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ORIGINAL ARTICLE

Tobacco extracts promote PD-L1 expression and enhance malignant biological differences via mTOR in gefitinib-resistant cell lines

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Cytokines; gefitinib-resistant; mTOR; PD-L1; tobacco extracts.

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Abstract

Background: The aim of this study was to investigate whether tobacco extracts could regulate PD-L1 expression and enhance malignant biological differences in gefitinib-resistant cell lines.

Methods: We constructed gefitinib-resistant cells and observed the biological differences in gefitinib-resistant cells. The cells were stimulated with medium containing 5% volume of tobacco extract, and the change in PD-L1 expression and the mammalian target of rapamycin (mTOR) and p-mTOR expression in gefitinib-resistant cells treated with tobacco extracts was observed. We discussed the relationship between PD-L1 and mTOR.

Results: Tobacco extracts could promote PD-L1 expression in the cell line. Western blot analysis showed that mTOR and p-mTOR were significantly enhanced in gefitinib-resistant cell lines cultured in the tobacco extracts. The mTOR signaling pathway was involved in PD-L1 expression and in regulating the expression of cytokines IL-6 and IL-23. In addition, the tobacco extracts could promote macrophage migration via mTOR/IL-6.

Conclusions: PD-L1 can transmit inhibitory signals and reduce the proliferation of CD8 + T cells in lymph nodes. Tobacco extracts upregulate PD-L1 expression via mTOR/IL-6. These results imply that lung cancer patients should not smoke and stay away from a smoke environment.

Introduction

Lung cancer is currently one of the most common malignant tumors and is the leading cause of cancer-related death.[1] Lung cancer is classified according to pathological features, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), of which NSCLC accounts for about 85%.[2] At present, the traditional treatments for lung cancer mainly include surgery, radiotherapy, chemotherapy, and target therapy.[3] Although these treatments can prolong the survival time of patients, the five-year overall survival rate of NSCLC patients is still about 16%.[4] These traditional treatment methods also cause serious toxic and side effects to the patients while killing the tumor cells, which seriously affects the quality of life of the patients. In recent years, with the further understanding of the body's own immune status and tumor immune microenvironment, tumor immunotherapy has become a treatment with providing prospects, and has shown good therapeutic effects in a variety of solid tumors.[5–7]

The rate of EGFR status has been found to vary by race in NSCLC; Caucasians is 5%–15%, and East Asians is 40%–55%.[8–10] The rate of PD-L1 expression is also different in NSCLC. It has been reported by Yang *et al.* that the positive rate of PD-L1 expression was 39.9%, which was higher in well-differentiated tumors according to data from Taiwan, China.[11] Cha *et al.* reported that the PD-L1 expression was 18.6% and higher expression of PD-L1 (≥50%) was more common in former or current smoker patients with NSCLC.[12] The co-occurrence of mutations in the *EGFR* gene and expression of PD-L1 remains controversial in lung cancer patients. The expression of the *EGFR* mutant cell line PD-L1 has been reported to be significantly higher than that of the *EGFR* wild-type cell line by

flow cytometric analysis,[13,14]RT-PCR and western blot analysis.[15] Similar results have been confirmed in advanced NSCLC patients.[16,17] However, it has been reported that the expression rate of PD-L1 in patients with *EGFR* mutation was significantly lower than that in patients with *EGFR* wild-type mutation.[18–20] When PD-L1 on tumor cell membrane binds to its receptor PD-1 on immune cells, such as T cells, tumor cells emit inhibitory signals, and T cells are then unable to recognize tumor cells and kill them, thus inhibiting the immune function of the body.[21–23] Immune checkpoint inhibitor treatment achieves an antitumor effect by releasing the immune inhibition and reactivating the immune response of T cells to the tumor.

The process of immunotherapy for lung cancer is facing many difficulties because of the low response rate for the special groups of patients with *EGFR* mutations. The simple application of PD-1/PD-L1 inhibitors has little benefit in these patients. Long-term application of EGFR-TKIs or EGFR-TKI-resistant patients results in changes in the tumor microenvironment. Some changes suggest that those patients may benefit from immunotherapy. Changes in the tumor immune microenvironment, such as FOXP3 + TIL density after EGFR-TKI treatment have been reported to be significantly lower than before therapy and TMB tends to be higher than before[24]; PD-L1 expression increased,[25] and EGFR-TKI gefitinib was able to prevent immune escape by upregulating the expression of NKG2D ligand on tumor cells and NKG2D on NK cells.[26] These studies suggest that patients with EGFR-TKI resistance or those having received long-term application of EGFR-TKIs may benefit from immunotherapy.

On the one hand, the effect of PD-1/PD-L1 inhibitor has a certain correlation with the patient's own PD-L1 expression status. Some patients already have immune suppression or immune dysfunction, which may be related to the PD-L1 expression of tumor cells. Combining PD-1 with PD-L1 tumor cells inhibits T lymphocyte anti-tumor effects. On the other hand, the effect of PD-1/PD-L1 inhibitor might be related to smoking history status. Subgroup analysis of clinical trials with anti-PD-1 mAbs (nivolumab or pembrolizumab) in NSCLC showed that the ever-smokers had better survival outcomes than that of the never-smokers.[6,27]In more than second-line setting, ICIs significantly prolonged OS compared with the chemotherapy in ever smokers with advanced NSCLC.[28] A meta-analysis of patients with advanced NSCLC showed that in the immunotherapy group, the OS benefit was similar between patients with smoking history and those without smoking history (HR = 0.69, 0.79, $P > 0.05$).[29] We speculate that smoking may change the expression of PD-L1 in tumor cells, and then participate in the regulation of tumor immunotherapy response. On the other hand, the substances in cigarettes can regulate the release of cytokines,[30] reshape the tumor immune microenvironment, adjust the lymphocyte components in the tumor microenvironment, and reconstruct the specific tumor immune microenvironment.[31,32] The change of reshaped tumor microenvironment might affect the therapeutic effect of antitumor in immunotherapy, but the detailed mechanism is currently unclear.

Smoking causes damage in bronchial mucosal endothelial cells and increases the risk of lung and cardiovascular disease. In addition, smoking can activate AKT protein, promote cell proliferation, and regulate biological behavior such as apoptosis.[33,34] The mammalian target of rapamycin (mTOR) is a downstream target gene of AKT, suggesting that smoking may regulate the mTOR activity of cells, which in turn affects the biological behavior of cells. mTOR activity has a certain regulatory effect on immune cells such as macrophages, dendritic cells[35] and T cells.[36,37] In addition, mTOR activity also has a certain regulatory effect on the release of cytokines in tumor and immune cells.[36]

At present, there are few studies on the role of smoking in immunotherapy with EGFR-TKI resistance. It is unknown that whether smoking can regulate PD-L1 and cytokines in EGFR-TKI-resistant cell lines. Therefore, the purpose of this study was to investigate whether tobacco extracts could regulate PD-L1 expression in gefitinib-resistant cell lines, and whether cigarette extracts regulate the release of cytokines.

Methods

Cell lines, reagents and antibodies

The NSCLC cell lines PC9 (19del [E746-A750]) (Keygenbio, Nanjing, China),HCC827 (19 del [E746-A750]) (Keygenbio, Nanjing, China) carrying *EGFR*-mutation were purchased and cultured in medium. The monocyte macrophage cell line THP1 (Keygenbio, Nanjing, China) was cultured in 1640 medium. The medium was supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) to maintain the requirements of the cell growth. The 37°C with 5% CO_2 and humidified atmosphere were used to incubate the cell lines. Gefitinib, an EGFR tyrosine kinase inhibitor, was purchased from Selleckchem (Munich, Germany), and mixed in the medium with increasing concentrations to generate and maintain the resistant cell lines for about six months. The Gefitinib resistant cell lines PC9/GR and HCC827/GR were cultured in the same cell incubation device with PC9. Rapamycin, an mTOR signal inhibitor, was purchased from Selleckchem (Houston, TX, USA).

The following primary antibodies were used: anti-GAPDH (Abcam, ab181602), anti-PD-L1 (Abcam,

ab205921), mTOR (CST, mAb #2983), Phospho-mTOR (Ser2448) (CST, mAb #5536), AKT (Abcam,ab32505), p-AKT (Abcam,ab192623).

Clonal formation

Cells were harvested using 0.05% trypsin and inoculated into six-well plates at 1000 cells per well. This was placed and cultured in an incubator at 37°C for 12–14 days, and terminated in the presence of macroscopic clones. The medium was removed and the cells were fixed in 4% paraformaldehyde for 15 minutes, stained with crystal violet stain for five minutes, washed and dried in 1 × PBS, a photographic record was taken and the data were analyzed.

Growth curve

The cells were digested with trypsin, the cell density was adjusted to $10 \times 10^{\sim 4}$, and they were then placed in a six-well plate. Three replicated wells were set up. The cells were harvested every 24 hours, and cell viability was determined by counting the number of cells.

Preparation of smoke extract medium

Cigarette smoke extract was prepared according to the method reported by Su *et al.* We used 15 mL of serum-free 1640 medium to absorb the smoke extract by passing it through a special device. Two cigarettes were ignited in sequence, and the cigarette was continuously inhaled with a vacuum pump to allow the smoke to pass through the medium. Each cigarette was ignited for about two minutes. The smoke extract medium was sterilized using a 0.22 μm filter, and stored at 4°C. This stock solution was considered to be 100%. The final concentration of CSE during use was calculated according to the following formula: final concentration (V/V) = smoke extract medium stock solution (mL)/final volume (mL) × 100%, final volume = smoke extract medium stock volume + 10% FBS 1640 medium.

Western blot

The cells were processed with RIPA buffer (Beyotime Institute of Biotechnology, Nanjing, China) and protein protease inhibitor (Beyotime Institute of Biotechnology, Nanjing, China) to obtain protein extraction. BCA protein assay kit (Biorad) was used to determine concentration. The different proteins were separated by SDS-PAGE (Beyotime Institute of Biotechnology, Nanjing, China) and the protein was transferred to PVDF membranes. The membranes carrying proteins were blocked with 5% milk medium for 1 hour, and then incubated with specific primary antibodies overnight, and followed with HRP-labeled secondary antibodies for 2 hours. The protein bands were detected using chemiluminescence.

PCR

Total RNA was isolated using Trizol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The PrimeScript RT kit (GeneCopoeia, Inc., USA) was used to synthesize complementary DNA from 1 ug of total RNA. We performed relative quantification of transcripts and calculated data using the ΔΔCt method based on SYBR Green Master Mix (GeneCopoeia, Inc., USA) and LightCycler 480 device (Roche Diagnostics). Expression of other genes in the transcript was normalized using GAPDH expression levels. No nonspecific products were observed. The primer sequences are as follows:

CCL20 F:GAGTTTGCTCCTGGCTGCTT, CCL20 RC CGTGTGAAGCCCACAATAA.

CXCL1F:ATCGAAAAGATGCTGAACAGTGAC CXCL 1R:TTCAGGAACAGCCACCAGTGA.

IL-6 F CCTGAACCTTCCAAAGATGGC IL-6R TTCA CCAGGCAAGTCTCCTCA.

IL-23 F TCAGGCTCAAAGCAAGTGGA IL-23 R AGC AGCAACAGCAGCATTAC.

PD-L1 F TCTGGACAAGCAGTGACCATC, PD-L1 R CAGTGTGCTGGTCACATTGAA.

ELISA

The concentrations of IL-6 were detected using a commercial human IL-6 ELISA kit (Multisciences (Lianke) Biotech Co. Ltd, Hangzhou, China), in accordance with the manufacturer's instructions.

Transwell assay

Cells were digested and collected, and resuspended in serum-free medium and counted. Then, 500 μL of complete culture medium was added to the lower chamber of the transwell chamber, and the chamber placed into a 24-well culture plate; 200 μL of serum-free medium containing 10 000 cells was added to the upper chamber of the transwell chamber (three replicates per group) and it was placed in a sterile constant temperature incubator at 37°C and 5% CO_2 volume fraction for 48 hours. The chamber was then removed, and the cells that had not passed through the chamber membrane were wiped with a cotton swab. The membrane was subsequently rinsed three times with PBS, and the cells were fixed with 4% paraformaldehyde solution for 15 minutes, The membrane was rinsed three times with PBS and dried; then, 0.5% crystal violet solution stain was used for five minutes, PBS was used to rinse the cells three times and they were then dried. The

cells were observed under an optical phase contrast microscope, with three fields of view selected and the number of cells passing through the membrane of the chamber counted.

Statistical analysis

Data were analyzed using GraphPad Prism V5.01 (GraphPad Software, Inc., California, USA) and represented as mean ± Standard Error of Mean (SEM). Student's *t*-test was used to compare the two groups. $P < 0.05$ was considered statistically significant.

Results

Construction of gefitinib-resistant cells and biological differences in gefitinib-resistant cells

The *EGFR*-mutant cell line PC-9 was used to construct a gefitinib-resistant cell line using a low continuous concentration induction method. The PC-9 parental cell line was continuously induced with a medium containing 0.1 μM gefitinib, and the concentration of gefitinib was increased every two days. The cells were divided under 1:3 every two days. The drug-resistant cell line grew freely in the medium of 12.5 μM gefitinib, and the IC50 of the gefitinib-resistant cells and the parental cells were detected. The IC50 of the gefitinib-resistant cells was found to be 4–5 times higher than that of the parental cells (Fig 1a), and gefitinib resistance was confirmed after six months. After the successful construction of the drug-resistant cells, the morphological changes in the drug-resistant and parental cells were compared. The cells of the drug-resistant strains increased in size, some were cord-shaped and grew in a diffuse manner, while the parental cells were small in size, the cells were round or similar to a circle, and likely to aggregate during the growth process (Fig 1b). According to the morphological changes and diffusion manner of gefitinib-resistant cells, we hypothesize that resistant cells may have higher malignant biological characteristics. As we expected, the proliferative capacity of gefitinib-resistant cells was significantly enhanced compared to the parental cells. It was confirmed in the colony formation experiment. The colony forming ability of the gefitinib-resistant cells was about twice that of the parental cells (Fig 1c,d). The cell growth curve was used to detect the proliferation ability of the cells, and it was found that the proliferation ability of gefitinib-resistant cells was higher than that of the parental cells (Fig 1e). It indicated that the resistant cell lines were much more malignant than the parental cells. The PD-L1 expression was higher in cell line PC-9/GR than cell line PC-9 (Fig 1f,g).

Ever-smokers had higher PD-L1 expression than never-smokers

To investigate PD-L1 expression in NSCLCs of other cohorts, we utilized the cancer microarray database Oncomine (www.oncomine.org). A total of 124 never-smokers and 118 ever-smokers were analyzed, and the ever-smoker patients had a much higher PD-L1 than never-smokers (Student's *t*-test, $P = 0.0188$; Fig 2).

Effect of tobacco extracts on PD-L1 expression

Immune checkpoint inhibitors are more effective in smokers than non-smokers carrying the EGFR-mutation, and we speculated that tobacco extracts may affect PD-L1 expression. PD-L1 plays an important role in inhibiting the function of effector T cells, so the changes of PD-L1 expression under tobacco extract stimulation will affect the efficacy of immunotherapy. The cells were stimulated with 5% volume of tobacco extract medium to observe whether PD-L1 would be changed in the cell line. Western blot analysis showed that the expression of PD-L1 protein in the cell line cultured in the tobacco extracts was higher than that of the cells cultured without the tobacco extracts in the PC9/GR cell line and HCC827/GR cell line and the effects of tobacco extracts were dose-dependent (Fig 3a) and time-dependent (Fig 3b). RT-qPCR also confirmed that tobacco extracts enhanced the transcription level of PD-L1 in PC9/GR cell line (Fig 3c) and HCC827/GR cell line (Fig 3d). These experimental results confirmed that the medium containing the tobacco extracts could stimulate the tumor cell PD-L1 expression and transcription.

mTOR signaling pathway involved in stimulation of PD-L1 expression by tobacco extracts

We hypothesized that the mTOR signaling pathway may be involved in promoting PD-L1 expression by tobacco extracts. We used a medium containing 5% volume of tobacco extract to culture cell lines, to mimic the stimulation of cells in smoking status individuals, and observed changes in the mTOR and AKT signaling pathways in cell lines. Western blot analysis showed that mTOR and p-mTOR were significantly increased in gefitinib-resistant cell lines cultured in the tobacco extracts in the PC9/GR and HCC827/GR cell lines (Fig 4a), p-AKT was also increased (Fig 4b), suggesting that the mTOR signaling pathway may be involved in PD-L1 expression. To further confirm this hypothesis, we used the mTOR inhibitor rapamycin to inhibit the function of mTOR in gefitinib-resistant cell lines cultured in the tobacco extracts. After

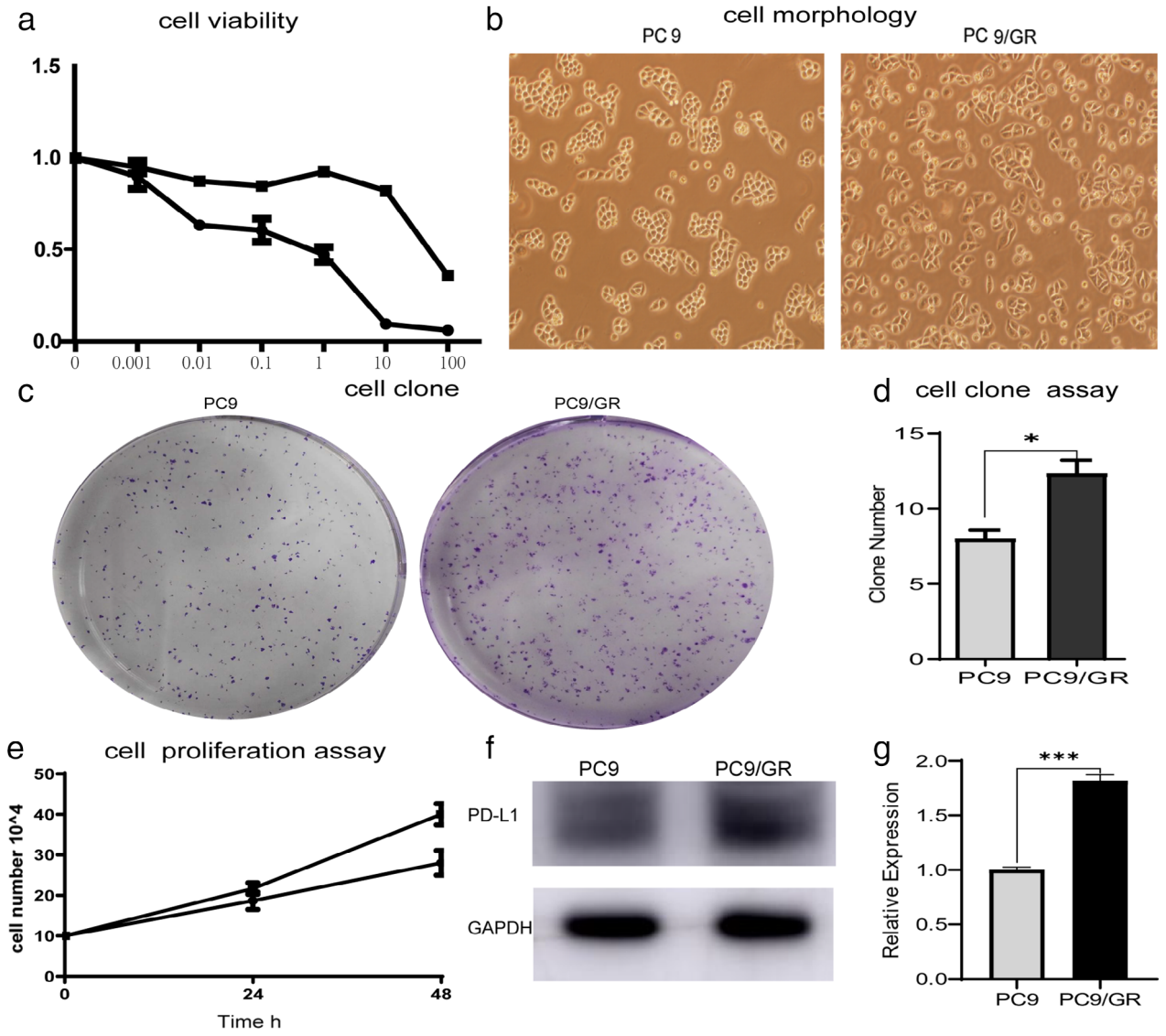


Figure 1 Biological differences between the parental cell line PC9 and the gefitinib-resistant cell line PC9/GR. (**a**) The IC50 of the gefitinib-resistant cell line PC9/GR was >10 μM, and the IC50 of the parental cell line PC9 was 1.288 μM; the drug sensitivity of the gefitinib was significantly decreased in PC9/GR. (**b**) Cell morphology changes in the gefitinib-resistant cell line PC9/GR were accompanied with the decreased sensitivity to gefinitib. The cell volume of the PC9/GR cell line was larger than that of the parental cell line PC9. The cells were fusiform and some appeared as pseudopods, while the parental cells were round. The gefitinib-resistant cells grew in a dispersed state, and the parental cells easily aggregated into clusters for growth. (**c, d**) Cell clone formation experiments confirmed that the colony-forming ability of gefitinib-resistant cells was significantly higher than that of the parental cells, PC9 = 8.000 ± 0.5774, PC9/GR = 12.33 ± 0.8819,**P* = 0.0147. (**e**) Cell proliferation curve verified that the cell proliferation ability of the gefitinib-resistant cells was significantly higher than that of the parental cells, PC9 = 28.00 ± 3.055, PC9/GR = 40.00 ± 2.646, **P* = 0.0412. (**f**) The PD-L1 expression were higher in the cell line PC-9/GR than the cell line PC-9. (**g**) The PD-L1mRNA were also increased (PC9 = 1.000 ± 0.01802, PC9/GR = 1.705 ± 0.01416,****P* < 0.0001). (**a**) (–●–) PC9, (–■–) PC9/GR; (**e**) (–●–) PC9, (–■–) PC9/GR.

inhibition of mTOR function (rapamycin, 5 ng/mL,10 ng/mL), the expression of PD-L1 protein in cell lines was significantly reduced in the PC9/GR and HCC827/GR cell lines (Fig 4c), the effects were time-dependent (Fig 4d) and the transcription level of mRNA was also decreased (Fig 4e,f).

The mTOR signaling pathway is involved in regulating the mRNA transcription of cytokines in gefitinib-resistant cell lines with the tobacco extracts. Studies have confirmed that the mTOR signaling pathway is involved in the regulation of various immunosuppressive cytokines and chemokine. To test this hypothesis, we examined the

transcription levels of CCL20, CXCL1, IL6 and IL-23 in tobacco extract-induced cell line PC9/GR cells, and found that IL-6 and IL-23 transcription levels were increased in gefitinib-resistant cell lines with the tobacco extracts (Fig 5a,c), CXCL1 decreased under the stimulation of the tobacco extract, and the change of CCL20 was not obvious (Fig 5e,g). To test whether these cytokines were regulated by the mTOR signaling pathway, we used mTOR inhibitor rapamycin (10 ng/mL) to treat gefitinib-resistant cell lines with the tobacco extracts and observed the changes of CCL20, CXCL1, IL6 and IL-23. The transcription levels of IL6 and IL-23 in PC9/GR cell lines were significantly decreased by rapamycin treatment in gefitinib-resistant cell lines with the tobacco extracts (Fig 5b,d), and CXCL1 was increased after treatment with rapamycin (Fig 5h), CCL20 did not change significantly (Fig 5f).

In another gefitinib-resistant cell HCC827/GR, it was found that the transcription levels of IL6 and IL-23 were significantly increased in gefitinib-resistant cell lines with the tobacco extracts (Fig 5i,k). The CXCL1, CCL20 were not obviously changed (Fig 5m, o). The transcription of CXCL1, CCL20, IL6 and IL-23 were found to be significantly reduced by rapamycin treatment in gefitinib-resistant cell lines with the tobacco extracts (Fig 5j,l,n,p). It is therefore suggested that the mTOR signaling pathway is involved in the transcriptional regulation of the cytokines IL6 and IL-23 in gefitinib-resistant cell lines with the tobacco extracts.

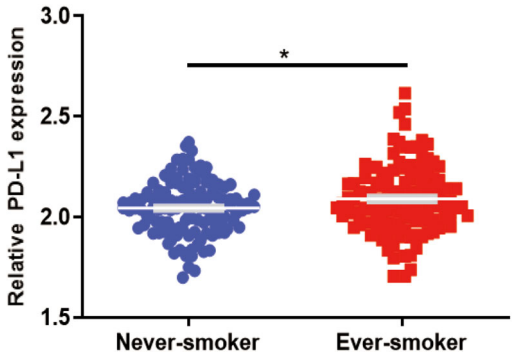


Figure 2 PD-L1 expression was higher in the ever-smoker than that of the never smoker patients. PD-L1 expression in an Oncomine report by PCR.

Tobacco extracts promote IL-6 expression via mTOR signaling pathway

We found that tobacco extracts could promote the mRNA transcription of IL-6. In order to observe the effect of tobacco extracts on IL-6 secretion level, we used ELISA to detect changes in IL-6 secretion and found that tobacco extracts could promote the release of IL-6. To test whether the increased IL-6 secretion came from mTOR, we combined the application of mTOR inhibitors in the tobacco extract-induced cell line and found that the release of IL-6

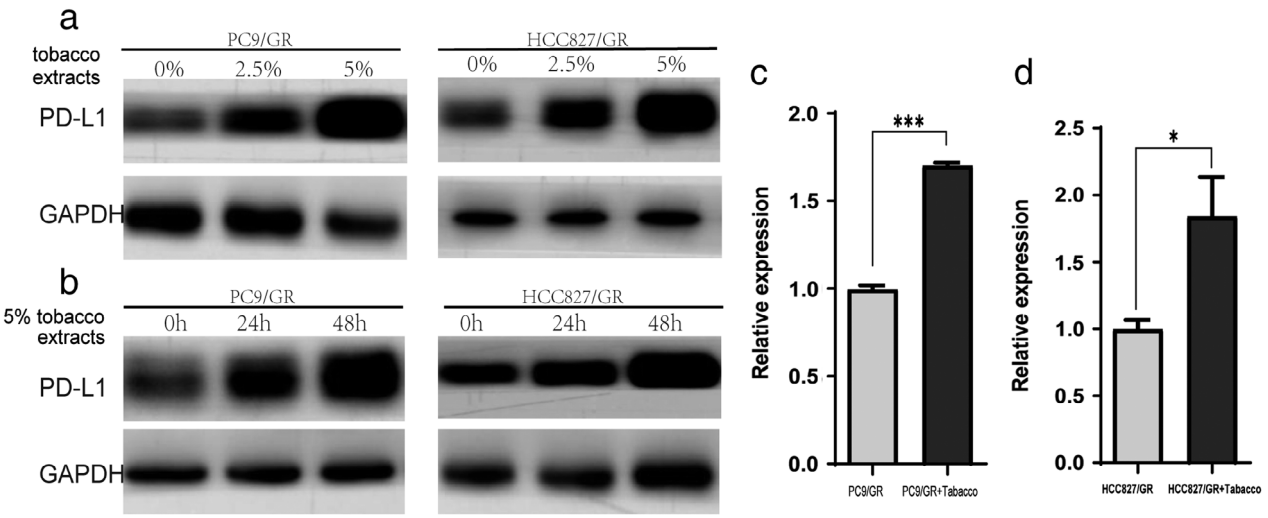


Figure 3 Tobacco extracts promoted the expression of PD-L1. (**a,b**) Western blot analysis showed PD-L1 expression was upregulated in the gefitinib-resistant cell line PC9/GR and HCC827/GR treated with tobacco extracts. (**c**) The relative expression of PD-L1 mRNA was also upregulated when the cell line PC9/GR was cultured with the medium treated with tobacco extracts, PC9/GR =1.000 ± 0.01802, PC9/GR + Tobacco = 1.705 ± 0.01416, ***$P < 0.0001$. (**d**) PD-L1 mRNA was also upregulated in the gefitinib-resistant cell line HCC827/GR treated with tobacco extracts HCC827/GR =1.000 ± 0.01802, HCC827/GR + Tobacco = 1.705 ± 0.01416, *$P = 0.0473$. PC9/GR group: gefitinib-resistant cell line PC9/GR; PC9/GR + Tobacco group: gefitinib-resistant cells PC9/GR treated with tobacco extracts; HCC827/GR group: gefitinib-resistant cell line HCC827/GR; HCC827/GR + Tobacco group: gefitinib-resistant cells HCC827/GR treated with tobacco extracts.

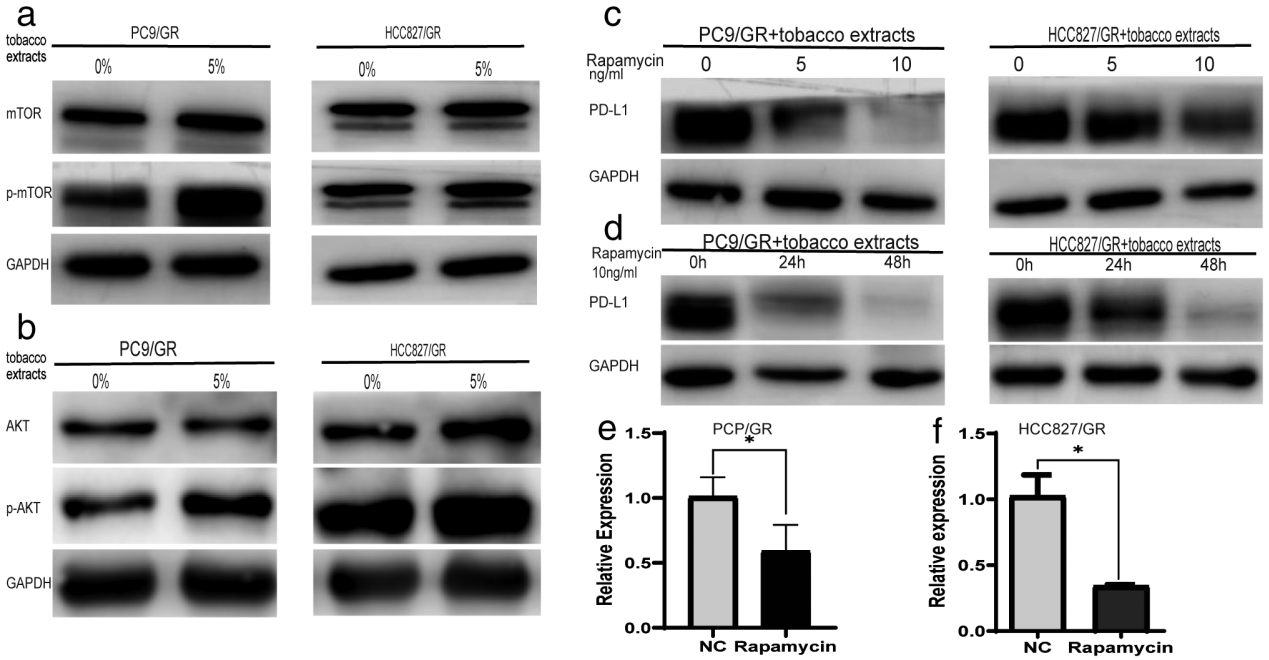


Figure 4 mTOR was upregulated by tobacco extracts and promoted the expression of PD-L1. (**a**) Western blot analysis showed mTOR and p-mTOR expression was upregulated in the gefitinib-resistant cell line PC9/GR and HCC827/GR treated with tobacco extracts. (**b**) Western blot analysis showed p-AKT expression was upregulated in the gefitinib-resistant cell line PC9/GR and HCC827/GR treated with tobacco extract. (**c, d**) Western blot analysis showed that mTOR inhibitor rapamycin inhibited the expression of PD-L1 which was induced by tobacco extracts in the gefitinib-resistant cell line PC9/GR and HCC827/GR. (**e**) The transcription of PD-L1 was downregulated by mTOR inhibitor rapamycin when the cell line PC9/GR was cultured with the medium treated with tobacco extract and rapamycin, PC9/GR+ Tobacco =1.008 ± 0.08771, PC9/GR + Tobacco+ m TOR inhibitor =0.5890 ± 0.1177, $*P = 0.0463$. (**f**) The transcription of PD-L1 was downregulated by mTOR inhibitor rapamycin when the cell line PC9/GR was cultured with the medium treated with tobacco extracts and rapamycin, HCC827/GR+ Tobacco =1.000 ± 0.01759, HCC827/GR + Tobacco+ mTOR inhibitor =0.4205 ± 0.0009726, $***P < 0.0001$. PC9/GR group: gefitinib-resistant cell line PC9/GR; PC9/GR + Tobacco group: gefitinib-resistant cells PC9/GR treated with tobacco extracts; HCC827/GR group: gefitinib-resistant cell line HCC827/GR; HCC827/GR + Tobacco group: gefitinib-resistant cells HCC827/GR treated with tobacco extracts;

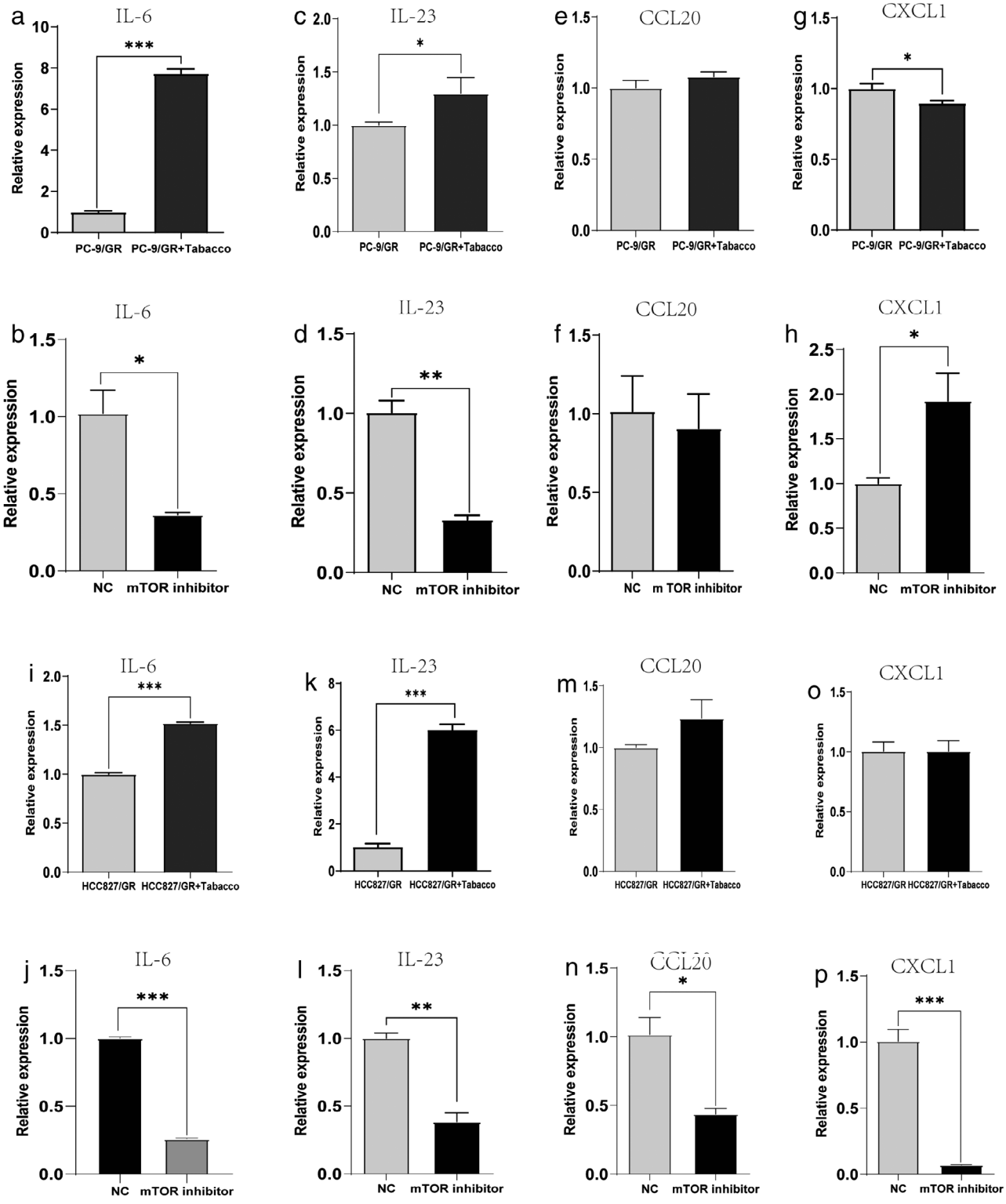
was reduced. In order to eliminate the effect of DMSO, a solvent used to dissolve rapamycin, on IL-6 release, we set up a DMSO group in the tobacco extracts group, and observed that there was no significant difference between the DMSO group and without DMSO group These results indicate that tobacco stimulates cells to promote the release of IL-6 by mTOR (Fig 6a,b).

mTOR regulates the expression of PD-L1 via IL-6

Western blot analysis showed that mTOR inhibitors reduce the expression of PD-L1. Western blot analysis showed that the expression of PD-L1 protein in the cell line cultured with IL-6 recombinant protein was higher than that of the cells cultured without IL-6 recombinant protein in the PC9/GR and HCC827/GR cell lines and the effects of tobacco extracts were dose-dependent (Fig 7a) and time-dependent (Fig 7b). In rescue experiments, the addition of IL-6 recombinant protein partially restored the ability of tobacco extracts to stimulate PD-L1 expression when cells were treated with mTOR inhibitors in the PC9/GR and HCC827/GR cell lines (Fig 7c,d).

Tobacco extracts promote macrophage migration via mTOR/IL-6

The above experiments confirmed that tobacco extracts promote the release of IL-6, and IL-6 was confirmed as a chemokine of attracting macrophages. We speculated that tobacco extract-induced cells would enhance the chemotactic ability of macrophages, and to test this hypothesis, we performed experiments on cell coculture to observe cell migration ability. We found that IL-6 recombinant protein (10 ng/mL) could improve macrophage (THP1-induced) migration capacity even without tobacco extract treatment in gefitinib-resistant cells PC9/GR (Fig 8a,b,g) and HCC827/GR (Fig 9a,b,g). We also found that tobacco extract-induced cells could enhance macrophage migration capacity in gefitinib-resistant cells PC9/GR (Fig 8a,c,g) and



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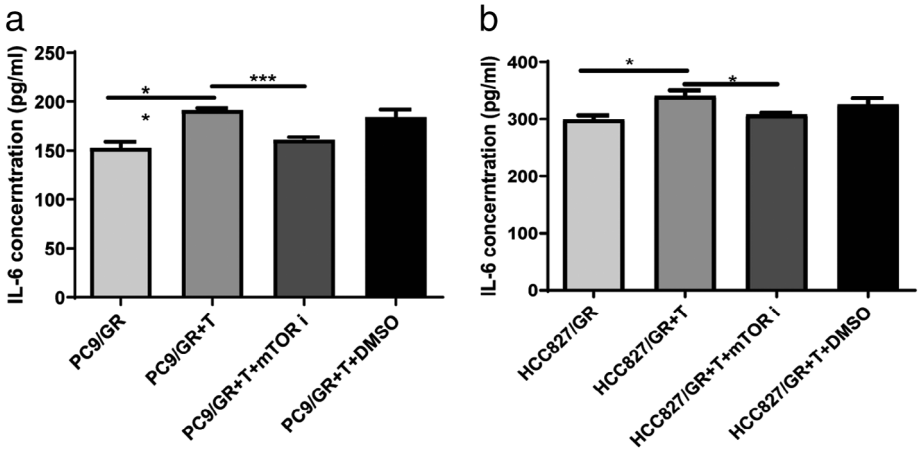

Figure 6 mTOR regulated the secretion of IL-6 detected by ELISA. T, tobacco; mTOR i, mTOR inhibitor. (**a**) Tobacco extract promoted cell supernatant IL-6 expression (PC9/GR, 152.9 ± 6.091; PC9/GR + T 191.5 ± 1.946; $^{**}P = 0.0038$ by two-tailed Student's *t*-test), and inhibiting mTOR reduced IL-6 secretion (PC9/GR + T, 191.5 ± 1.946; PC9/GR + T + mTOR i, 160.9 ± 2.855; $^{***}P = 0.0009$ by two-tailed Student's *t*-test). DMSO served as a control corresponding to the mTOR-inhibited group. (**b**) Tobacco extract promoted the expression of IL-6 in the cell supernatant (HCC827/GR, 299.2 ± 7.831; HCC827/GR + T, 340.8 ± 9.702; $^{*}P = 0.0289$ by two-tailed Student's *t*-test), and inhibition of mTOR decreased IL-6 secretion (HCC827/GR + T, 340.8 ± 9.702; HCC827/GR + T + mTOR i, 308.2 ± 3.043; $^{*}P = 0.0326$ by two-tailed Student's *t*-test). DMSO served as a control corresponding to the mTOR-inhibited group. Data are presented as mean ± SEM.

HCC827/GR (Fig 9a,c,g). The above experiments confirmed that mTOR could promote the secretion of IL-6. We speculated that the ability of tobacco extract cells to attract macrophages may come from tobacco extracts/mTOR/IL-6. To test this hypothesis, we conducted experiments on mTOR intervention and IL-6 blocking, and rescue experiments on cell coculture with tobacco extracts to observe cell migration ability. We found that after inhibiting mTOR, the migration capacity of macrophages was significantly reduced in gefitinib-resistant cells

Figure 5 Effects of tobacco extracts and rapamycin on cytokines CCL20, CXCL1, IL-6 and IL-23 in gefitinib-resistant cell lines. The results of PC9/GR are shown in Fig **a–g** and the results of HCC827/GR are shown in Fig **i–p**. (**a**) The transcription of IL-6 was significantly upregulated treated with tobacco extracts in gefitinib-resistant cells PC9/GR, PC9/GR = 1.001 ± 0.03201,PC9/GR + Tobacco =7.730 ± 0.1280,$^{***}P < 0.0001$; (**b**) mTOR signaling pathway inhibitors rapamycin inhibited IL-6 transcription, NC =1.022 ± 0.1507, mTOR inhibitor = 0.3641 ± 0.01482,$^{*}P = 0.0122$; (**c**) Transcription of IL-23 was significantly upregulated in the strain PC9/GR = 1.000 ± 0.01656, PC9/GR + Tobacco =1.298 ± 0.08518,$^{*}P = 0.0265$; (**d**) mTOR signaling pathway inhibitors inhibited IL-23 transcription, NC =1.006 ± 0.07515, mTOR inhibitor =0.3334 ± 0.02589,$^{**}P = 0.0011$. (**e**) Tobacco extract had no obvious effect on cytokine CCL20, PC9/GR = 1.001 ± 0.03079, PC9/GR + Tobacco = 1.080 ± 0.01951, $P > 0.05$; (**f**) mTOR signaling pathway inhibitor had no obvious effect on CCL20, NC = 1.016 ± 0.1299, mTOR inhibitor = 0.9063 ± 0.1265, $P > 0.05$; (**g**) Tobacco extract inhibited CXCL1 transcription in the gefitinib-resistant cell line PC9/GR, PC9/GR = 1.000 ± 0.02067, PC9/GR + Tobacco =0.8972 ± 0.01093, $^{*}P = 0.0116$; (**h**) mTOR signaling pathway inhibitor rapamycin promoted the transcription of CXCL1, NC =1.004 ± 0.06000,mTOR inhibitor =1.927 ± 0.3070,$^{*}P = 0.0419$. (**i**) IL-6 transcription was significantly upregulated in the gefitinib-resistant cell line HCC827/GR cultured with tobacco extract, HCC827/GR = 1.000 ± 0.01623, HCC827/GR + Tobacco = 1.523 ± 0.009293 $^{***}P < 0.0001$; (**j**) mTOR signaling pathway inhibitors inhibited IL-6 transcription, NC = 1.000 ± 0.006103, mTOR inhibitor = 0.2583 ± 0.003303,$^{***}P < 0.0001$; (**k**) The transcription of IL-23 was significantly upregulated in the gefitinib-resistant cell line HCC827/GR cultured with tobacco extract HCC827/GR = 1.022 ± 0.1443, HCC827/GR + Tobacco = 6.030 ± 0.2285, $^{***}P < 0.0001$; (**l**) mTOR signaling pathway inhibitor rapamycin inhibited IL-23 transcription NC = 1.001 ± 0.03796, mTOR inhibitor = 0.3849 ± 0.06552,$^{**}P < 0.0001$. (**m**) There was no obvious effect on cytokine CCL20 treated with tobacco extract in the gefitinib-resistant cell line HCC827/GR,HCC827/GR = 1.001 ± 0.02406, HCC827/GR + Tobacco = 1.237 ± 0.1512, $^{*}P > 0.05$; (**n**) mTOR signaling pathway inhibitor could inhibit the transcriptional activity of CCL20, NC = 1.017 ± 0.1242, mTOR inhibitor = 0.4380 ± 0.04006,$^{**}P = 0.0114$; (**o**) Tobacco extract did not affect CXCL1 transcription in the gefitinib-resistant cell line HCC827/GR, HCC827/GR = 1.005 ± 0.07582, HCC827/GR + Tobacco = 1.005 ± 0.08815,$^{*}P > 0.05$; (**p**) mTOR signaling pathway inhibitors did not affect CXCL1 transcription, NC = 1.007 ± 0.08836, mTOR inhibitor = 0.07201 ± 0.001878,$^{**}P = 0.0114$; PC9/GR group: gefitinib-resistant cell line PC9/GR; PC9/GR + Tobacco group: gefitinib-resistant cells PC9/GR treated with tobacco extract;HCC827/GR group: gefitinib-resistant cell line HCC827/GR; HCC827/GR + Tobacco group: gefitinib-resistant cells HCC827/GR treated with tobacco extract; NC group: negative control, medium including DMSO; m TOR inhibitor group: rapamycin, medium including DMSO and rapamycin.

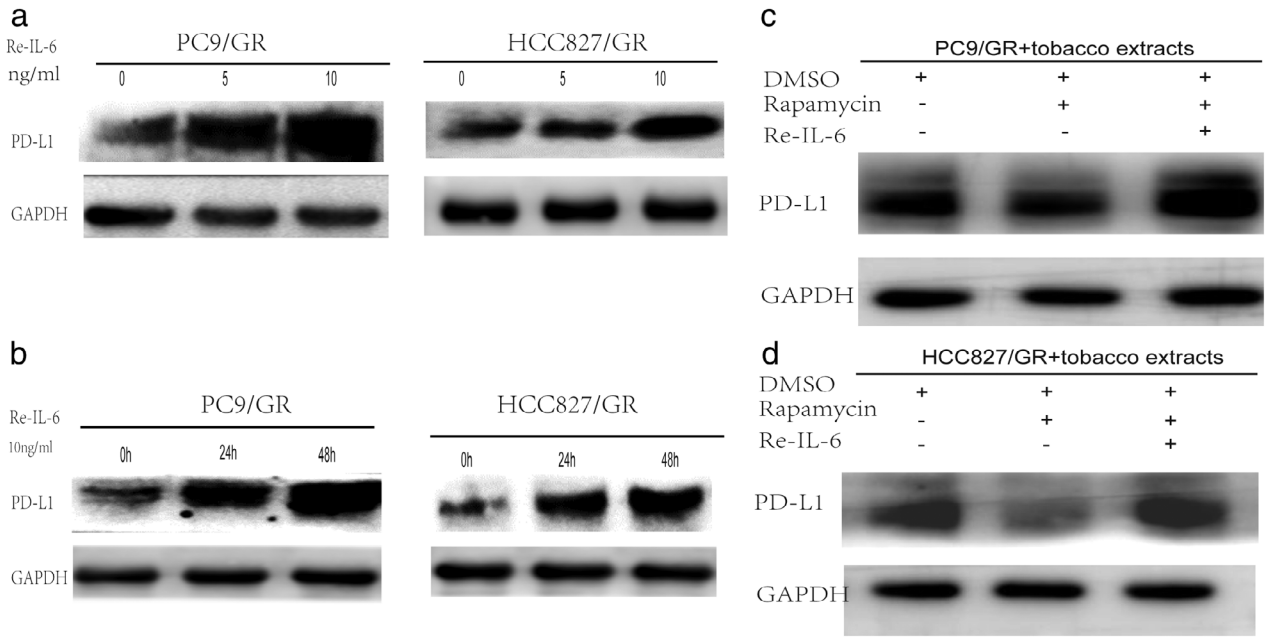

Figure 7 mTOR/IL-6 regulated the expression of PD-L1. (**a**) IL-6 recombinant proteins promote the expression of PD-L1 with dose-dependent. (**b**) IL-6 recombinant proteins promote the expression of PD-L1 with time-dependent. (**c**) PC9/GR + T: In PC9/GR cell lines, western blot analysis showed that mTOR inhibitors reduced the expression of PD-L1. In rescue experiments, the addition of IL-6 recombinant protein partially restored the ability of tobacco extracts to stimulate PD-L1 expression when cells were treated with mTOR inhibitors. (**d**) HCC827/GR + T: In HCC827/GR cell lines, mTOR inhibitors reduced PD-L1 expression. In rescue experiments, the addition of IL-6 recombinant protein partially restored the ability of tobacco extracts to stimulate PD-L1 expression when cells were treated with mTOR inhibitors. PC9/GR + T: PC9/GR cells were treated with tobacco extracts. HCC827/GR + T: HCC827/GR cells were treated with tobacco extracts.

PC9/GR (Fig 8a,d,h) and HCC827/GR (Fig 9a,d,h). After blocking IL-6 (2 μg/mL Anti-hIL-6 IgG), the migration capacity of macrophages also decreased in gefitinib-resistant cells PC9/GR (Fig 8a,e,h) and HCC827/GR (Fig 9a,e,h). In a salvage experiment, it was found that inhibiting mTOR and adding IL-6 recombinant protein would reverse the decrease in the migration capacity of macrophages, and then increase the migration capacity of macrophages in gefitinib-resistant cells PC9/GR (Fig 8e,f,i) and HCC827/GR (Fig 9e,f,i).

Discussion

We successfully constructed EGFR-TKI resistant cells. The gefitinib-resistant cells and parental cell lines were not only significantly different in IC50, but we also found significant differences in morphology and biological behavior. Compared with the parental cell line, the TKI-resistant cells were of great volume and enhanced proliferation ability. On the basis of gefitinib-resistant cell lines, we induced gefitinib-resistant cells with a medium containing 5% tobacco extracts, and found that the biological behavior of the cells was also changed after stimulation with tobacco extracts. This was partly due to the changes in the mTOR signaling pathway.

The expression of PD-L1 is regulated by a variety of signal pathway molecules, and studies have confirmed that IL-6,[15] ROS,[38,39] TGF-beta,[40] ALIX, NF-kappaB[14] and other factors such as osimertinib[41] can also regulate the expression of PD-L1. However, there are few studies on the regulation of the expression of PD-L1 under tobacco extract stimulation.

In the present study, the expression of PD-L1 was upregulated in gefitinib-resistant cell lines with the tobacco extracts, and the upregulation of PD-L1 inhibited the antitumor effect of tumor-infiltrating T lymphocytes. In patients with high PD-L1 expression in clinical studies, PD-L1 inhibitors have been found to be more effective than patients with low PD-L1 expression in solid tumors. In this study, it was found that tobacco extracts could stimulate the expression of PD-L1 in drug-resistant cells. This study also partly confirms that the efficacy of immunotherapy in smoker patients is higher than that in non-smokers in clinical studies. However, further experiments are needed to confirm this conclusion. On the other hand, patients with EGFR-TKI resistance are advised to give up smoking and stay far away from the smoking environment

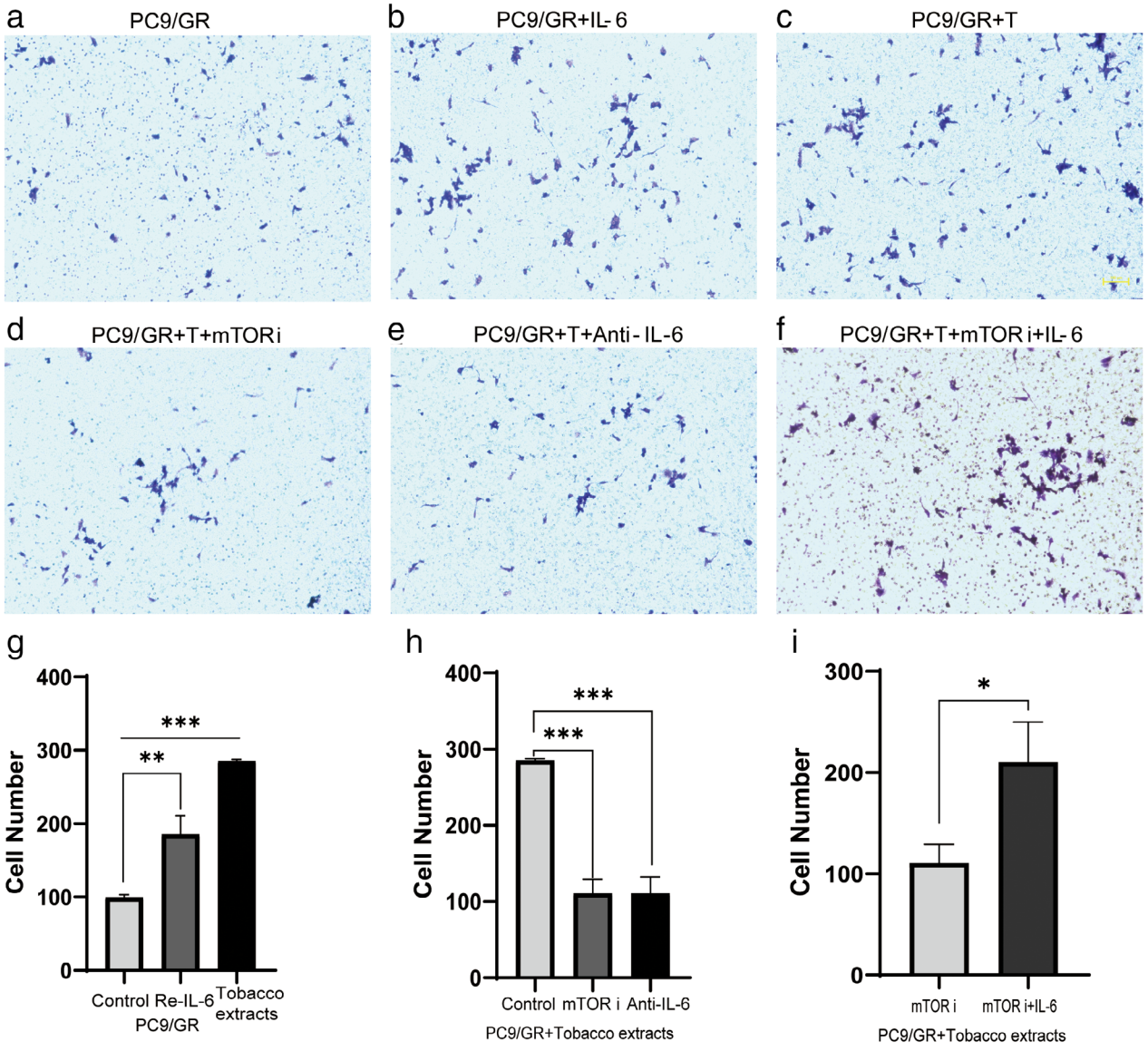

Figure 8 Tobacco extract promoted the migration of macrophages via mTOR/IL-6 in PC9/GR. Transwell migration assay image. (**a**) PC9/GR. Gefitinib-resistant cell line PC9/GR without treatment. (**b**) PC9/GR + IL-6. Gefitinib-resistant cell line PC9/GR treated with IL-6 recombinant protein (10 ng/mL). (**c**) PC9/GR + T. Gefitinib-resistant cell line PC9/GR treated with tobacco extracts. (**d**) PC9/GR + T + mTOR i. Gefitinib-resistant cell line PC9/GR treated with tobacco extracts and mTOR inhibitors. (**e**) PC9/GR + T + Anti -IL-6. Gefitinib-resistant cell line PC9/GR treated with tobacco extracts and Anti-hIL-6 IgG (2 μg/mL) blocking antibody. (**f**) PC9/GR + T + mTOR i + IL-6. Gefitinib-resistant cell line PC9/GR treated with tobacco extracts and mTOR inhibitors and IL-6 recombinant protein (10 ng/mL). Transwell migration assay data analysis. (**g**) IL-6 and tobacco extracts promoted the migration of macrophages. IL-6 promoted the migration of macrophages (PC9/GR, 99.67 ± 2.028; PC9/GR + IL-6185.3 ± 14.66; $^{**}P = 0.0044$ by two-tailed Student's *t*-test). Tobacco extracts promoted macrophage migration ability (PC9/GR, 99.67 ± 2.028; PC9/GR + T 285.3 ± 1.202; $^{***}P < 0.0001$ by two-tailed Student's *t*-test). (**h**) mTOR inhibitors and blocking of IL-6 inhibited the migration ability of macrophages. mTOR inhibitors reduced the migration ability of macrophages stimulated by tobacco extracts (PC9/GR + T, 285.3 ± 1.202; PC9/GR + T + mTOR i 110.7 ± 10.73; $^{***}P < 0.0001$ by two-tailed Student's *t*-test). Blocking of IL-6 could also inhibit the migration ability of macrophages stimulated by tobacco extracts (PC9/GR + T, 285.3 ± 1.202; PC9/GR + T + Anti -IL-6110.7 ± 12.57; $^{***}P = 0.0002$ by two-tailed Student's *t*-test). (**i**) IL-6 recombinant protein reversed the mTOR inhibitor of reducing macrophage migration. In rescue experiments, the addition of IL-6 recombinant protein will reverse the mTOR inhibitor's ability to reduce macrophage migration and partially restore its ability to be stimulated by tobacco extracts (PC9/GR + T + mTOR i, 110.7 ± 10.73; PC9/GR + T + mTOR i + IL-6210.7 ± 22.67; $^{*}P = 0.0163$ by two-tailed Student's *t*-test).

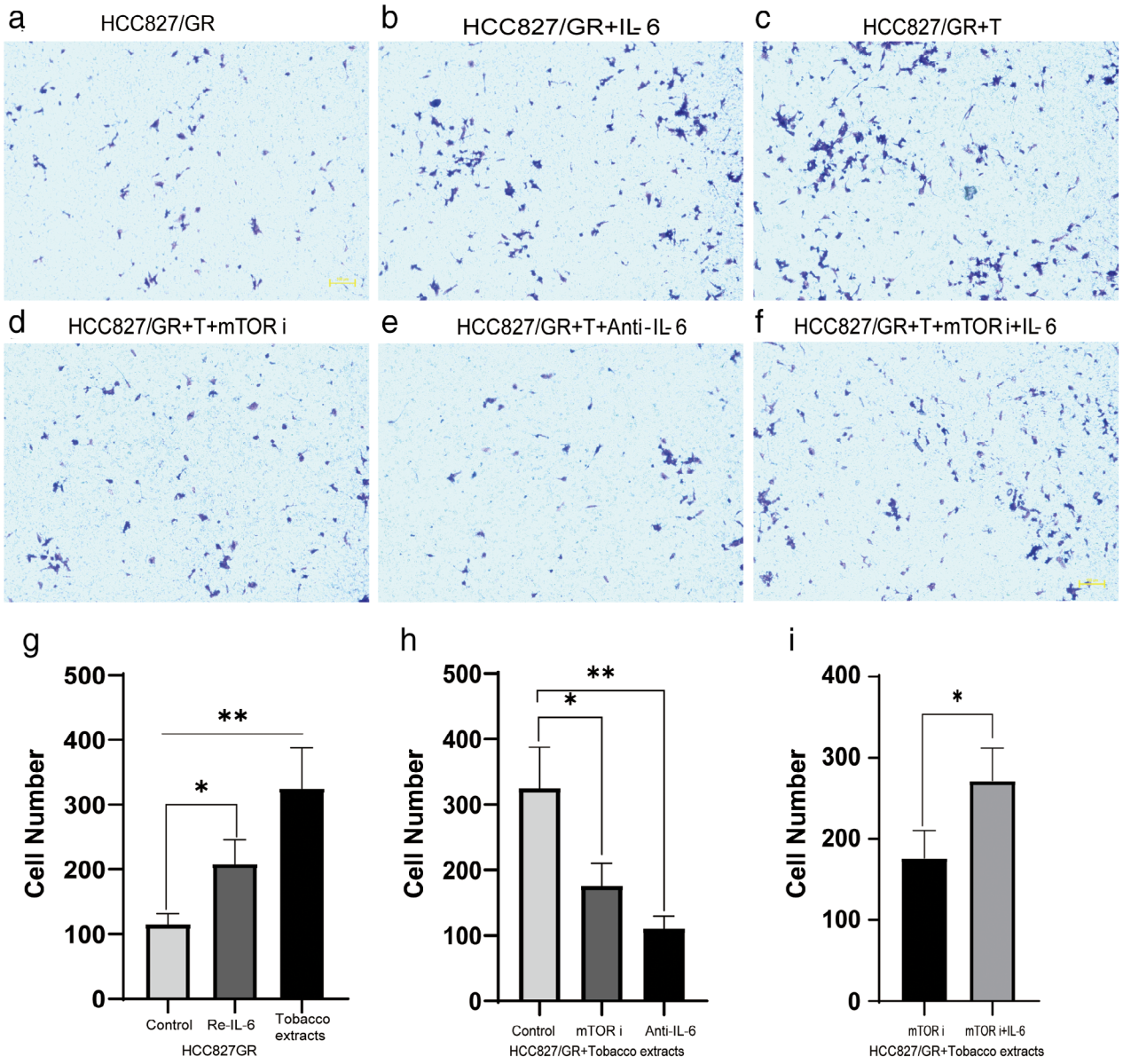

Figure 9 Tobacco extract promoted the migration of macrophages via mTOR/IL-6 in HCC827/GR. Transwell migration assay image. (**a**) HCC827/GR. Gefitinib-resistant cell line HCC827/GR without treatment. (**b**) HCC827/GR + IL-6. Gefitinib-resistant cell line HCC827/GR treated with IL-6 recombinant protein (10 ng/mL). (**c**) HCC827/GR+ T. Gefitinib-resistant cell line HCC827/GR treated with tobacco extract. (**d**) HCC827/GR + T + mTOR i. Gefitinib-resistant cell line HCC827/GR treated with tobacco extract and mTOR inhibitors. (**e**) HCC827/GR + T + Anti -IL-6. Gefitinib-resistant cell line HCC827/GR treated with tobacco extract and Anti-hIL-6 IgG (2 μg/mL) blocking antibody. (**f**) HCC827/GR + T + mTOR i + IL-6. Gefitinib-resistant cell line HCC827/GR treated with tobacco extract and mTOR inhibitors and IL-6 recombinant protein (10 ng/mL). Transwell migration assay data analysis. (**g**) IL-6 and tobacco extracts promoted macrophage migration. IL-6 could promote the migration of macrophages (HCC827/GR, 115.0 ± 9.539; HCC827/GR + IL-6208.3 ± 21.87; $*P = 0.0044$ by two-tailed Student's *t*-test), tobacco extract promoted macrophage migration ability (HCC827/GR, 115.0 ± 9.539; HCC827/GR + T 325.0 ± 36.30; $**P < 0.0001$ by two-tailed Student's *t*-test), (**h**) mTOR inhibitors and blocking of IL-6 inhibit macrophage migration. mTOR inhibitor reduced macrophage migration ability stimulated by tobacco extract (HCC827/GR, 325.0 ± 36.30; HCC827/GR + T + mTOR i 176.3 ± 19.46; $*P < 0.0001$ by two-tailed Student's *t*-test), the blocking of IL-6 also inhibited the migration ability of macrophages stimulated by tobacco extract (HCC827/GR, 325.0 ± 36.30; HCC827/GR + T + Anti-IL-6111.0 ± 10.82; $**P = 0.0002$ by two-tailed Student's *t*-test). (**i**) IL-6 recombinant protein reversed the mTOR inhibitor of reducing macrophage migration. In salvation experiments, the addition of IL-6 recombinant protein reversed the mTOR inhibitor's ability to reduce macrophage migration and partially restored its ability to be stimulated by tobacco extract (HCC827/GR + T + mTOR i, 176.3 ± 19.46; HCC827/GR + T + mTOR i + IL-6271.7 ± 23.21; $*P = 0.0163$ by two-tailed Student's *t*-test). Data are presented as mean ± SEM.

as much as possible to avoid increasing the expression of PD-L1 which can inhibit the anti-tumor effect of tumor-infiltrating T lymphocytes. At the same time, it provides a theoretical basis for smoker cancer patients in smoking cessation propaganda.

Our study found that tobacco extracts stimulated the mTOR signal pathway, which in turn promoted PD-L1 expression in tumor cells. The literature also confirms that tobacco extracts can stimulate the mTOR signal pathway. This experiment explores the regulation of tobacco extracts/mTOR/PD-L1. This study found that mTOR has a certain regulatory relationship with PD-L1, suggesting that mTOR signaling pathways and related downstream regulatory factors might be used as therapeutic targets for tumor immunotherapy in patients.

Weichhar *et al.* confirmed that rapamycin can promote dendritic cells to release proinflammatory cytokines IL-6/12/23, IL-1β, and TNF-α.[42,43] Studies have also shown that the secretion of the cytokine IL-23 may be increased in macrophages.[44] However, studies have also shown that rapamycin inhibits the secretion of proinflammatory cytokines.[45,46] Whilst these studies are proven in mononuclear/macrophage systems, the results are inconsistent. The role of mTOR inhibitor in anti-tumor via regulation the related cytokines is less well known.

We also found that some of the immune-related cytokines regulated by mTOR, such as CCL20, CXCL1, IL-6 and IL-23, underwent different changes under tobacco extract stimulation in two gefitinib-resistant cell lines.

Our study revealed that tobacco extract stimulation was able to alter the release of the cytokines associated with immune response, and that this may be due to the changes in the mTOR signaling pathway. The changes in these cytokines may be involved in the remodeling of the tumor microenvironment, partly providing an explanation as to why smoker patients achieve a better immune response when applying immunotherapy than non-smokers. IL-6 and IL-23 were elevated in both two gefitinib-resistant cell lines with the tobacco extracts, and decreased after application of the mTOR signaling pathway inhibitor rapamycin, suggesting that the changes induced by these two cytokines in gefitinib-resistant cell lines with the tobacco extracts came from the change in the mTOR signaling pathway.

Cytokine combined immunotherapy has previously been demonstrated to play an important role in synergistic anti-tumor effects,[47,48] suggesting that cytokines such as IL-6 can be targets in the process of immunotherapy.

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Disclosure

The authors declare that they have no competing interests.

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