Sequential Treatment of Advanced-stage Lung Adenocarcinoma Harboring Wild-type *EGFR* Gene: Second-line Pemetrexed Followed by Third-line Erlotinib *versus* the Reverse Sequence

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Abstract. *Background: Pemetrexed and erlotinib represent novel agents for the treatment of non-small cell lung cancer (NSCLC). The role of sequential treatment in NSCLC has not been elucidated yet. We compared the efficacy of second-line pemetrexed followed by third-line erlotinib (P-E) to treatment with the reverse sequence (E-P). Patients and Methods: We analyzed data of 57 patients with advanced-stage (IIIB/IV) lung adenocarcinoma harboring wild-type epidermal growth factor receptor (EGFR) gene; 31 patients were treated with P-E and 26 patients with the E-P sequence. Results: The median progression-free survival (PFS) for patients treated with P-E was 3.6 months vs. 7.8 months for patients treated with E-P (p=0.029). The median overall survival (OS) for patients treated with P-E was 7.9 months vs. 26.3 months for patients treated with E-P (p=0.006). Conclusion: The results proved a significant improvement of both PFS and OS for patients treated with the E-P sequence as compared to the P-E sequence.*

Non-small cell lung cancer (NSCLC) is one of the most common human malignant diseases (1). Adenocarcinoma is the dominant histological type of NSCLC. Rapid progress in the treatment of NSCLC has recently led to the approval of several new effective agents such as pemetrexed and erlotinib. Pemetrexed is a cytostatic agent, targeting several folate-dependent enzymatic pathways, which has demonstrated efficacy in combination with platinum derivative- in the first-line setting (3, 4) and also as monotherapy in pre-treated patients with advanced-stage non-squamous NSCLC (5). Erlotinib is a tyrosine kinase inhibitor (TKI) targeting the epidermal growth factor receptor (EGFR), which demonstrated efficacy in patients with advanced-stage NSCLC pre-treated with chemotherapy (6, 7) and also in the first-line setting for the treatment of those harboring activating *EGFR* mutations (8, 9). Given the evidence-based data, pemetrexed, erlotinib and docetaxel are currently recommended for second-line treatment of patients with advanced-stage lung adenocarcinoma; erlotinib is recommended for third-line treatment.

In daily clinical practice, it is common that patients with a good performance status who progressed on erlotinib in second line to be treated with chemotherapy beyond the second line, and pemetrexed presents a reasonable option for those with non-squamous histology. The optimal choice of treatment in the second and third line, respectively, is a great challenge and there is still a lack of clinical data regarding this topic. Thus we conducted this retrospective study, based on clinical experience, aiming to compare two possible treatment strategies, second-line pemetrexed followed by third-line erlotinib *versus* second-line erlotinib followed by third-line pemetrexed. To avoid potential bias, we analyzed only patients harboring wild-type *EGFR* gene.

Patients and Methods

Study design and treatment. We retrospectively analyzed data of 57 patients with cytologically or histologically confirmed locally advanced (IIIB) or metastatic (IV) lung adenocarcinoma harboring a wild-type *EGFR* gene whose disease had progressed on standard first-line chemotherapy and who were subsequently treated with pemetrexed in second line followed by erlotinib in third line, or with the reverse sequence between 2007 and 2013. The primary objective

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of the study was to compare progression-free survival (PFS) and overall survival (OS) between the two patient groups according to the treatment sequence in the second and third line, respectively. PFS was defined in two ways: a) from the date of second-line treatment initiation until the date of first documented progression on third-line treatment, or death; and b) from the date of second-line or third-line treatment initiation to first progression preceding initiation of the next line of treatment or death in case of third-line therapy. The main exclusion criteria were as follows: switch to second-, third- or fourth-line treatment without documented progression; previous exposure to pemetrexed or EGFR-TKIs prior to second-line treatment, pemetrexed-based combined chemotherapy or chemoradiotherapy. Pemetrexed was administered intravenously at the standard approved dose of 500 mg/m^2 on day 1 every three weeks; the treatment was scheduled up to six cycles unless development of intolerable toxic effects or disease progression occurred. Erlotinib was administered orally at the standard approved dose of 150 mg daily; dose interruption or reduction was permitted in the event of treatment-related toxicity; the treatment continued until disease progression or development of intolerable toxicity.

Clinical assessments and statistics. The treatment was prospectively monitored and the clinical course of patients was continuously assessed at specific time points. Clinical follow-up including physical examination, plain chest X-ray and routine laboratory tests was performed every 3-4 weeks; computed tomography (CT) or positron emission tomography - (PET)-CT was performed after 2-3 cycles of pemetrexed and after two or three months of treatment with erlotinib, respectively. Standard summary statistics were used to describe the sample data set. The significance of differences in baseline characteristics, as well as treatment response, was determined using the Fisher's exact test or Chi-square test with respect to the number of categories (in the case of categorical variables) or Mann-Whitney test (in the case of continuous variables). The terminations of PFS and OS, as well as the estimations of survival probabilities, were performed using Kaplan Meier survival curves; all point estimates were accompanied by 95% confidence intervals. The differences in survival were tested using the log-rank test. As a level of statistical significance, p=0.05 was used.

EGFR mutation analysis. The tumor specimens acquired during initial bronschoscopy were evaluated by a senior cytologist using standard giemsa staining. In a few cases, a tumor biopsy was processed into formalin-fixed paraffin-embedded (FFPE) histological sections. The cytology slides or, eventually, the FFPE sections, were submitted for molecular genetic testing, which included detection of somatic mutations of *EGFR* genes. If necessary, tumor cells were carefully selected and removed from the samples by laser microdissection using a P.A.L.M. microlaser instrument (Carl Zeiss MicroImaging GmbH, Jena, Germany). The microdissected cells were collected directly into the polymerase chain reaction (PCR) buffer and processed without a special DNA extraction step. In all other cases, the DNA was extracted from tissue cells by a standard spin-column procedure using JetQuick Tissue DNA Issolation Kit (Genomed GmbH, Loehne, Germany). Mutations in exons 19 and 21 of the *EGFR* gene were tested by Genoscan mutation detection kits (Genomac International, Prague, Czech Republic) utilizing a denaturing capillary electrophoresis (DCE) technique on an ABI PRISM 3100 16-capillary genetic analyzer (Applied Biosystems, Foster City, CA, USA). Detected mutations were confirmed by Sanger DNA sequencing using a BigDye v 3.0 chemistry (Applied Biosystems). In rare cases, where the overall fraction of mutated DNA was below the 20% threshold for DNA sequencing, mutation was identified indirectly after forming only a homoduplex fragment with a given known mutation reference standard.

Table I. *Baseline clinical characteristics of patients treated with second-line pemetrexed followed by third-line erlotinib (P-E) and those treated with second-line erlotinib followed by third-line pemetrexed (E-P).*

	P-E sequence (n=31)	E-P sequence (n=26)	*p*-Value
Gender, n (%)			
Male	17 (54.8)	12 (46.2)	0.599
Female	14 (45.2)	14 (53.8)	
Age (years)			
Median (5-95%)	59 (30-79)	66 (48-78)	0.007
Smokig status, n (%)			
Current-smoker	24 (77.4)	10 (38.5)	0.011
Former-smoker	3 (9.7)	6 (23.1)	
Never-smoker	4 (12.9)	10 (38.5)	
Stage, n (%)			
IIIB	3 (9.7)	6 (23.1)	0.275
IV	28 (90.3)	20 (76.9)	
ECOG PS, n (%)			
0	3 (9.7)	1 (3.8)	0.123
1	27 (87.1)	20 (76.9)	
2	1 (3.2)	5 (19.2)	

Results

Baseline patient characteristics. Clinical data of all patients treated with erlotinib and pemetrexed were properly analysed according to the inclusion criteria mentioned above. The whole cohort of 57 patients was divided into two groups. The first group included 31 patients treated with pemetrexed in second line followed by erlotinib in third line (P-E); the group consisted of 14 women and 17 men, 24 current smokers, 3 former smokers and 4 never-smokers, 3 with stage IIIB and 28 with stage IV disease. The second group included 26 patients treated with erlotinib in the second line followed by third-line pemetrexed (E-P); the group consisted of 14 women and 12 men, 10 current-smokers, 6 former-smokers and 10 never-smokers, 6 with stage IIIB and 20 stage with IV patients. The baseline patient characteristics are summarized in Table I. Both groups differed significantly with regard to patients' age and smoking status at the time of initiation of second-line treatment. The group treated with the P-E sequence included more patients in younger age (p=0.007) and those of positive smoking history (p=0.011) and *vice versa* in the group treated with the E-P sequence. The groups did not significantly differ in performance status (p=0.123), nor in sex representation (p=0.599).

Table II. *Progression-free (PFS) and overall (OS) survival data for patients treated with second-line pemetrexed followed by third-line erlotinib (P-E) and those treated with second-line erlotinib followed by third-line pemetrexed (E-P).*

	n	Median PFS	6-months PFS probability	12-months PFS probability	*p*-Value	Median OS	12-months OS probability	24-months OS probability	*p*-Value
		(95% CI)	(%; 95% CI)	(%; 95% CI)	(Log-rank)	(95% CI)	(%; 95% CI)	(%; 95% CI)	(Log-rank)
P-E sequence	31	3.6 months (3.2; 4.0)	26.7 (10.8; 42.5)	10.0 (0.1; 20.7)	0.029	7.9 months (1.3; 14.5)	41.5 (22.6; 60.4)	20.7 (2.9; 38.6)	0.006
E-P sequence	26	7.8 months (4.8; 10.7)	76.9 (60.7; 93.1)	26.6 (8.3; 44.9)		26.3 months (16.7; 35.9)	74.5 (56.7; 92.3)	55.0 (31.6; 78.5)	

CI: Confidence interval.

Survival according to treatment sequence. The median PFS for patients treated with the P-E sequence was 3.6 months *vs*. 7.8 months for patients treated with the E-P sequence; the difference was statistically significant (p=0.029) (Figure 1A). The 6-month PFS probability for patients treated the P-E sequence was 26.7% *vs*. 76.9% for patients treated with the E-P sequence. The median OS for patients treated with the P-E sequence was 7.9 months *vs*. 26.3 months for patients treated with the E-P sequence; the difference was statistically significant (p=0.006) (Figure 1B). The 12-month OS probability for patients treated with the P-E sequence was 41.5% *vs*. 74.5% for patients treated with the E-P sequence. The summary of survival data for both groups is listed in Table II.

Comparison of PFS according to single-agent treatment line. The median PFS for patients treated with pemetrexed in the second line was 1.5 months *vs*. 3.1 months for patients treated with pemetrexed in the third line; the difference was of borderline statistical significance (p=0.054) (Figure 2A). The median PFS for patients treated with erlotinib in the second line was 2.9 months *vs*. 1.8 months for patients treated with erlotinib in the third line; the difference was not statistically significant (p=0.115) (Figure 2B).

Discussion

Sequential treatment strategies have become a very interesting topic in current oncology research. The concept of sequential treatment has been recently successfully established for renal cell cancer (10-14). The role of sequential treatment in advanced-stage NSCLC has not been elucidated yet. Hong *et al*. have recently published a similar comparison of both treatment sequences in a cohort of unselected Chinese patients with lung adenocarcinoma. Their study, similarly to ours, proved significantly longer OS for patients treated with second-line erlotinib followed by third-line pemetrexed (23.6 *vs*. 16.3 months; p=0.042). The difference in PFS between the two sequential strategies was not significant (17.5 *vs*. 11.6 months; p=0.191) (15); however, interpretation of the results could be affected by the fact that the *EGFR* mutation status, which is known to be a strong predictor of the efficacy of EGFR-TKIs in NSCLC (16, 17), was unknown. Moreover, it is probable that a large proportion of patients with *EGFR*-mutation positive tumors were included, given the fact that *EGFR* mutations are frequently found in patients with adenocarcinoma and those of Asian ethnicity (18-21). Thus, we decided to include only patients harboring the wild-type *EGFR* gene, who represent the predominant population in Caucasians.

The study proved approximately two-fold longer PFS (3.6 *vs*. 7.8 months; p=0.029) and three-fold longer OS (7.9 *vs*. 26.3 months; p=0.006) for patients treated with second-line erlotinib followed by third-line pemetrexed compared to those treated with the reverse sequence. Our findings suggest that second-line erlotinib increased sensitivity to third-line pemetrexed, probably *via* modulation of several signaling pathways. Giovanetti *et al*. has previously reported that erlotinib significantly inhibited the activity of thymidylate synthase (TYMS) in NSCLC (22), while high expression of TYMS correlates with pemetrexed resistance both *in vitro* and *in vivo* (23, 24). Moreover Li *et al*. has reported that pemetrexed induced phosphorylation of protein kinase B (AKT), while the aberrant activation of AKT signaling correlates with resistance to EGFR-TKIs in NSCLC (25-27). These pre-clinical studies support our findings. In our study, we observed longer PFS with third-line pemetrexed following second-line erlotinib compared to second-line pemetrexed (1.5 *vs*. 3.1 months; p=0.054) and longer PFS with second-line erlotinib compared to third-line erlotinib following second-line pemetrexed (2.9 *vs*. 1.8 months; p=0.115), although the difference was not statistically significant.

The principal limitations of our study are its retrospective design and relatively small number of patients included. In our study, it was not possible to avoid some selection bias. The group treated with second-line erlotinib followed by third-line pemetrexed involved more never-smokers and

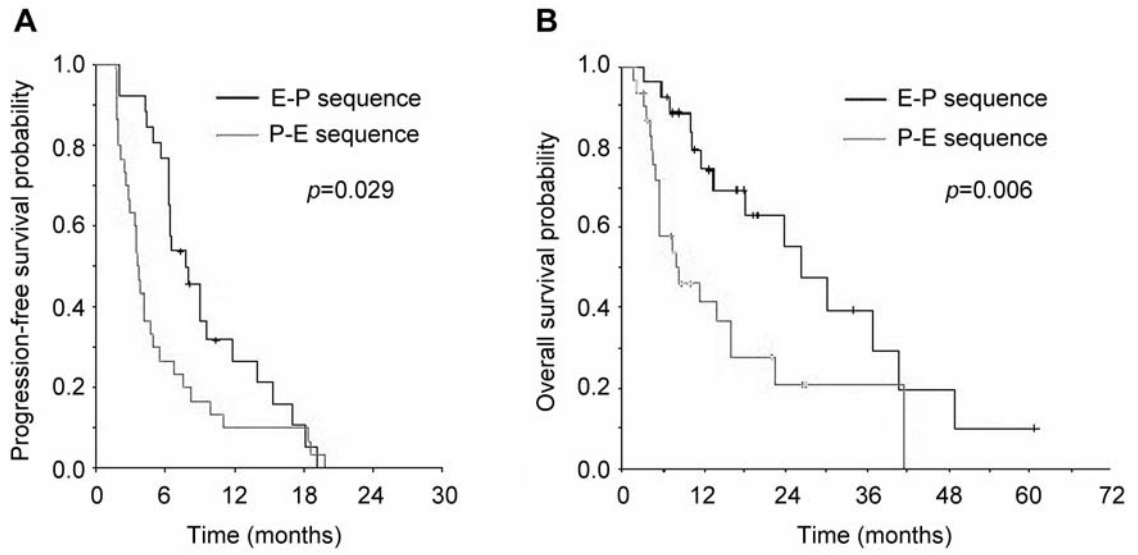

Figure 1. *Comparison of progression-free (A) and overall (B) survival between patients treated with second-line pemetrexed followed by third-line erlotinib (P-E) and those treated with second-line erlotinib followed by third-line pemetrexed (E-P).*

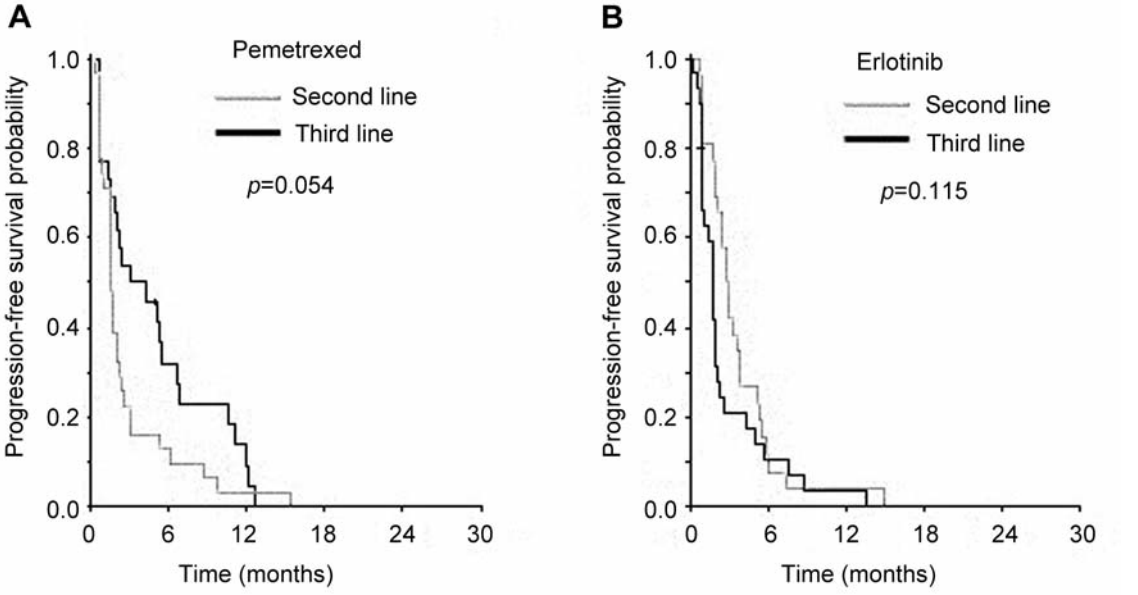

Figure 2. *Comparison of progression-free survival for pemetrexed (A) and erlotinib (B) between patients treated in the second line and third line setting.*

patients in older age categories compared to the group treated with the reverse sequence.

In conclusion, our study proved a significant improvement of both PFS and OS for patients treated with second-line erlotinib followed by third-line pemetrexed as compared to the reverse sequence. To our knowledge, this is the first study assessing the role of the sequential treatment with erlotinib and pemetrexed in patients with advanced-stage lung adenocarcinoma harboring wild-type *EGFR* gene. Although the study was limited, its findings could have a great impact on the treatment of advanced-stage NSCLC. The role of the sequential treatment of NSCLC should be further investigated in a prospective randomised study in the future.

Conflicts of Interest

All Authors declare that they have no actual or potential conflict of interest including any financial, personal or other relationship with other people or organizations that could inappropriately influence this work.

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