Clinical and biological significance of erlotinib therapy after pemetrexed in non-small cell lung cancer with wild-type EGFR

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Pemetrexed is a multi-targeted anti-folate agent that confers favorable benefits to patients with non-small cell lung cancer (NSCLC). However, the optimal use including treatment schedule of pemetrexed and other drugs in clinical practice remains to be determined, particularly for NSCLC with wild-type *epidermal growth factor receptor (EGFR)*. The present study investigated a potential therapeutic strategy for NSCLC patients with wild-type *EGFR* who were treated with pemetrexed. To identify factors associated with a survival, medical record data from 130 patients were retrospectively reviewed, using the Kaplan-Meier method with log-rank test. Factors identified in the clinical analysis were further investigated within *in vitro* studies. Patients who underwent the treatment schedule of erlotinib at the time of progression after pemetrexed-based chemotherapy prolonged overall survival, compared to those treated with other schedules (*p*=0.010; hazard ratio, 0.418). This survival benefit was also observed in the treatment schedule of pemetrexed monotherapy and subsequent erlotinib (*p*=0.008; hazard ratio, 0.220). As a treatment at the time of progression after pemetrexed-based chemotherapy, erlotinib conferred a survival benefit when compared to docetaxel (*p*=0.024; hazard ratio, 0.377). The cell growth assay confirmed that treatment with pemetrexed followed by erlotinib significantly inhibited proliferation of NSCLC cells regardless of *EGFR* mutation status. In conclusion, use of erlotinib at the time of progression after pemetrexed therapy confers a survival benefit in NSCLC patients with wild-type *EGFR*. The result of this study provides an important clue to the optimal treatment schedule for NSCLC.

Key words: erlotinib, non-small cell lung cancer, pemetrexed, survival benefit, treatment schedule, wild-type EGFR

Lung cancer is the leading cause of cancer death worldwide. Approximately 80% of lung cancer consists of non-small cell lung cancer (NSCLC), more than three-quarters of which is diagnosed at an advanced stage [1]. Drug therapy, the standard of care for advanced lung cancer, is determined according to the molecular profile of the tumor. At present, epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) and anaplastic lymphoma kinase (ALK)-TKIs are preferentially used for NSCLC with mutant *EGFR* and *ALK* rearrangements, respectively. However, more than half of NSCLC with negative or unknown oncogenic drivers require cytotoxic chemotherapy. Based on the guidelines of the American Society of Clinical Oncology (ASCO) and the National Comprehensive Cancer Network (NCCN), platinum-based doublets are recognized as the standard first-line chemotherapy for these patients, and monotherapy

with docetaxel, pemetrexed, or erlotinib is proposed as the standard second-line treatment for patients in whom the first-line chemotherapy is ineffective [2]. In fact, studies have demonstrated that the efficacy was similar when comparing each of these first-line chemotherapies with platinum plus third-generation cytotoxic drugs [3, 4], and there were no significant differences in the therapeutic effects of docetaxel, pemetrexed, and erlotinib in patients with previously treated NSCLC [5-8]. In clinical practice, these therapies are currently used in order, and further treatment is sometimes implemented according to patient preference, as the effectiveness of pemetrexed in treatment beyond second-line chemotherapy has been reported [9, 10]. On the other hand, accumulating evidence shows that treatment schedule of anti-tumor drugs against NSCLC may have an impact on the therapeutic effect [10-14]. A comprehensive survey of drug use throughout

a treatment course is therefore required to assess the survival benefit for patients with NSCLC in clinical practice.

Of the third-generation cytotoxic drugs, pemetrexed is a multi-targeted anti-folate that inhibits thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyltransferase [15]. Previous phase III trials showed that pemetrexed-based chemotherapy provided favorable outcomes in NSCLC, especially for those with the non-squamous cell type, in response to the use of pemetrexed as first- and second-line treatments [4, 5]. Based on clinical evidence, pemetrexed is currently one of the most commonly used drugs against advanced NSCLC. However, there is very limited information about the optimal use including a treatment schedule for pemetrexed. Along with concerns regarding the cost of pemetrexed [16, 17], determination of an optimal treatment strategy for pemetrexed may help improve therapeutic outcomes as well as the cost-effectiveness of pemetrexed therapy, leading to advances in management for NSCLC. Parameters that remain to be determined in this regard include identification of prognostic markers and determination of which drug is the optimal partner to pemetrexed.

To address these issues, we conducted a retrospective analysis of 130 patients with wild-type *EGFR* among 201 NSCLC patients who had undergone pemetrexed-based chemotherapies, and then used *in vitro* studies to confirm the clinical results. The present study advocates a specific treatment model in NSCLC patients with wild-type *EGFR* who are treated with pemetrexed according to practical use, and warrant further prospective studies.

Patients and methods

Study population. This study enrolled 201 patients with advanced NSCLC who were treated with pemetrexed-based chemotherapies at the Saitama Medical University International Medical Center from 2009 to 2010. Of these patients, 130 patients (65%) who had wild-type *EGFR* were subjected to the analysis. The presence of *EGFR* mutations was examined using the peptide nucleic acid-locked nucleic acid polymerase chain reaction clamp method [18]. The medical records of these patients were retrospectively analyzed after approval of the institutional review board.

Treatment schedules. All patients were treated with pemetrexed, carboplatin/pemetrexed, cisplatin/pemetrexed, or carboplatin/pemetrexed/bevacizumab. Pemetrexed was administered as 500 mg/m^2 every 3 weeks in each regimen, and dose reduction or cessation of the treatment was decided by attending physicians based on the onset of adverse events and disease progression. Maintenance pemetrexed monotherapy was employed for patients eligible after more than four courses of combination chemotherapies with platinum. Treatments administered just before and after treatment regimens including pemetrexed were investigated to evaluate the impact of the treatment schedule. These treatments consisted of one or more of the following drugs: cisplatin, carboplatin, paclitaxel,

docetaxel, gemcitabine, vinorelbine, bevacizumab, tegafur/gimeracil/oteracil potassium (S1), gefitinib, and erlotinib.

Clinicopathological characteristics. Patients were classified into two groups according to age (≥ 70 years, < 70 years). Performance status was assessed by the criteria of Eastern Cooperative Oncology Group (ECOG). Smoking status was classified into a "never smoker" group and a smoker group; the latter group was further divided into a light smoker group (0-20 pack-year) and a heavy smoker group (> 20 pack-year). Using the Response Evaluation Criteria in Solid Tumors (RECIST guideline version 1.1), maximal effects were classified into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) [19]. CR+PR was defined as objective response (OR), and CR+PR+SD was defined as disease control (DC). Parameters evaluated included the objective response rate (ORR), the disease control rate (DCR), and the time from the initiation of the corresponding treatment to the confirmation of disease progression (progression-free survival; PFS) or to death of the patient (overall survival; OS). Adverse events were evaluated according to the Common Terminology Criteria for Adverse Events version 4.0 (CTCAE v4.0).

Cell cultures. Lung cancer cell lines were obtained from the sources as follows: A549 cells (adenocarcinoma without *EGFR* mutations) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan); PC9 cells (adenocarcinoma with delE746-A750 activating *EGFR* mutation) were purchased from Immuno-Biological Laboratories (Gunma, Japan); and NCI-H1975 cells (adenocarcinoma with both L858R activating and T790M resistant *EGFR* mutations) were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) in a humidified chamber with air containing 5% CO_2.

Cytotoxic assay. The cytotoxic activity of each drug was evaluated using the Cell Counting Kit-8 (CCK-8) (Dojindo, Kumamoto, Japan). According to the manufacturer's instructions, each cell type was seeded into 96-well dishes at 0.3 × 10^4 cells/well. After incubation for 24 h, cells were treated with each drug for 72 h. Thereafter, 10 μL of CCK-8 was added to each well, followed by further incubation for 1.5–2 h at 37°C. The optimal density of each well was measured at 450 nm on a 1420 multilabel counter ARVO MX (PerkinElmer, Waltham, MA, USA). The cell-growth inhibitory rate was expressed as the percentage of cell viability calculated as follows: [(mean absorbance of drug-treated wells minus mean absorbance of blank wells)/(mean absorbance of control wells minus mean absorbance of blank wells)] × 100. IC$_{50}$ was defined as the drug concentration that induced 50% of growth inhibition.

In vitro cell growth assay. To evaluate the *in vitro* therapeutic efficacy of drugs administered before and after pemetrexed, three types of NSCLC cell lines were treated with candidate drugs before and after pemetrexed treatment. According to

the conditions in the previous reports regarding treatment schedule [20, 21], each cell type was treated with medium containing the IC_{50} concentration of pemetrexed disodium (Toronto Research Chemicals, Ontario, Canada) for 72 h, washed and incubated in medium without anti-tumor drugs for 72 h, and then treated with medium containing the IC_{50} concentration of candidate drugs for 72 h. The reverse treatment schedule was applied to the assay of drug use followed by pemetrexed. Next, 10 μL of CCK-8 was added to each well, which was further incubated for 1.5–2 h at 37°C. Then, the optimal density of each well was measured at 450 nm, and the cell-growth inhibitory rate was expressed as the percentage of cell viability, as described above. Each experiment was performed independently and in triplicate.

Statistical analysis. PFS and OS for each parameter were calculated using the Kaplan-Meier method, and the log-rank test was used to compare groups. Multivariate logistic regression analysis was used to adjust for potential confounding factors. Differences in the cell growth assay were assessed by the Mann-Whitney U test. A p value of < 0.05 was considered to represent statistical significance.

Results

Patient characteristics. The clinicopathological characteristics of 130 NSCLC patients with wild-type *EGFR* who received pemetrexed-based chemotherapy are shown in Table 1. Most patients had good performance status, adenocarcinoma, and stage IV.

Treatment regimen and schedule. Treatment regimens and schedules used for pemetrexed-based chemotherapy are shown in Table 2. Thirty-two of 81 patients who received combination chemotherapies with platinum and pemetrexed (40%) underwent maintenance treatment with pemetrexed. Furthermore, 48% of 130 patients received subsequent

Table 1. Characteristics of patients who received pemetrexed-based chemotherapy (n=130)

Parameters		No. of Patients
Age		
Median (range)		66 (37-82)
	< 70	93
	≥ 70	37
Sex		
Male		90
Female		40
Performance status (ECOG)		
0		73
1		41
2		10
3		6
Smoking history		
Never smoker		35
Light smoker	(Pack year ≤ 20)	8
Heavy smoker	(Pack year > 20)	87
Histological type		
Adenocarcinoma		115
Squamous cell carcinoma		2
Large cell carcinoma		3
Adenosquamous carcinoma		1
Large cell neuroendocrine carcinoma		1
Pleomorphic carcinoma		1
Diagnosed only as NSCLC		7
Clinical stage		
IIIB		9
IV		121

ECOG, eastern cooperative oncology group; NSCLC, non-small cell lung cancer

Table 2. Treatment history and regimen

Treatment		No. of Patients
Regimen		
Cisplatin/Pemetrexed		13
Carboplatin/Pemetrexed		61
Pemetrexed		49
Carboplatin/Pemetrexed/Bevacizumab		7
Course		
Median		4
	1-3	55
	≥ 4	75
Line		
	1st	69
	≥ 2nd	61
Preceding treatment regimen of pemetrexed		
No treatment		69
Carboplatin/Paclitaxel		12
Cisplatin/Docetaxel		6
Cisplatin/Vinorelbine		6
Carboplatin/Paclitaxel/Bevacizumab		5
Carboplatin/Docetaxel		2
Carboplatin/Gemcitabine		1
Carboplatin/S1		1
Carboplatin/Vinorelbine		1
Docetaxel/S1		3
Gemcitabine/Vinorelbine		1
Erlotinib		11
Gefitinib		3
Docetaxel		6
Gemcitabine		2
S1		1
Treatment regimen following pemetrexed		
No treatment		68
Cisplatin/S1		1
Docetaxel/S1		14
Docetaxel		19
Erlotinib		26
Gefitinib		1
Gemcitabine		1

S1, Tegafur/gimeracil/oteracil potassium

treatment with other drugs after failure of pemetrexed. Of treatment regimens just after pemetrexed, docetaxel-based chemotherapy including docetaxel monotherapy (15%) and docetaxel/S1 (11%) was most frequently employed. As a monotherapy, erlotinib (20%) was most frequently employed. The median elapsed time from the preceding pemetrexed-based chemotherapy to erlotinib and docetaxel in this clinical analysis were 13 and 19 days, respectively. Compared to pemetrexed monotherapy, platinum-combined pemetrexed chemotherapy elicited favorable therapeutic responses, whereas there were no significant differences in survival between both therapy types as shown in Table 3; these data are comparable to those from previous reports [4, 5]. There were no fatal toxicities, while the occurrence of grade 3–4 toxicities as follows: nephrotoxicity (0.7%), anorexia (0.7%), neutropenia (19.2%), anemia (11.5%), and thrombocytopenia (15.4%) for grade 3; neutropenia (3.1%) and thrombocytopenia (0.7%) for grade 4.

Analysis of survival benefit. Kaplan-Meier survival curves and log-rank tests showed that the following factors had a significant effect on survival: maintenance treatment, performance status, ORR, and DCR for PFS for pemetrexed-based therapy; and DCR, treatment course, pemetrexed monotherapy, and performance status for OS for pemetrexed-based therapy. These survival analyses were also applied for the treatment schedule employed in this study as follows: erlotinib, docetaxel, carboplatin/paclitaxel, cisplatin-based chemotherapy, or carboplatin-based chemotherapy as treatment before pemetrexed; and erlotinib, gefitinib, and docetaxel-based chemotherapy as treatment after pemetrexed. None of the drugs used before treatment with pemetrexed-based chemotherapy were associated with significant OS benefit. Of treatments initiated at the time of progression after pemetrexed-based chemotherapy, erlotinib mono-therapy that was independently associated with significant OS benefit (Figure 1A) produced the same result even after pemetrexed monotherapy (Figure 1B). On the other hand, there were no statistical differences in PFS for only preceding pemetrexed-based chemotherapy between erlotinib-treated patients and others (data not shown). Of interest, erlotinib
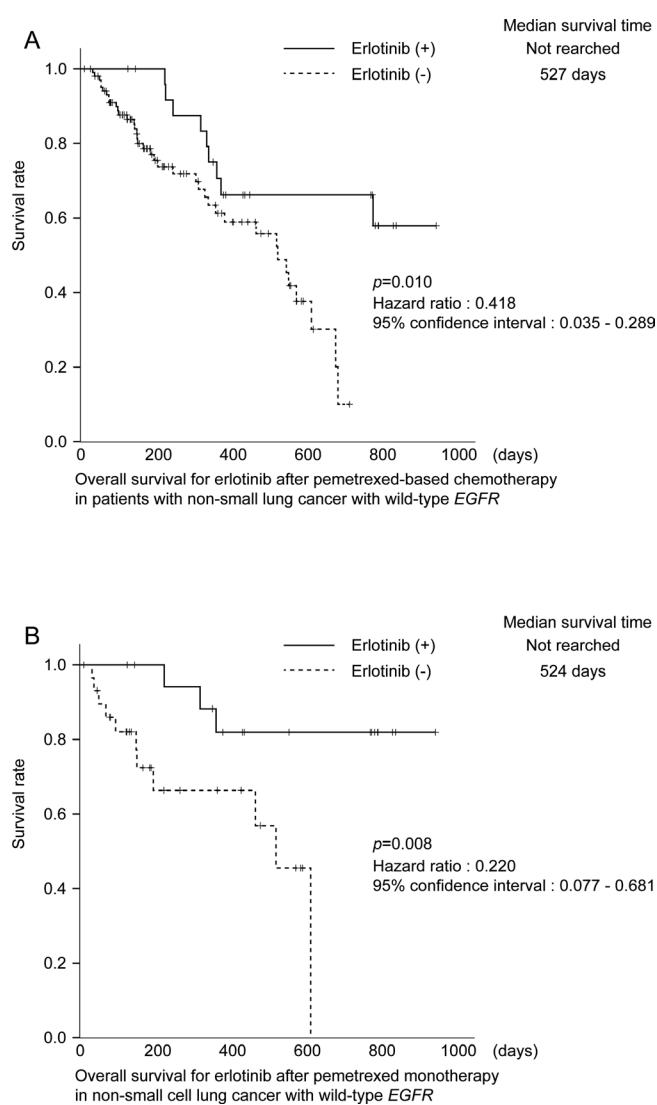

Figure 1. Kaplan-Meier survival curves with log-rank tests for treatment with erlotinib after pemetrexed. Erlotinib at the time of progression after pemetrexed-based chemotherapy (A) and pemetrexed monotherapy (B) significantly prolonged overall survival in non-small cell lung cancer with wild-type *EGFR*.

Table 3. Therapeutic response and survival time according to therapeutic regimens

Treatment regimen	Therapeutic response (number)				ORR (%)	DCR (%)	PFS (days [95% CI])	OS (days [95% CI])
	CR	PR	SD	PD				
Total (n = 130)	3	27	60	40	23	69	133 [114-152]	556 [462-650]
Platinum-combined therapy (81)	3	23	35	20	32	75	150 [121-179]	527 [320-734]
Cisplatin/Pemetrexed (13)	2	2	7	2	31	85	226 [103-349]	375 [155-606]
Carboplatin/Pemetrexed (61)	0	17	26	18	28	70	131 [115-147]	550 [305-795]
Carboplatin/Pemetrexed/Bevacizumab (7)	1	4	2	0	71	100	213 [129-297]	341 [208-474]
Pemetrexed (49)	0	4	25	20	8	59	124 [71-177]	NR

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; ORR, objective response rate; DCR, disease control rate; PFS, progression-free survival; OS, overall survival, NR; not reached

monotherapy at the time of progression after pemetrexed-based chemotherapy resulted in better PFS and OS even when compared to docetaxel-based chemotherapy as well as docetaxel monotherapy (Figure 2). Similar analyses in pemetrexed monotherapy were not applied because of only 3 patients who had received docetaxel monotherapy after pemetrexed monotherapy. Multivariate logistic regression analysis revealed that the use of erlotinib monotherapy after pemetrexed was associated with smoking status (never-smoker), treatment course (more than four courses), and OR

in pemetrexed (Table 4), whereas no parameters other than treatment course that showed a negative association provided a survival benefit (data not shown).

***In vitro* evaluation of erlotinib efficacy after pemetrexed.** The *in vitro* cell growth assay was used to evaluate erlotinib efficacy after treatment with pemetrexed. To clarify erlotinib efficacy, the assay was also applied for docetaxel. Pemetrexed, erlotinib, or docetaxel (Focus Biomolecules, Plymouth Meeting, PA, USA) after pemetrexed treatment was administered at 72-h intervals with the IC_{50} concentrations for each drug in
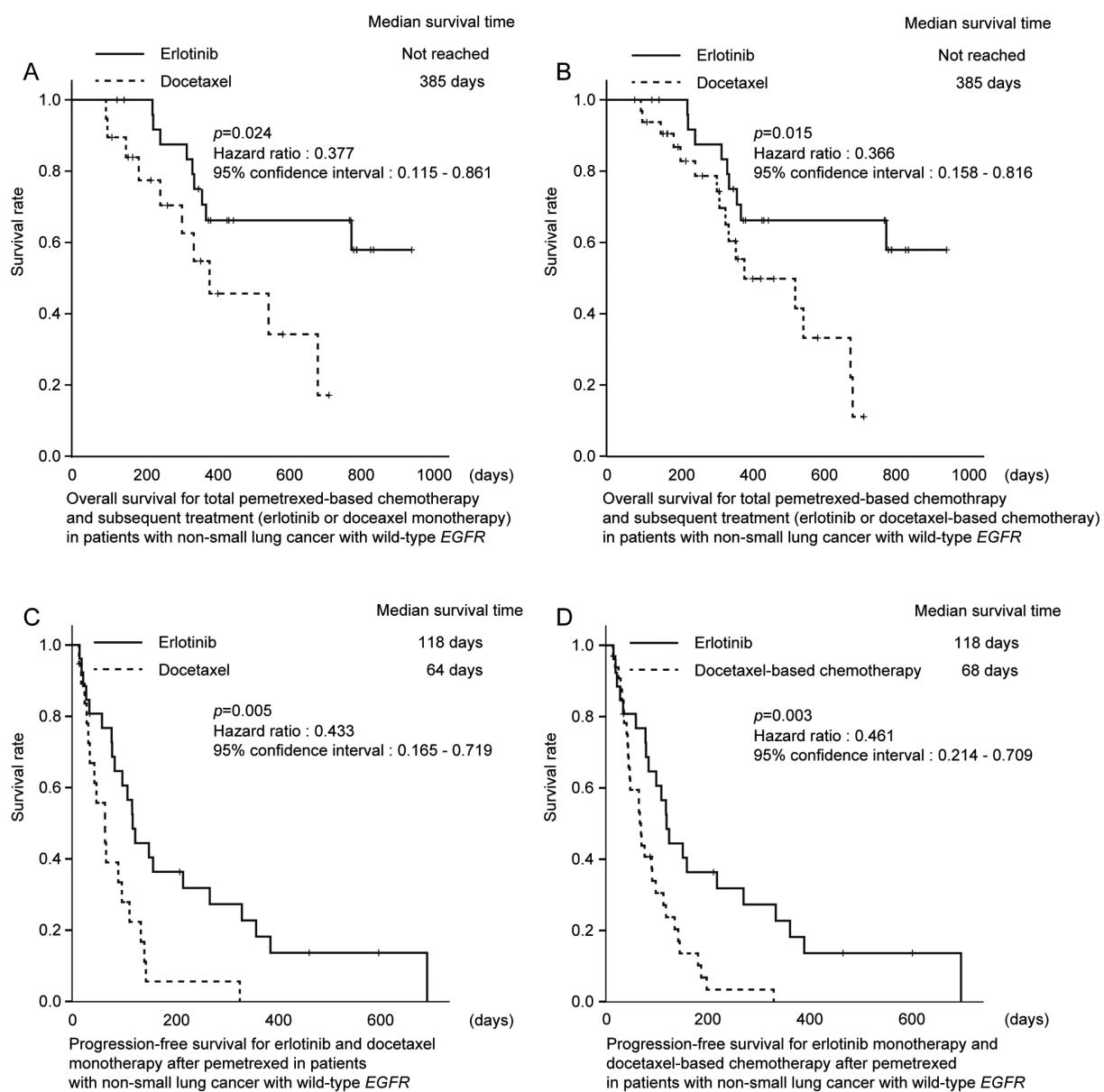

Figure 2. Kaplan-Meier survival curves with log-rank tests for treatment with erlotinib versus docetaxel at the time of progression after pemetrexed-based chemotherapy. Overall survival for total pemetrexed-based chemotherapy and subsequent erlotinib was significantly longer than that for total pemetrexed-based chemotherapy and docetaxel monotherapy (A) or docetaxel-based chemotherapy (B). Progression-free survival confined to erlotinib was also longer than docetaxel monotherapy (C) or docetaxel-based chemotherapy (D).

individual cells (Table 5). Cells that were treated with pemetrexed again after pemetrexed exposure were used as a control group (pemetrexed → pemetrexed group; P → P). The growth inhibitory rates of each treatment schedule in individual cells compared to control groups were as follows (median value ± standard deviation): Treatment with erlotinib after pemetrexed (pemetrexed → erlotinib group; P → E), 0.702 ± 0.066 and treatment with docetaxel after pemetrexed (pemetrexed → docetaxel group; P → D), 0.931 ± 0.267 in A549 cells; P → E, 0.581 ± 0.141 and P → D, 0.822 ± 0.131 in PC9 cells; P → E, 0.599 ± 0.083 and P → D, 0.819 ± 0.309 in NCI-H1975 cells (Figure 3). The pemetrexed → erlotinib group resulted in significant growth inhibition of all types of cells when compared to the pemetrexed → docetaxel group and the control group. There were no significant differences in the growth inhibitory rate in the reverse schedules, respectively.

Discussion

The present study provided both clinical and biological evidence that erlotinib at the time of progression after pemetrexed therapy prolonged survival in NSCLC patients with wild-type *EGFR*. The previous retrospective study reported the superiority of the reverse treatment sequence [22]. However, previous *in vitro* studies show that EGFR/Akt phosphorylation, low thymidylate synthase (TS) expression, and cell cycle-dependent cytotoxicity mediate the synergistic effect of pemetrexed and subsequent erlotinib [20, 21, 23-26]. These findings may account for a direct molecular mechanism underlying favorable outcome from treatment sequence of erlotinib after pemetrexed regardless of *EGFR* mutation status. Along with such evidences, our biological data exemplify the survival benefit from the treatment schedule of erlotinib following pemetrexed compared to the reverse treatment sequence.

While these reports regarding treatment sequences, there is very limited information about which partner for pemetrexed as a treatment schedule is optimal. In this context, erlotinib also conferred a survival advantage compared to docetaxel, when used as treatment after pemetrexed. Some recent trials for previously treated NSCLC with wild-type *EGFR* suggested superior outcomes of docetaxel over erlotinib [27, 28], others reported that the effect of EGFR-TKIs is equivalent to that of docetaxel [6, 8]. Different drugs had been administered before docetaxel or erlotinib in these trials, whereas patients who received the preceding pemetrexed therapy have been exclusively the subject of the present study. This focused approach may sensitively reflect the effectiveness of the treatment schedule for pemetrexed compared to the previous trials. One of other potential explanations for our results is that erlotinib may be better tolerated than the cytotoxic drugs, leading to long-term administration [8, 29, 30]. Since more emphasis has been placed on palliative than curative intent when using treatment after first-line chemotherapy [31], preferred reduced toxicity in treatment may have induced early termination of the cytotoxic drugs which had a similar toxicity profile to

Table 4. Logistic regression analysis for erlotinib monotherapy following pemetrexed

	Odds ratio	95% Confidence interval	*p*-Value
Age (70 >)	1.808	0.463 – 7.050	0.394
Smoking status (20 pack/year >)	0.082	0.018 – 0.374	0.001
Sex (Female)	1.460	0.319 – 6.674	0.626
Histology (Adenocarcinoma)	0.627	0.117 – 3.368	0.587
Treatment course of pemetrexed (Four ≥)	8.157	1.718 – 38.723	0.008
Treatment line of pemetrexed (1st line)	0.232	0.052 – 1.031	0.055
Performance status (0-1)	3.707	0.872 – 15.754	0.076
Objective response of pemetrexed (CR+PR)	0.115	0.017 – 0.759	0.025
Disease control of pemetrexed (CR+PR+SD)	0.721	0.157 – 3.303	0.674
Maintenance treatment (+)	1.229	0.243 – 6.217	0.804

CR, complete response; PR, partial response; SD, stable disease

Table 5. IC50 for each drug in individual cell lines (µM)

Cell type	Drug		
	Pemetrexed	Erlotinib	Docetaxel
A549 (wild-type EGFR)	1.0	1.7	0.006
PC9 (delE746-A750 activating EGFR mutation)	0.25	0.5	0.003
NCI-H1975 (L858R activating and T790M resistant EGFR mutations)	10.0	10.0	0.002

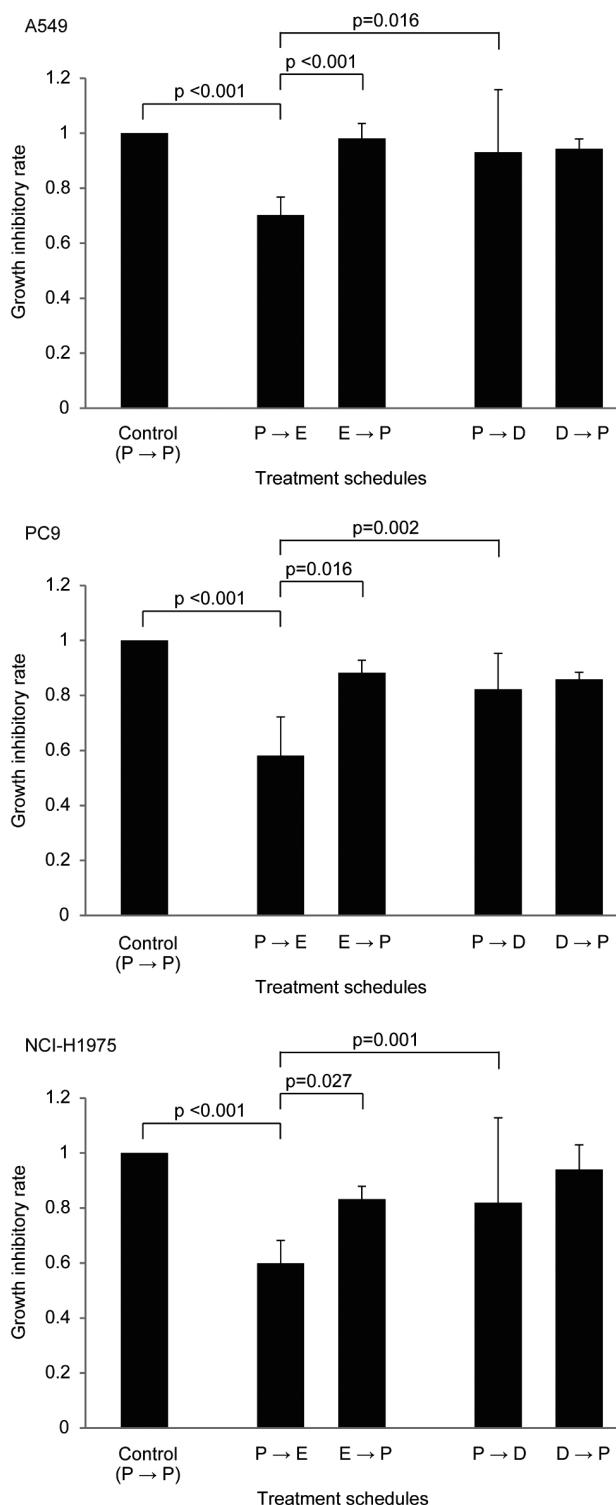IC50, 50% growth inhibitory concentration

Figure 3. The *in vitro* cell growth assays for treatment schedules of pemetrexed → pemetrexed (P → P; control), pemetrexed → erlotinib (P → E), erlotinib → pemetrexed (E → P), pemetrexed → docetaxel (P → D), and docetaxel → pemetrexed (D → P) using three types of NSCLC cell lines, A549 (wild-type EGFR), PC9 (delE746-A750 activating *EGFR* mutation), and NCI-H1975 cells (both L858R activating and T790M resistant *EGFR* mutations). Data represent median values ± standard deviations.

pemetrexed. The choice of EGFR-TKI after a cytotoxic drug may improve clinical outcome.

On the other hand, one limitation of the present study is that reciprocal treatment sequences of pemetrexed and erlotinib were not directly compared. As another limitation, the present study consists of a heterogenous population including patients who had received the different preceding treatments and were treated with pemetrexed at different treatment-lines. However, neither preceding treatments nor treatment-lines were associated with a survival benefit of pemetrexed therapy. Furthermore, no drugs other than erlotinib provided a survival benefit as the treatment following pemetrexed. Multivariate analysis also showed that no potential confounding factors positively affects erlotinib therapy.

In conclusion, use of erlotinib at the time of progression after pemetrexed therapy confers a survival benefit in NSCLC patients with wild-type *EGFR*. Our findings provide an important clue to accessing the significance of the treatment schedule and advocate the possibility of a novel therapeutic approach, although further validation requires large-scale prospective trials that consist of a homogenous population and minimized a potential bias.

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