

Macrophage renewal modes affect acquired resistance to gefitinib in EGFR-mutant lung cancer PC-9 cells

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Abstract. EGFR tyrosine kinase inhibitors (TKIs) have been successfully used in lung cancer treatment. Nevertheless, tumor cells may develop resistant phenotypes in the tumor microenvironment after a period of treatment with any generation of EGFR TKIs. Macrophages play a pivotal role in carcinogenesis and development. At the same time, macrophages renew continuously, and their polarized phenotype changes dynamically. Although macrophage polarization is generally considered to be involved in secondary resistance, it would be time consuming if renewed macrophages would only protect tumor cells from drug attacks after polarization. It is unclear how dynamic renewal of macrophage contributes to acquired resistance. By using two co-culture approaches, the present study modeled two types of renewal of macrophages: Migrated macrophages (Mm) and locally self-renewal resident macrophages (Mr). The EGFR-mutant lung cancer cell line PC-9 was induced to develop resistance to gefitinib in these co-culture approaches and without macrophages

co-culture. PC-9 cells induced in Mm co-culture recovered fast from gefitinib attack, and formed the gefitinib resistant (GR) subline PC-9/Mm/GR with the strongest resistant ability. PC-9/Mm/GR cells exhibited epithelial characteristics, including stable expression of EGFR, phosphorylated EGFR, insulin-like growth factor 1 receptor, TGF- β II receptor and E-cadherin. PC-9 cells induced in Mr co-culture and those without co-culture developed similar weak resistance to gefitinib. TGF- β secretion was inhibited when PC-9 cells were in Mr co-culture, but not in Mm co-culture. Macrophages were not polarized in either co-culture type. The present findings suggested that macrophages may affect resistance acquirement by different renewal modes. Mm could promote resistance acquirement by stabilizing tumor cell phenotypes, which would happen before polarization. It would be helpful to monitor the response to EGFR-TKIs and design novel treatment strategies if macrophages from two renewal modes were distinguished effectively.

Introduction

Lung cancer is a malignant tumor with the second highest morbidity rate and the leading mortality rate worldwide (1). EGFR tyrosine kinase inhibitor (TKI) therapy has made a great progress in non-small cell lung cancer treatment with a longer patient survival time and fewer adverse effects. However, acquired resistance is inevitable after a period of time treatment (2). The third generation EGFR-TKIs have met the same fate, though they were designed to surmount some resistant mutations (3). Tumor cells are spared from drug attack after obtaining resistant phenotype, either by on-target resistance, such as ATP affinity alteration by EGFR tyrosine kinase domain mutation (mainly T790M), or by off-target resistance, such as bypassing signaling transduction pathway activation (4). Either way, tumor resistance acquirement is inseparable with tumor microenvironment (TME). Macrophages and other cells of the TME may provide various growth factors and extracellular signals to orchestrate between tumor cells and host body, and thus mold the malignancy of tumor cells (5).

Macrophages coordinate immunity and tumor development with their constant migration and renewal. They are associated

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Abbreviations: TKI, tyrosine kinase inhibitor; TME, tumor microenvironment; Mr, resident macrophage; Mm, migrated macrophage; PC-9/Mr/GR, gefitinib-resistant sublines induced with PC-9 cells in Mr co-culture; PC-9/Mm/GR, gefitinib-resistant sublines induced with PC-9 cells in Mm co-culture; PC-9/GR, gefitinib-resistant sublines induced with PC-9 cells without macrophage co-culture; PC-9/Mr, PC-9 cells co-cultured with Mr; PC-9/Mm, PC-9 cells co-cultured with Mm; ELISA, Enzyme-linked immunosorbent assay; PMA, phorbol-12-myristate-13-acetate; GR, gefitinib resistant; p-EGFR, phosphorylated-EGFR; IGF-1R, insulin-like growth factor 1 receptor; TGF- β IIR, TGF- β II receptor; HLA, human leukocyte antigen; HRM, high-resolution melting

Key words: macrophage, renewal mode, second resistance, tyrosine kinase inhibitor, tumor microenvironment

with carcinogenesis, tumor cell proliferation, metastasis and even influence the response to anti-tumor therapy (6-10). Though some research has indicated that EGFR-TKIs treatment interferes with macrophage immunoreaction, it is still unclear how macrophages are involved in acquirement of resistance to EGFR-TKIs (11,12). A number of studies have focused on macrophage polarization. It is considered that M2 macrophages assist in obtaining secondary resistance to gefitinib, and high recruitment of M1 macrophages is associated with good response to gefitinib and longer survival (13-16). However, macrophage phenotypes change with TME changes, and the M1 and M2 specific marker may be co-expressed in some macrophages (6,13,17). Moreover, short-lived macrophages need to constantly renew themselves, and in either renewal way, new macrophages can polarize into M1 and M2 macrophages (6,18). It would be time consuming if new macrophages didn't perform protection until polarization.

Macrophage renewal ways are mainly determined by their origins. Resident macrophages renew by local proliferation, which are from embryonic precursors of the yolk sac (6,17). Monocyte-derived macrophages are recruited at the tumor site after emigrating through the blood stream, originating from hematopoietic stem cells (6,19). Macrophage refreshing potentially leads to various temporal and spatial distributions, and the distribution environment instead will affect their functions (20,21). Resident macrophages are more involved in tumor initiation, whereas monocyte-derived macrophages are more associated with tumor metastasis (6,13,22). Although the origin of the macrophage endows some inheritance characters, macrophages with the same origin may possess functional heterogeneity (20). The functional status of macrophages is closely associated with their different renewal processes. Previous studies have demonstrated that monocyte-derived macrophages commonly recruited in intratumoral area and intratumoral macrophages contribute to the epithelial-mesenchymal transition phenotype of tumor cells, which is a mechanism of secondary resistance (7,20,22-25). Notably, macrophage refreshing affects secondary resistance to EGFR-TKIs.

The present study induced resistance to gefitinib in PC-9 cells with an EGFR deletion mutation by alternative treatment with gefitinib and recovery in drug-free medium for ~10 months in macrophage co-culture systems. Renewal modes were stimulated by controlling macrophage co-culture ways as follows: i) Co-culture started before PC-9 cells treatment; meaning that co-culture continued all along from treatment to recovery, and was used as self-renewal resident macrophages (Mr); and ii) co-culture started after PC-9 cells treatment; meaning that co-culture presented only in recovery, was used as migrated macrophages (Mm). The aim of the present study was to investigate the dynamic role of macrophages in the acquired resistance to gefitinib in PC-9 cells, which are sensitive to gefitinib. To the best of our knowledge, this is the first report to build an *in vitro* model to simulate macrophage renewal ways.

Materials and methods

Reagents and cell lines. Gefitinib powder was purchased from Selleck Chemicals (cat. no. S1025). The human lung cancer

cell line PC-9 (cat. no. 90071810; European Collection of Authenticated Cell Cultures; STR analysis was used to confirm the cell line STR loci including D5S818, D13S317, D7S820, D16S539, VWA, TH01, AM, TPOX and CSE1PO were all matched) with EGFR-mutation (exon 19 deletion, E746-A750 deletion) and the human acute monocyte leukemia cell line THP-1 (TIB-202™; American Type Culture Collection) were used. Both of them were provided by Cobioer Biosciences Co., Ltd. PC-9 cells were cultured in RPMI 1640 medium (Hyclone; Cytiva) with 10% fetal calf serum (PAN Biotech UK, Ltd.). The THP-1 cells were cultured in RPMI 1640 medium with 10% fetal calf serum supplemented with 0.05 mM 2-mercaptoethanol (Sigma-Aldrich; Merck KGaA) and differentiated into macrophages using 320 nM phorbol-12-myristate-13-acetate (PMA; Abcam) for 48 h at 37°C. All cells were maintained in a 5% CO₂ humidified incubator at 37°C.

Inducing gefitinib-resistant sublines of PC-9 cells with and without macrophages co-culture. PC-9 cells and macrophages differentiated from THP-1 cells were co-cultured in Transwell system with 3-μm Biopore™ membrane (MilliporeSigma) inserted in six-well plates. PC-9 cells were seeded into lower chamber (5×10^5 cells/well) and macrophages in the upper chamber (6.25×10^4 cells/well) in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Gefitinib-resistance was induced in PC-9 cells by alternating gefitinib treatment for 24 h and recovery in drug-free medium at 37°C. When PC-9 cells were at 50-70% confluency, they were sub-cultured 1-2 times, then the processes of treatment and recovery were repeated. Gefitinib was added with increasing concentration from 5 to 35 μM by 5 μM each step. Macrophages were renewed when co-cultured PC-9 cells were passaged. Local self-renewal macrophages and migrated macrophages were stimulated using two co-culture ways. In one group, co-culture of macrophages and PC-9 cells began before gefitinib treatment, therefore, macrophages accompanied by PC-9 cells all along, during both gefitinib treatment or recovery in drug-free medium. Macrophages in this group were called resident macrophages (Mr). In the other group, co-culture began after gefitinib treatment during the first recovery in drug-free medium. Macrophages only presented in the drug free medium recovery process. Macrophages in this group were called migrant macrophages (Mm). As a control group, resistant was induced in PC-9 cells without co-culture with macrophages (Fig S1). After 10 months, PC-9 cells induced in Mr co-culture, in Mm co-culture and without co-culture were marked as PC-9/Mr/GR, PC-9/Mm/GR and PC-9/GR, respectively.

Cell proliferation curves. The cell viability was measured by trypan blue dye (Spectrum Laboratory Products, Inc.) exclusion method (26). Cell counting was performed manually using a hemacytometer. PC-9 cell growth co-cultured with macrophage in different ratios were recorded and charted with cell proliferation curves. Growth of PC-9 cells and the three gefitinib-resistance sublines (PC-9/Mr/GR, PC-9/Mm/GR and PC-9/GR) under the circumstances of different gefitinib concentration were also measured using cell proliferation curves. Cells were counted in triplicate.

MTT assay. The growth inhibition of PC-9 cells and three gefitinib-resistance sublines (PC-9/Mr/GR, PC-9/Mm/GR and PC-9/GR) to gefitinib was detected using MTT (Sigma-Aldrich; Merck KGaA) assay. After treated with gefitinib in two-time serial dilution (20-1.25 μ M for resistance sublines, 0.04-0.0025 μ M for PC-9 cells) at 37°C for 48 h, growth inhibition was detected using MTT assay, as described previously (27). Optical density measurements were repeated in triplicate. Growth inhibition ratios of the four types of cells at different concentration of gefitinib were charted.

Flow cytometry analysis. The cell cycle (G_2 , S and G_0/G_1 phase) distributions were analyzed using flow cytometry. The parental PC-9 cells and three resistant sublines (PC-9/Mr/GR, PC-9/Mm/GR and PC-9/GR) were treated with or without gefitinib (5 μ M) for 24 h at 37°C, then harvested and fixed in 70% ethanol at 4°C overnight. After staining with PI/RNase solution (Nanjing KeyGen Biotech Co., Ltd.) for 30 min in a 37°C water bath, according to the manufacturer's instructions, cell cycle distribution was detected using a FACSCalibur™ flow cytometer (BD Biosciences).

Macrophage phenotypes were identified using fluorescence antibodies anti-human leukocyte antigen (HLA)-DR-FITC (1:50; cat. no. 130-095-295; Miltenyi Biotec GmbH) for M1 and CD206-PE for M2 (1:50; cat. no. 130-095-220; Miltenyi Biotec GmbH). Phenotype alteration of macrophages before and after co-culture with PC-9 cells as well as after treatment with low concentration gefitinib (5 μ M) were analyzed using a FACSCalibur™ flow cytometer (BD Biosciences). After the debris was excluded with forward and side scatter, the data were presented using x- and y-axis biexponential display. Cells close to the x-axis were corrected. The cell distribution was compensated to provide an even visual. The gate of negative and positive of markers was set manually, since the quadrant gate was shifted after compensation. Because the shift mainly occurred in x-axis, the gate was set according to FITC channel histogram. The same gate was applied in all groups. All analysis was performed using BD FACSDiva software (version 8.0; BD Biosciences). All processes were performed using the manufacturer's protocol and checked by an expert in flow cytometry technology.

High-resolution melting analysis and sequencing. Genomic DNA of parental PC-9 cells and all resistant sublines (1×10^6 cells) were extracted using a E.Z.N.A.® tissue DNA kit (Omega Bio-Tek, Inc.) according to manufacturer's protocol for cultured cells. PCR was performed in a total volume of 10 μ l reaction mixture with a 15 μ l mineral oil overlay in each tube on a 72-well rotor. The reaction mixture contained 1x PCR buffer (Takara Bio, Inc.) with $MgCl_2$ (2.0 mM for EGFR exon 19 and 20; 2.5 mM for EGFR exon 18 and 21), 200 μ M dNTPs, 0.25 U Hot Start Ex Taq polymerase (Takara Bio, Inc.), 10 ng genomic DNA, 0.5 μ M primers and 1x LCGreen PLUS® (BioFire Diagnostics). Primer sequences are presented in Table SI. PCR was performed on a Rotor-Gene Q (Qiagen, Inc.) instrument with 35 cycles of 98°C for 20 sec, 58-67°C for 20 sec (58°C for exon 19; 62°C for exon 18; 67°C for exons 20, 21) and 72°C for 20 sec. Then high-resolution melting (HRM) was run from 60-95°C and other parameters were defaulted. Instrumental commercial software (Rotor-Gene Q Series

software; version 2.3.4; Qiagen, Inc.) was used for HRM analysis (28,29).

EGFR exon 18-21 fragments for sequencing were amplified on a Mastercycler (Eppendorf) with 35 cycles of 98°C for 30 sec, 61°C for 30 sec and 72°C for 30 sec. The reaction mixture contained 1x PCR buffer (Takara Bio, Inc.) with 2.0 mM $MgCl_2$, 200 μ M dNTPs, 0.25 U Hot Start Ex Taq polymerase (Takara Bio, Inc.), 10 ng genomic DNA and 0.5 μ M primers. Primer sequences are presented in Table SI. In order to double check the results from HRM analysis and confirm deletion mutation sequence of the 4 fragments, Sanger sequencing of PCR products was performed blindly, which was performed by General Biol., Inc. (www.generalbiol.com).

Western blot analysis. PC-9 cells, PC-9/Mr/GR, PC-9/Mm/GR and PC-9/GR were collected, and treated with cell lysis buffer (Beyotime Institute of Biotechnology). Whole proteins were quantified using a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology). Aliquots of cell lysate containing 60 μ g protein were separated by 10% SDS-PAGE (Beyotime Institute of Biotechnology) and transferred to polyvinylidene difluoride membranes (MilliporeSigma). After blocking with 5% skim milk for 2 h at room temperature, the membranes were incubated with anti-EGFR (rabbit monoclonal antibody; 1:1,000; cat. no. #4267; Cell Signaling Technology, Inc.), anti-phosphorylated (p)-EGFR (rabbit monoclonal antibody, Try1068; 1:1,000; cat. no. #3777; Cell Signaling Technology, Inc.), anti-HER2 (rabbit polyclonal antibody; 1:2,000; cat. no. 18299-1-AP; ProteinTech Group, Inc.), anti-insulin-like growth factor 1 receptor (IGF-1R; mouse monoclonal antibody; 1:2,000; cat. no. 66283-1-Ig; ProteinTech Group, Inc.), anti-TGF- β II receptor (TGF- β IIIR; rabbit monoclonal antibody; 1:1,000; cat. no. ab184948; Abcam) and anti- β actin (mouse monoclonal antibody; 1:500; cat. no. TA328071; OriGene Technologies, Inc.) at 4°C overnight. Afterwards, they were treated with IRDye 800CW goat anti-rabbit (cat. no. #926-32211) and anti-mouse (cat. no. #926-32210) secondary IgG antibodies (1:5,000; LI-COR Biosciences) for 1 h at room temperature, and the blots were visualized using an Odyssey CLX (LI-COR Biosciences). The gray-level of bands were analyzed using Image Studio (version 4.0; LI-COR Biosciences).

Immunofluorescence staining. PC-9 cells, PC-9/Mr/GR, PC-9/Mm/GR and PC-9/GR were cultured on coverslips. After fixing with 4% formaldehyde in PBS for 15 min and blocking with 50 μ l goat serum (OriGene Technologies, Inc.) for 30 min at room temperature, cells were incubated with anti-E-cadherin (mouse monoclonal antibody; 1:50; 60335-1-Ig; ProteinTech Group, Inc.) and anti-Vimentin (rabbit polyclonal antibody; 1:50; 10366-1-AP; ProteinTech Group, Inc.) at 4°C overnight, respectively. Then they were incubated with Rhodamine (TRITC)-conjugated goat anti-rabbit IgG (1:50; cat. no. SA00007-2; ProteinTech Group, Inc.) and fluorescein (FITC)-conjugated goat anti-mouse IgG (1:100; cat. no. SA00013-1; ProteinTech Group, Inc.) for 1 h at room temperature, and DAPI staining solution 5 min at room temperature. Samples were imaged using an immunofluorescence microscope (Leica DMI 3000B; Leica Microsystems, Inc.).

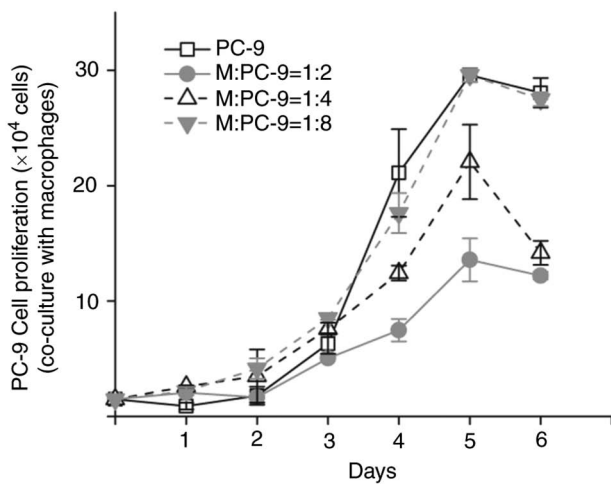


Figure 1. Proliferation curves of PC-9 cells when co-cultured with macrophages at various ratios. PC-9 cells began to proliferate rapidly on Day 2 after seeding, and reached to peak on Day 5. When co-cultured with macrophages differentiated from THP1 cells at varied ratios, PC-9 cell number was markedly decreased with the increasing macrophage percentage. High percentage of macrophages inhibited PC-9 cells proliferation. The inhibition was weakened with the decreasing percentage of macrophages. When the ratio of macrophages to PC-9 cells were 1:8, PC-9 cells growth curves were similar compared with those without macrophages co-culture.

Enzyme-linked immunosorbent assay (ELISA). TGF- β 1 levels in cell culture supernatants of transient treatment and long term induction were detected. TGF- β 1 levels were measured two consecutive days after discarding gefitinib using a Human TGF β 1 ELISA kit (cat. no. ab100647; Abcam) according to the manufacture instruction. In the transient treatment group, PC-9 cells in Mr co-culture (PC-9/Mr), in Mm co-culture (PC-9/Mm) as well as without co-culture (PC-9) were treated with 5 μ M gefitinib at 37°C for 24 h. In the long term induction group, three gefitinib-resistant sublines were treated with 35 μ M gefitinib at 37°C for 24 h.

Statistical analysis. All data are presented as mean $\pm$ standard deviation (SD). One way analysis of variance (ANOVA) and Tukey's HSD test were used to compare the difference of cell numbers in various environments and cell cycle distribution among four cell types. All calculations were performed using Excel (Microsoft Office 2013; Microsoft Corporation) or R software version 3.5.2 (30). $P < 0.05$ was considered to indicate a statistically significant difference. All of the assays were repeated at least two times.

Results

Macrophages affect acquirement of PC-9 cell gefitinib-resistance. Macrophages and PC-9 cells were co-cultured in a Transwell system using two approaches: Co-culture starting before (Mr) or after gefitinib treatment (Mm) to induce PC-9 cell resistance to gefitinib. The macrophages differentiated from THP1 cells and were not polarized specifically. PC-9 cells proliferation was inhibited in the co-culture system, compared with those without co-culture. The inhibition was stronger with increasing macrophage percentage (Fig. 1 and Table SII).

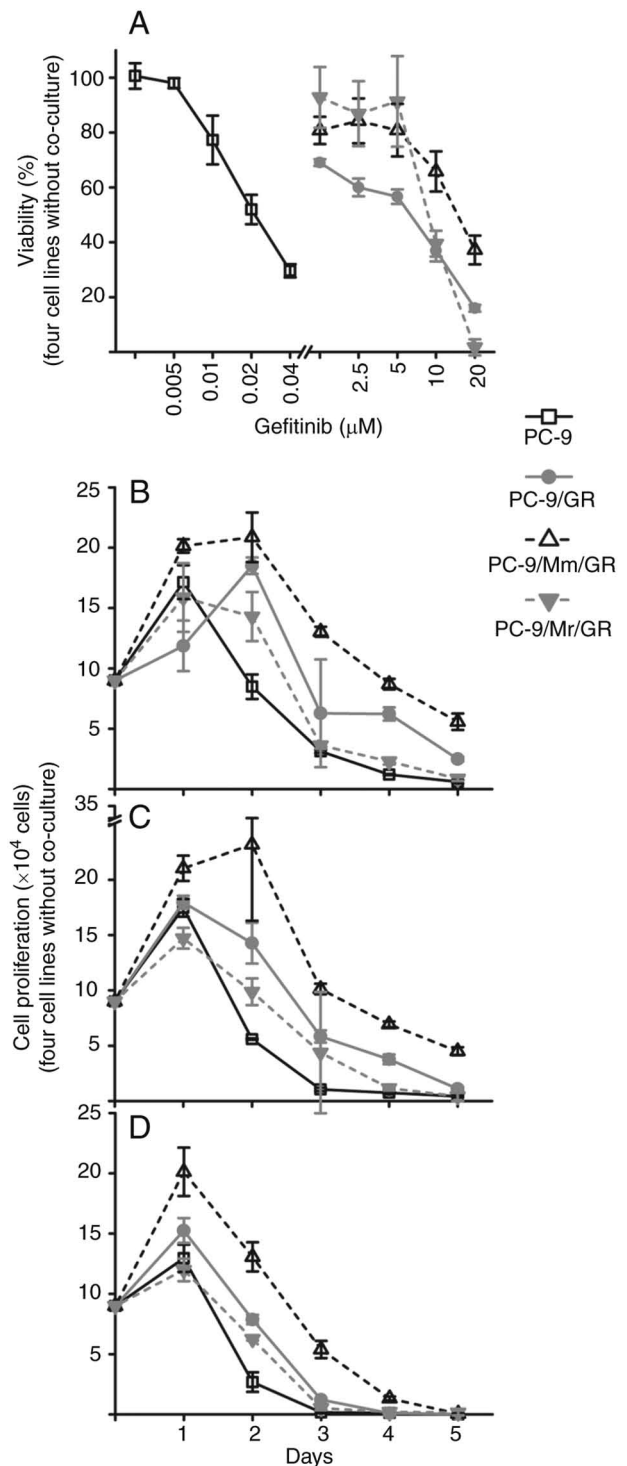


Figure 2. Gefitinib-resistant sublines and parental PC-9 cells inhibited by gefitinib. (A) Inhibition curves of PC-9/Mr/GR, PC-9/Mm/GR, PC-9/GR and PC-9 at different concentrations of gefitinib. (B-D) Growth curves when treated with gefitinib at (B) 5, (C) 10 and (D) 20 μ M. Mr, resident macrophage; Mm, migrated macrophage; PC-9/Mr/GR, gefitinib-resistant sublines induced with PC-9 cells in Mr co-culture; PC-9/Mm/GR, gefitinib-resistant sublines induced with PC-9 cells in Mm co-culture; PC-9/GR, gefitinib-resistant sublines induced with PC-9 cells without macrophage co-culture.

When the ratio of macrophages to PC-9 cells was 1:8, PC-9 cell proliferation in the co-culture system was not markedly different compared with the control PC-9 cells. Therefore, the co-culture system was built at a ratio of 1:8 (M:PC-9).

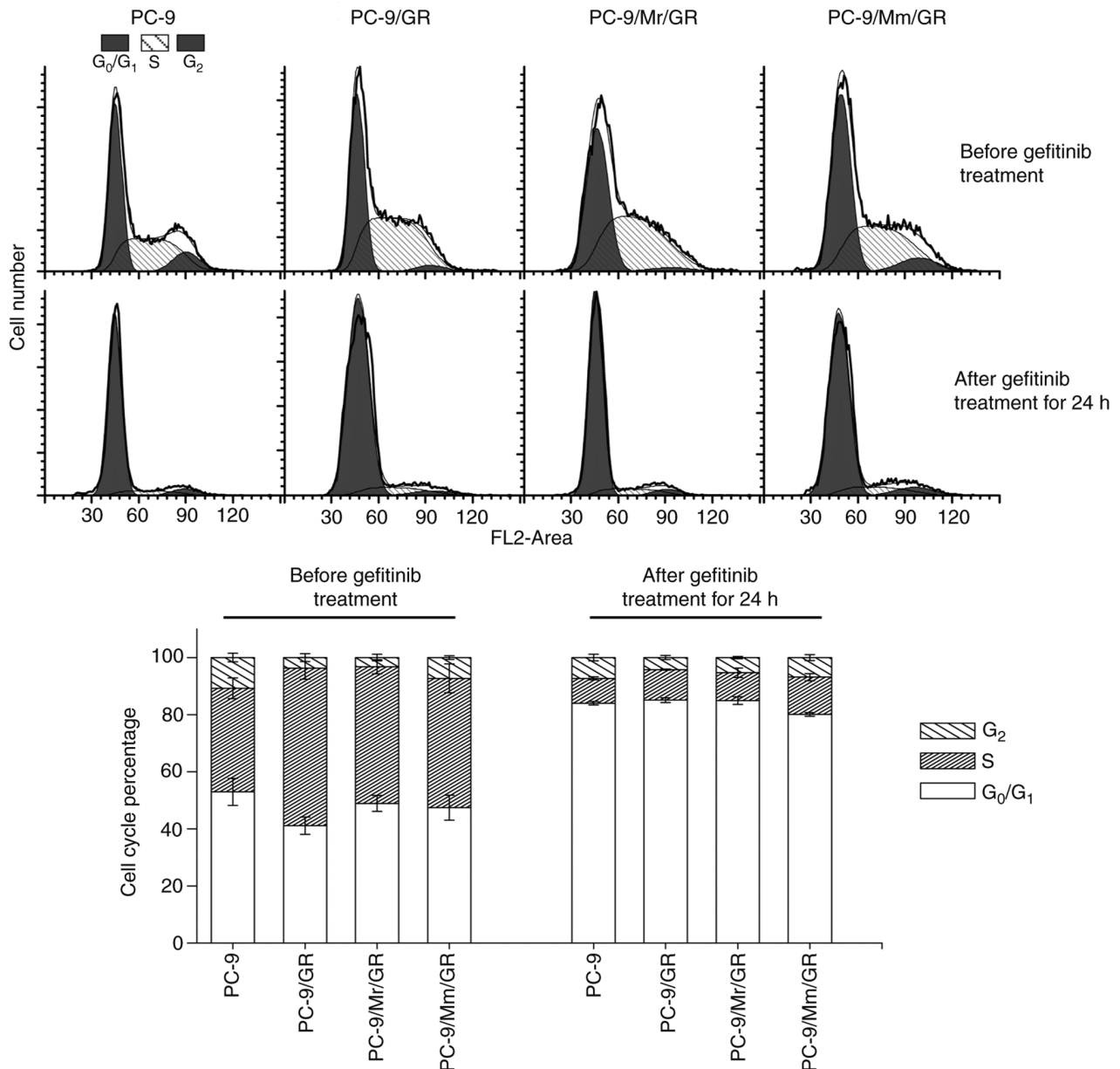


Figure 3. Cell cycle distribution before and after gefitinib treatment in PC-9 and three resistant sublines. PC-9/Mr/GR and PC-9/GR had significantly higher percentage of S phase compared with PC-9 cells before gefitinib treatment ($P=0.024$ and $P=0.002$, respectively), but cell cycle distribution was similar between PC-9/Mm/GR and PC-9 cells. After gefitinib treatment, PC-9/Mm/GR remained a higher percentage of S and G₂ phase than others. Mr, resident macrophage; Mm, migrated macrophage; PC-9/Mr/GR, gefitinib-resistant sublines induced with PC-9 cells in Mr co-culture; PC-9/Mm/GR, gefitinib-resistant sublines induced with PC-9 cells in Mm co-culture; PC-9/GR, gefitinib-resistant sublines induced with PC-9 cells without macrophage co-culture.

Following gefitinib treatment in each step, PC-9 cells in Mr and Mm co-culture as well as without co-culture recovered at different speeds. It took about 9-13 days for PC-9 cells to recover and reach confluency in Mm co-culture, about 13-17 days in Mr co-culture, and about 10-14 days without co-culture after each step treatment from 5 to 30 μM gefitinib (data not shown). When treated with 35 μM gefitinib for 24 h, PC-9/Mm/GR cells took 8.9 ± 2.0 days to reach confluency again in drug-free medium, which was significantly shorter compared with the time needed by PC-9/Mr/GR cells (14.7 ± 2.7 days) and PC-9/GR cells (11.0 ± 1.7 days). By contrast, parental PC-9 cells could not recover even after 60 days (data not shown). After 10 months, the PC-9/Mr/GR, PC-9/Mm/GR and PC-9/GR sublines all acquired resistance

to gefitinib as shown in Fig. 2A and Table SIII. Half maximal inhibition concentration (IC_{50}) of PC-9 was $\sim 0.02 \mu\text{M}$. PC-9/Mm/GR had the largest IC_{50} at $\sim 20 \mu\text{M}$. IC_{50} of PC-9/GR was slightly decreased compared with that of PC-9/Mr/GR, and both of them located between 5 and 10 μM . At low concentration of gefitinib ($< 10 \mu\text{M}$), PC-9/Mr/GR had a higher viability compared with PC-9/GR (Fig. 2A).

PC-9/Mm/GR had the strongest resistance ability. PC-9/Mr/GR displayed stronger resistant ability compared with PC-9/GR at low concentration of gefitinib ($< 10 \mu\text{M}$). The proliferation curves of the three resistant sublines and the parental PC-9 cells in the presence of 5, 10 and 20 μM gefitinib are presented in Fig. 2B-D and Table SIV. PC-9 cells had proliferative ability only on day 1 in three gefitinib

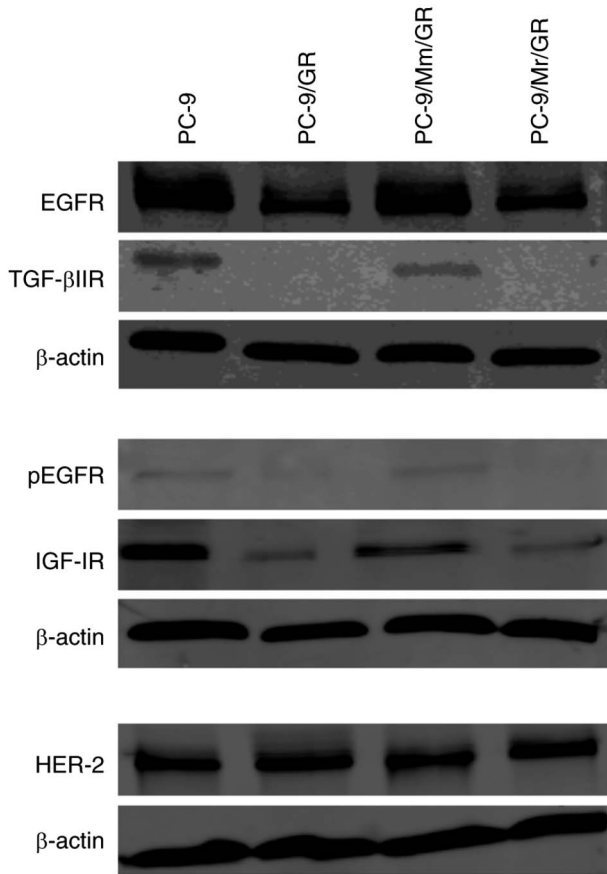


Figure 4. Growth receptor expression levels in PC-9 and three resistant sublines with western blotting. Blots in each black square were exposed at the same time with β -actin as a reference. Bands in each lane of each panel were from the same loading sample. Mr, resident macrophage; Mm, migrated macrophage; PC-9/Mr/GR, gefitinib-resistant sublines induced with PC-9 cells in Mr co-culture; PC-9/Mm/GR, gefitinib-resistant sublines induced with PC-9 cells in Mm co-culture; PC-9/GR, gefitinib-resistant sublines induced with PC-9 cells without macrophage co-culture; p-EGFR, phosphorylated-EGFR; TGF- β IIIR, TGF- β II receptor; IGF-1R, insulin-like growth factor 1 receptor.

concentration environments, which then began to be inhibited. By contrast, the three resistant sublines could keep proliferating on Day 2 in 5 μ M gefitinib environment, while PC-9/Mm/GR proliferated on Day 2 even in 10 μ M gefitinib environment. PC-9/Mm/GR maintained the largest cell numbers from Day 1 in three gefitinib environments. The three gefitinib-resistant sublines had a similar proliferation trend to that of parental PC-9 cells in a gefitinib environment. PC-9/Mm/GR showed the strongest proliferation ability from the beginning, and PC-9/GR demonstrated increased proliferation compared with PC-9/Mr/GR.

Features of gefitinib-resistant sublines are induced in three ways. Gefitinib-resistant sublines induced in three ways displayed different features. PC-9/Mm/GR had similar a cell cycle distribution to that of parental PC-9 cells (the G_2 , S and G_0/G_1 phase percentage was $10.7 \pm 1.5\%$ vs. $7.3 \pm 0.6\%$, $P=0.075$; $36.3 \pm 3.6\%$ vs. $45.3 \pm 5.0\%$, $P=0.112$ and $52.9 \pm 4.7\%$ vs. $47.5 \pm 4.4\%$, $P=0.433$, respectively in PC-9 vs. PC-9/Mm/GR cells), while PC-9/Mr/GR and PC-9/GR showed a significantly higher percentage of S

phase and lower percentage of G_2 phase (specifically, the G_2 , S and G_0/G_1 phase percentage was $3.2 \pm 1.1\%$, $P=0.001$; $47.9 \pm 2.5\%$, $P=0.024$ and $48.9 \pm 2.8\%$, $P=0.577$, respectively, in PC-9/Mr/GR cells, and $3.8 \pm 1.4\%$, $P=0.001$; $55.1 \pm 3.8\%$, $P=0.002$ and $41.1 \pm 3.0\%$, $P=0.024$, respectively, in PC-9/GR cells) compared with those of parental PC-9 cells (Fig. 3 and Tables SV). After treatment with 5 μ M gefitinib for 24 h, the majority of cells, PC-9 cells and three resistant sublines, tended to stay in G_0/G_1 phase and decreased markedly in S phase. The cell cycle distribution was comparable among parental PC-9 cells and the three resistant sublines. PC-9/Mm/GR displayed the strongest proliferation activity with the largest S and G_2 phase percentages ($19.9 \pm 0.6\%$ in PC-9/Mm/GR vs. $16.0 \pm 0.6\%$ in PC-9, $P=0.003$ and $14.9 \pm 0.8\%$ in PC-9/GR vs. PC-9, $P=0.487$ and $15.1 \pm 1.3\%$ in PC-9/Mr/GR vs. PC-9, $P=0.610$; Tables SVI).

After 10 months of induction, no new mutation on the EGFR exon 18-21 was revealed in any of the three resistant sublines, except for harboring a deletion mutation on exon 19 from parental PC-9 cells (Figs. S2 and S3). However, resistant sublines exhibited varied phenotypes. Compared with the findings in parental PC-9 cells, PC-9/GR and PC-9/Mr/GR lost expression of certain membrane receptors, including EGFR, p-EGFR, IGF-1R and TGF- β IIIR (Fig. 4). E-cadherin expression was also decreased, while vimentin expression did not markedly change (Figs. 5A-D, G, H and S4A-D, G and H). PC-9/Mm/GR maintained EGFR, p-EGFR, IGF-1R and TGF- β IIIR expression similarly to parental PC-9 cells (Fig. 4). In addition, PC-9/Mm/GR sustained both E-cadherin and vimentin expression (Figs. 5E and F and S4E and F).

The TGF- β content in the supernatant of PC-9 cells in Mr and Mm transient co-culture as well as without co-culture was measured for 2 days continuously after treatment with gefitinib (5 μ M) for 24 h. In the supernatant of PC-9 cells in Mr co-culture (PC-9/Mr), the TGF- β content remained close to the basic level (1,114.9 pg/ml). However, in the supernatant of PC-9 cells without co-culture (PC-9) and in Mm co-culture (PC-9/Mm), the TGF- β content increased and was higher compared with that of PC-9/Mr in days 1 ($P=0.0131$) and 2 ($P=0.007$) after discarding the drugs (Fig. 6A and Table SVII). The TGF- β level in the supernatant of the gefitinib-resistant sublines PC-9/Mr/GR, PC-9/Mm/GR and PC-9/GR was also detected. After treatment with gefitinib (35 μ M) for 24 h, the TGF- β level in the supernatant was similar among the three sublines at day 1 after discarding the drugs ($P=0.381$). However, on day 2, the TGF- β level decreased significantly in PC-9/Mr/GR compared with the other two sublines ($P=0.027$; Fig. 6B and Table SVIII).

Macrophage polarization in co-culture systems. Following differentiation from THP-1 with PMA, ~25% of macrophages expressed HLA-DR while nearly none expressed CD206. As shown in Fig. 7, the percentage of macrophages with HLA-DR expression increased slightly when treated with gefitinib for 24 h. When co-cultured with PC-9 cells, the percentage of macrophages with HLA-DR expression decreased to 4.9 and 1.6% at a ratio of macrophages to PC-9 cells of 1:8 and 1:1, respectively, whereas no CD206-expressing macrophages appeared. In either co-culture way and in either co-culture cell

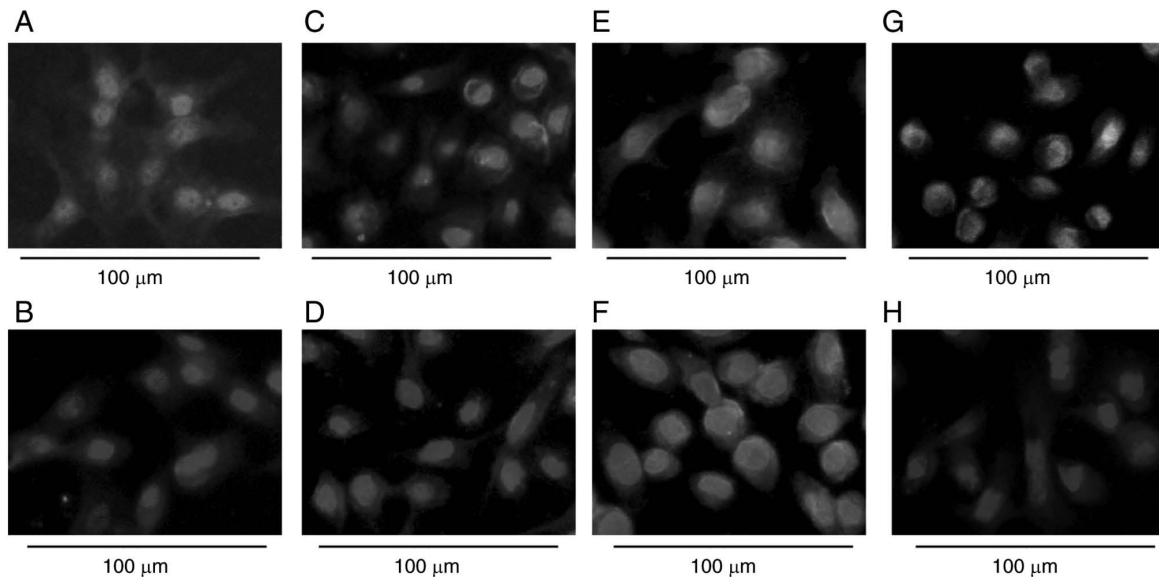


Figure 5. E-cadherin and vimentin expression with immunofluorescence shown in gray scale. E-cadherin and vimentin expression in (A and B) PC-9, (C and D) PC-9/GR, (E and F) PC-9/Mm/GR and (G and H) PC-9/Mr/GR (scale bar, 100 μ m). PC-9/GR, gefitinib-resistant sublines induced with PC-9 cells without macrophage co-culture; PC-9/Mr/GR, gefitinib-resistant sublines induced with PC-9 cells in Mr co-culture; PC-9/Mm/GR, gefitinib-resistant sublines induced with PC-9 cells in Mm co-culture.

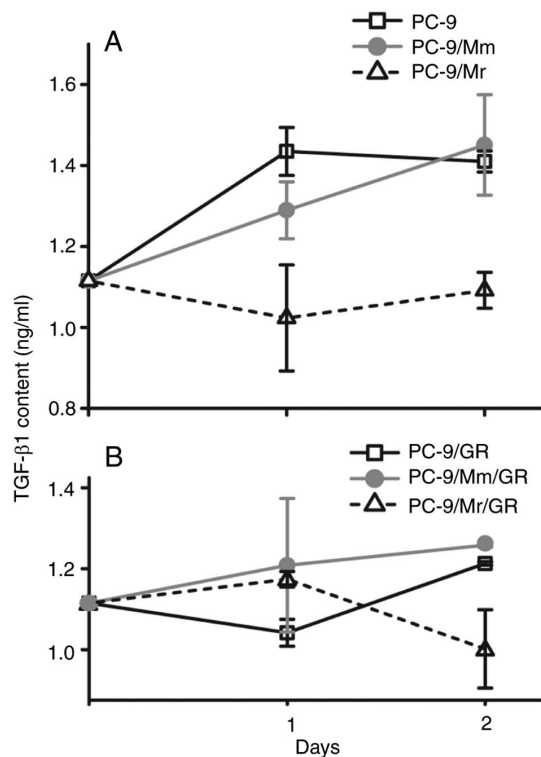


Figure 6. TGF- β levels in the supernatant of PC-9 and three resistant sublines. (A) After PC-9 cells alone and transient co-culture in Mr way (PC-9/Mr) or Mm way (PC-9/Mm) were treated with gefitinib (5 μ M) for 24 h, TGF- β levels in the supernatant of PC-9/Mr were significantly lower compared that in others at the first and second day after discarding drugs ($P=0.0131$ and $P=0.007$, respectively). (B) After resistant sublines of PC-9/GR, PC-9/Mr/GR and PC-9/Mm/GR were treated with gefitinib (35 μ M), TGF- β level was not significantly different among three groups ($P=0.381$) at the first day, while it was significantly lower in PC-9/Mr/GR compared with that of others at the second day ($P=0.027$). Mr, resident macrophage; Mm, migrated macrophage; PC-9/Mr, PC-9 cells co-cultured with Mr; PC-9/Mm, PC-9 cells co-cultured with Mm; PC-9/Mr/GR, gefitinib-resistant sublines induced with PC-9 cells in Mr co-culture; PC-9/Mm/GR, gefitinib-resistant sublines induced with PC-9 cells in Mm co-culture.

ratio, there were not macrophages polarized to M1 (HLA-DR positive) or M2 (CD206-positive).

Discussion

Tumor cell activities change the microenvironment, and the tumor microenvironment also shapes tumor cell characteristics. The present study treated PC-9 cells with gefitinib in three different microenvironments. It was revealed that PC-9 cells always acquired resistance to gefitinib, either without macrophages co-culture or with macrophages co-culture in two ways. Similar to patients with lung cancer in gefitinib treatment who present individual differences, in the present study, PC-9 cells indicated different features after being exposed to gefitinib for a period of time with the same dose and frequency in different environments. PC-9/GR and PC-9/Mr/GR were similar. Both decreased EGFR activation, which is the binding target of gefitinib, and lost the expression of other proliferated-related membrane receptors, such as IGF-1R and TGF- β IIR. It was not strange that PC-9/GR and PC-9/Mr/GR cells were not sensitive to gefitinib when the binding target was missing. EGFR, IGF-1R and TGF- β IIR triggered by corresponding ligands will activate downstream signaling pathways, including the Ras-MAPK and PI3K signaling pathways, which in turn promotes cell proliferation and cell survival (31,32). Due to reduced stimulation of proliferation signaling, the aforementioned two sublines mostly accumulated in phases prior to G_2 in the cell cycle. This 'low proliferation status' was the result of receptors closing, which could help the cells to avoid from further gefitinib treatment.

Notably, all the three resistant sublines showed different resistance ability, but none of them showed the most common EGFR exon 20 mutation (T790M). In our previous study, PC-9 cells were induced to exhibit resistance to gefitinib with another administration regimen, the alteration of high-dose stimulation and low-dose maintenance, which led to the appearance of the T790M mutation in the resistant subline

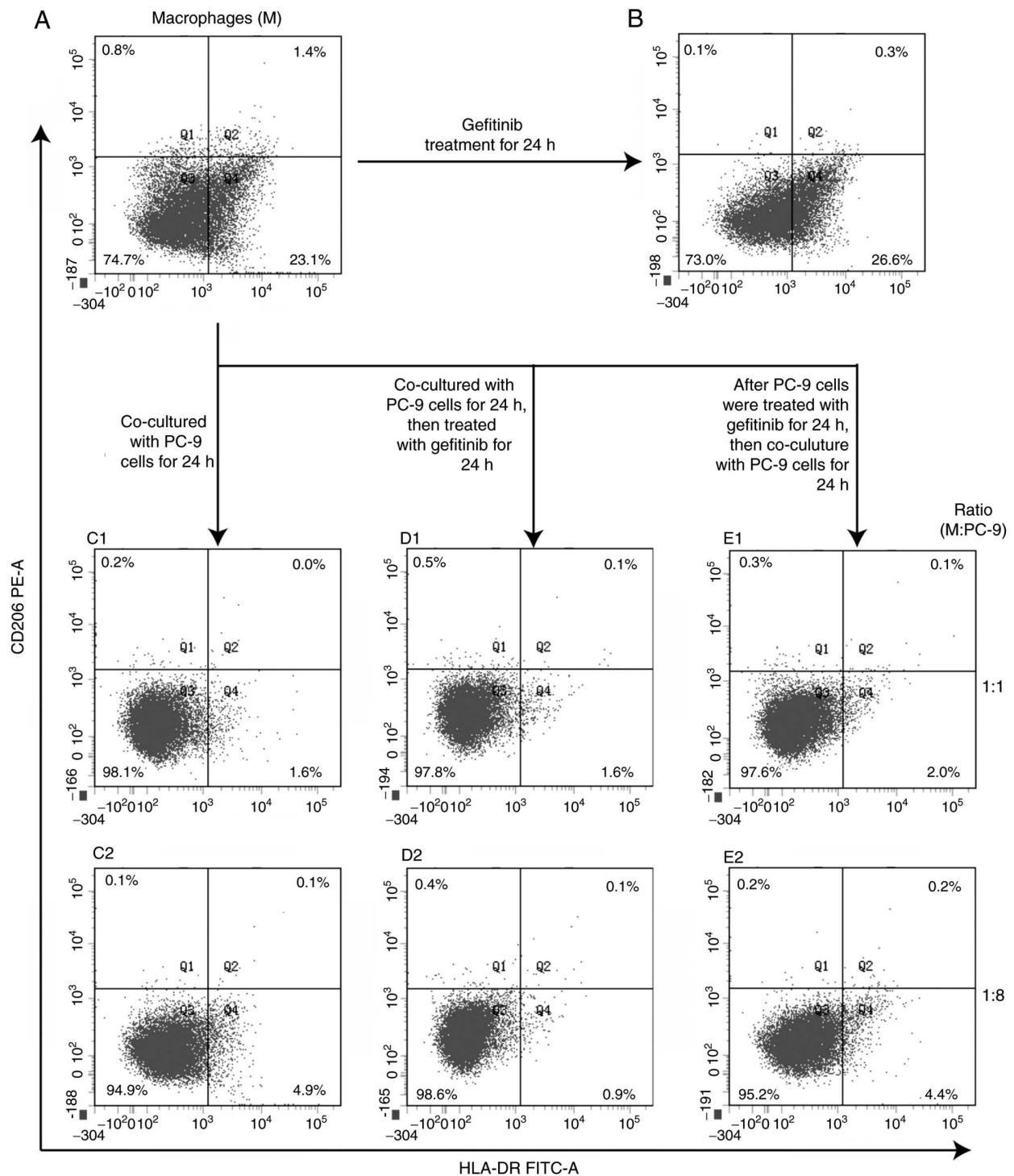


Figure 7. Macrophage polarization after gefitinib treatment and co-culture with PC-9 cells. HLA-DR (M1-like macrophage marker) and CD206 (M2-like macrophage marker) positive macrophages were detected with flow cytometry. (A) After THP-1 was differentiated with PMA (320 nM) for 48 h, there were ~1/4 HLA-DR-positive macrophages, while very few CD206 positive cells. (B) Macrophages differentiated from THP-1 were treated with gefitinib for 24 h, the percentage of HLA-DR positive macrophages increased slightly. (C1 and C2) When co-cultured with PC-9 cells in different ratio, the percentage decreased. (D1 and D2) After macrophages co-cultured with PC-9 for 24 h, then macrophages and PC-9 were treated with gefitinib together (Mr co-culture way), HLA-DR-positive percentage remained low at either co-culture ratio. (E1 and E2) If PC-9 cells were treated with gefitinib first, then co-cultured with macrophages (Mm co-culture way), HLA-DR-positive macrophages remained low. There were few CD206-positive macrophages during the whole process. HLA, human leukocyte antigen; PMA, phorbol-12-myristate-13-acetate; Mr, resident macrophage; Mm, migrated macrophage.

(Zhao *et al*, unpublished data). While the mechanism is unclear, changes in the environment modulate tumor cells to develop secondary resistance, which has been shown in the clinic where the resistance mechanism is individually characterized, even among patients with lung cancer who harbored

the same sensitive mutation with the same administration regimen (2,4,33).

Macrophages renewing locally and migrating from the blood vessels change the microenvironment continuously (6). The present study explored how macrophage

renewal affected PC-9 cells to develop secondary resistance to gefitinib. Macrophages that renew locally always share the same environment with tumor cells (6,19); thus, co-culture was performed, which started before PC-9 treatment with gefitinib (Mr) to let macrophages always accompany PC-9 cells. Cell damage is the common signal to attract macrophages, either monocyte-derived or resident macrophages, to carry out clearance activity and reparative response (6,17,20,21). Thus, the co-culture starting after PC-9 cell treatment (Mm) simulated this renewal mode. Notably, in Mm co-culture, macrophages markedly assisted PC-9 cell recovery from gefitinib treatment and developed resistance rapidly.

However, if macrophages appeared before gefitinib treatment, they hardly protected PC-9 cells from gefitinib treatment. It seemed that resident (Mr) macrophages could not recognize PC-9 cells exposure to gefitinib as an abnormal event as Mm macrophages did. Unlike cytotoxic killing agents, gefitinib inhibits PC-9 cells proliferation, while not killing directly (34). Both PC-9 cells and gefitinib-resistant sublines mainly showed elongation of G₀/G₁ phase after gefitinib treatment in the first 24 h. The gradual proliferation inhibition resembled slow physiological proliferation, and could mislead macrophages (Mr) in the same microenvironment with PC-9 cells from turning on protection. By contrast, if macrophages (Mm) could recognize it as a damage signal they may help PC-9 cells to survive. It is not clear how Mm macrophages protected PC-9 cells from gefitinib. TGF- β secretion was increased in the Mm group, while it was inhibited in the Mr group. At the same time, PC-9/Mm/GR maintained TGF- β IIR expression, while PC-9/Mr/GR did not. This indicated that TGF- β /TGFR signal transduction could be activated in PC-9/Mm/GR. Normally, TGF- β contributes to tumor initiation, progression, metastasis and chemotherapy resistance by regulating inflammatory factors, fibrotic process and epithelial mesenchymal transition, besides promoting proliferation (35-37). The present study revealed a relatively strong vimentin expression in PC-9/Mm/GR cells compared with that in other sublines, which suggested that the cells exhibited certain mesenchymal character.

In the future, it would be helpful to detect inflammatory factors and fibrosis *in vivo* or co-culture fibroblasts and lymphocytes *in vitro*. In the present study, Mm macrophages not only assisted PC-9 cells to keep a stable phenotype compared with that of their parental cells, but they displayed a relative strong vimentin expression, which could be regulated by TGF- β signaling and may be the potential reason for the PC-9/Mm/GR acquisition of the fastest and strongest resistance ability observed. Decreasing TGF- β expression or inhibiting its binding with receptor to inhibit TGF- β signal transduction are needed to confirm the function of TGF- β in the process, which is a limitation of the current study. Renewal modes of macrophages may lead to different distribution in time and space. Migrated macrophages, while not resident ones, may play a protective function, which could affect the development of resistance. If macrophage infiltration prior to gefitinib treatment and migrated macrophages to the TME were monitored, development of secondary resistance could be predicted accurately.

Previous clinical studies aimed to investigate the association between the efficacy of EGFR-TKIs treatment

and the density of macrophages, but the results are controversial (13,14,22,23,38). The discrepancy in results is not unexpected, since the majority of samples are obtained by surgical excision, while operations are usually performed prior to EGFR-TKIs treatment. The macrophages in the above tissues are likely to be associated with tumorigenesis and malignant potential, rather than treatment response (24,25). High levels of certain inflammatory cytokines and chemokines are found to be associated with EGFR-TKIs resistance, but the clinical data on concentration changes before and after treatment are not enough yet (39,40). In numerous studies, macrophages are categorized into M1 or M2 polarization (13-17). The present study did not induce macrophage polarization. Upon treatment of PC-9 cells with gefitinib in Mr or Mm co-culture, macrophages did not polarize. This suggested that macrophages may be activated to protect tumor cells prior to polarization. Considering that migrated and resident macrophages play their own roles in individual inflammatory status and are associated with smoking, infection, carcinogenesis and toxic reaction after treatment (6,18,21,22,25,41), the present study attempted to exclude other interference. However, the role of the renewal type *in vivo* was not evaluated, which is a limitation of the present study.

From traditional chemotherapy to target therapy, cancer treatment strategies always aim to fight tumor cells directly. Although the latter causes less injury to the host body, resistance always develops after the interaction of tumor cells and the TME (2-4,42). Immunotherapy has been largely developed, but the tactic remains to avoid or limit harmful effects in the host body. Macrophages may be a prospective target to be 'trained tumor killing cells' by host immunoreaction (43,44). Despite several cases showing good responses, it is not clear if novel resistance-like process will develop or whether adverse events will occur (45,46). Since cell killing is followed by resistance and host injury, it should be considered changing the treatment strategy to quarantine tumor cells to mold them to be regulated by the host body or limit their harmful activity. Macrophages could be a prospective target to be 'trained' to regulate the TME.

In summary, macrophage renewal modes could interfere with the development of secondary resistance by altering their distribution in time and space. The present study may provide a different perspective to identify novel approaches to monitor EGFR-TKIs response and design new treatment strategies against lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The data generated in the present study may be found in the Figshare at the following URL: https://figshare.com/articles/online_resource/Sanger_sequencing_results_rar/21680843.

Authors' contributions

BZ and ML designed the outline of the study. BZ and YZ conducted the experiments and data analysis. ML wrote the manuscript. BZ, SL and ML supervised the study and contributed to data interpretation and manuscript revision. BZ and ML confirmed the authenticity of all raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patients consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

- Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 71: 209-249, 2021.
- Ohashi K, Maruvka YE, Michor F and Pao W: Epidermal growth factor receptor tyrosine kinase inhibitor-resistant disease. *J Clin Oncol* 31: 1070-1080, 2013.
- Konig D, Prince SS and Rothschild SI: Targeted therapy in advanced and metastatic non-small cell lung cancer. An update on treatment of the most important actionable oncogenic driver alterations. *Cancers (Basel)* 13: 804-840, 2021.
- Bivona TG and Doebele RC: A framework for understanding and targeting residual disease in oncogene-driven solid cancers. *Nat Med* 22: 472-478, 2016.
- Lin A, Wei T, Meng H, Luo P and Zhang J: Role of the dynamic tumor microenvironment in controversies regarding immune checkpoint inhibitors for the treatment of non-small cell lung cancer (NSCLC) with EGFR mutations. *Mol Cancer* 18: 139, 2019.
- Loyher PL, Hamon P, Laviro M, Meghraoui-Kheddar A, Goncalves E, Deng Z, Torstensson S, Bercovici N, de Chanville CB, Combadière B, *et al*: Macrophages of distinct origins contribute to tumor development in the lung. *J Exp Med* 215: 2536-2553, 2018.
- Bonde AK, Tischler V, Kumar S, Soltermann A and Schwendener RA: Intratumoral macrophages contribute to epithelial-mesenchymal transition in solid tumors. *BMC Cancer* 12: 35, 2012.
- Mantovani A, Marchesi F, Malesci A, Laghi L and Allavena P: Tumour-associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol* 14: 399-416, 2017.
- De Palma M and Lewis CE: Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell* 23: 277-286, 2013.
- Mantovani A and Allavena P: The interaction of anticancer therapies with tumor-associated macrophages. *J Exp Med* 212: 435-445, 2015.
- Jia Y, Li X, Jiang T, Zhao S, Zhao C, Zhang L, Liu X, Shi J, Qiao M, Luo J, *et al*: EGFR-targeted therapy alters the tumor microenvironment in EGFR-driven lung tumors: Implications for combination therapies. *Int J Cancer* 145: 1432-1444, 2019.
- Wang DH, Lee HS, Yoon D, Berry G, Wheeler TM, Sugarbaker DJ, Kheradmand F, Engleman E and Burt BM: Progression of EGFR-mutant lung adenocarcinoma is driven by alveolar macrophages. *Clin Cancer Res* 23: 778-788, 2017.
- Garrido-Martin EM, Mellows TWP, Clarke J, Ganesan AP, Wood O, Cazaly A, Seumois G, Chee SJ, Alzetani A, King EV, *et al*: M1(hot) tumor-associated macrophages boost tissue-resident memory T cells infiltration and survival in human lung cancer. *J Immunother Cancer* 8: e000778, 2020.
- Xiao F, Liu N, Ma X, Qin J, Liu Y and Wang X: M2 macrophages reduce the effect of gefitinib by activating AKT/mTOR in gefitinib-resistant cell lines HCC827/GR. *Thorac Cancer* 11: 3289-3298, 2020.
- Zhang B, Zhang Y, Zhao J, Wang Z, Wu T, Ou W, Wang J, Yang B, Zhao Y, Rao Z and Gao J: M2-polarized macrophages contribute to the decreased sensitivity of EGFR-TKIs treatment in patients with advanced lung adenocarcinoma. *Med Oncol* 31: 127, 2014.
- Chung FT, Lee KY, Wang CW, Heh CC, Chan YF, Chen HW, Kuo CH, Feng PH, Lin TY, Wang CH, *et al*: Tumor-associated macrophages correlate with response to epidermal growth factor receptor-tyrosine kinase inhibitors in advanced non-small cell lung cancer. *Int J Cancer* 131: E227-E235, 2012.
- Gentek R, Molawi K and Sieweke MH: Tissue macrophage identity and self-renewal. *Immunol Rev* 262: 56-73, 2014.
- Mascia F, Lam G, Keith C, Garber C, Steinberg SM, Kohn E and Yuspa SH: Genetic ablation of epidermal EGFR reveals the dynamic origin of adverse effects of anti-EGFR therapy. *Sci Transl Med* 5: 199ra110, 2013.
- Ginhoux F and Guillemins M: Tissue-resident macrophage ontogeny and homeostasis. *Immunity* 44: 439-449, 2016.
- Xu-Vanpala S, Dehake ME, Wheaton JD, Parker ME, Juvvadi PR, MacIver N, Ciofani M and Shinohara ML: Functional heterogeneity of alveolar macrophage population based on expression of CXCL2. *Sci Immunol* 5: eaba7350, 2020.
- Du J, Li G, Jiang L, Zhang X, Xu Z, Yan H, Zhou Z, He Q, Yang X and Luo P: Crosstalk between alveolar macrophages and alveolar epithelial cells/fibroblasts contributes to the pulmonary toxicity of gefitinib. *Toxicol Lett* 338: 1-9, 2021.
- Mukaida N, Nosaka T, Nakamoto Y and Baba T: Lung macrophages: Multifunctional regulator cells for metastatic cells. *Int J Mol Sci* 20: 116, 2018.
- Li Z, Maeda D, Yoshida M, Umakoshi M, Nanjo H, Shiraishi K, Saito M, Kohno T, Konno H, Saito H, *et al*: The intratumoral distribution influences the prognostic impact of CD68- and CD204-positive macrophages in non-small cell lung cancer. *Lung Cancer* 123: 127-135, 2018.
- Casanova-Acebes M, Dalla E, Leader AM, LeBerichel J, Nikolic J, Morales BM, Brown M, Chang C, Troncoso L, Chen ST, *et al*: Tissue-resident macrophages provide a pro-tumorigenic niche to early NSCLC cells. *Nature* 595: 578-584, 2021.
- Cotechini T, Atallah A and Grossman A: Tissue-resident and recruited macrophages in primary tumor and metastatic microenvironments: Potential targets in cancer therapy. *Cells* 10: 960, 2021.
- Strober W: Trypan blue exclusion test of cell viability. *Curr Protoc Immunol* 111: A3.B.1-A3.B.3, 2015.
- Zhao BX, Wang J, Song B, Wei H, Lv WP, Tian LM, Li M and Lv S: Establishment and biological characteristics of acquired gefitinib resistance in cell line NCI-H1975/gefitinib-resistant with epidermal growth factor receptor T790M mutation. *Mol Med Rep* 11: 2767-2774, 2015.
- Li M, Zhang Q, Liu L, Liu Z, Zhou L, Wang Z, Yue S, Xiong H, Feng L and Lu S: The different clinical significance of EGFR mutations in exon 19 and 21 in non-small cell lung cancer patients of China. *Neoplasia* 58: 74-81, 2011.
- Sun Xuerui WY, Jialin G and Li Mei: Difference between LCGreen PLUS and EvaGreen in detecting EGFR mutation with HRM. *J Dalian Med Univ* 42: 391-394, 2020.
- R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org/>.
- Hakuno F and Takahashi SI: IGF1 receptor signaling pathways. *J Mol Endocrinol* 61: T69-T86, 2018.

32. Ark AV, Cao J and Li X: TGF- β receptors: In and beyond TGF-beta signaling. *Cell Signal* 52: 112-120, 2018.
33. Rotow J and Bivona TG: Understanding and targeting resistance mechanisms in NSCLC. *Nat Rev Cancer* 17: 637-658, 2017.
34. Paez JG, Janne PA, Lee JC, Tracy S, Greulich H, Gabriel S, Herman P, Kaye FJ, Lindeman N, Boggon TJ, *et al*: EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science* 304: 1497-1500, 2004.
35. Batlle E and Massague J: Transforming growth factor- β signaling in immunity and cancer. *Immunity* 50: 924-940, 2019.
36. Caja L, Dituri F, Mancarella S, Caballero-Diaz D, Moustakas A, Giannelli G and Fabregat I: TGF- β and the tissue microenvironment: Relevance in fibrosis and cancer. *Int J Mol Sci* 19: 1294, 2018.
37. Yu X, Buttgereit A, Lelios I, Utz SG, Cansever D, Becher B and Greter M: The cytokine TGF-beta promotes the development and homeostasis of alveolar macrophages. *Immunity* 47: 903-912 e904, 2017.
38. Wang H, Wang X, Li X, Fan Y, Li G, Guo C, Zhu F, Zhang L and Shi Y: CD68(+)HLA-DR(+) M1-like macrophages promote motility of HCC cells via NF- κ B/FAK pathway. *Cancer Lett* 345: 91-99, 2014.
39. Yao Z, Fenoglio S, Gao DC, Camiolo M, Stiles B, Lindsted T, Schleiderer M, Johns C, Altorki N, Mittal V, *et al*: TGF-beta IL-6 axis mediates selective and adaptive mechanisms of resistance to molecular targeted therapy in lung cancer. *Proc Natl Acad Sci USA* 107: 15535-15540, 2010.
40. Fukuoka M, Yoshioka K and Hohjoh H: NF- κ B activation is an early event of changes in gene regulation for acquiring drug resistance in human adenocarcinoma PC-9 cells. *PLoS One* 13: e0201796, 2018.
41. Wan L, Wang Y, Tang Y, Tan Y, He F, Zhang Y, Yang K, Chen Z, Song C, Gu R, *et al*: Gefitinib-induced cutaneous toxicities in brown norway rats are associated with macrophage infiltration. *Inflammation* 43: 2137-2146, 2020.
42. Bukowski K, Kciuk M and Kontek R: Mechanisms of multidrug resistance in cancer chemotherapy. *Int J Mol Sci* 21: 3233, 2020.
43. Anderson NR, Minutolo NG, Gill S and Klichinsky M: Macrophage-based approaches for cancer immunotherapy. *Cancer Res* 81: 1201-1208, 2021.
44. Kennedy LB and Salama AKS: A review of cancer immunotherapy toxicity. *CA Cancer J Clin* 70: 86-104, 2020.
45. Im JS, Herrmann AC, Bernatchez C, Haymaker C, Molldrem JJ, Hong WK and Perez-Soler R: Immune-modulation by epidermal growth factor receptor inhibitors: Implication on anti-tumor immunity in lung cancer. *PLoS One* 11: e0160004, 2016.
46. Hussaini S, Chehade R, Boldt RG, Raphael J, Blanchette P, Vareki SM and Fernandes R: Association between immune-related side effects and efficacy and benefit of immune checkpoint inhibitors-A systematic review and meta-analysis. *Cancer Treat Rev* 92: 102134, 2021.