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ORIGINAL RESEARCH

Impact of gefitinib in early stage treatment on circulating cytokines and lymphocytes for patients with advanced non-small cell lung cancer

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Objectives: The impact of epidermal growth factor receptor (EGFR) tyrosine-kinase inhibitors (TKIs) on the human immune system remains undefined. This study illustrates the immunomodulatory effect of gefitinib in patients with advanced non-small cell lung cancer (NSCLC) and its relevant prognostic significance.

Patients and methods: Peripheral blood samples were collected from 54 patients at baseline and after 4 weeks of gefitinib treatment. Circulating lymphocyte populations and cytokine levels were measured. Pilot investigation of the impact of gefitinib on programmed cell death ligand-1 (PD-L1) expression was conducted by immunohistochemistry (IHC).

Results and conclusion: A significant increase of peripheral natural killer cells and interferon-gamma (INF-γ) after 4 weeks of gefitinib treatment (P=0.005 and 0.02, respectively). In addition, circulating interleukin (IL)-6 was significantly decreased, especially in patients sensitive to gefitinib (P<0.001). Higher levels of IL-6 at baseline independently correlated with poorer progression-free survival. Experiments with NSCLC specimens illustrated that PD-L1 expression were downregulated after 4 weeks of gefitinib treatment. In summary, it was found that gefitinib treatment can alter circulating cytokines and lymphocytes. Dynamic changes of circulating lymphocytes, cytokines, and even PD-L1 IHC expression around gefitinib treatment support the specific immunomodulatory effect of this agent for advanced NSCLC.

Keywords: non-small cell lung cancer, gefitinib, PD-L1, lymphocyte, cytokine

Introduction

Recent insights into genetic aberrations and the role of the immune system in non-small cell lung cancer (NSCLC) have ushered in a new era of rapidly evolving targeted therapy and immune-based treatments.[1–3] Tyrosine-kinase inhibitors (TKIs) targeting epidermal growth factor receptor (EGFR) are efficacious as targeted therapy for NSCLC.[4–7] Although the progression-free survival (PFS) and the overall survival (OS) have been significantly improved, patients inevitably develop acquired resistance, and durable responses for advanced NSCLC have only been reported with immunologic therapy.[8,9]

Very recent findings include the mechanism of immunoediting and the complexity of immune escape mechanisms in cancer.[10] Programmed cell death protein-1 (PD-1) and its ligand-1 (PD-L1) are key immunological checkpoints mediating immune escape of cancer cells and limiting the anticancer immune response.[11,12] Blocking PD-1 or PD-L1 can restore the functions of tumor-specific T cells, which will further be reactivated to initiate direct killing of tumor cells, and the secretion of immuno-stimulatory cytokines such as interferon-gamma (INF-γ), interleukin (IL)-2, and tumor necrosis

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factor alpha (TNF-α).[13,14] The immunological checkpoint PD-L1 is regarded as an important biomarker for tailoring immunotherapy.[15]

Increasing evidence has suggested that EGFR-TKIs may have important immunological functions.[16] In fact, the enhanced antitumor effect seen in patients with skin rash may reflect a more functional antitumor immune response in these individuals.[17] Surprisingly, the therapeutic efficacy of several targeted agents seems to rely in part on off-target mechanisms, some of which are mediated by the immune system.[18] However, little is known about the immunoregulatory effects of EGFR-TKIs for NSCLC patients. This exploratory study illustrates the impact of gefitinib on peripheral inflammatory cytokines and lymphocytes. The potential prognostic value was also explored. Pilot investigation of PD-L1 expression was also performed on the basis of the available paired tissues pre- and posttreatment.

Patients and methods

Patient characteristics and treatment scheme

Patients diagnosed with NSCLC at the Cancer Center of Sun Yat-Sen University from March 2014 to March 2015 were screened for enrollment. The mutation status of EGFR was determined by amplification refractory mutation system-polymerase chain reaction. Patients were eligible to participate if they were between 18 and 75 years of age, pathologically diagnosed as advanced NSCLC, harbored EGFR-activating mutation, without T790M mutation, and treatment naive. Those patients who had 1) infection fever, inflammatory or autoimmune disease; 2) recent history of steroid, recent or current intake history of immunosuppressive drugs, opioid use or alcohol or illegal substance abuse; or 3) severe cardiac, respiratory, neurologic, or psychiatric diseases were excluded from this study.

Patients received gefitinib (IRESSA®, AstraZeneca, Macclesfield, UK) treatment at the standard dose (orally, 250 mg/day). Treatment interruptions and dose modifications were carried out according to the general recommendations. Computed tomography scans of the chest and abdomen and magnetic resonance imaging of the brain were performed at baseline, 4 weeks, and every 8 weeks thereafter or as clinically indicated for follow-up. Response evaluation was performed following the standard Response Evaluation Criteria in Solid tumors (RECIST version 1.0). Patients with a complete or partial response (PR) were regarded as objective response. PR refers to at least a 30% decrease in the sum of the longest diameter of target lesions. Systemic disease progression or the appearance of new lesions during treatment was considered disease progression.

Fasting blood samples were separately taken before (within 1 week) and 4 weeks after continuous gefitinib treatment. Serum samples were collected for cytokine analysis, and heparin plasma samples were collected for lymphocyte analysis.

Cytokine measurements

Blood samples were left to stand at room temperature for 30 min. Serum was isolated from blood samples after centrifugation (3,500 rpm for 15 min) and then stored at −80°C for cytokine measurement. Cytokines in serum samples were measured with a BD CBA Th1/Th2 Cytokine Kit (Catalog No 551809; BD Biosciences, San Jose, CA, USA). The kit was used for the simultaneous detection of IL-2, IL-4, IL-6, INF-γ, and IL-10 in a single sample. This assay kit provided a mixture of capture beads with distinct fluorescence intensities that have been coated with capture antibodies specific for each cytokine. The assays were performed according to the manufacturer's instructions. Individual cytokine concentrations were determined by measuring the fluorescent intensities of the corresponding capture beads. Concentrations of all cytokines were reported as pg/mL.

Lymphocyte phenotype analyses by flow cytometry

CD3 FITC was used for the identification of T lymphocytes, CD16, and CD56 PE for the identification of natural killer (NK) lymphocytes, CD45 PerCP-Cy™5.5 to allow for lymphocyte population gating, CD4 PE-Cy™7 for detecting T-helper/inducer lymphocytes, CD19 APC to identify B lymphocytes, and CD8 APC-Cy7 for the identification of suppressor/cytotoxic T lymphocytes (BD Multitest™ 6-color TBNK reagent, Catalog No 644611; BD Biosciences). The cells were analyzed by a FACSCalibur flow cytometer using BD FACSDiva clinical software (BD Biosciences) as indicated by the manufacturer. The results corresponding to each lymphocyte were presented as percentages (%) in human peripheral blood.

Immunohistochemistry (IHC) analyses

Paired, formalin-fixed, paraffin-embedded specimens were available from two patients with metastatic NSCLC. Both of them had a deletion of EGFR exon 19. The metastatic lesions were collected for comparison. The expression of PD-L1 was measured by immunohistochemical staining using a rabbit monoclonal antihuman antibody (E1L3N™; Cell Signaling Technology, Danvers, MA, USA, 1:200). Two pathologists

who were blinded to the clinical or pathological information of these patients independently assessed the expression of PD-L1. A semiquantitative H-score (maximum value of 300 corresponding to 100% of tumor cells positive for PD-L1 with an overall staining intensity score) was defined as the product of the percentage of stained cells and an intensity score (0, absent; 1, weak; 2, moderate; and 3, strong).

Ethics statements

The study protocol and patients' informed consent were approved by the Ethics Committee of Cancer Center of Sun Yat-Sen University (SYSUCC, Guangzhou, People's Republic of China). All the patients provided written informed consent to participate in this study. All procedures performed in the study involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The studies conducted in laboratory were performed under exploratory research principles. The raw data can be provided per request.

Table 1 The characteristics of enrolled NSCLC patients (n=54)

Parameter case	N (%)
Gender	
Female	28 (52)
Male	26 (48)
Smoking	
Never	36 (67)
Ever	18 (33)
Stage	
IIIB	3 (6)
IV	51 (94)
Pathology	
ADC	48 (89)
Non-ADC	6 (11)
EGFR mutation status	
Exon 19 del	33 (61)
Exon 21 L858R	17 (32)
Others*	4 (7)
Gefitinib efficacy	
Partial response	41 (76)
Stable disease	9 (17)
Progression disease	4 (7)

Notes: Non-ADC includes four squamous carcinoma and two adenosquamous carcinoma. *Other EGFR-activating mutations without EGFR exon T790M. Two cases harbor exon 18 (Gly719Ser and Gly719Ala) and another two cases with exon 21 (Leu861Gln), respectively.
Abbreviations: ADC, adenocarcinoma; EGFR, epidermal growth factor receptor; NSCLC, non-small cell lung cancer.

Statistical analysis

Nonparametric quantitative data were presented as median and interquartile range (IQR). The Wilcoxon test was used for the comparison of paired nonparametric data. Pearson's chi-squared test or Fisher' exact test was used to assess the correlation between immunological parameters and the efficacy of gefitinib. PFS was calculated from the start of gefitinib treatment until disease progression, death, or the last follow-up. All of the statistical analyses were performed with SPSS 20.0 for Windows (IBM Corporation, Armonk, NY, USA). A two-sided *P*-value of <0.05 was considered statistically significant.

Results

The characteristics of 54 patients with advanced NSCLC receiving gefitinib are listed in Table 1. Twenty-six patients (48%) were male and 28 patients (52%) were female. The median age was 57 (39–88) years old. The objective response rate (ORR) was 76% (n=41, all cases were partial remissions), and another nine patients (16%) had stable disease. The median tumor size at baseline was 56.5 mm (IQR: 38.0–80.8 mm). After gefitinib treatment, the median value decreased to 41.5 (IQR: 27.8–57.0). This change was significant according to Wilcoxon test ($P<0.001$). The median PFS was 10.4 months (95% confidence interval [CI], 10.1–10.7 months) with a median follow-up of 14.2 months. The percent of peripheral lymphocyte subsets at baseline and after 4 weeks of gefitinib treatment was determined by flow cytometry. The dynamic changes are presented in Figure 1. The percent of total T cells ($CD4^+$ T cells) was significantly decreased after 4 weeks ($P=0.003$). An inverse relationship was found in effector T cells ($CD8^+$ T cells); however, the difference was not significant ($P=0.48$). Additionally, a pronounced induction of peripheral NK cells ($CD3^-CD16^+CD56^+$) was detected after gefitinib treatment ($P=0.005$), which was considered as an augmentation of innate immunity. Whether regulatory $CD4^+CD25^+$ T cells were influenced by gefitinib treatment was also explored. A slight increase in total T cells was induced by gefitinib, but the change was not statistically significant ($P=0.18$).

Whether gefitinib could modulate the peripheral levels of pro-inflammatory cytokines was further explored. The levels of circulating IL-6 were significantly decreased after gefitinib treatment ($P<0.001$, Figure 2A). Moreover, 4 weeks later, a significant increase of peripheral INF-γ levels was detected ($P=0.02$, Figure 2D). In order to elucidate the influence of the treatment on the dynamic changes of INF-γ and IL-6 levels, the results were distinguished according to gefitinib efficacy. Significant changes in IL-6 (Figure 2B and C) and INF-γ (Figure 2E and F) were present only in the response group. The correlation between the alteration in immunological parameters and the efficacy of gefitinib was further analyzed.

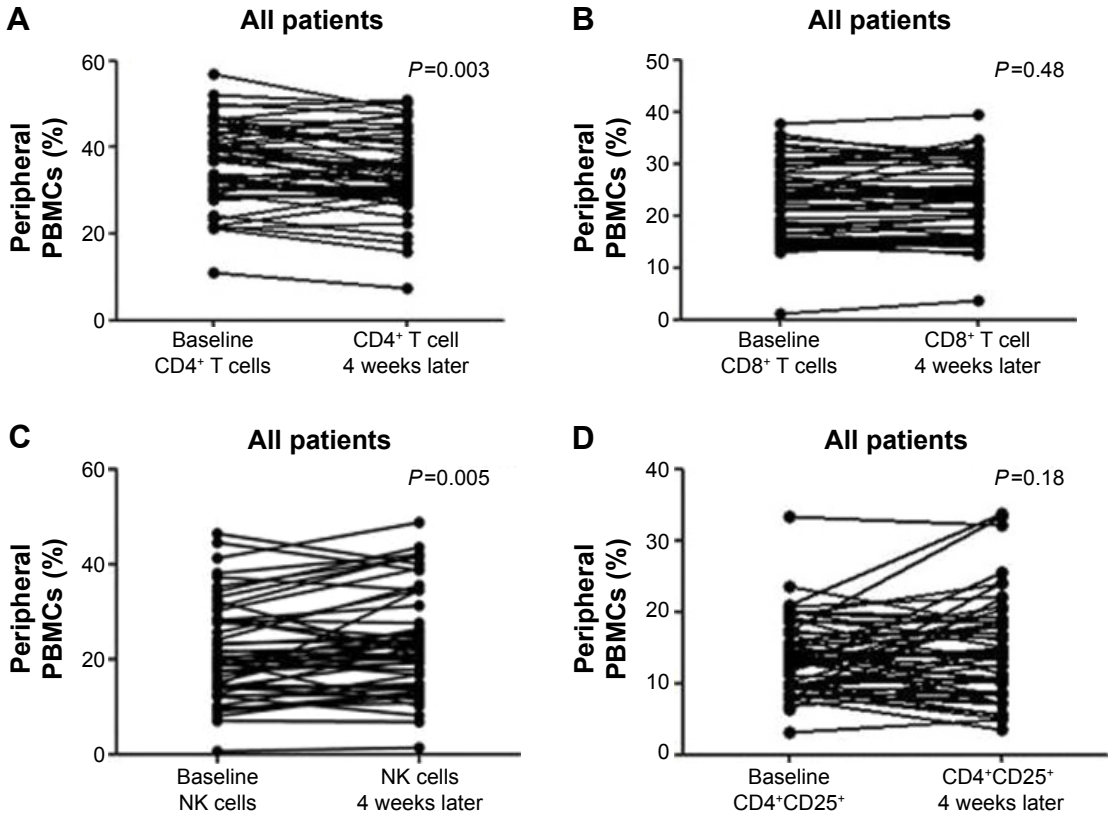

Figure 1 Gefitinib-induced changes of the peripheral lymphocyte subsets: (**A**) $CD4^+$ T cells, (**B**) $CD8^+$ T cells, (**C**) NK cells, and (**D**) $CD4^+CD25^+$ T cells.
Note: The changes of lymphocyte in terms of percent of PBMCs were analyzed for all patients (n=54) at baseline and 4 weeks after gefitinib treatment.
Abbreviation: PBMC, peripheral blood mononuclear cell.

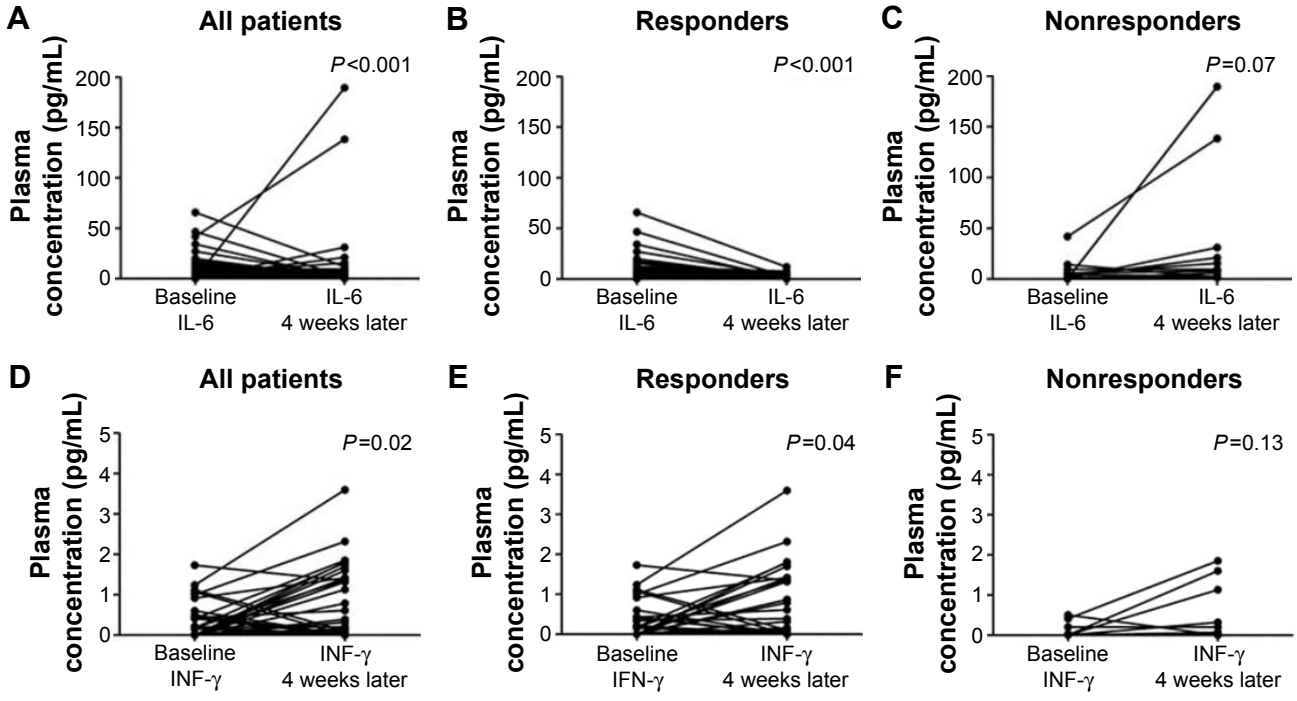

Figure 2 Gefitinib-induced decrease of circulating IL-6 and increase of INF-γ.
Notes: (**A**) IL-6, all patients. (**B**) IL-6, responders to gefitinib. (**C**) IL-6, nonresponders to gefitinib. (**D**) INF-γ, all patients. (**E**) INF-γ, responders to gefitinib. (**F**) nonresponders to gefitinib. All patients (n=54); responders to gefitinib (n=41); nonresponders to gefitinib (n=13).
Abbreviations: IL, interleukin; INF-γ, interferon-γ.

The dynamic feature was classified according to the change caused by the treatment. No apparent changes in circulating IL-2, IL-4, and IL-10 were detected after gefitinib treatment (*P*-values were 0.12, 0.17, and 0.70, respectively; Figure S1). Despite no significant association between changes in IL-2, IL-4, IL-10, and INF-γ and treatment efficacy, reduced IL-6 levels were significantly correlated with objective response to gefitinib (Table 2).

The prognostic significance of circulating IL-6 on PFS was then investigated. Changes in plasma IL-6 during gefitinib treatment were classified into reduced or nonreduced groups. Although reduced IL-6 levels might predict a response to gefitinib, it failed to present as an independent predictor for PFS by Cox proportional hazards regression models (Table S1). In order to demonstrate this finding, baseline and posttreatment plasma levels were also analyzed. In each circumstance, median values were used as the cutoff between the higher and lower groups separately. Kaplan–Meier survival curves are presented in Figure 3. Higher levels of IL-6 at baseline correlated significantly with shorter PFS compared to the group with lower levels of IL-6 at baseline, with a median PFS of 8.4 and 11.4 months, respectively (Table 3). However, this difference was not as large in posttreatment plasma IL-6 levels, in which the median PFS was 10.2 and 11.4 months, respectively (Table S2 and Figure S2).

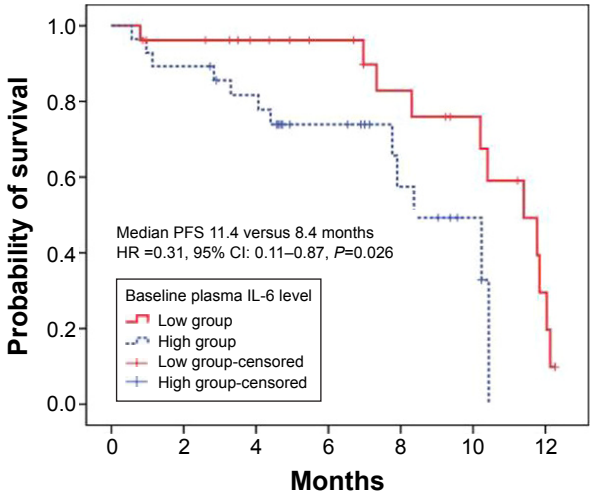

Figure 3 The prognostic value of IL-6 for PFS.
Note: Higher level (≥5.5 pg/mL) versus lower level (<5.5 pg/mL) of IL-6 at baseline.
Abbreviations: CI, confidence interval; HR, hazard ratio; IL, interleukin; PFS, progression-free survival.

Table 2 The dynamic change of several immunological parameters and the efficacy of gefitinib

Parameters	Response (N)	Nonresponse (N)	χ^2	*P*-value
IL-2				
Reduced	15	5	0.02	1.00
Nonreduced	26	8		
IL-4				
Reduced	13	6	0.90	0.51
Nonreduced	28	7		
IL-6				
Reduced	31	5	6.13	0.02
Nonreduced	10	8		
IL-10				
Elevated	18	4	–	0.52[a]
Nonelevated	23	9		
INF-γ				
Elevated	19	3	–	0.20[a]
Nonelevated	22	10		
$CD4^+CD25^+$ T cells				
Reduced	23	5	1.23	0.27
Nonreduced	18	8		
Natural killer cells				
Elevated	24	11	2.94	0.09
Nonelevated	17	2		
$CD4^+$ T cells				
Reduced	28	10	–	0.73[a]
Nonreduced	13	3		
$CD8^+$ T cells				
Elevated	22	7	<0.001	1.00
Nonelevated	19	6		

Notes: The dynamic feature was classified according to the change trend that was observed in the study. [a]*P*-value was calculated with Fisher's exact test; all others were calaculated with Pearson chi-squared test.
Abbreviations: IL, interleukin; INF-γ, interferon-γ.

Finally, a pilot investigation was conducted to verify whether the expression of PD-L1, an immunomodulatory checkpoint molecule, could be altered by gefitinib in the early treatment stage. The results revealed that gefitinib treatment reduced the expression of PD-L1 4 weeks later compared to that at baseline. The H-score of PD-L1 changed from 120 and 80 to 40 in both the cases. Images of gefitinib-treated tumor cells stained for PD-L1 are presented in Figure S3.

Discussion

In the current era of rapidly evolving targeted therapies and immune-based treatments, limited evidence for gefitinib or erlotinib as immunomodulatory drugs remains. Although some insight into the interaction between EGFR-TKIs and immunological checkpoints has been reported, little is known about the immunoregulatory effects of these targeted agents in vivo. This study demonstrated for the first time that gefitinib could alter the composition of peripheral lymphocytes and the levels of circulating pro-inflammatory cytokines. The increased innate immunity, increased systemic INF-γ, and reduced circulating IL-6 suggested that an immune-related mechanism may be involved in mediating the anticancer effects of gefitinib. In addition, the immunomodulatory

Table 3 Univariate and multivariate survival analyses of 54 NSCLC patients

Variables	Category	Median PFS (months)	PFS analyses					
			Univariate analyses			Multivariate analyses		
			HR	95% CI	*P*-value	HR	95% CI	*P*-value
Gender	Female/male	11.8 vs 7.9	0.25	0.09–0.69	0.008	0.24	0.07–0.83	0.025
Age	<57/≥57 years	11.4 vs 7.9	0.39	0.17–0.90	0.028	0.24	0.08–0.66	0.006
Smoking history	No/yes	10.4 vs 7.9	0.35	0.14–0.91	0.031	1.58	0.50–5.26	0.452
EGFR status	Exon 19 del/others	11.8 vs 8.4	0.31	0.12–0.79	0.014	0.34	0.13–0.91	0.032
IL-6 level*	Lower/higher	11.4 vs 8.4	0.31	0.11–0.87	0.026	0.19	0.05–0.66	0.009
Response to gefitinib	PR/not PR	10.4 vs 10.2	0.45	0.18–1.12	0.086	0.17	0.05–0.53	0.002

Note: *Patients were dichotomized on the basis of the median value (5.5 pg/mL) of baseline plasma IL-6.
Abbreviations: CI, confidence interval; EGFR, epidermal growth factor receptor; HR, hazard ratio; IL, interleukin; NSCLC, non-small cell lung cancer; PFS, progression-free survival; PR, partial response.

effects were also reflected in the downregulation of PD-L1 expression after 4 weeks of gefitinib treatment.

NK cells are key components of innate immunity against viral infection and neoplastic cells.[19] NK cells can eliminate cancer cells via direct killing, induction of apoptosis, or INF-γ secretion.[20,21] He et al[22] reported that gefitinib could greatly enhance NK-cell cytotoxicity by restoring receptor–ligand interactions between NK cells and human lung cancer cells with EGFR L858R and T790M resistance mutations. Moreover, NK-cell-based immunotherapy is regarded as a promising way to eliminate tumor cells.[23] The results of this study were in accordance with the previous reports. After 4 weeks of gefitinib treatment, the percentage of NK cells was significantly increased from 21.3% to 23.7% (P=0.005; Table S3). This change in the innate immune system was likely due to enhanced receptor–ligand interactions described earlier.

Moreover, the impact of gefitinib on adoptive cellular immunoreaction was also explored. Peripheral percentages of $CD4^+$ T cells were significantly decreased after 4 weeks of gefitinib treatment, whereas no apparent alteration in the percentage of effector T cells was observed. A research study previously revealed that erlotinib could impair T-cell-mediated immune responses both in vitro and in vivo by inhibiting T-cell proliferation and activation.[24] This immunosuppressive activity was believed to be due to the downregulation of the c-Raf/ERK cascade and the Akt signaling pathway. The Ras/Raf/ERK and PI3K/Akt pathways are critical in the signaling networks that regulate cell proliferation, differentiation, and survival.[25,26] Of note, drug sensitivity to gefitinib is closely correlated with EGFR-dependent ERK1/2 and Akt activation.[27] Our controversial finding was presumably due to different degrees of inhibition mediated by gefitinib or different mechanisms than the one by which erlotinib acts against the T-cell-mediated immune response. Despite the complexity of the human immune system, evidence at clinical level to demonstrate the immunomodulatory impact of gefitinib has been provided.

Immune cells have a broad impact on tumor initiation, progression, and drug resistance. Several proinflammatory cytokines are considered to be vital in the aforementioned process.[28,29] INF-γ and IL-6, for instance, are pleiotropic cytokines with complex functions in various immune and inflammatory reactions.[30,31] Moreover, INF-γ is an important effector cytokine produced by NK cells, and it is vital for the immune surveillance of tumors.[32] IL-6 has been reported to be associated with tumor progression, and it acts to inhibit cancer cell apoptosis and stimulate angiogenesis.[33] IL-6 was shown to induce an epithelial-to-mesenchymal transition (EMT) and promote metastasis in various human cancers.[34–36] Several studies have reported resistance to anti-NSCLC agents that is mediated by IL-6.[36,37] TGF-β/IL-6 axis mediates selective and adaptive mechanisms of resistance to erlotinib in lung cancer.[37] In addition, previous data have already suggested a statistically significant difference between lung cancer patients and the healthy control group regarding circulating IL-6.[38] Results from Songur et al showed a relationship between elevated serum IL-6 levels and malnutrition and bad prognosis in patients with advanced NSCLC.[39] The results of the present study also demonstrated that gefitinib significantly increased systemic levels of INF-γ and decreased the circulating levels of IL-6. This alteration was more apparent in those that were sensitive to gefitinib. There is evidence for circulating IL-6 as a survival predictor in advanced NSCLC patients treated with chemotherapy.[40] A similar result was found in terms of PFS for NSCLC patients receiving gefitinib. However, the differences found in this study were only at higher baseline levels of IL-6, which correlated with shorter PFS, whereas the change types (reduced versus nonreduced) and posttreatment levels were not independent predictors. However, this study cannot conclude whether the changes

are specific to gefitinib or a universal phenomenon when receiving chemotherapeutic agents.

Indeed, preclinical data have demonstrated that the continuing activation of EGFR contributes to the upregulation of *p*-STAT3, which leads to the increased expression of IL-6.[41] The blocking of EGFR activation induced by TKI consequently generates the downregulation of IL-6. Given the biologic rationale for therapeutic anti-IL-6 activity and preliminary clinical evidence that targeted anti-IL-6 antibodies are well tolerated in cancer patients, the findings of this study provide further insight into a potential strategy for improving gefitinib anticancer efficacy and prolonging PFS by combining an anti-IL-6 agent with gefitinib.[42,43]

Along with the enthusiastic promotion of PD-1/PD-L1 checkpoint blockades, most biomarker investigation has focused on the tumor microenvironment, especially the immunohistochemical expression of PD-L1.[44] This study verified that EGFR-TKIs, such as gefitinib, can downregulate tumor PD-L1 expression in patients with NSCLC. This validation was concordant with previously published data.[13–15] Erlotinib could downregulate PD-L1 expression in cell lines with activating EGFR mutations but not in those with wild type PD-L1.[13] Meanwhile, our previous work showed that inhibiting EGFR with EGFR-TKIs could attenuate the inhibition of T cells mediated by PD-L1 and enhance the production of INF-γ.[14] Besides, the changes of aforementioned circulating parameters were probably a consequence of the influence of mediators released from disintegrated cancer cells or enhanced immune system during gefitinib treatment. Therefore, the immunomodulatory effect may be due to the alteration of the PD-1/PD-L1 interaction caused by downregulating PD-L1 expression and increasing INF-γ levels and NK-cell activation.[14,45] In addition, the results of the present study demonstrated that PD-L1 expression is not constant during EGFR-TKIs treatment. The clinical significance here is that physicians should be cautious while applying PD-L1 status or expression level in pretreatment specimens as a prognostic factor or biomarker for immunotherapy. At present, targeted therapy directed at oncogenic signaling pathways is an attractive candidate in combination with immune checkpoint blockade agents.[42,46] Nevertheless, synergistic antitumor effect was not observed with combined EGFR-TKIs and an anti-PD-1 antibody treatment in a coculture system.[14] The positive immunoregulatory effect of gefitinib observed in the present study weakens the demand for the combination of gefitinib with immunotherapy. However, more investigation and prospective clinical studies are needed to verify this hypothesis.

This study was limited by its exploratory nature and the relatively small number of participants. Paired tissues for PD-L1 detection were a limited resource, which restricts these experiments as a pilot investigation. In addition, the causality between PD-L1 reduction and the alteration of lymphocytes or cytokines is not identified in the present work. Despite these limitations, a paired comparison was used during early gefitinib treatment and, for the first time, the specific immunomodulatory effects of gefitinib were detected.

Collectively, this study demonstrated that gefitinib treatment at early stages leads to distinct changes of circulating lymphocytes, cytokines, and even PD-L1 IHC expression around treatment. All these data support that certain immunomodulatory effects exist for gefitinib in advanced NSCLC.

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Author contributions

LZ, WFF, and JS designed the study. XL collected the blood samples, and SX and JHZ conducted the experiment. YXM collected the raw data and with NNZ together analyzed it. JS, WFF, LZ, YH, and HYZ wrote the manuscript. All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

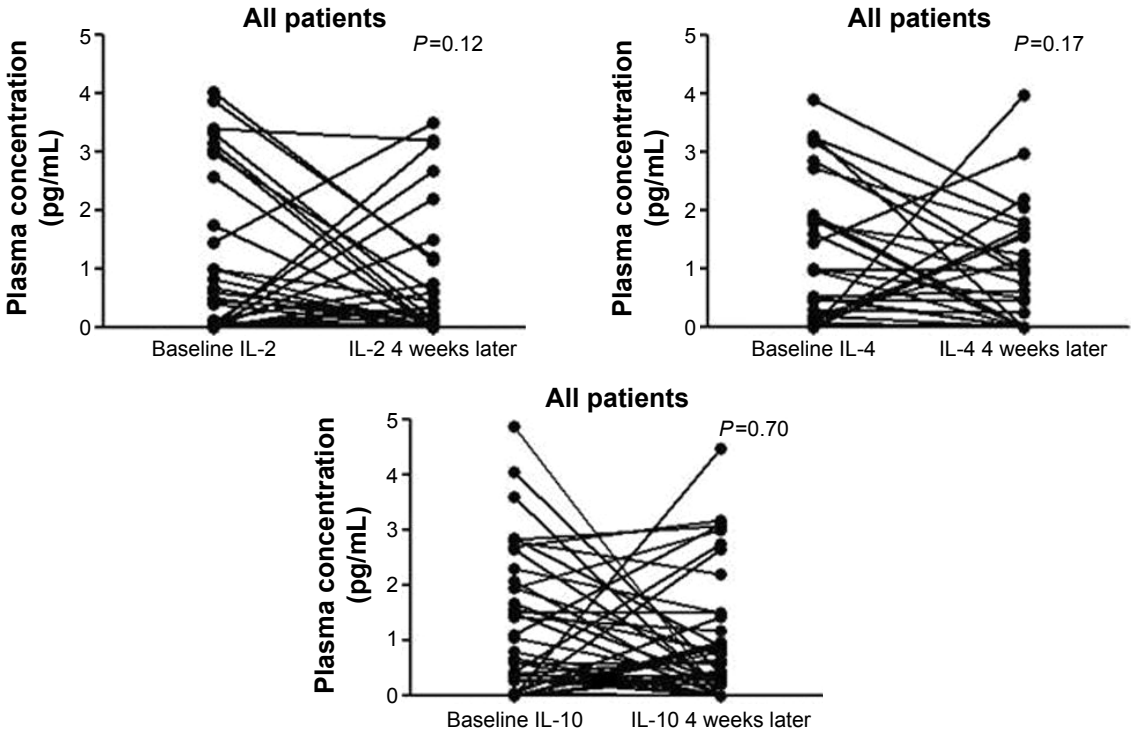

Figure S1 The changes of several circulating cytokines induced by gefitinib treatment.
Note: These were analyzed for all patients (n=54) with a baseline and 4 weeks after gefitinib treatment.
Abbreviation: IL, interleukin.

Table S1 Univariate and multivariate survival analyses involving plasma IL-6 change types

Variables	Category	Progression-free survival analyses					
		Univariate analysis			Multivariate analysis		
		HR	95% CI	*P*-value	HR	95% CI	*P*-value
Gender	Female/male	0.25	0.09–0.69	0.008	0.30	0.09–1.02	0.055
Age	<57/≥57 years	0.39	0.17–0.90	0.028	0.31	0.12–0.83	0.019
Smoking history	No/yes	0.35	0.14–0.91	0.031	0.75	0.21–2.67	0.654
EGFR status	Exon 19 del/others	0.31	0.12–0.79	0.014	0.28	0.10–0.77	0.013
IL-6 change types*	Reduced/nonreduced	0.36	0.11–1.14	0.083	0.52	0.14–1.95	0.335

Note: *Patients were dichotomized based on the change type during gefitinib treatment.
Abbreviations: CI, confidence interval; EGFR, epidermal growth factor receptor; HR, hazard ratio; IL, interleukin.

Table S2 Univariate and multivariate survival analyses involving posttreatment plasma IL-6

Variables	Category	Progression-free survival analyses					
		Univariate analysis			Multivariate analysis		
		HR	95% CI	*P*-value	HR	95% CI	*P*-value
Gender	Female/male	0.25	0.09–0.69	0.008	0.23	0.06–0.82	0.024
Age	<57/≥57 years	0.39	0.17–0.90	0.028	0.30	0.11–0.81	0.017
Smoking history	No/yes	0.35	0.14–0.91	0.031	1.69	0.45–6.45	0.439
EGFR status	Exon 19 del/others	0.31	0.12–0.79	0.014	0.28	0.10–0.76	0.013
IL-6 level*	Lower/higher	0.57	0.25–1.33	0.200	0.47	0.16–1.33	0.153

Note: *Patients were dichotomized based on the median value (1.7 pg/mL) of posttreatment plasma IL-6.
Abbreviations: CI, confidence interval; EGFR, epidermal growth factor receptor; HR, hazard ratio; IL, interleukin.

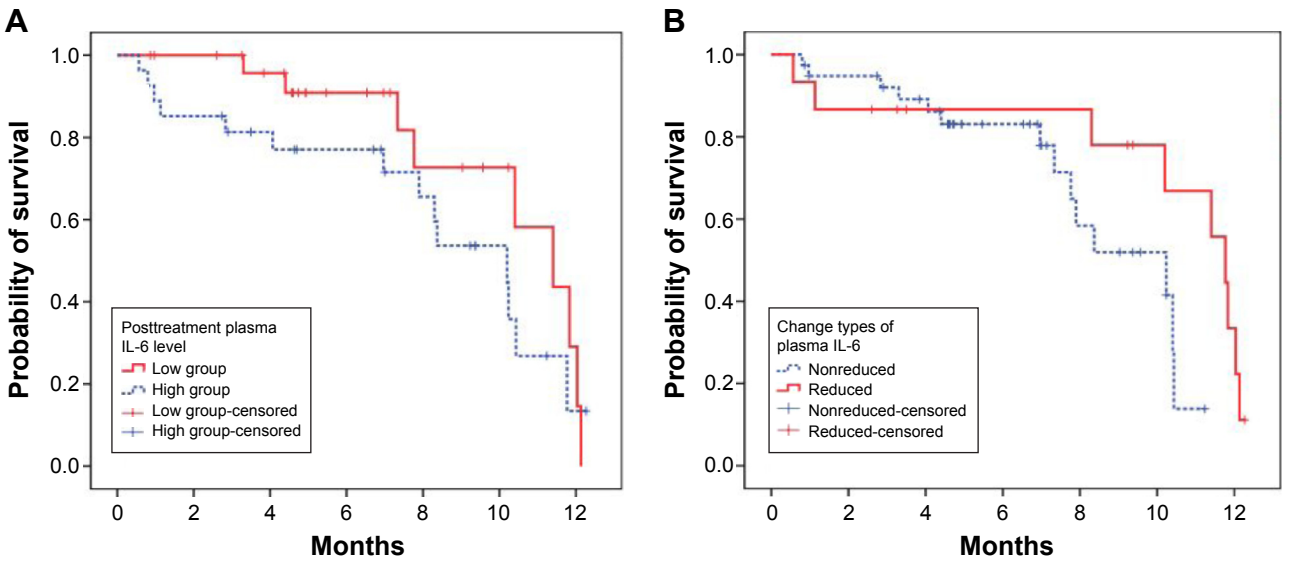

Figure S2 The prognostic value of IL-6 for progression-free survival.
Note: Higher level (≥1.7 pg/mL) versus lower level (<1.7 pg/mL) of IL-6 (**A**) and IL-6 reduced versus nonreduced after gefitinib treatment (**B**).
Abbreviation: IL, interleukin.

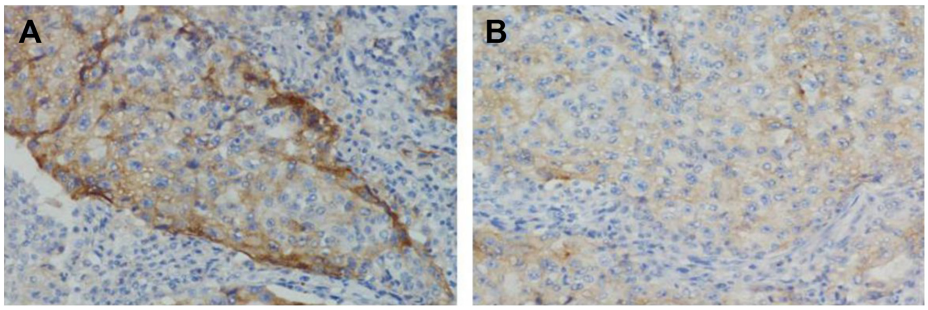

Figure S3 The expression of PD-L1 by immunohistochemical staining with a membranous pattern.
Note: Baseline staining (**A**) and 4 weeks after gefitinib treatment (**B**).
Abbreviation: PD-L1, programmed cell death ligand-1.

Table S3 The levels of peripheral lymphocytes at baseline or after 4 weeks of gefitinib treatment

Lymphocyte subgroup	Percent at baseline (%)	Percent after 4 weeks (%)	*P*-value
$CD4^+$ T cells	36.5±9.1	34.4±9.2	0.003
$CD8^+$ T cells	22.3±7.3	22.6±7.4	0.48
CD4/CD8 ratio	1.8±0.7	1.7±0.7	0.08
NK cells	21.3±10.0	23.7±10.9	0.005
$CD4^+CD25^+$ T cells	13.7±5.3	14.8±7.0	0.18

Note: The results of percent of lymphocytes of PBMC are presented as mean ± standard deviation.
Abbreviations: NK, natural killer; PBMC, peripheral blood mononuclear cell.