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Potential influence of interleukin-6 on the therapeutic effect of gefitinib in patients with advanced non-small cell lung cancer harbouring EGFR mutations

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A B S T R A C T

Although epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) are a key therapy used for patients with EGFR-mutant non-small cell lung cancer (NSCLC), some of whom do not respond well to its therapy. Cytokine including IL-6 secreted by tumour cells is postulated as a potential mechanism for the primary resistance or low sensitivity to EGFR-TKIs. Fifty-two patients with advanced EGFR-mutant NSCLC who had received gefitinib were assessed retrospectively. The protein expression of IL-6 in the tumour cells was assessed by immunostaining and judged as positive if $\geq$ 50 of 100 tumour cells stained positively. Of the 52 patients, 24 (46%) and 28 (54%) were defined as IL-6-postitive (group P) and IL-6-negative (group N), respectively. Group P had worse progression-free survival (PFS) than that of group N, which was retained in the multivariate analysis (hazard ratio: 2.39; 95 %CI: 1.00–5.68; $p < 0.05$). By contrast, the PFS after platinum-based chemotherapy did not differ between groups P and N ($p = 0.47$). In cell line-based model, the impact of IL-6 on the effect of EGFR-TKIs was assessed. The combination of EGFR-TKI and anti-IL-6 antibody moderately improved the sensitivity of EGFR-TKI in lung cancer cell with EGFR mutation. Interestingly, suppression of EGFR with EGFR-TKI accelerated the activation of STAT3 induced by IL-6. Taken together, tumour IL-6 levels might indicate a subpopulation of EGFR-mutant NSCLC that benefits less from gefitinib monotherapy.

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1. Introduction

Following the discovery of epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer (NSCLC), first-generation EGFR-tyrosine kinase inhibitor (EGFR-TKI) (gefitinib or erlotinib) therapy was shown to yield better progression-free survival (PFS) (medians: 10.8–5.7 vs. 5.8–5.2 months, respectively) and comparable overall survival (OS) (medians: 30.5–21.6 vs. 23.6–21.9 months, respectively), compared with standard cytotoxic chemotherapy, in chemo-naïve patients with EGFR-mutant tumours. Furthermore, afatinib, a 2nd-generation EGFR-TKI, yielded a better OS (median: 27.3 vs. 24.3 months, respectively; $p = 0.037$) and PFS (median: 11.1 vs. 6.9, respectively; $p = 0.001$) than those using standard cytotoxic chemotherapy [1–8]. These results suggest that EGFR-TKIs are key agents in EGFR-mutant tumours; however, resistance to this promising agent is inevitably encountered during its therapy [9,10], suggesting the limited efficacy of EGFR-TKIs.

Overexpression of interleukin (IL)-6 in tumour cells is postulated to be a potential mechanism for such primary resistance or low sensitivity to EGFR-TKI in preclinical models [11,12]; an

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increased IL-6 secretion induced by the activation of transforming growth factor (TGF)-beta signaling weakened sensitivity to EGFR-TKIs in EGFR-mutated NSCLC cells [11]. Furthermore, reduction of the tumour IL-6 level by an IL-6-neutralizing antibody combined with EGFR-TKI successfully led to the tumour growth inhibition in EGFR-mutated, IL-6 overexpressing NSCLC cells resistant to EGFR-TKI monotherapy. Other researchers reported that mutant EGFR could activate the gp130/JAK/STAT3 pathway by means of IL-6 upregulation in primary human lung adenocarcinoma cells [12].

To date, the role of IL-6 in the sensitivity to EGFR-TKIs in EGFR-mutant NSCLC has not yet been fully assessed clinically. Based on preclinical study results, we evaluated here, using clinical specimens, whether the tumor IL-6 level can affect the effect of EGFR-TKI therapy in NSCLC patients with EGFR-mutant tumours.

2. Methods

2.1. Patients and treatments

We retrospectively assessed patients registered in the database during the study period, from 2005 to 2013, at our hospital. The patients included in the study were those who 1) had locally advanced, metastatic or postoperative recurrent NSCLC, 2) had EGFR-mutant tumours, 3) were deemed unfit for curative surgery and irradiation, 4) underwent gefitinib monotherapy (250 mg/body/day, orally), irrespective of the line of treatment, 5) had no prior EGFR-TKI therapy, and 6) had tumour samples (formalin-fixed, paraffin-embedded [FFPE]) that were sufficient for IL-6 immunostaining. The tumour EGFR mutation status was assessed by direct sequencing or the polymerase chain reaction (PCR) clamp method, as described previously [9,10]. This study was approved by the institutional review board of Okayama University (No. 1506-002).

2.2. Data abstraction

The following information regarding the patients was retrospectively extracted from medical charts: age, gender, performance status (PS), disease stage, smoking status, tumour histology, and type of EGFR mutations. For this study, we basically assessed the tumour response every 2 or 3 months in each patient according to the Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1. The diagnosis of brain metastasis was based on magnetic resonance imaging or computed tomography.

2.3. Immunostaining of IL-6 in tumour and serum samples

Serial 4-μm-thick sections were sliced from FFPE tissue blocks and subjected to haematoxylin and eosin staining and immunohistochemistry. We stained each tumour specimen with the anti-human IL-6 antibody (mouse monoclonal antibody; Leica Biosystems, Newcastle Upon Tyne, UK; product code: NCL-L-IL6), using the Leica BOND-MAX automated immunostainer (Leica Microsystems, Wetzlar, Germany) [13,14]. This antibody was applied at a 1:200 dilution for 15 min at room temperature. Sections were scored independently by two physicians (YK and TT) and one pathologist (TT), without any information about the clinical course and outcome of the patients; the intensity of staining was scored negative or positive if the proportion of stained cells was 0–50% or 51-100% out of 100 cells, referring to prior relevant criteria [15]. We defined group P as those with IL-6-positive tumours and group N as those with IL-6-negative tumours.

Peripheral blood samples, collected from 11 (21%) of the 52 patients before receiving gefitinib treatment, were stored at −80 °C until use. The serum IL-6 level was measured by chemiluminescent enzyme immunoassay (CLEIA) using the Quanti Glo Human IL-6 Immunoassay 2nd Generation kit (R&D Systems, Inc., Minneapolis, MN, USA).

2.4. IL-6 expression and sensitivity to gefitinib and/or anti-IL-6 antibody in preclinical models

To check the expression levels of IL-6 protein in 10 EGFR-mutant NSCLC cell lines [16,17], the supernatant IL-6 levels in the cell culture were measured by the enzyme-linked immunosorbent assay (ELISA), using Human IL-6 Quantikine ELISA Kit D6050 (R&D Systems, MN, USA). Sensitivities of each NSCLC cell to gefitinib and anti-IL-6 antibody (Biolegend, CA, USA) were evaluated by MTT assay.

2.5. Immunoblotting analysis

Cells and frozen tissue were lysed in a radio-immunoprecipitation assay buffer [1% Triton X-100, 0.1% SDS, 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L β-glycerolphosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate-containing protease inhibitor tablets (Roche Applied Sciences GmbH, Mannheim, Germany)]. Proteins were separated by electrophoresis on polyacrylamide gels, transferred onto nitrocellulose membranes, and probed with specific antibodies followed by detection with Enhanced Chemiluminescence Plus (GE Healthcare Biosciences, Piscataway, NJ, USA).

2.6. Statistical analyses

We primarily assessed the causal relationship between the tumour IL-6 expression level and therapeutic efficacy, particularly progression-free survival (PFS). Differences between groups were assessed using the chi-squared test. PFS was defined as the duration from the date of initiation of EGFR-TKI treatment or cytotoxic chemotherapy to the date of documented relapse. OS was defined as the duration from the date of initiation of EGFR-TKI treatment to the date of death. PFS and OS curves were constructed using the Kaplan–Meier product-limit method. Differences between Kaplan–Meier curves were evaluated by the log-rank test. The logistic regression model and Cox proportional regression model were applied to the overall response and survival analysis, respectively, to evaluate the potential impact of confounders on the association, using the forward and backward stepwise method, with threshold p-values of 0.05 and 0.20 for entry into and removal from the model, respectively. Age, gender, smoking status, disease stage, Eastern Cooperative Oncology Group (ECOG) PS, type of EGFR mutations, and lines of gefitinib monotherapy were adjusted for in the multivariate analysis. All p-values were obtained from two-sided tests, and significance was set at $p < 0.05$. Statistical analyses were conducted using STATA software (ver. 13; StataCorp, College Station, TX, USA).

3. Results

3.1. Patient demographics and gefitinib treatment

Fig. 1 shows the study flow. We identified 52 patients with advanced NSCLC harboring EGFR mutations for further assessments. The patient demographic information is listed in Table 1. Most of the patients had a PS of 0–1 (n = 48, 92.3%) and exhibited adenocarcinoma histology (n = 49, 94.2%). Regarding the type of EGFR mutation, there were 29 patients with exon 19 tumours, 18 exon 21 tumours, and five other mutations.

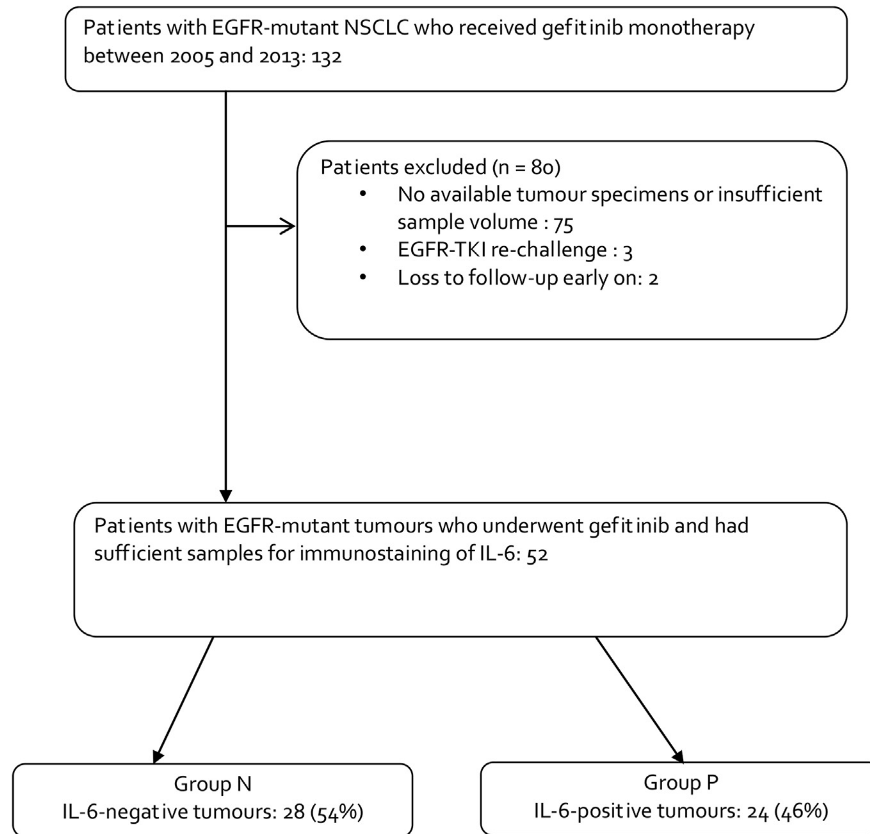


Fig. 1. Flow chart showing the progress of the study.
Fourteen of 28 and 11 of 24 received platinum-based chemotherapy in Group N and P, respectively.
Abbreviations: EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; NSCLC, non-small cell lung cancer; IL-6, interleukin 6.

Table 1
Clinicopathological characteristics.

Variable	Total no. of Patients (N = 52)	Group N		Group P	
		IL-6 negative (N = 28)		IL-6 positive (N = 24)	
		No.	%	No.	%
Age (median; range)		67 (40-81)	–	66 (36-85)	–
Sex					
Male	24	11	39	13	54
Female	28	17	61	11	46
Smoking history					
ever	28	13	46	15	63
never	24	15	54	9	38
Stage					
postoperative recurrence	29	17	61	12	50
III	1	0	0	1	4
IV	22	11	39	11	46
Histology					
Ad	49	28	100	21	88
Sq	3	0	0	3	13
Type of EGFR mutation					
exon 19	29	17	61	12	50
exon 21	18	9	32	9	38
other	5	2	7	3	13
PS					
0-1	48	27	96	21	88
2	4	1	4	3	13
Line of gefitinib therapy					
first-line	28	16	57	12	50
second-line	21	10	36	11	46
third-line	3	2	7	1	4

Values are presented as n (%), except for age.
Abbreviations: EGFR, epidermal growth factor receptor; PS, performance status; IL-6, interleukin 6; Ad, adenocarcinoma; Sq, squamous cell carcinoma.

At the time of this analysis, 29 (55.8%) of the 52 patients developed PD following treatment. Of the 52 patients, 25 (48.1%) were treated with platinum-based chemotherapy before or after gefitinib treatment during their treatment course (Supplemental Table 1).

3.2. Tumour IL-6 expression

The proportion of IL-6-positive cells among the tumour cells of each patient ranged from 0% to 97.5% (median: 39.6%). As described above, expression was defined to be positive if ≥ 50% of 100 tumour cells stained positively; 24 (46%) and 28 (54%) of the 52 patients were judged as IL-6-positive (group P) (Fig. 2A–C) and IL-6-negative (Group N) (Fig. 2D–F), respectively (Fig. 2G). The characteristics of the two groups were somewhat different (Table 1); Group P included lower proportions of female patients with poor PS, never-smokers, and non-squamous cell carcinoma, despite no statistically significant differences.

3.3. IL-6 and survival

Among the whole 52 patients, 26 achieved partial response (50%; 95% confidence interval [CI]: 36-64%). Group P had low response rate as compared with Group N (9 [38%] of 24 v 17 [61%] of 28; $p = 0.095$). Multivariate analysis also revealed a similar trend with odds ratio of 0.39 and its 95% CI of 0.13–1.20 ($p = 0.098$).

The median follow-up time of the patients was 50.9 months. In the whole 52 patients, 6-month-PFS rate and median PFS were 84.1% and 18.0 months, respectively, whereas OS rate at 1, 2 and 3 years were 88.5%, 72.9%, and 67.8%, respectively. When stratified by the IL-6 staining, group P experienced a worse PFS than that of group N (74.5% vs. 92.4% at 6 months, and median of 13.5 months vs. not reached, respectively; $p = 0.045$) (Fig. 3A), and the significance was retained in the multivariate analysis (hazard ratio [HR]:

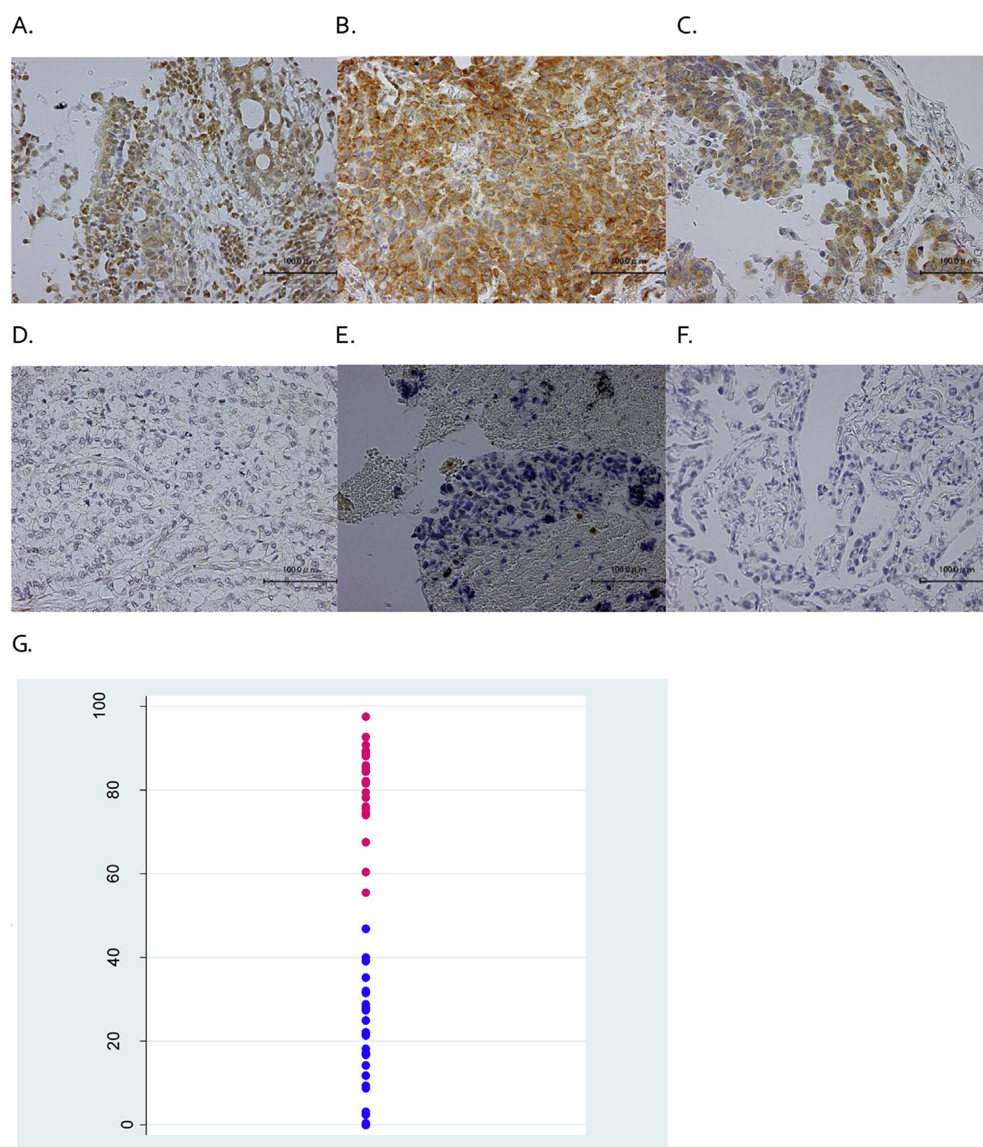


Fig. 2. Tumour IL-6 immunostaining.
A-F. Representative microscopic findings of IL-6 staining (A-C; Group P, × 200, D-F; Group N, × 200).
G. Proportions of IL-6-positive cells among the tumour cells of each patient. Red and blue plots represent groups P and N, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

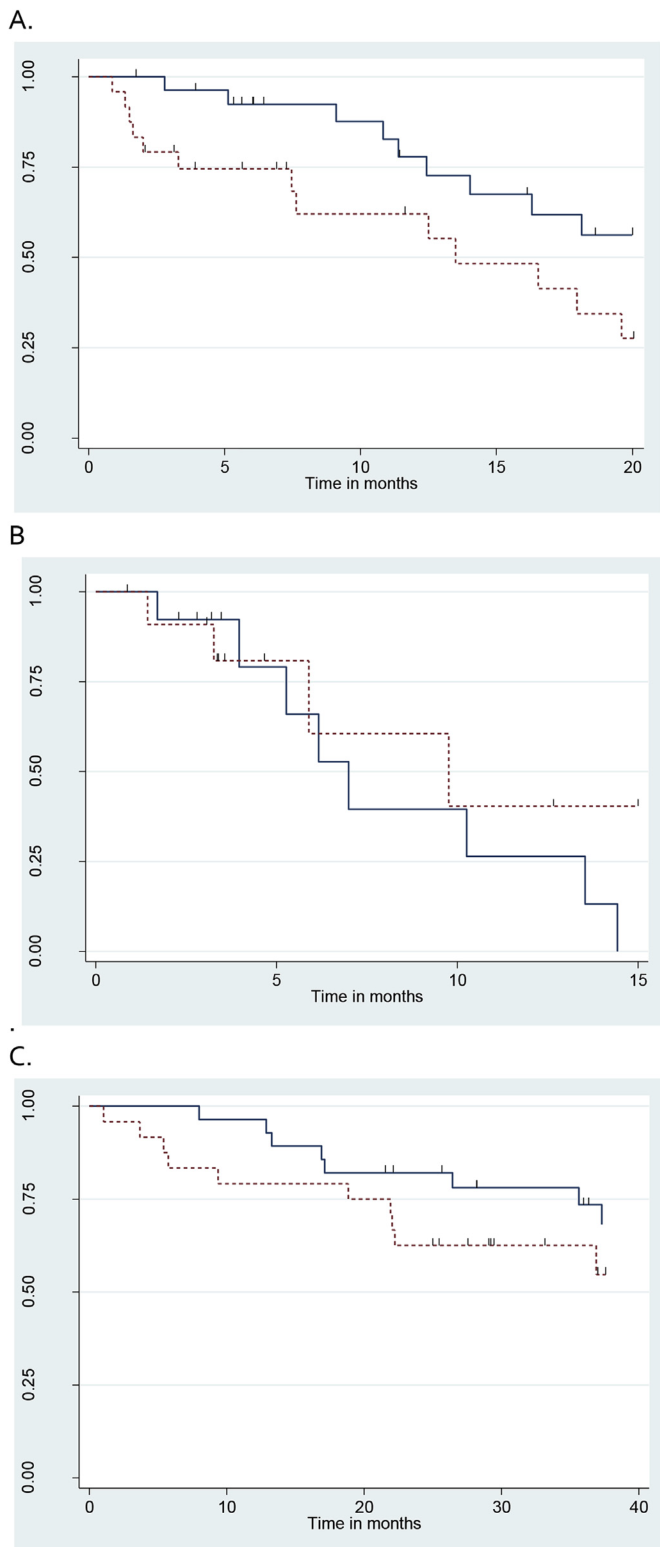


Fig. 3. Survival curves.
A. Dotted and solid lines represent the progression-free survival (PFS) curves for gefitinib monotherapy in groups P and N, respectively.
B. Dotted and solid lines represent the PFS curves of the platinum-based chemotherapy in groups P and N, respectively.
C. Overall survival curves in groups P and N, represented by dotted and solid lines, respectively; Overall survival was defined as the duration from the initiation of EGFR-TKI treatment until death.

2.39; 95% CI: 1.00–5.68; p = 0.049).

Next, we examined the relationship between the tumour IL-6 status and PFS after platinum-based chemotherapy in 25 patients of the same cohort (Supplemental Table 1). Eleven (44%) and fourteen (56%) of 25 patients who received the platinum-based chemotherapy during their treatment course possessed IL-6-positive and -negative tumours, respectively. The PFS after platinum-based chemotherapy did not differ between groups P and N (median; 9.8 vs. 7.0 months, respectively; p = 0.475) (Fig. 3B).

The OS curves according to IL-6 expression status are illustrated in Fig. 3C. Group P tended to have a worse OS than that of group N (79.2% vs. 96.4%, 62.5% vs. 82.1%, and 62.5% vs. 73.5%, at 1, 2, and 3 years, respectively), despite the lack of a significant difference (p = 0.342).

3.4. Potential impact of the tumour IL-6 cut-off level on PFS

We performed sensitivity analysis to assess potential variations and uncertainty regarding the categorisation of IL-6. We chose an IL-6 score cut-off level of 50% (Supplemental Table 2), which provided an almost identical HR for PFS as those observed when 20%, 25%, 30%, 35%, 40%, 45%, 55%, 60%, 65%, 70% and 75% cut-off levels were used. This observation suggests that the use of an IL-6 cut-off level of 50% in our retrospective study was robust for detecting subpopulations that would benefit less from gefitinib monotherapy.

3.5. Serum IL-6 level by CLEIA

The serum IL-6 level was assessed in 11 (21%) of 52 patients. The serum IL-6 level ranged from 0.75 to 23.80 pg/ml (median: 2.90 pg/ml) (Supplemental Fig. 1A), which rarely correlated with that in the tumour cells (regression coefficient: 1.69, p = 0.294) (Supplemental Fig. 1B). Additionally, we failed to find any significant relationship between the serum IL-6 level and PFS after gefitinib monotherapy (regression coefficient: −0.53, p = 0.055) (Supplemetal Fig. 1C).

3.6. IL-6 expression and sensitivity to gefitinib and/or anti-IL-6 antibody in preclinical models

Among 10 EGFR-mutated NSCLC cells, the 11–18 cells harboured high IL-6 protein expression, whilst PC-9 cells had a low expression (Fig. 4A). Both of PC-9 and 11–18 had sensitivity to gefitinib, but the latter cells possessed lower sensitivity than PC-9 cells (IC 50; 131 nM vs. 30 nM, respectively) (Fig. 4B and C). Unexpectedly, the effect of monotherapy with anti-IL-6 antibody was observed neither in 11–18 nor PC-9 cells *in vitro* (Fig. 4D and E). In contrast, the combination of an anti-IL-6 antibody with EGFR-TKIs, gefitinib, erlotinib or rociletinib, showed moderate improvement on the inhibitory effect of cell proliferation in 11–18 cells (Fig. 4F and Supplemental Fig. 2A). The combination effect was observed neither in PC-9 cells nor H1975 cells with low level expression of IL-6 protein (Supplemental Fig. 2B and 2C). The combination of an anti-IL-6 antibody with EGFR-TKIs had no additive inhibitory effect in A549 with KRAS mutations (Supplemental Fig. 2D). In this model, activation of STAT3, downstream of IL-6 signaling pathway, was inhibited with anti-IL-6 antibody in 11–18 cells (Fig. 5A). Consistent with the previous reports [11,12,18], an addition of recombinant IL-6 activated phosphorylation of STAT3 in PC-9 cells, which was further accelerated by suppression of EGFR with gefitinib (Fig. 5B).

4. Discussion

The present small-scale study demonstrated that the proportions of IL-6-positive cells among tumour cells in the patients

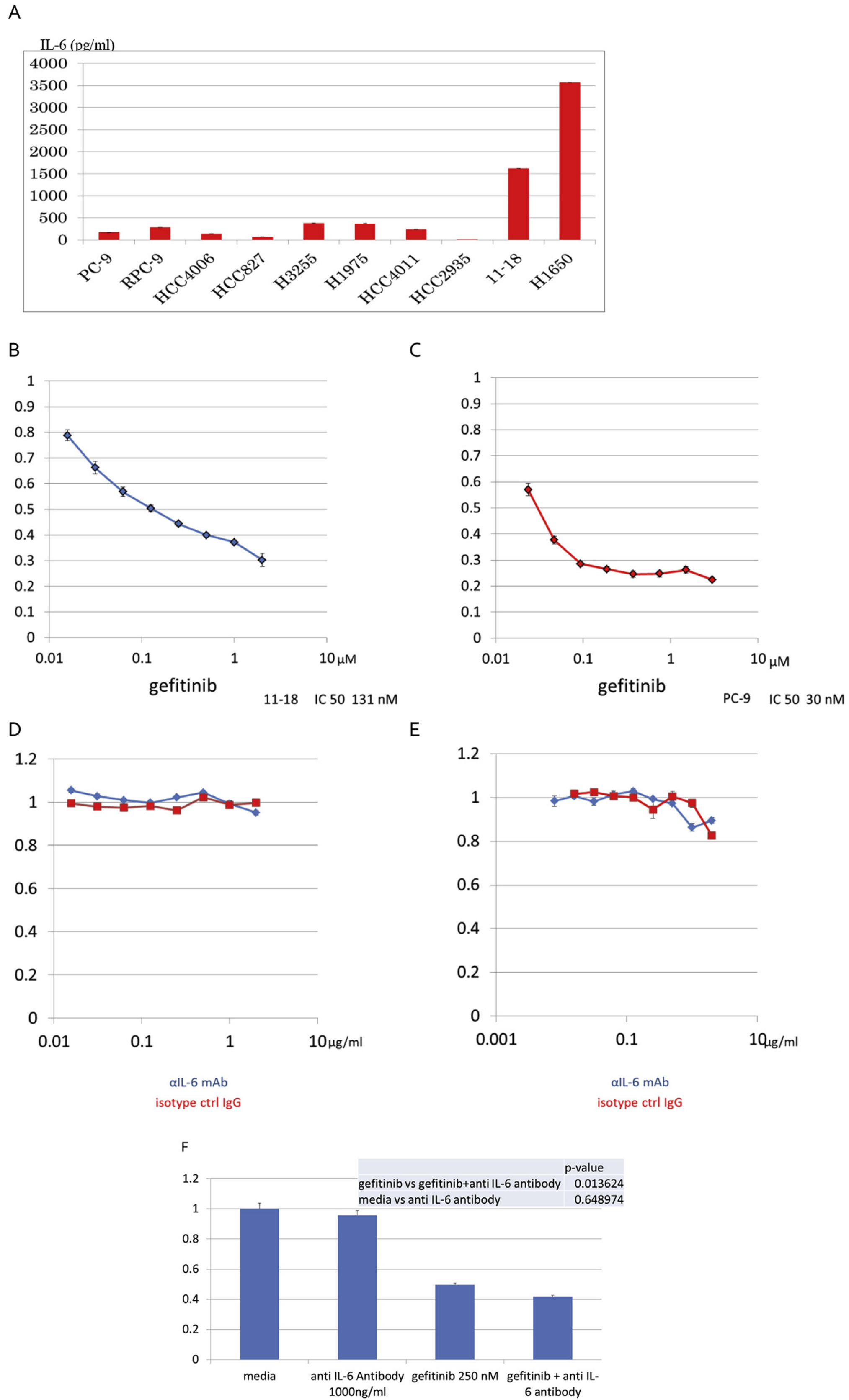


Fig. 4. IL-6 and sensitivity of EGFR-TKI in EGFR-mutant tumour cells.
A. Expression of IL-6 in the supernatant of culture medium.
B. The sensitivity of gefitinib in 11–18 cells.
C. The sensitivity of gefitinib in PC-9 cells.
D. The sensitivity of anti-IL-6 antibody (blue line) and isotype control IgG (red line) in 11–18 cells.

ranged from 0% to 97.5% (median: 39.6%). Even among those with EGFR-mutant tumours whose standard therapy was EGFR-TKI, Group P exhibited both low response rate (38% v 61%; $p = 0.095$) and worse PFS (75% v 92% at 6 months; $p = 0.045$), which was retained in the multivariate analysis (odds ratio: 0.39, 95% CI: 0.13–1.20; $p = 0.098$ and HR: 2.39, 95% CI: 1.00–5.68; $p = 0.049$, respectively). By contrast, PFS in the platinum-based chemotherapy did not differ between groups P and N ($p = 0.475$). In the preclinical model, the NSCLC cell line (11–18) with high IL-6 level showed relatively low sensitivity to gefitinib, but the combination of an anti-IL-6 antibody with gefitinib improved the sensitivity (Fig. 4B, C and 4F). Furthermore, suppression of EGFR accelerated the activation of STAT3 induced by IL-6 in PC-9 cells (Fig. 5B).

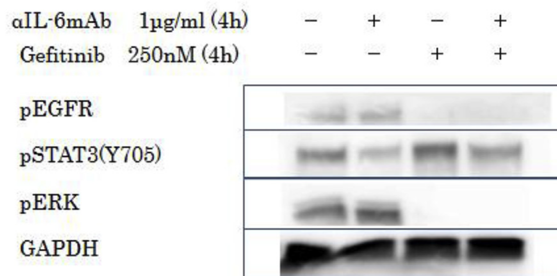
We found a worse PFS after EGFR-TKI monotherapy in group P (IL-6-positive tumours) (Fig. 3A), supporting previous preclinical results of limited growth inhibition by EGFR-TKI alone via protection from apoptosis in EGFR-mutant cell lines overexpressing IL-6 [11]. These NSCLC cells were dependent on the IL-6 axis for long-term proliferation/survival [11,12]. By contrast, PFS after platinum-based chemotherapy did not differ between groups P and N (Fig. 3B), indicating that the tumour IL-6 expression might clinically affect the effect of EGFR-TKI monotherapy. All of these results suggest that the inhibition of IL-6 expression is additionally needed to inhibit tumour growth, particularly in patients harboring EGFR-mutant tumours with high IL-6 expression. Our clinical and preclinical data may lead to a novel strategy for overcoming resistance or low sensitivity to EGFR-TKI therapy [19,20]; that is, an IL-6 neutralizing antibody might modify its sensitivity via a decrease in IL-6-mediated signaling, particularly in IL-6-positive, EGFR-mutant NSCLC, hopefully leading to further survival improvement.

We were very interested in the potential role of serum IL-6 levels in predicting tumour IL-6 expression levels and outcome. Unfortunately, the serum IL-6 level did not correlate to either (Fig. 4B), but an elevated serum IL-6 level was related to disease severity and outcome in several cancers, including prostate, ovarian, and pancreatic cancers [21]. This discordance could arise from the following. First, the serum level is readily affected by any inflammation, in addition to the tumour expression level. Indeed, IL-6, a pleiotropic cytokine with varied systemic functions, was originally shown to play a major role in inflammatory processes [20]. Second, in our series, the timing of obtaining serum and tumour samples was not uniform. Third, our series was restricted by a small patient population, preventing definitive results. Thus, a future, well-designed, large-scaled cohort study is warranted.

This study possesses several limitations. We used a small sample and a retrospective design; thus, the study is suitable only for hypothesis generation. The clinicopathological characteristics of the two groups were somewhat different, although the multivariate analysis we conducted possibly adjusted the confounding factors. Additional limitations included the low PFS maturity of 55.8% and the lack of a uniform procedure for patient follow-up. Furthermore, we did investigate here IL-6 expression level but not TGF-beta level or relevant signaling expressions. The most critical should be the lack of validation in the cut-off level of tumour IL-6 expression. We applied the previous relevant criteria to the current study [15]. Also, though there is the statistical difference in the PFS between the two groups treated with gefitinib, both groups appeared to have benefited from the medication. Indeed, even for Group P, the median PFS is 13.5 months, suggesting a prolonged response to the therapy. Therefore, we still question whether differentiating IL-6 expression at the 50% cut-off point would really ultimately make a difference in clinical decision making. More work needs to be done for this issue in the future, using a larger prospective cohort data. Furthermore, in this study, we could not describe whether there was any heterogeneity in regards to IL-6 expression between primary tumours and metastatic sites, because we did not conduct the biopsies from multiple sites. Regarding the serum IL-6 analysis by CLEIA, the number of samples was too small to make any claims and it was not split into the two groups either for analysis. As for efficacy data of gefitinib monotherapy in the 52 patients, overall response rate, we did have information about PFS but not overall response rate. All these strongly suggest that our results should be interpreted with caution, but our results raise critical issues to be resolved regarding how blocking IL-6 expression levels can potentially overcome resistance to EGFR-TKI.

In conclusion, patients in group P benefited less from gefitinib therapy, even though this has been a standard therapy, even for such a population. Our pilot preclinical data also revealed IL-6 expression could have some roles in the sensitivity to gefitinib.

A.



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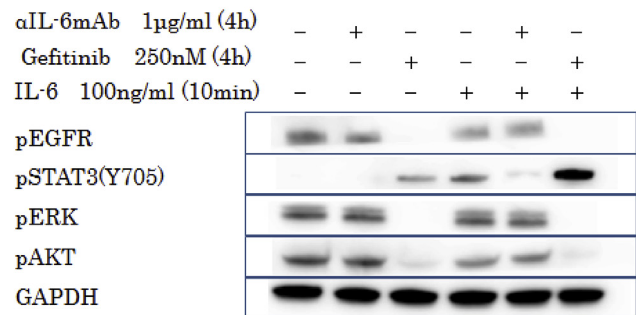

Fig. 5. IL-6/STAT3 axis and EGFR-TKI in lung cancer with EGFR mutations.
A. Anti-IL-6 antibody inhibited activation of STAT3 in 11–18 cells.
B. Suppression of EGFR with gefitinib accelerated the activation of STAT3 induced by IL-6 in PC-9 cells.

E. The sensitivity of anti-IL-6 antibody (blue line) and isotype control IgG (red line) in PC-9 cells.
F. The sensitivity of combination of anti IL-6 antibody and gefitinib in 11–18 cells.
Abbreviations: NSCLC, non-small cell lung cancer; IL-6, interleukin 6. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This finding might support the potential role of IL-6 in active cancers and provide a rationale for targeted therapeutic investigations.

Conflict of interest statement

KH received honoraria outside the current work from AstraZeneca, Ono Pharmaceutical, Astellas, Novartis, BMS, MSD, Eli Lilly Japan, Daiichi-Sankyo Pharmaceutical, Boehringer-Ingelheim, Nihon Kayaku, Taiho Pharmaceutical, and Chugai Pharmaceutical. KH also has received research funding outside the current work from AstraZeneca, Boehringer-Ingelheim, Ono Pharmaceutical, Astellas, Novartis, BMS, Eli Lilly Japan, MSD, and Chugai Pharmaceutical. YK received honoraria from Eli Lilly and Daiichi-Sankyo Pharmaceutical. KO received honoraria from AstraZeneca, Ono pharmaceutical and Chugai Pharmaceutical. KO also received research funding from Novartis Pharmaceuticals, Eli Lilly, and Boehringer-Ingelheim. KK received honoraria from Eli Lilly Japan, Nihon Kayaku, AstraZeneca, Daiichi-Sankyo Pharmaceutical, Chugai Pharmaceutical, Taiho Pharmaceutical, and Sanofi-Aventis. All other authors declare no conflicts of interest regarding this study.

The role of funding sources

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Acknowledgments

The interpretation and reporting of the data are the sole responsibility of the authors. TT, YK and KH had full access to all of the data in the study, were responsible for the integrity of the data and accuracy of the data analysis, and contributed to the study design, data collection, analyses, and manuscript writing. All other co-authors contributed to manuscript writing. This study has been conducted with support from the Center for Innovative Clinical Medicine, Okayama University Hospital.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2017.10.175.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2017.10.175.

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