant tumor with about 2.2 million new cases in 2020, occupying 11.4% of cancer incidence [1]. Non-small cell lung cancer (NSCLC) as well as SCLC are two major kinds of lung cancer, and 80%-85% of patients are diagnosed with NSCLC. NSCLC is separated into lung adenocarcinoma (LUAD) together with lung squamous cell carcinoma, and LUAD belongs to the most prevalent NSCLC subtype [2]. Surgery, radiotherapy, chemotherapy and targeted therapy are the main treatment options for lung cancer patients. Nevertheless, the treatment outcomes are limited due to the late diagnosis in most patients with advanced or widely metastatic tumors, and the 5-year survival rate of NSCLC patients presents just 15% [3]. Therefore, it is essential to discover promising therapeutic methods for lung cancer treatment.

Drug resistance remains a negative factor limiting the treatment outcomes in patients with cancer. Epidermal growth factor receptor (EGFR) gene somatic mutations are observed in 30-40% of NSCLC patients in Asia [4]. EGFR tyrosine kinase inhibitors (EGFR-TKIs) containing gefitinib with favorable response rate are recommended to be the first-line therapy for advanced NSCLC patients with EGFR mutations [5, 6]. Nevertheless, patients usually develop resistance to EGFR-TKIs followed by long-time therapy of approximately 9-12 months [7, 8]. Hence, it is of vital significance to probe the potential mechanism of gefitinib resistance in LUAD and develop effective strategies to promote the sensitivity of cancer cells to gefitinib [9]. Combination therapy can confer synergistic clinical benefits compared with monotherapy. Lenalidomide is an analog of thalidomide with immunomodulatory and anti-angiogenic activities and is commonly used in the treatment of multiple hematological malignancies such as myeloma [10]. Lenalidomide is also revealed with potent

* Corresponding author.
E-mail address: taomin@suda.edu.cn (M. Tao).
# These authors contributed equally
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clinical effects on the treatment of solid tumors [11], and the combined treatment of lenalidomide with docetaxel is effective in disease stabilization of solid tumors compared with monotherapy with single agent [12]. Lenalidomide combined with tafasitamab is also demonstrated to improve the treatment outcome in prolonging the survival of relapsed/refractory diffuse large B-cell lymphoma patients [13]. Moreover, research has reported that lenalidomide enhances the cytolytic effects of T cells with chimeric antigen receptors targeting the EGFRvIII antigen on glioma cells expressing EGFRvIII [14]. Thus, we assumed that the combined treatment with gefitinib and lenalidomide may exert synergistic anticancer effects in LUAD.

Lenalidomide and gefitinib exert anti-tumor activities via various pathways. PI3K/AKT/mTOR signaling pathway is critically implicated the lung cancer aggressiveness and cell survival [15, 16]. Moreover, increasing evidences have also displayed that the PI3K/AKT/mTOR signaling activation is linked to gefitinib resistance in lung cancer [17-19]. In addition, lenalidomide has been reported to inactivate the PI3K/AKT pathway in mantle cell lymphoma cells [20]. Whether lenalidomide and gefitinib affected LUAD progression via the PI3K/AKT/mTOR signaling still requires further investigation.

In the present research, we delved into the impacts of combined treatment of lenalidomide and gefitinib on LUAD and explored the underlying mechanisms. We assumed that PI3K/AKT/mTOR signaling is crucial for the synergistic effects of lenalidomide and gefitinib in LUAD, which might provide novel therapeutic strategies for LUAD patients.

2. Materials and methods

2.1. Cell culture and treatment

Procell Life Science & Technology Co., Ltd. (Wuhan, China) offered NSCLC cell lines (H460, PC9), which were cultivated in RPMI-1640 medium (Sigma-Aldrich, USA) containing 10% FBS along with 1/100 Penicillin-Streptomycin at 37°C with 5% CO_2. Gefitinib-resistant PC9 cells (PC9/GR) were induced by adding 10 nmol/L gefitinib to the culture medium of PC9 cells and hatched for 48 h. Subsequently, PC9 cells were rinsed and cultured in culture medium without gefitinib to reach 80% confluence. Next, cells were stimulated by increasing doses of gefitinib and cells stably survived in 1 μmol/L gefitinib were harvested as PC9/GR cells. For gefitinib or lenalidomide treatment, cells were exposed to 0.25, 0.5, 1, 2, 4, 8 and 16 μmol/ L of gefitinib or lenalidomide for 24, 48 as well as 72 h, and cells viability could be subject to CCK-8 assays.

2.2. Cell transfection

Short hairpin RNA (shRNA) targeting ADRB2 (sh-ADRB2) was obtained from the GeneChem (Shanghai, China) with scrambled shRNA adopted to be the negative control (sh-NC). PC9/GR cells received transfection with the sh-NC or sh-ADRB2 plasmids with Lipofectamine 3000 (Invitrogen, USA). 48 h later, cells were harvested for follow-up experiments.

2.3. qRT-PCR

Extraction of total RNA was implemented with a TRIzol kit (Sigma-Aldrich, USA). Then RNA reverse transcription was implemented with a reverse transcription kit (TaKaRa, Japan). PCR was implemented with a SYBR Green master kit (Applied Biosystems, USA). Relative gene expression received calculation with the $^{2-\Delta\Delta Ct}$ method normalized to GAPDH. The primer sequences were as below: sense: 5'-GTCTACTCCAGGGTCTTTCAG-3', antisense: 5'-CCTCAGATTTGTCAATCTTCTGG-3'; GAPDH: sense: 5'-TCATTTCCTGGTATGACAACGA-3', antisense: 5'-GTCTTACTCCTTGGAGGCC-3'.

2.4. Western blot

Cells after indicated treatment were lysed by RIPA lysis buffer (Sigma-Aldrich). The concentration of proteins was examined with a BCA kit. Then the protein sample could be loaded on the 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by shifting to the nitrocellulose filter membranes (Millipore). The 5% skimmed milk was adopted for blocking the membranes. Next, the membranes received treatment with the primary antibodies at 4°C overnight. Subsequently, the secondary antibodies were treated into cells for incubation after washing. An enhanced chemiluminescence detection kit (Thermo Fisher, USA) could be applied for target protein blot development, and ImageJ software was used for protein density quantification.

2.5. Cell Counting Kit-8 (CCK-8) assays

H460 as well as PC9/GR cells could be collected and planted into 96-well plates (Corning, NY, USA) at 10000 cells/well, and incubated at 37°C, 5% CO_2 for 24 h, 48 h and 72 h. Subsequently, 10 μL CCK-8 solution could be supplemented into each well and hatched at 37°C for 2 h. A microplate reader (Thermo Fisher, USA) could be implemented to determine the absorbance OD value at 450 nm. The half-maximal inhibitory concentrations (IC50) of gefitinib or lenalidomide were calculated with GraphPad Prism 8 software.

2.6. Colony formation assays

The proliferative abilities of H460 together with PC9/GR cells were evaluated with colony formation assays. Cells were grown in 6-well plates at 1000-2000 cells/well. Then cells were cultured for 2 weeks at 37°C. Next, paraformaldehyde (Thermo Fisher) was adopted for fixing the cells and crystal violet was applied for staining the cells. Finally, the colony number was counted manually under a light microscope (Olympus, Japan).

2.7. Transwell assays

A transwell membrane (0.8 μm pore size, FALCON, USA) was implemented for conducting the migration and invasion assays. A transwell membrane precoated with Matrigel (2 mg/mL, Corning, USA) was implemented for the cell invasion assay (2×10^5 cells). Totally 2×10^4 cells were adopted for the migration assays with non-coated transwell membrane. Cells in serum-free medium were grown in the top chamber. The bottom chamber was added with 750 μL medium including 10% FBS. After 24 h-incubation, the remaining cells on the top surface were wiped off with a cotton swab. The migrated or invaded cells were fastened with 4% paraformaldehyde and subject to crystal violet staining (0.1%, Beyotime, China). Cell number was counted under a light microscope in 3 randomly chosen fields.

2.8. Flow cytometry analysis

An Annexin V-FITC Apoptosis Detection Kit (Beyo-

time, China) was applied for cell apoptosis evaluation. Cells after indicated treatment were harvested, rinsed and adjusted in binding buffer (5×10^5/mL). Then Annexin V and PI were added and cultured for 15 min avoiding light. A flow cytometer (Beckman-Coulter) could be applied to determine the apoptosis rate of cells. For cell cycle distribution analysis, cells could be stained with PI, followed by the analysis with a flow cytometer. The Cell Quest software (BD Biosciences) was implemented for cell cycle distribution quantification.

2.9. Mouse xenograft model

Female BALB/c nude mice (5 weeks, 18-20 g) were provided by the Vital River (Beijing, China). PC9/GR cells (1×10^6/mL) received subcutaneous injection into the right flank of BALB/c nude mice to build tumor-bearing mouse models. When the average tumor size achieved 50 mm^3, the mice were randomized into the Control, Lena, Gefi and Lena+Gefi groups (n=14 per group). Mice were administrated with the PBS in the control group; received oral lenalidomide (50 mg/kg/day) in the Lena group; treated with gefitinib (30 mg/kg) by oral gavage in the Gefi group, and 50 mg/kg lenalidomide combined with 30 mg/kg gefitinib in the Lena+Gefi group. Tumor size was monitored with a digital caliper every week. After 4 weeks, mice (n=4/group) were sacrificed to isolate the tumors. The remaining mice were implemented for survival analysis. The animal study was approved by the Ethics Committee of our hospital.

2.10. HE staining

Mouse lung tissues were collected, processed with 4% paraformaldehyde, embedded in paraffin, and then sliced into 5-μm thickness. For HE staining, the sections received staining with hematoxylin and eosin and then observed and photographed with a microscope.

2.11. Immunohistochemistry (IHC)

The paraffin-embedded mouse tumor sections were processed with xylene for 15 min, rehydrated in graded ethanol and heated for 10 min at 105°C in citric acid buffer. After washing with PBS and processing with 3% hydrogen peroxide solution, the sections were sealed with 10% normal goat serum at 37°C. Then Ki-67 antibody was added to culture at 37°C. Next, the sections were rinsed and then cultured with the secondary antibody. Next, the sections were taken to stain with DAB substrate kit and hematoxylin was adopted to stain the nuclei for 4 min. The Ki-67 positive cells (brown staining) were imaged under a light microscope (Olympus).

2.12. TUNEL assays

The paraffin-embedded tumor sections could be dewaxed with xylene and rehydrated in graded alcohol, followed by treatment with proteinase K (Beyotime, 20 μg/ml). Then the sections were processed with 3% H_2O_2 in PBS at room temperature, followed by washing. Then sections could be treated with TUNEL assay kit (Beyotime). The sections were developed by DAB chromogenic solution and counterstained using hematoxylin (Beyotime), and the apoptosis was assessed using fluorescence microscopy (Nikon, Japan).

2.13. Statistical analysis

Statistical analysis could be processed with GraphPad Prism 8.0 software (GraphPad Prism; San Diego, CA, USA). Data were exhibited as the mean ± SD from at least 3 independent assays. The statistical differences were analyzed by student's t-test or one-way ANOVA followed. $P<0.05$ was defined as the significant difference.

3. Results

3.1. Lenalidomide and gefitinib synergistically inhibited NSCLC cell growth

The LUAD cell line (H460) together with gefitinib-resistant LUAD cell line (PC9/GR) was chosen for cell growth assessment. H460 together with PC9/GR cells received lenalidomide or gefitinib treatment alone or combined treatment for 24, 48 as well as 72 h. As revealed by CCK-8 assays, the viability of H460 was reduced by lenalidomide and gefitinib treatment in a dose- and time-dependent way. After treatment for 72 h, H460 cells viability presented lower after the combined treatment of lenalidomide and gefitinib than monotherapy. Additionally, higher doses of lenalidomide and gefitinib treatment (over 8 μmol/L) tended to enhance the viability of 460 cells (Figures 1A, C, E). However, the viability of PC9/GR cells showed dose-independent growth suppression when treated with gefitinib for 24 h (Figure 1B). Lenalidomide treatment was demonstrated to induce significant reduction of PC9/GR cell viability at 8 μmol/L (Figure 1D). Moreover, we demonstrated that the combined treatment with over 4 μmol/L lenalidomide and gefitinib significantly decreased PC9/GR cell growth, and PC9/GR cell viability was lower followed by exposure to lenalidomide and gefitinib at 8 μmol/L for 72 h compared with each treatment alone (Figure 1F). The IC50 values of lenalidomide, gefitinib and

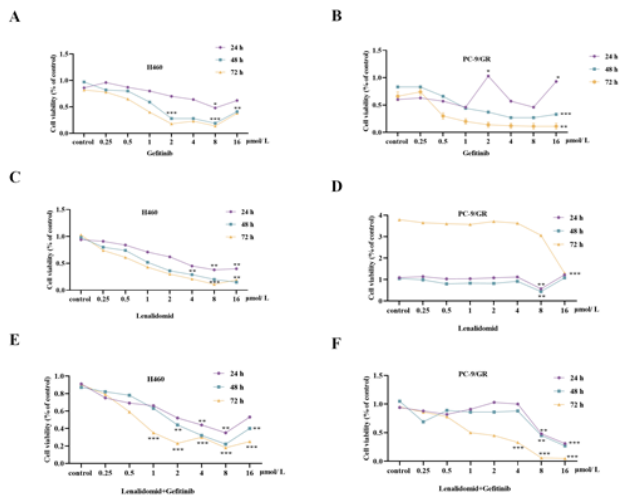

Fig. 1. Lenalidomide and gefitinib synergistically inhibit LUAD cell growth. CCK-8 assays detected the viability of (A) H460 cells and (B) PC9/GR cells after treatment with gefitinib at 0.25, 0.5, 1, 2, 4, 8 and 16 μmol/ L for 24, 48 as well as 72 h. CCK-8 assays detected the viability of (C) H460 cells and (D) PC9/GR cells exposed to different concentrations of lenalidomide for 24, 48 and 72 h. CCK-8 assays measured the viability of (E) H460 cells and (F) PC9/GR cells after combined lenalidomide and gefitinib treatment at indicated concentrations for 24, 48 as well as 72 h. *P<0.05, **P<0.01, ***P<0.001.

combined treatment are shown in Figure 1. Overall, the lenalidomide at 3 μmol/L or gefitinib at 19 μmol/L was used for following monotherapy, while the lenalidomide at 2 μmol/L and gefitinib at 2 μmol/L was used for following combined treatment.

3.2. Lenalidomide and gefitinib synergistically suppressed NSCLC cells proliferation as well as induced cell apoptosis

As revealed by colony-formation assays, the treatment of lenalidomide and gefitinib both decreased the proliferative potential of H460 cells and PC9/GR cells. Moreover, the combined treatment showed enhanced suppressive effects compared with each treatment alone (Figure 2A). Flow cytometry analysis displayed that H460 and PC9/GR cells apoptosis was significantly elevated after exposure to lenalidomide or gefitinib, and the cells that survived in the combined treatment group were reduced relative to each monotherapy (Figure 2B). Overall, the combined treatment repressed the resistance of PC9/GR cells to gefitinib and more effectively suppressed the proliferation of both gefitinib-sensitive and resistant cells relative to each treatment alone. The lenalidomide and gefitinib showed synergistic anti-tumor effects on NSCLC cell proliferation and apoptosis.

3.3. Lenalidomide and gefitinib synergistically repressed NSCLC cell migration and invasion

Transwell assays demonstrated that H460 together with PC9/GR cells migration and invasion were decreased followed by stimulating lenalidomide or gefitinib, and the combined lenalidomide and gefitinib treatment significantly enhanced the suppressive effects compared with each treatment alone (Figure 3A, B). The results indicated the synergistic antitumor effects of Lenalidomide and gefitinib on NSCLC cell migration and invasion.

3.4. Lenalidomide and gefitinib synergistically suppressed the cell cycle progression of NSCLC cells

We discovered that the lenalidomide or gefitinib treatment alone significantly promoted cell cycle arrest at the G0/G1 phase, while combined lenalidomide and gefitinib treatment showed enhanced elevation on cell cycle distribution at the G1 phase (Figure 4A). Moreover, western blot unveiled that Cyclin D1, Cdk4 along with Cdk6 expression exhibited significant reduction in the Lena and Gefi groups, and their protein levels presented lower in the Lena + Gefi group relative to the Lena or Gefi group (Figure 4B). Overall, these results indicated that combination of lenalidomide and gefitinib synergistically suppressed the cell cycle process of NSCLC cells sensitive or resistant to gefitinib.

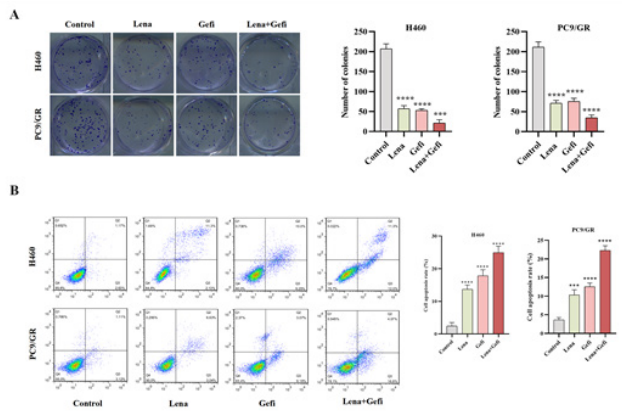

Fig. 2. Lenalidomide and gefitinib synergistically repressed the proliferation while promoting the apoptosis of NSCLC cells. (A) Colony-formation assays measured H460 and PC9/GR cell proliferation followed by treatment with lenalidomide, gefitinib or both. (B) Flow cytometry analysis examined NSCLC cell apoptosis in each group. ***P<0.001.

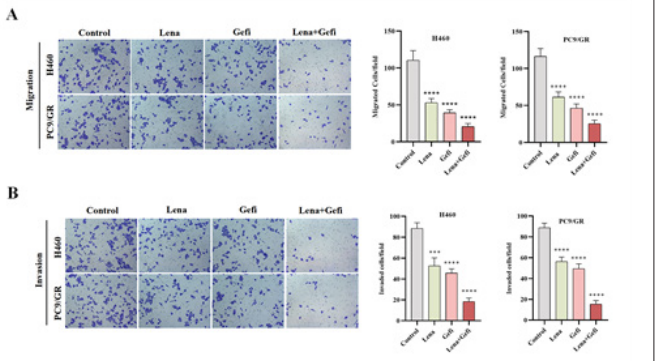

Fig. 3. Lenalidomide and gefitinib synergistically suppressed NSCLC cell migration and invasion. Transwell assays detected the (A) migration and (B) invasion abilities of H460 cells and PC9/GR cells after exposure to lenalidomide, gefitinib or both. *P<0.05, **P<0.01, ***P<0.001.

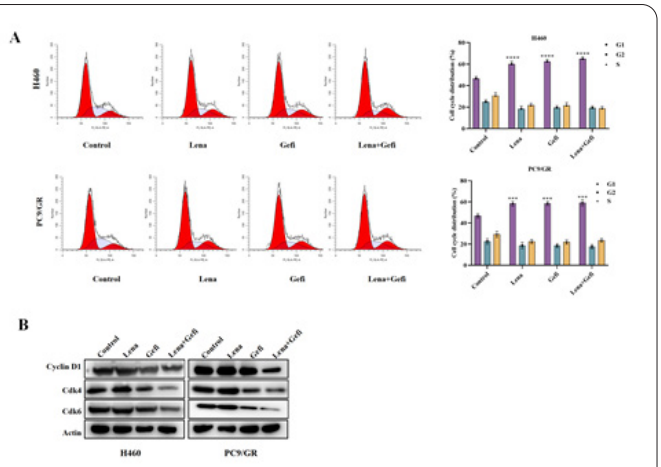

Fig. 4. Lenalidomide and gefitinib synergistically suppressed the cell cycle progression of NSCLC cells. (A) Flow cytometry analysis measured the cell cycle distribution of H460 and PC9/GR cells in each group. (B) Western blot detected Cyclin D1, Cdk4 and Cdk6 protein levels in each group. *P<0.05, **P<0.01, ***P<0.001.

3.5. Lenalidomide and gefitinib synergistically restrained tumor growth while increased the survival of tumor-bearing mice

Xenograft mouse models were generated using PC9/GR cells, and the impacts of lenalidomide and gefitinib on tumorigenesis were explored. Mice could be sacrificed after 4 weeks, and the size of tumors was evidently smaller in the Lena and Gefi groups in comparison with the control. The combined administration with lenalidomide and gefitinib significantly lessened the volume of mouse tumors compared with each treatment alone (Figure 5A). The tumor weight was reduced by lenalidomide or gefitinib treatment and most significantly decreased in the combined treatment group (Figure 5B). The survival analysis

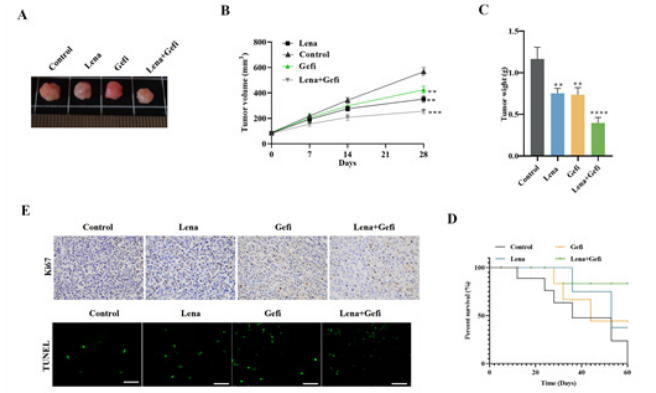

Fig. 5. Lenalidomide and gefitinib synergistically restrained tumor growth along with increased the survival of tumor-bearing mice. (A) Representative tumor images in the control, Lena, Gefi and Lena+Gefi groups. (B) Tumor volume and (C) weight in indicated groups. (D) Survival rate of mice in each group (n=10 per group). (E) Representative HE staining, Ki-67 staining along with TUNEL staining images of mouse tumor specimens in indicated groups. *P<0.05, **P<0.01, ***P<0.001.

of tumor-bearing mice suggested that mice in the combined treatment showed the most favorable survival outcome compared with monotherapy (Figure 5C, D). The pathological alterations in mouse tumor tissues were attenuated by the combined administration of lenalidomide and gefitinib. The IHC results indicated that Ki-67-positive cells in mouse tumor tissues showed a reduction in the Lena or Gefi group, and were most significantly decreased in the combined treatment group. Additionally, TUNEL assays showed that tumor cells apoptosis was repressed by the lenalidomide or gefitinib treatment, and the highest apoptosis rate was detected in the combined treatment group relative to each monotherapy (Figure 5E). These results indicated that lenalidomide and gefitinib synergistically inhibited tumorigenesis *in vivo*.

3.6. ADRB2 was low-expressed in PC9/GR cells

According to the GSE169513 dataset, ADRB2 was identified to be low-expressed in the gefitinib-resistant PC9/GR cells relative to gefitinib-sensitive PC9 cells (Figure 6A). Moreover, the GEPIA database also indicated that ADRB2 was low-expressed in LUAD tumor tissues when compared with normal tissues (Figure 6B). Kaplan-Meier Plotter depicted that LUAD patients with high ADRB2 expression were linked to favorable survival outcomes (HR=0.64, P=3.4e-13) (Figure 6C). We also confirmed that ADRB2 was lowly expressed in PC9/GR cells when compared with PC9 cells (Figure 6D). Moreover, qRT-PCR displayed that ADRB2 expression increased by the lenalidomide or gefitinib treatment in PC9/GR cells, and most significantly elevated in the combined treatment group (Figure 6E). Whether ADRB2 was essential for the lenalidomide or gefitinib-mediated suppression on PC9/GR cell growth was further explored. The expression of ADRB2 showed significant decrease after the transfected of shRNAs targeting ADRB2 (Figure 6F). Based on CCK-8 as well as colony-formation assays, PC9/GR cells viability and proliferation were reduced in the PC9/GR group were significantly elevated after ADRB2 silencing, which suggested that lenalidomide and gefitinib inhibited gefitinib-resistant tumor cell growth by elevating the expression of ADRB2 (Figure 6G, H).

3.7. Lenalidomide and gefitinib inactivated the mTOR/PI3K/AKT pathway to suppress NSCLC cell malignancy

Previous literatures have unveiled that the mTOR/PI3K/AKT pathway is linked to the treatment resistance to EGFR-TK inhibitors in NSCLC [15, 21, 22]. We found that the treatment of lenalidomide or gefitinib lessened p-PI3K, p-AKT, as well as p-mTOR levels, and the combined lenalidomide and gefitinib treatment, showed the most significant suppression on the p-PI3K, p-AKT as well as p-mTOR levels in H460 together with PC9/GR cells (Figure 7A, B). Next, the SC79, a PI3K activator was applied to stimulate the PC9/GR cells to explore whether lenalidomide and gefitinib synergistically inhibited NSCLC cell growth in an mTOR/PI3K/AKT-dependent way. It was discovered that the decrease in PC9/GR cell viability and proliferation after combined lenalidomide and gefitinib stimulation was significantly elevated after SC79 treatment

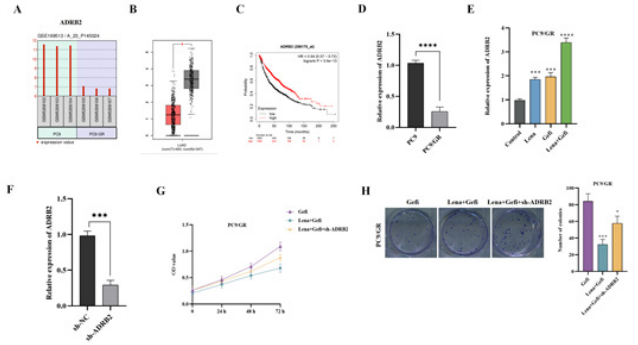

Fig. 6. ADRB2 is downregulated in PC9/GR cells. (A) ADRB2 expression in gefitinib-sensitive PC9 cells along with gefitinib resistant PC9/GR cells based on the GSE169513 dataset. (B) The expression profile of ADRB2 in LUAD tumor tissues as well as normal tissues in the GEPIA database. (C) The survival curves of LUAD patients with high or low levels of ADRB2 on the Kaplan-Meier Plotter database (http://kmplot.com/analysis/). (D) qRT-PCR detected the expression of ADRB2 in PC9 as well as PC9/GR cells. (E) qRT-PCR measured the expression of ADRB2 in PC9/GR cells stimulated by lenalidomide, gefitinib or both. (F) qRT-PCR detected ADRB2 expression in PC9/GR cells after ADRB2 silence. (G) CCK-8 assays detected PC9/GR cell viability in the gefi, lena + gefi, or lena + gefi +sh-ADRB2 groups. (H) Colony-formation assays detected PC9/GR cell proliferation in each group. **P<0.01, ***P<0.001, ##P<0.01.

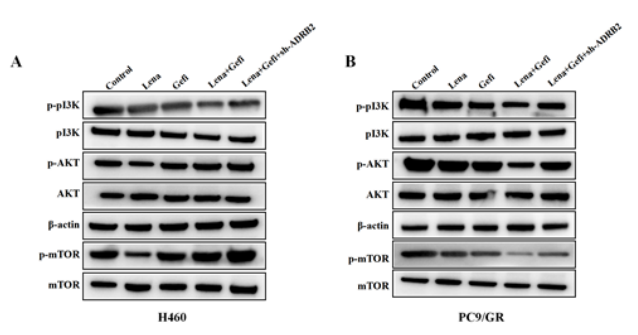

Fig. 7. Lenalidomide and gefitinib inactivated the mTOR/PI3K/AKT pathway. (A,B) Western blot measured mTOR/PI3K/AKT pathway-linked key protein levels in H460 and PC9/GR cells. *P<0.05, **P<0.01, ***P<0.001.

(Figure 8A-C). The apoptotic rate of PC9/GR cells was elevated in the Lena+Gefi group relative to the Gefi group and showed significant reduction in the Lena+Gefi+SC79 group in comparison with the Lena+Gefi group (Figure 8D, E). Moreover, Transwell assays showed that the reduced migration and invasion induced by lenalidomide and gefitinib stimulation were rescued after SC79 treatment (Figure 8F). Flow cytometry analysis also indicated that lenalidomide and gefitinib elevated the cell cycle distribution at the G1 phase, which was impaired after SC79 treatment (Figure 8G). Overall, these outcomes demonstrated that lenalidomide and gefitinib synergistically suppressed the malignant phenotypes in NSCLC via inactivating the mTOR/PI3K/AKT pathway.

4. Discussion

Gefitinib is recommended to be the first-line treatment for NSCLC patients with EGFR mutations [23]. Nevertheless, the therapeutic effect is limited in the long term by the acquired resistance to gefitinib [24]. In this research, it was discovered that the combined lenalidomide and gefitinib treatment showed synergistic anti-tumor potentials on gefitinib-sensitive or resistant NSCLC cells relative to monotherapy. Moreover, lenalidomide and gefitinib treatment was revealed to upregulate ADRB2 and inactivate the mTOR/PI3K/AKT pathway in PC9/GR cells, and further analysis implied that lenalidomide and gefitinib synergistically suppressed PC9/GR cell growth by elevating ADRB2 expression and inactivating the mTOR/PI3K/AKT pathway.

Lenalidomide with antiangiogenic and immunomodulatory activities is reported to repress the proliferation along with angiogenesis of cancer cells [25, 26]. Lenalidomide is also indicated to augment the antiproliferative effects of cisplatin on urothelial carcinoma [27]. Lenalidomide combined with sorafenib treatment induces tumor cell apoptosis, inhibits tumor growth and improves survival in animal models. Lenalidomide is indicated to enhance the suppressive effects of sorafenib on hepatocellular carcinoma by modulating the immune response [28]. In our study, the lenalidomide treatment was also demonstrated to suppress the viability of gefitinib-sensitive NSCLC cells, while the viability of gefitinib-resistant PC9/GR cells showed no significant changes until lenalidomide concentration increased to 8 μmol/ L. Moreover, we revealed that the combined lenalidomide and gefitinib treatment reduced H460 and PC9/GR cell viability compared with each treatment alone, which indicated that lenalidomide enhanced the antitumor activities of gefitinib on gefitinib-sensitive and resistant NSCLC cells growth.

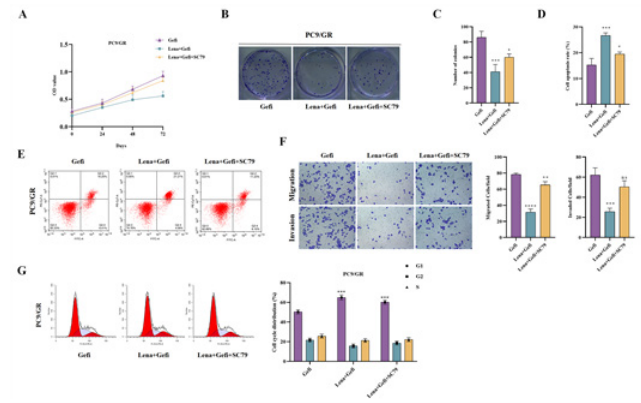

Fig. 8. Lenalidomide and gefitinib synergistically suppressed NSCLC progression by inactivating the mTOR/PI3K/AKT pathway. (A) CCK-8 assays examined PC9/GR cell viability in the Gefi, Lena+Gefi and Lena+Gefi+SC79 groups. (B,C) Colony-formation assays detected the proliferative capacity of PC9/GR cells in each group. (D,E) Flow cytometry analysis measured PC9/GR cell apoptosis in indicated groups. (F) Transwell assays detected PC9/GR cell migration and invasion after indicated treatment. (G) Flow cytometry analysis measured the cell cycle distribution of PC9/GR cells followed by indicated treatment. *P<0.05, **P<0.01, ***P<0.001; #P<0.05, ##P<0.01, ###P<0.001.

Gefitinib is an EGFR-TKI widely applied in the clinical therapy of NSCLC. Gefitinib is revealed to effectively improve the clinical outcomes of advanced NSCLC patients, and the combination with chemotherapy is reported to enhance the therapy efficacy for lung cancer [29]. In the present study, gefitinib treatment was demonstrated to limit the proliferation, cell cycle, migration and invasiveness while promoting the apoptosis of H460 together with PC9/GR cells. Furthermore, the combined treatment with lenalidomide showed high antitumor activities on NSCLC cells compared with gefitinib treatment alone. Additionally, the tumor-bearing mouse models were built using PC9/GR cells, and the outcomes suggested that the combined lenalidomide and gefitinib treatment showed enhanced suppressive impacts on mouse tumor growth and improved mouse survival relative to each monotherapy, which was consistent with the previous findings.

However, the treatment outcome of gefitinib is challenged by the acquired resistance. Increasing literatures have been conducted to elucidate the potential mechanism involved in this process. For instance, Sun et al. have pointed that FGL1 is high-expressed in PC9/GR cells relative to PC9 cells and is upregulated by gefitinib stimulation in a dose-dependent way. Moreover, FGL1 silencing is revealed to overcome the gefitinib resistance in PC9/GR cells [30]. STAT3/ZEB1 signaling pathway is revealed to participate in the gefitinib resistance in NSCLC and facilitate proliferation and invasion. Targeting STAT3 is suggested as a promising option against gefitinib resistance in NSCLC [31]. In our study, we probed the differentially expressed genes in PC9/GR cells based on bioinformatics analysis. ADRB2 was identified to be low-expressed in PC9/GR cells as well as LUAD tumor tissues. LUAD patients with high ADRB2 expression have favorable survival outcomes, which is in line with the previous research [32]. Nevertheless, the role of ADRB2 in NSCLC progression still requires further exploration. We found that ADRB2 is involved in the gefitinib resistance in NSCLC. ADRB2 expression was significantly elevated by the combined lenalidomide and gefitinib treatment in PC9/GR cells. ADRB2 deficiency is indicated to reverse the inhibition induced by combined lenalidomide and gefitinib treatment on PC9/GR cell growth.

Accumulating evidence also reveals that the mTOR/PI3K/AKT signaling is crucially linked to the treatment effects and resistance of gefitinib in lung cancer [33-35]. The activated mTOR/PI3K/AKT signaling is a key element to regulate lung cancer cell proliferation and metastasis [16]. In this research, we discovered that the mTOR/PI3K/AKT signaling is significantly inhibited after the cotreatment with lenalidomide and gefitinib in H460 and PC9/GR cells. Additionally, the SC79 was used to activate

the signaling in PC9/GR cells, and we discovered that the anti-tumor effects induced by cotreatment of lenalidomide and gefitinib on PC9/GR cells were significantly reversed after SC79 administration, suggesting that lenalidomide and gefitinib synergistically regulated PC9/GR cell growth by inactivating the mTOR/PI3K/AKT pathway.

In conclusion, lenalidomide and gefitinib synergistically suppressed cell growth, migration, invasion, and cell cycle process along with induced cell apoptosis of gefitinib-resistant NSCLC cells by upregulating ADRB2 and inactivating the PI3K/AKT signaling pathway in NSCLC.

Authors' contributions

Tao M and Liang RR contributed to the study concept and design; Zhou SS performed the main experiments and wrote and revised the manuscript; Deng MY, Bian XY and Shi JD partly contributed to the experiments, data analysis, and interpretation. All authors read and approved the final manuscript.

Declaration of conflicting interests

All the authors contributed to the creation of this manuscript. We declare that there are no conflicts of interest, and no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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Research ethics and patient consent

The present study was approved by the Ethics Committee of the First Affiliated Hospital of Soochow University. Informed consent was obtained from all patients before the start of this study.

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