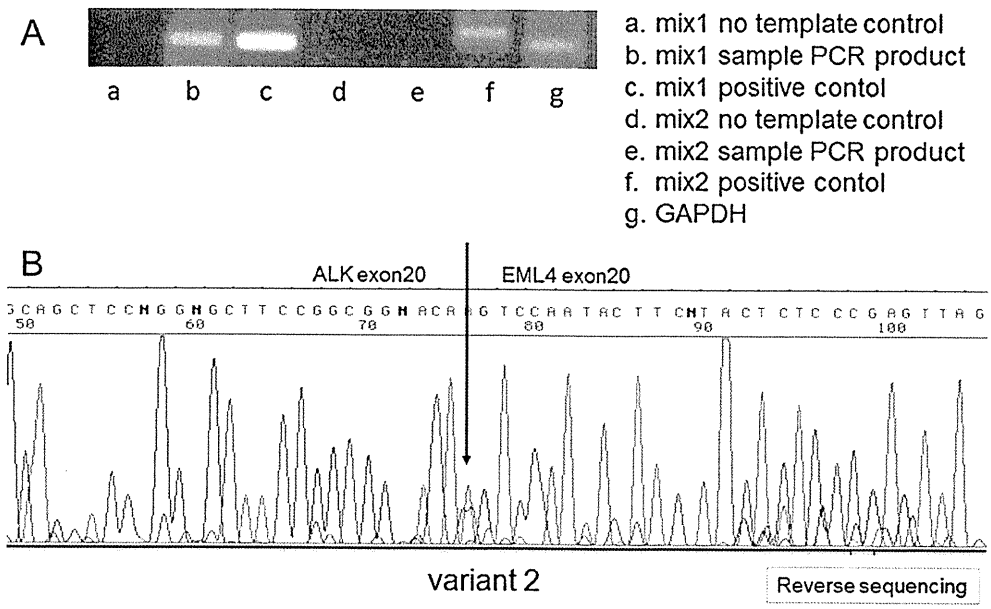

Fig. 3. Genetic analyses. (A) Multiplex RT-PCR to capture all in-frame fusions between *EML-4* and *ALK* messages was conducted with the primers. Mix1 contains variants 1, 2, 4, 7, and KIF5B. Mix2 contains variants 3, 5, and 6. (B) The transition was confirmed to be the *EML4–ALK* inversion-variant 2 by a direct sequencing method.

its except for an elevated serum level of carcinoembryonic antigen (CEA) (15.6 ng/ml).

Because of the elevated level of CEA and the results of the radiological examination, the lesion was clinically suspected to be a primary lung cancer. The clinical TNM classification was T1bN1M0; cStage IIA. Intraoperative aspiration cytology for the primary tumor revealed carcinoma, so a right lower lobectomy and lymph node dissection were performed.

Macroscopically, the well-demarcated tumor contained a yellowish gelatinous substance, thus indicating the presence of an abundant amount of mucin in the tumor (Fig. 2A). Histopathologically, two-thirds of the tumor consisted of mucin pools that distended the alveoli and floating foci of mucinous epithelia, some of which resembled signet ring cells (Fig. 2B and C). The rest of the tumor was composed of acinar adenocarcinoma and signet ring adenocarcinoma with fibrous stroma (Fig. 2D). Columnar mucinous epithelial cells were not evident in this tumor. Immunohistochemically, the tumor cells were positive for CEA, cytokeratin 7 (CK7), and thyroid transcription factor-1 (TTF-1), whereas they were negative for cytokeratin 20 (CK20), MUC5AC, cluster of differentiation 10 (CD10), and CDX-2 (Figs. 2E and F). No tumor cells were detected in the dissected lymph nodes. As a result, the tumor was diagnosed to be a primary signet ring cell adenocarcinoma, and the pathological TNM classification was T1bN0N0; stage IA.

The tumor was examined for inversion of the *EML4-ALK* gene by multiplex RT-PCR analysis of the frozen section, and the result was confirmed by direct sequencing. The sequences of these primers were modified following the previous report [8]. This case was positive for variant 2 of the *EML4-ALK* gene (Fig. 3). The tumor was negative for mutations of the epidermal growth factor receptor (EGFR).

There were no postoperative complications. The patient was discharged on the 8th postoperative day and is doing well at 10 months after the surgery.

3. Discussion

This case was strongly suggested to be MC macroscopically, because the cut surface was yellowish and gelatinous, like MC. Histologically, our case showed the presence of abundant mucin pools, floating foci of the tumor cells, and expansion of the alveolar spaces. These findings seemed to be compatible with the histological features of MC [2]. However, a well differentiated columnar mucinous epithelium could not be detected in the tumor. On the contrary, foci of signet ring cell adenocarcinoma were recognized in the fibrous stroma, accompanied by floating signet ring cells in the mucin pools. Therefore, the tumor was finally diagnosed to be primary signet ring cell carcinoma mimicking MC.

Rossi et al. divided MC into the two distinct types: the classic goblet cell-type and the signet-ring cell type [2]. The classic goblet cell-type is more frequent and consists of prominent pools of mucin-disrupting alveoli that invade the adjacent lung. Columnar mucinous neoplastic elements float into the mucus and line the alveolar structures. The signet-ring cell type is rare, and shows rich mucin pools expanding the alveolar spaces. Neoplastic cells floating in mucin pools have a signet ring cells appearance with significant cytologic atypia. Histologically, our case is considered to mimic the pattern of the signet ring cell cancer. The signet ring cell type of MC shows positive immunostaining for TTF-1, CK-7, and MUC-5AC [2]. Our case showed positive immunostaining for TTF-1and CK-7, and negative staining for CDX-2, MUC5AC, CK-20, and MUC2. On the other hand, signet ring cell carcinoma has been reported to demonstrate positive immunostaining for TTF-1 and CK-7, while it is negative for MUC5 [9]. Therefore, the immunohistochemical features indicate signet ring cell carcinoma rather than MC. Immunohistochemical findings might therefore play an important role in distinguishing signet ring cell carcinoma from the signet ring cell type of MC.

Activating mutations within the *EGFR* have been identified in lung cancers, and chemical inhibitors of the kinase activity of EGFR have been found to be effective in the treatment of a subset of lung cancer patients harboring such mutations [10–12]. Recently, tumors with the *EML4–ALK* gene fusion have also been receiving attention because they may be the target of new molecular targeting therapy. In fact, *EML4–ALK*-dependent cells undergo apoptosis when treated with an *ALK*-inhibitor [4,13].

A variety of histological features reported to be associated with *EML4–ALK* gene fusion positive lung cancers were reported in two

articles [7,14]. Inamura et al. reported that the acinar pattern was mostly associated with *EML4–ALK* gene fusion positive lung adenocarcinomas in an Asian population [7]. *EML4–ALK* gene fusion positive lung adenocarcinomas comprised 11 (4%) of 253 patients in their series [7]. According to the predominant subtypes of adenocarcinomas, 6 of 11 *EML4–ALK* gene fusion positive lung cancers (55%) were subclassfied as acinar adenocarcinomas, and the other 5 cancers (45%) were subclassfied as papillary adenocarcinomas. On the other hand, Rodig et al. reported that the solid pattern and the signet-ring cell histology were most commonly associated with this gene fusion in Western patients [14]. The *EML4–ALK* gene fusion positive lung adenocarcinomas were found on 16 (5%) of their 326 patients. With regard to the predominant subtypes of adenocarcinomas, 11 of the 16 *EML4–ALK* gene fusion positive lung cancers (69%) in this study were subclassfied as solid adenocarcinomas, while the other 4 cancers (25%) were subclassfied as acinar adenocarcinomas, and the other tumor was subclassfied as bronchioloalveolar carcinoma. Of these 16 tumors, 12 (75%) had the signet ring cells. Similarly, Shaw et al. proposed that there is a close relationship between the signet ring cell pattern and *EML4–ALK* [6]. The present report was subclassfied as a signet ring adenocarcinoma mimicking MC with the *EML4–ALK* gene fusion. On the other hand, no report has yet diagnosed an MC with the *EML4–ALK* gene fusion. In the future, the further analysis of the expression of such genes as *EML4–ALK* and *EGFR* is strongly recommended as an important part of the histopathological classification of lung malignancies.

4. Conclusions

This report describes a case of *EML4–ALK*-positive signet ring cell adenocarcinoma of the lung mimicking MC.

Conflict of interest statement

All authors have declared that they have no conflict of interest.

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Reaction of plasma hepatocyte growth factor levels in non-small cell lung cancer patients treated with EGFR-TKIs

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Hepatocyte growth factor induces resistance to epidermal growth factor receptor tyrosine kinase inhibitors. It has been hypothesized that epidermal growth factor receptor tyrosine kinase inhibitors administration may influence the levels of plasma hepatocyte growth factor. Patients with advanced non-small cell lung cancer and relapsed after chemotherapies were eligible. Plasma hepatocyte growth factor levels were analyzed on pretreatment and post-treatment day 15 and 30. We also investigated the correlation between plasma hepatocyte growth factor levels and sensitivity to epidermal growth factor receptor tyrosine kinase inhibitors, tissue immunoreactivity for hepatocyte growth factor and *MET* gene status. Thirty-one patients were enrolled. Plasma hepatocyte growth factor levels on post-treatment day 15 (630.1 ± 366.9 pg/ml) were significantly higher ($p = 0.029$) than the pretreatment plasma hepatocyte growth factor levels (485.9 ± 230.2 pg/ml). Plasma hepatocyte growth factor levels on the post-treatment day 30 (581.5 ± 298.1 pg/ml) tend to be higher than those before treatment ($p = 0.057$). Pretreatment plasma hepatocyte growth factor levels in patients with progressive disease (724.1 ± 216.4 pg/ml) were significantly higher than those in patients with stable disease (396.5 ± 148.3 pg/ml; $p = 0.0008$) and partial response (381.7 ± 179.0 pg/ml; $p = 0.0039$). The optimal pretreatment plasma hepatocyte growth factor cut-off value for diagnosis of responder was 553.5 pg/ml, and its sensitivity and specificity were 90% and 65%, respectively. Pretreatment plasma hepatocyte growth factor levels had no correlation with tissue immunoreactivities for hepatocyte growth factor, *MET* gene status and active *EGFR* mutations. Administration of epidermal growth factor receptor tyrosine kinase inhibitors significantly increased plasma hepatocyte growth factor levels. High levels of pretreatment plasma hepatocyte growth factor indicated intrinsic resistance to epidermal growth factor receptor tyrosine kinase inhibitors. Plasma hepatocyte growth factor can serve as a useful biomarker for the early diagnosis of tumor relapse treated with epidermal growth factor receptor tyrosine kinase inhibitors.

Key words: EGFR-TKIs, hepatocyte growth factor, non-small cell lung cancer, EGFR mutation, MET

Abbreviations: DAB: 3,3′-diaminobenzidine tetrahydrochloride; DAPI: 4,6-diamidino-2-phenylindole; EGFR-TKIs: epidermal growth factor receptor-tyrosine kinase inhibitors; FISH: fluorescence in situ hybridization; FITC: fluorescein isothiocyanate; HGF: Hepatocyte growth factor; NSCLC: non-small cell lung cancer; PBS: phosphate buffered saline; PD: progressive disease; PI3K: phosphatidylinositol-3-kinase; PNA-LNA PCR: peptide nucleic acid-locked nucleic acid polymerase chain reaction; PR: partial response; ROC: receiver-operating characteristic; SD: stable disease; SD: standard deviation

Grant sponsor: Chugai Pharmaceutical
DOI: 10.1002/ijc.25799
History: Received 11 Aug 2010; Accepted 20 Oct 2010; Online 2 Dec 2010

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Lung cancer is the leading cause of cancer-related death worldwide, largely because most patients are diagnosed at advanced stages.[1,2] Recent strategies for treating non-small cell lung cancer (NSCLC) have focused on the development of molecular-targeted therapies, such as those including epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) of erlotinib and gefitinib. The tumors with active *EGFR* mutations are extremely sensitive to EGFR-TKIs. However, disease progression usually occurs in most patients at 6–12 months after EGFR-TKI treatments. Acquired EGFR-TKIs resistance has been associated with the development of a secondary mutation of T790M in *EGFR*.[3,4] In addition, *MET* amplification has been identified as another mechanism of acquired resistance.[5,6]

Recently, Yano *et al.* reported that hepatocyte growth factor (HGF) induces EGFR-TKIs resistance by restoring the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway *via* phosphorylation of MET.[7] HGF was first identified in the serum of hepatectomized rats as a potent growth factor of hepatocytes.[8] HGF is secreted in response to injury by many

organs such as the mammary gland, kidneys, liver and lungs.[9,10] HGF is produced by various cells, especially tumor cells, fibroblast cells and endothelial cells.[11,12] HGF induces multiple biological effects in target cells, including proliferation and survival, angiogenesis, cell migration and invasion and morphogenesis and tissue organization.[13,14] Tumor tissue specimens that stain strongly for HGF indicate poor outcome after NSCLC resection.[15] However, the mechanism regulating plasma HGF level in NSCLC patients has been investigated to a limited extent. It has been hypothesized that EGFR-TKIs administration may influence the levels of plasma HGF. In this study, we evaluated the pretreatment and post-treatment plasma HGF levels after EGFR-TKIs administration in advanced NSCLC. We also investigated the correlation between plasma HGF levels and sensitivity to EGFR-TKIs, tissue immunoreactivity for HGF and *MET* gene status. In addition, we determined a cut-off value of pretreatment plasma HGF level to discriminate sensitive from resistant populations, and evaluated the association between the resultant cut-off value and presence of active *EGFR* mutations.

Material and Methods

Patients

Patients with histologically or cytologically confirmed advanced NSCLC and relapsed after one or two prior chemotherapies were eligible. Each patient was required to meet the following criteria: adequate organ functions, performance status of 0-2, and no other active malignancies. Mutations in the tyrosine kinase domain (exons 18–21) of *EGFR* were identified by peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp assay. Written informed consent was obtained from all patients. This study was approved by the Osaka City University Hospital Institutional Review Boards.

Treatment, response and clinical outcome

EGFR-TKIs (erlotinib 150 mg/day, gefitinib 250 mg/day) were administered once daily. The treatment was continued until the disease progression or the patient developed intolerable toxicity or withdrew his/her consent to continue participation in the study. The objective tumor responses of each examined lesion were assessed every 4 weeks after EGFR-TKIs administration according to the Response Evaluation Criteria in Solid Tumors, ver1.0. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria Version 3.0 criteria. The grade 3 and 4 of EGFR-TKIs-related nonhematologic toxicities were managed by reducing the dose of EGFR-TKIs.

Plasma HGF analysis

Plasma samples were collected pretreatment and on post-treatment days 15 and day 30. Venous blood (7 ml) was collected in EDTA (anticoagulant)-containing tubes and immediately centrifuged at 3,000 rpm for 15 minutes. Plasma samples were frozen at −80°C until analysis. Plasma HGF levels were analyzed by Luminex 200 PONENT system (Milliplex MAP kits; Millipore) according to the manufacturer's instructions. Plasma HGF levels were estimated as previously reported;[16] in brief, 25 μl of plasma was incubated with antibody-linked beads overnight at 4°C, washed twice with the washing solution, and incubated for 1 hour with biotinylated secondary antibodies. Data acquisition using the Luminex system was performed after a final incubation of 30 minutes with streptavidin-phycoerythrin. The minimum detectable concentration of HGF was 19.2 pg/ml. All the samples were assayed in duplicate.

Immunohistochemical staining of HGF

Immunohistochemical staining of HGF was performed as previously reported.[17] Briefly, we used a 1:20 dilution of a rabbit polyclonal antibody against HGF-α (IBL, Gunma, Japan). Immunohistochemical staining was performed using formalin-fixed paraffin-embedded tissue sections obtained from our NSCLC patients. Endogenous peroxidase was blocked with 3% aqueous H_2O_2 solution for 30 minutes, and the sections (4-μm thick) were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Microwave antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0). The sections were treated with primary antibodies for 1 hour at room temperature, washed with phosphate buffered saline (PBS) and treated with EnVision/HRP polymer reagent (Dako, Glostrup, Denmark) for 30 minutes at room temperature. The immunostained sections were treated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (Dako, Glostrup, Denmark). Omission of primary antibodies served as negative controls. The immunoreactivities of the samples were independently evaluated by 2 investigators (KT and MN).

The scoring index was obtained as the product of percentage and intensity of the immunostained sections in the range 0–300, according to the method of Turke *et al.* with slight modifications.[18,19] In brief, the tissue immunoreactivity for HGF was evaluated as the percentage of cancer cells with positive cytoplasmic and/or membranous staining (0–100%), and the model intensity of the positively stained cells was determined on a scale of 0 to 3+ (0, complete absence of staining; 1+, staining weaker than that of the normal bronchial epithelium; 2+, staining similar to the that of the normal bronchial epithelium; 3+, staining clearly more intense than that of the normal bronchial epithelium).

Fluorescence in situ hybridization analyses of MET

MET fluorescence *in situ* hybridization (FISH) analyses were performed as previously reported.[19] Briefly, 4-μm thick serial sections from each tissue block were analyzed by a dual-color FISH by using a MET/CEP7 probe cocktail (Kreatech Diagnostics, Amsterdam, The Netherlands). After deparaffinization and dehydration, the slides were immersed in 0.2N HCl, incubated in 1 M NaSCN for 30 minutes at 80°C, and immersed in a pepsin solution for 15–45 minutes at 37°C.

The DNA probe set was applied onto the slides; subsequently, the slides were incubated on a hot plate at 80°C for 5 minutes to allow attachment of the target DNA and the probe, and incubated again at 37°C for 16 hours to achieve hybridization. Then, posthybridization washes were performed using 0.4× SSC/0.3% NP-40 for 2 minutes at 72°C and subsequently with 2×SSC/0.1% NP-40 for 1 minute at room temperature. The slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and an antifade compound (*p*-phenylenediamine). The FISH signals were enumerated in at least 100 nonoverlapping tumor cell nuclei by using an epifluorescence microscope with single interference filters sets for green (fluorescein isothiocyanate, FITC), red (Texas red) and blue (DAPI), and using dual (red/green) and triple (blue, red and green) band pass filters. According to the copy number of the *MET* gene, the following 6 categories were established: disomy (≤2 copies in >90% of cells); low trisomy (≤2 copies in ≥40% of cells, 3 copies in 10–40% of cells and ≥4 copies in <10% of cells); high trisomy (≤2 copies in ≥40% of cells, 3 copies in ≥40% of cells and ≥4 copies in <10% of cells); low polysomy (≥4 copies in 10–40% of cells); high polysomy (≥4 copies in ≥40% of cells); and gene amplification (presence of tight *MET* gene clusters and *MET* gene to chromosome 7 ratio of ≥2 or ≥15 copies of Met per cell in ≥10% of cells).[20,21] The *MET* gene status was further classified into three groups: *MET* FISH-negative (disomy, low trisomy, high trisomy and low polysomy), *MET* FISH-positive with high polysomy, and *MET* FISH-positive with gene amplification.

Statistical analysis

Statistical analyses were performed by using the Student's *t*-test (Stat View5.0 and JMP8.0 statistical program). All values are expressed as mean and standard deviation (SD). The association between pretreatment plasma HGF concentrations and responses to EGFR-TKIs was compared by Kruskal-Wallis test. The best cut-off value for diagnosis of responder (PR plus SD patients) was investigated by receiver-operating characteristic (ROC) analysis, and its sensitivity and specificity were calculated. The association between the resultant cut-off value and presence of active *EGFR* mutations was performed with Fisher's exact test. The relation between plasma HGF concentrations and scoring indexes of both HGF immunoreactivity and *MET* gene status was investigated by Pearson's correlation coefficient test. A p value of less than 0.05 was considered statistically significant.

Results

Patient characteristics

Between September 2008 and October 2009, 31 patients were enrolled in this study. We could not obtain adequate plasma samples for analyses from 3 patients on pretreatment, 6 patients on the post-treatment day 15, and 8 patients on the post-treatment day 30. *EGFR* mutation status was positive in 19 patients, negative in 9 patients, and unknown in 3 patients. Initially, no patients with *EGFR* mutations had the secondary T790M mutation. The patient population profile is provided in Tables 1 and 2.

Table 1. Patient characteristics (n = 31)

Characteristic	Number of patients
Age	
Median (range)	67 (54–86)
Gender	
Male/female	17/14
Performance status	
0/1/2	3/22/6
Clinical stage	
IIIB/IV	6/25
Histology	
Adenocarcinoma	28
Squamous	2
Large cell	1
Smoking history	
Never	11
Former	20

Plasma HGF levels before and after EGFR-TKIs treatment

Plasma HGF levels were analyzed in 28 of the 31 patients before treatment, in 25 patients on the post-treatment day 15, and in 23 patients on the post-treatment day 30. The mean pretreatment plasma HGF level was 485.9 ± 230.2 pg/ml. The association between plasma HGF levels and EGFR-TKIs treatment is shown in Figure 1. Plasma HGF levels on the post-treatment day 15 (630.1 ± 366.9 pg/ml) were significantly increased as compared to the pretreatment plasma HGF levels (p = 0.029). Plasma HGF levels on the post-treatment day 30 (581.5 ± 298.1 pg/ml) tend to be higher than those before treatment (p = 0.057).

Association between pretreatment plasma HGF levels and the efficacy of EGFR-TKIs treatment

We analyzed the association between pretreatment plasma HGF levels and the efficacy of EGFR-TKIs treatment in 28 patients (Fig. 2). The response to EGFR-TKIs treatment was partial response (PR) in 8 cases, stable disease (SD) in 12 cases and progressive disease (PD) in 8 cases. The 8 PR cases, 8 of 12 SD cases and 3 of 8 PD cases had *EGFR* mutations. Pretreatment plasma HGF levels in PD patients (724.1 ± 216.4 pg/ml) were significantly higher as compared to those in SD patients (396.5 ± 148.3 pg/ml; p = 0.0008) and PR patients (381.7 ± 179.0 pg/ml; p = 0.0039).

In the ROC analysis, the optimal pretreatment plasma HGF cut-off value for diagnosis of responder (PR plus SD patients) was 553.5 pg/ml. In Figure 2, the horizontal dash

Table 2. Summary of pretreatment plasma HGF and tissue samples from patients with NSCLC

Case	Response to EGFR-TKIs	Pretreatment plasma HGF (pg/ml)	*EGFR* mutation	HGF IHC	*MET*
1	PR	112.3	Ex21 (L858R)	Low	Positive
2	PR	245.0	Ex19 (E746-A750Del)	Strong	Negative
3	PR	257.6	Ex21 (L858R)	Low	Negative
4	PR	303.3	Ex21 (L858R)	High	Positive
5	PR	449.6	Ex19 (E746-A750Del)	High	Amplification
6	PR	508.5	Ex21(L858R) +Ex19(E746-A750Del)	Low	Negative
7	PR	538.0	E746-A750Del	Low	Negative
8	PR	639.5	Ex21 (L858R)	Low	Negative
9	SD	185.8	Ex19 (E476-A750Del)	N.E	N.E
10	SD	188.1	Ex21 (L858R)	Low	Negative
11	SD	208.0	Negative	Low	Negative
12	SD	331.3	Ex21 (L858R) + Ex19 (E747-S752Del)	Low	Negative
13	SD	335.4	Ex19 (E746-A750Del)	Strong	Negative
14	SD	421.0	Ex21 (L858R)	High	Negative
15	SD	432.1	Ex19 (E746-A750Del)	Low	Negative
16	SD	481.1	Negative	Low	Negative
17	SD	487.8	Ex19	High	Positive
18	SD	494.6	Negative	N.E	N.E
19	SD	553.5	Ex19	Low	Negative
20	SD	639.5	Negative	N.E	N.E
21	SD	N.E	Unknown	N.E	N.E
22	SD	N.E	Unknown	N.E	N.E
23	PD	432.1	Negative	Strong	Positive
24	PD	481.1	Negative	Low	Positive
25	PD	639.5	Ex19	High	Negative
26	PD	678.0	Ex21 (L858R)	Low	Positive
27	PD	688.0	Negative	High	Positive
28	PD	882.0	Negative	Low	Unknown
29	PD	989.1	Negative	Low	Negative
30	PD	1003.1	Ex18	High	Positive
31	PD	N.E	Unknown	N.E	N.E

Abbreviations: N.E: not evaluated; PD: progressive disease; SD: stable disease; PR: partial response; L858R: L858R point mutation; Del: in-frame deletion.

line showed the cut-off value of 553.5 pg/ml. Its sensitivity and specificity were 90% and 65%, respectively. The area under the curve for pretreatment plasma HGF levels was 0.888. Furthermore, we investigated whether this cut-off value is associated with presence of active *EGFR* mutations. However, pretreatment plasma HGF cut-off value had no correlation with presence of active *EGFR* mutation (p=0.405).

The most frequent adverse events were rash and diarrhea. Grade 2 rash and diarrhea occurred in 9 (29%) and 4 (13%) patients, respectively. Grade 3 diarrhea occurred in 1 (3%) patient. Adverse events had no correlation with the pretreatment plasma HGF levels.

Tissue immunoreactivities for HGF and MET gene status

To clarify the source of plasma HGF, we evaluated the tissue immunoreactivity for HGF and the *MET* status by FISH in 25 of the 31 patients. Six patients were diagnosed NSCLC only cytologically. Tumor samples were collected from 25 patients, including surgically resected specimens from 8 patients, transbronchial biopsy specimens from 15 patients

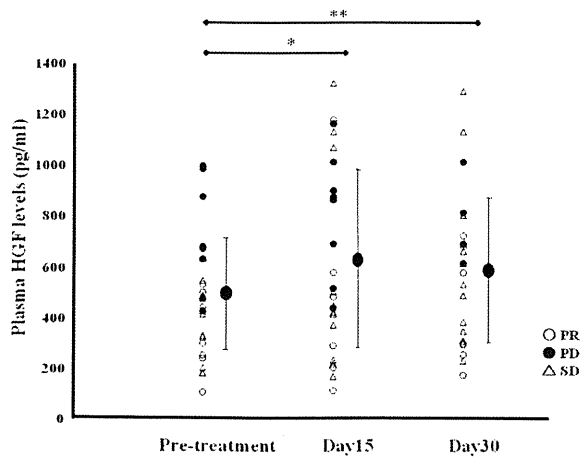

Figure 1. Changes in plasma HGF levels before and after EGFR-TKIs treatment in lung cancer patients. Plasma HGF levels on post-treatment day 15 (630.1 ± 336.9 pg/ml) were significantly increased as compared to the pretreatment HGF levels (485.9 ± 230.2 pg/ml) (*$p = 0.029$). Plasma HGF levels on post-treatment day 30 (581.5 ± 298.1 pg/ml) were higher than those before treatment (**$p = 0.057$).

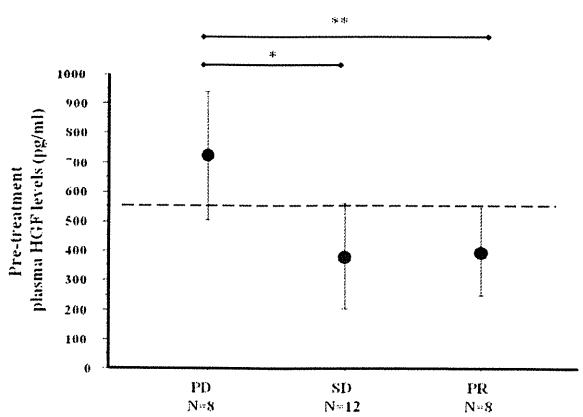

Figure 2. Relationship between pretreatment plasma HGF levels and the efficacy of EGFR-TKIs treatment. Pretreatment plasma HGF levels in PD patients ($n = 8$; 724.1 ± 216.4 pg/ml) were significantly higher as compared to those in PR patients ($n = 8$; 381.7 ± 179.0 pg/ml; **$p = 0.0039$) and SD patients ($n = 12$; 396.5 ± 148.3 pg/ml; *$p = 0.0008$). The horizontal dash line showed the optimal pretreatment plasma HGF cut-off value of 553.5 pg/ml for diagnosis of responders (PR plus SD patients).

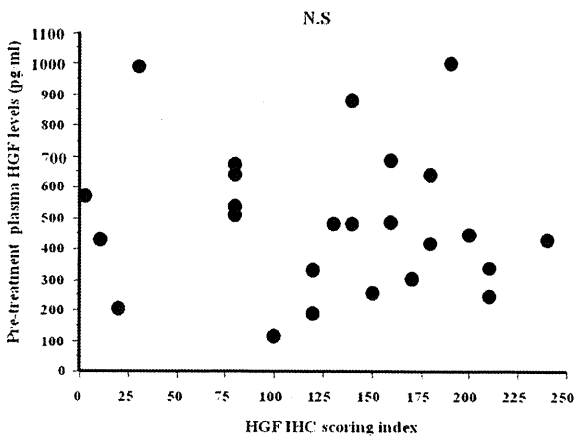

Figure 3. Pretreatment plasma HGF levels had no correlation with the scoring indexes of HGF immunoreactivity ($r = -0.088$, $p = 0.678$).

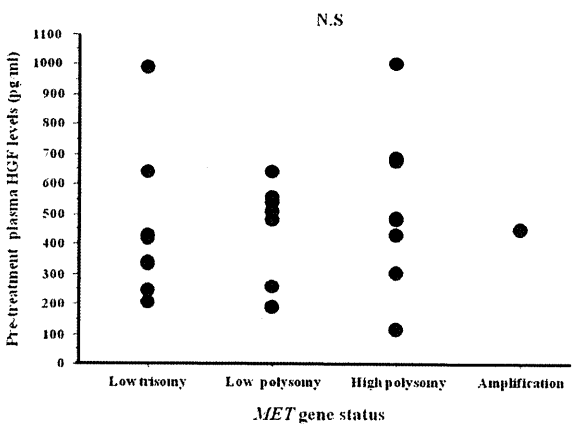

Figure 4. Pretreatment plasma HGF levels had no correlation with MET gene status ($r = 0.176$, $p = 0.414$).

and biopsy specimens from vertebra and skin in each 1 patient. High HGF expression (index score over 150) was detected in 10 of the 25 (40%) specimens and strong expression in 3 specimens (12%) with HGF immunoreactivity scoring index of more than 200.

FISH revealed that 9 patients (36%) were *MET* positive (1 with gene amplification and 8 with high polysomy). Pretreatment plasma HGF levels had no correlation with the scoring indexes of both HGF immunoreactivity ($r = -0.088$, $p = 0.678$) and *MET* gene status ($r = 0.176$, $p = 0.414$) (Figs. 3 and 4). Further, response to EGFR-TKIs was not associated with the scoring indexes of HGF immunoreactivity and *MET* gene status.

Discussion

We have shown that plasma HGF levels on the post-treatment day 15 were significantly increased as compared to the pretreatment plasma levels, and the HGF concentration continued to remain high up to day 30. Pretreatment plasma

HGF levels had no correlation with the scoring indexes of both HGF immunoreactivity and *MET* gene status. Recently, Wei Wang *et al.* reported that treatment of the *EGFR* mutant human lung adenocarcinoma cell lines, PC-9 (del E746_A750) and HCC827 (del E746_A750), by gefitinib induced the recruitment of fibroblast cells.[22] HGF is a predominant fibroblast-derived factor.[23] These findings indicate that other cells such as fibroblast cells and endothelial cells secrete HGF to a greater extent than the tumor cells; the former cell types secrete HGF to protect the lung cancer cells from and repair the damage caused by EGFR-TKIs.

Several trials have reported that patients with *EGFR* mutations exhibit a response rate of 70–75%.[24–27] However, some patients are intrinsically resistant to EGFR TKIs even though they have *EGFR* mutations. Among patients with intrinsic resistance, only 0.5 and 3% patients have the secondary T790M mutation and *MET* amplification, respectively.[6,28] HGF have been reported as third mechanism of EGFR-TKIs resistance.[7] Kasahara *et al.* reported that lung adenocarcinoma patients with *EGFR* mutation did not affect serum HGF levels and that the serum HGF levels in PD patients were higher than those in SD and PR patients, irrespective of their *EGFR* mutation status.[29] In our study, the cut-off value of pretreatment plasma HGF concentrations was over 553.5 pg/ml in 6 of the 8 PD patients with its sensitivity and specificity of 90% and 65%, respectively. Even though 3 of the 8 PD patients had *EGFR* mutations and high concentrations of plasma HGF, they had intrinsic resistance to EGFR-TKIs. None of these patients had secondary T790M mutation and *MET* amplification. Besides, the pretreatment plasma HGF levels in PD patients were significantly higher as compared to those in SD patients and PR patients, and the HGF cut-off level had no correlation with presence of active *EGFR* mutations. Our results suggest that high plasma HGF concentrations may be involved in EGFR-TKIs resistance, irrespective of the *EGFR* mutation status.

Further, we investigated the correlation between plasma HGF concentration, tissue immunoreactivity for HGF, *MET* gene status and the efficacy of EGFR-TKIs treatment. The pretreatment plasma HGF levels had no association with tissue immunoreactivity for HGF and *MET* gene status. In addition, response to EGFR-TKIs treatment had no association with tissue immunoreactivity for HGF and *MET* gene status. Cappuzzo *et al.* reported that patients with primary resistance to gefitinib therapy could not be identified by *MET* FISH analysis of pretreatment tumor biopsy specimens.[19] Our results may provide the evidence that plasma HGF levels are a better indicator of intrinsic EGFR-TKIs resistance than tissue immunoreactivity for HGF or *MET* gene status.

Our study has certain limitations. First, plasma HGF has been influenced from various factors such as renal or liver dysfunction, interstitial pneumonia, and other active malignancies. We excluded the patients who had the possible interfering factors in the analysis of plasma HGF levels. However, we could not completely exclude the influencing factors such as other growth factors and cytokines. Second, the number of patients enrolled in this study was small. Third, we collected blood samples at only 3 time points (pretreatment and on post-treatment days 15 and 30). By measuring plasma HGF levels at more time points, we could evaluate the relationship between acquired resistance to EGFR-TKIs and plasma HGF levels. Finally, in the evaluation of the tissue immunoreactivity for HGF, there was some difference in evaluating the percent positivity for HGF between the small biopsy specimen of transbronchial tissue and the large specimen of surgically resected tissue.

In conclusion, statistically significant increase in plasma HGF levels was observed on administering EGFR-TKIs. High levels of pretreatment plasma HGF, which cut-off level was 553.5 pg/ml, indicated intrinsic resistance to EGFR-TKIs. This cut-off value is useful in defining a case as positive or negative for EGFR-TKIs therapy for relevant clinicians. Further studies are needed to clarify the mechanisms of plasma HGF and HGF signaling in relation to the acquired resistance to EGFR-TKIs. Plasma HGF levels can be easily measured and are sufficient for objective analysis of the patient condition. Plasma HGF levels might be a useful biomarker for the early diagnosis of relapsed patients treated with EGFR-TKIs.

Acknowledgements

The authors thank Ms. Maki Nakai and Ms. Mari Okamoto for their secretarial assistance. Dr. S. Yano has received commercial research support from Chugai Pharmaceutical Co., Ltd.

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Clinical Study

Chemotherapy

Chemotherapy 2011;57:357–362
DOI: 10.1159/000330481

Received: March 11, 2011
Accepted after revision: July 4, 2011
Published online: September 6, 2011

Randomized Phase II Study of Two Schedules of Carboplatin and Gemcitabine for Stage IIIB and IV Advanced Non-Small Cell Lung Cancer (JACCRO LC-01 Study)

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Key Words
Lung cancer · Randomized phase II study · Gemcitabine · Carboplatin · Thrombocytopenia

Abstract
Background: Gemcitabine combined with carboplatin (CG) is one of the regimens used widely for advanced non-small cell lung cancer. Improvement in its toxicity may result in good clinical outcomes. ***Methods:*** A new schedule of gemcitabine and carboplatin (CG8) was compared with the standard one (CG1). Both are 3-weekly regimens, but carboplatin is administered on day 1 in CG1 and on day 8 in CG8. ***Results:*** The response rate of CG1 was 29.2%, which was higher than that of CG8 (22.2%). Median survival times in CG1 and CG8 were 348 and 455.5 days, respectively. Grade ≥3 leukopenia, thrombocytopenia and anemia were observed in 56.0, 72.0 and 36.0% of patients with CG1 and in 33.3, 25.9 and 14.8% of patients with CG8, respectively. Whereas grade ≥3 elevation of asparatate aminotransferase, alanine aminotransferase and alkaline phosphatase was seen mainly in CG8, grade ≥3 non-hematologic toxicities such as febrile neutropenia, infection, appetite loss, diarrhea and eruption were observed only in CG1. ***Conclusion:*** CG1 is superior in response rate, but CG8 shows improved toxicities and a tendency of prolonged survival.

Introduction

Cytotoxic chemotherapy still plays a pivotal role in the management of advanced non-small cell lung cancer (NSCLC), whereas recently, the combination of platinum doublets and molecular targeted drugs has been extensively studied. Among several platinum doublets, the combination of carboplatin and gemcitabine (CG) is a popular regimen. Carboplatin is commonly used in a practical setting compared with cisplatin, although the antitumor effect of carboplatin is suggested to be somewhat inferior to cisplatin [1–3]. Several studies have shown that platinum doublets with 3rd-generation cytotoxic drugs were similar in antitumor activities but different in toxicities [4]. A recent trial showed that pemetrexed/cis-

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platin produced better survival compared with gemcitabine/cisplatin in NSCLC with non-squamous (Non-Sq) histology [5]. In contrast, Grønberg et al. [6] showed that the superiority in survival of pemetrexed over gemcitabine is not clear when carboplatin was combined with these drugs. Hematologic toxicities of CG were more severe than those of pemetrexed/carboplatin: grade ≥3 leukopenia 46 and 23% (p = 0.001), neutropenia 51 and 40% (p = 0.024) and thrombocytopenia 56 and 24% (p = 0.001) in CG and pemetrexed/carboplatin, respectively. More patients in the CG arm received transfusions of red blood cells and platelets. If hematologic toxicities of CG regimens are improved, its application will be extended in the 1st-line treatment for Sq NSCLC or as an alternative of pemetrexed/carboplatin for Non-Sq NSCLC.

Clinical trials including CG regimens initially adopted a 4-week regimen. Subsequently, 3-week schedules in which gemcitabine was administered on days 1 and 8 were evaluated. In phase II studies, 3-week regimens showed comparable efficacy with the 4-week schedules [7]. In the 4-week arm, carboplatin at an area under the curve (AUC) of 5 mg/ml × min was administered on day 8 and gemcitabine 1,000 mg/m^2 was given on days 1 and 8. In the 3-week arm, carboplatin at AUC 5 was given on day 1, and gemcitabine at 1,000 mg/m^2 was administered on days 1 and 8. Obasaju et al. [8] compared 3- and 4-week schedules of CG in 472 advanced NSCLC patients. Although statistically not significant, 2% complete responses and 38% partial responses produced by the 3-week schedule are better than no complete response and 23% partial responses by the 4-week schedule. In contrast, the frequency of grade 3 or 4 thrombocytopenia, which is particularly problematic in CG, was 14% in the 3-week schedule and 8% in the 4-week schedule. A Japanese comparative phase II study, where 3-week schedule CG was compared with the combination of gemcitabine and vinorelbine, also showed a high incidence of dose reduction and early withdrawal due to myelosuppression, mainly thrombocytopenia, in CG [9].

These results indicate that hematologic toxicities of the 3-week CG regimen should be improved. We already reported a new regimen, in which gemcitabine of 1,000 mg/m^2 was administered on days 1 and 8 and carboplatin of AUC 5 on day 8 every 21 days. A schedule-dependent synergistic effect of gemcitabine combined with pemetrexed was reported in the treatment of NSCLC cell lines [10]. A similar schedule-dependent synergy may be observed in the CG combination. In a preceding phase II trial with 31 patients with stage IIIB or IV NSCLC, the response rate was 22.6%, including one complete response, and median time to progression and median survival time were encouraging, i.e. 161 and 454 days, respectively [11]. In this regimen, the criteria to start new cycles and those to perform day 8 infusion were specially defined in order to improve myelosuppression and to maintain dose intensity: white blood cell count (WBC) ≥2,500/mm^3 and platelet count (Plt) ≥750,000/mm^3 to start new cycles and WBC ≥3,000/mm^3 and Plt ≥100,000/mm^3 to perform day 8 infusion. As reported previously, these criteria were proved to work well, resulting in grade 3/4 thrombocytopenia only in 2 patients (6.5%; one grade 3 and one grade 4). Since this phase II study was performed in a single institution, we have compared the 3-week CG regimen that we developed with the standard 3-week CG regimen in a multi-institutional randomized phase II study.

Patients and Methods

Eligibility

Patients were eligible for study participation when they met the following criteria: age <75 years; histologic or cytologic diagnosis of NSCLC; clinical stage IIIB not amenable to curative treatment or stage IV by the Union for International Cancer Control TNM classification version 6; first-line treatment; Eastern Cooperative Oncology Group performance status 0–1; measurable disease in Response Evaluation Criteria in Solid Tumors (RECIST); adequate bone marrow reserve (neutrophil count ≥1,500/mm^3, Plt ≥10 × 10^4/mm^3, hemoglobin ≥9.0 g/dl); acceptable hepatic (serum bilirubin <1.5 mg/dl, transaminases less than twice the upper limit of normal) and renal function (normal serum creatinine and creatinine clearance determined by Cockcroft equation ≥50 ml/min), and a life expectancy of at least 3 months. Patients were excluded from the study when they met one of the following conditions: active uncontrolled infection; unstable concomitant disease (ischemic heart disease, hypertension, arrhythmia, cirrhosis and diabetes mellitus); active concomitant malignant disease; massive effusion; concomitant interstitial lung disease; superior vena cava syndrome; brain metastasis, and pregnancy or breastfeeding. Written informed consent was obtained from all patients.

Study Design

The eligible patients were randomized to the CG1 or the CG8 arm. In the CG1 arm, carboplatin of AUC 5 calculated using the Calvert formula with creatinine clearance evaluation by the Cockcroft equation and gemcitabine of 1,000 mg/m^2 were administered as an intravenous injection on day 1 and on days 1 and 8, respectively (CG1). In the CG8 arm, the same doses of CG were administered as an intravenous injection on day 8 and on days 1 and 8, respectively (CG8). Treatment was repeated every 3 weeks. The two arms are similar, but developed independently. Therefore, we adopted independent hematologic criteria suitable for each arm to start new cycles and to perform day 8 infusion: in the CG1 arm, WBC ≥3,000/mm^3 and Plt ≥10 × 10^4/mm^3 were required to start new cycles, and WBC ≥2,000/mm^3 and Plt ≥100,000/mm^3 were necessary to perform day 8 infusion. In the CG8 arm, corresponding hematologic criteria were the same as

those in our preceding phase II study described in the Introduction section: WBC ≥2,500/mm^3 and Plt ≥750,000/mm^3 to start new cycles, and WBC ≥3,000/mm^3 and Plt ≥100,000/mm^3 to perform day 8 infusion. When one of these criteria was not met, treatment was skipped. A dose reduction of up to two times was permitted in the case of a leukocyte count <1,000/mm^3, Plt <25,000/mm^3, febrile neutropenia, grade >2 non-hematologic toxicity, or skip of day 8 administration in the preceding cycle. The dose of gemcitabine was reduced to 800 mg/m^2 in the first dose reduction and that of carboplatin to AUC 4 in the second one. After withdrawal from the study, subsequent treatment was decided by the investigator.

This study was performed by JACCRO (Japanese Cancer Clinical Research Organization) as an LC-01 study.

Evaluation of Toxicity and Response

Toxicity was scored every 3 weeks during treatment and every month thereafter according to the National Cancer Institute Common Toxicity Criteria version 2.0. Response was evaluated every 4 weeks during treatment and every 6 months until disease progression thereafter, according to RECIST criteria [12]. Brain MRI, chest CT scan and abdominal CT scan were performed at any time if assessment for disease progression was necessary. Objective responses were required to be confirmed after at least 4 weeks.

Endpoint and Statistical Analysis

The primary endpoint of this study was the response rate. Secondary endpoints included overall survival, toxicities, completion rate of 1–3 cycles, and dose intensity during 1–3 cycles. If the threshold response rate and the expected response rate were to be 20 and 35%, respectively, the study has 90% power to detect the better arm with 90% confidence using Simon's selection design when 29 patients were included in each arm. The Kaplan-Meier method was used to plot overall survival.

Table 1. Patient characteristics

	Arm A	Arm B
Patients	25	27
Age, years		
Mean	59.2	61.4
Range	40–74	40–73
Men/women	19/6	15/12
Performance status 0/1	10/15	14/13
Stage (IIIb/IV/after operation)	5/18/2	4/21/2
Histology (Ad/Sq/La)	22/2/1	21/4/2

Ad = Adenocarcinoma; Sq = squamous cell carcinoma; La = large cell carcinoma.

Table 2. Treatment delivery and dose intensity

	Arm A	Arm B
Completion of 3 cycles, %		
Overall	52.0	66.7
Without skips	40.0	33.3
With skips	12.0	33.3
Dose intensity, % of planned dose		
Carboplatin	76.3	68.1
Gemcitabine	67.2	74.4

Results

Patient Characteristics

From February 2005 to April 2007, 55 patients were enrolled in the study. Protocol amendment was done to prolong accrual time because of slow accrual, but the entry of the patients was finally stopped before completing the planned accrual of 60 patients. One patient was ineligible because of preceding chemotherapy, and 2 patients who had been allocated to the CG1 arm received CG8. Excluding these 3 patients, 25 patients in the CG1 arm and 27 patients in the CG8 arm were analyzed. Patient backgrounds are shown in table 1. Although ages, stages and histology are well balanced between the two arms, the ratios of men and the patients with a performance status of 1 seem to be slightly higher in the CG1 arm.

Treatment Delivery

The median number of the cycles administered was 3 in both arms. Whereas more patients underwent the 2nd to 4th cycle in the CG8 arm compared with the CG1 arm, the percentage of the patients who received the 5th and 6th cycle was higher in the CG1 arm. As shown in table 2, 52.0% of the patients in the CG1 arm and 66.7% in the CG8 arm received at least 3 cycles of chemotherapy, and 40.0% of the patients in the CG1 arm and 33.3% in the CG8 arm did without skips of administration schedule. Skipping administration on day 8 occurred in 36 and 48.1% in CG1 and CG8, and dose reduction was necessary in 56.0 and 40.7% in CG1 and CG8, respectively. Reflecting these modifications, the relative dose intensity of gemcitabine was 67.2% of the planned dose in CG1 and 74.4% in CG8, and that of carboplatin was 76.3% in CG1 and 68.1% in CG8 in the first 3 cycles (table 2).

Efficacy Results

The response rate in the CG1 arm was 29.2% and is higher than that in the CG8 arm (22.2%; table 3). The response rates of both arms exceeded the threshold of 20%, but that of the better one did not reach the expected value of 35%. The rate of progression was higher in CG1 than

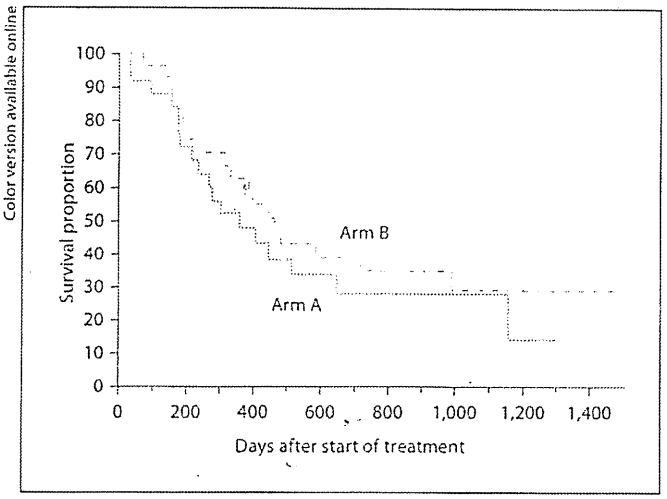

Fig. 1. Survival curves. Survival curves of arms A and B were drawn by the Kaplan-Meier method. The difference is not statistically significant (p values are 0.40 and 0.41 by the log-rank test and Wilcoxon test, respectively).

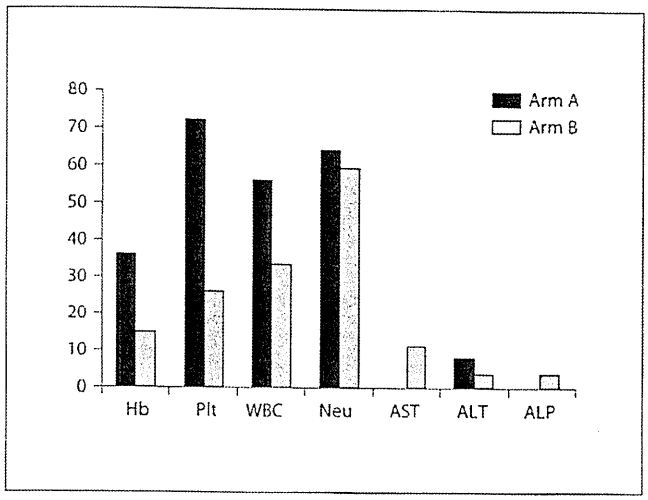

Fig. 2. Hematologic toxicity and abnormal blood chemistry of grade 3 and 4. Toxicities in blood hemoglobin value (Hb), WBC and neutrophil counts (Neu) are shown. AST = Asparatate aminotransferase; ALT = alanine aminotransferase; ALP = alkaline phosphatase.

Table 3. Response and survival

	Arm A	Arm B
Response rate, %	29.2	22.2
CR	4.0	0
PR	24.0	22.2
SD	40.0	51.8[1]
PD	28.0	25.9
NE	4.0	0
Median survival time[2], days	348	455.5

CR = Complete response; PR = partial response; SD = stable disease; PD = progressive disease; NE = not evaluable.

[1] Including one unconfirmed partial response.

[2] p = 0.40 by the log-rank test; p = 0.41 by the Wilcoxon test.

that in CG8. Overall survival curves were shown in figure 1. Median overall survival time was 348 and 455.5 days in the CG1 and CG8 arm, respectively.

Toxicity

Toxicity profiles are summarized in figure 2 and table 4. Hematologic toxicities were generally milder in CG8 than in CG1: grade 3/4 leukopenia was observed in 56.0 and 33.3% of the patients, grade 3/4 thrombocytopenia in 72.0 and 25.9%, and grade 3/4 anemia (hemoglobin) in 36.0 and 14.8% in the CG1 and CG8 arm, respectively, whereas the frequency of grade 3/4 neutropenia was comparable between the two arms (64.0 and 59.2%, respectively). Grade 4 thrombocytopenia occurred in 4.0% in CG1, whereas no grade 4 thrombocytopenia was observed in CG8. Abnormal blood chemistry was sporadically observed but not severe in both arms. Table 4 summarizes non-hematologic toxicities of grade ≥3 except those in blood chemistry. Whereas the grade ≥3 elevation of asparatate aminotransferase, alanine aminotransferase and alkaline phosphatase was seen mainly in CG8, grade ≥3 non-hematologic toxicities such as febrile neutropenia, infection, appetite loss, diarrhea and eruption were observed only in CG1.

Discussion

Recently, there has been progress in medical treatment for advanced NSCLC, incorporating molecular targeted drugs such as epidermal growth factor receptor tyrosine kinase inhibitors and bevacizumab, an angiogenesis inhibitor. However, cytotoxic drugs are still playing a pivotal role acting as themselves or as platforms with which molecular targeted drugs are combined. Less toxic chemotherapy regimens will have the advantages to maintain the quality of life of the patients

Table 4. Non-hematologic toxicities

	Arm A, %		Arm B, %	
	G2	G3	G2	G3
Febrile neutropenia	–	4.0	–	0
Infection	0	8.0	0	0
Body weight loss	0	0	3.7	0
Fever	0	0	3.7	0
General fatigue	0	0	7.4	0
Dyspnea	8.0	0	3.7	0
Nausea	8.0	0	7.4	0
Vomiting	4.0	0	0	0
Appetite loss	0	4.0	0	0
Diarrhea	4.0	4.0	3.7	0
Stomatitis	0	0	3.7	0
Constipation	16.0	0	11.1	0
Atrial fibrillation	4.0	0	0	0
Eruption	12.0	4.0	7.4	0

G2 = Grade 2; G3 = grade 3. No grade 4 and 5 toxicities were observed. Adverse events were graded by the National Cancer Institute Common Toxicity Criteria version 2.0.

and to combine them with molecular targeted drugs. The CG combination was tested in various situations of NSCLC patients such as in an adjuvant setting, for the elderly and as a second line for selected patients, showing encouraging effects [13–15]. In addition, an acceptable toxicity and promising median overall survival was reported in the combination of CG and bevacizumab for Non-Sq NSCLC [16]. The management of carboplatin doublets is shown to be improved by introducing a clinical pathway [17].

We studied a 3-week CG regimen in which carboplatin is administered on day 8 and gemcitabine on days 1 and 8. In the present study, we compared two 3-week CG regimens, CG1 and CG8. The results show that the response rate, the primary endpoint of this study, is higher in CG1 than in CG8. The response rate in CG8 in our preceding phase II study described above was 22.6%, which was reproduced in this study. Since this study was conducted to select the better arm with regard to the response rate, one main conclusion of this study is that CG1 should be selected in subsequent studies.

In addition to the difference in response rate, our study also clarifies several useful differences between CG1 and CG8. In both hematologic and non-hematologic toxicities, CG8 is less toxic than CG1. Hematologic toxicities, especially thrombocytopenia, of CG8 are milder than those of CG1. Survival of the patients with CG8 is better than that of patients with CG1. Median survival time in the CG1 group is 348 days and is comparable to that of paclitaxel/carboplatin in the Four Arm Chemotherapy Study, a phase III study for advanced NSCLC performed in Japan [18]. Median survival time in the CG8 group is 455.5 days and is longer than that of CG1, although statistically not significant. The overall survival time of CG8 patients in our preceding phase II trial (454 days) was again reproduced. The difference in overall survival between CG1 and CG8 is hard to explain, because of the comparable dose intensity between the two arms and a better response rate in CG1. Reduced toxicity together with increased dose intensity of gemcitabine in CG8 may contribute to some extent to a possible prolonged survival. Increased dose intensity without deteriorating the patients' conditions may lead to prolonged survival and improvement in quality of life. Therefore, dose intensity could be selected as a primary endpoint in this study. The results show that dose intensities of both arms are comparable; the dose intensity of carboplatin is higher in CG1 and that of gemcitabine is higher in CG8. Progression-free survival time or overall survival time might have been a better endpoint. It may be possible to select tumor markers such as CYFRA21-1 or carcinoembryonic antigen as an endpoint in certain trials, because it is reported that they are valuable in evaluating chemotherapy in NSCLC [19].

CG was one of the least toxic platinum doublets. Recently, pemetrexed was introduced in the treatment of Non-Sq NSCLC. Pemetrexed/carboplatin produced comparable efficacy and showed statistically significant improvement in hematologic toxicities, when compared with CG1. Although pemetrexed/platinum is the first-choice regimen for Non-Sq NSCLC, CG is considered to be used for Sq NSCLC and even for Non-Sq NSCLC in an individual patient setting. In future chemotherapy for NSCLC, molecular targeted drugs will be used in combination with cytotoxic regimens. There are growing evidences showing that some molecular targeted drugs may require a suitable selection of cytotoxic regimens to exhibit cooperation [20–22]. Therefore, several kinds of platinum doublets, such as pemetrexed/platinum, paclitaxel/carboplatin and gemcitabine/platinum, should be tested in combination with new drugs. Our study may improve the usage of CG regimens, alone and in combination with molecular targeted drugs.

Conclusion

Whereas a standard 3-week schedule of CG (CG1) should be selected in subsequent studies because of a higher response rate, the new schedule (CG8) seems less toxic and shows a tendency of better survival.

Acknowledgment

This work is supported by JACCRO (Japan Clinical Cancer Research Organization).

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Med Oncol
DOI 10.1007/s12032-011-0034-6

ORIGINAL PAPER

Long-term chemotherapy may prolong survival in advanced non-small-cell lung cancer among responders to first-line chemotherapy

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Received: 19 May 2011 / Accepted: 22 July 2011

Abstract Survival in patients with advanced non-small-cell lung cancer (NSCLC) has substantially improved. Long-term chemotherapy with epidermal growth factor tyrosine kinase inhibitors (EGFR-TKIs) and other agents has been associated with long survival. We retrospectively examined the associations between overall survival (OS) and clinical variables in patients with advanced NSCLC who received at least one dose or course of outpatient chemotherapy in our institution. Of 360 patients who received first-line chemotherapy between January 1, 2004 and December 31, 2007, 185 subsequently received additional outpatient chemotherapy and 175 underwent inpatient chemotherapy only. Of the 185 patients, 147 (79.5%), 96 (51.9%), and 60 (32.4%) received second-line, third-line, and fourth-line chemotherapy, respectively. Patients who received outpatient chemotherapy had significantly longer median OS (22.3 months) than did those undergoing inpatient chemotherapy only (7.6 months; $P < 0.0001$). In univariate analysis of the 185 patients, sex, performance status (PS), smoking status, stage, best response to first-line chemotherapy, use of docetaxel, and EGFR-TKIs were significantly associated with OS (P values: 0.0019, 0.0066, 0.0001, 0.0231, 0.0011, 0.0250, and 0.0023, respectively). In multivariate analysis, PS, stage, best response to first-line chemotherapy, and use of docetaxel were significantly associated with OS (P values: 0.0272, 0.0030, 0.0022, and 0.0376, respectively). Survival was significantly longer among patients who responded to docetaxel and/or EGFR-TKIs. Long-term chemotherapy did not increase cumulative hospitalization. In patients with advanced NSCLC, an effective long-term chemotherapy regimen might prolong survival in responders to first-line chemotherapy.

Keywords Non-small-cell lung cancer · Advanced stage · Survival · Long-term chemotherapy · Outpatient chemotherapy

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Introduction

Although lung cancer is the leading cause of cancer-related death worldwide [1], the prognosis in patients with advanced non-small-cell lung cancer (NSCLC) is improving [2]. In Japan, median survival time (MST) before the year 2000 in patients with NSCLC who received platinum doublets was 8–10 months [3]. Since then, third-generation agents such as vinorelbine, docetaxel, paclitaxel, irinotecan, and gemcitabine have improved survival. In Japan, the

Published online: 19 August 2011

Four Arms Cooperative Study (FACS) [4] showed no significant differences in MST between patients receiving cisplatin plus irinotecan (14.2 months), carboplatin plus paclitaxel (12.3 months), cisplatin plus gemcitabine (14.8 months), and cisplatin plus vinorelbine (11.4 months). In contrast with the finding from Japanese patients, the Eastern Cooperative Oncology Group (ECOG) study noted an MST of only 7–8 months among patients receiving four third-generation regimens [5], and a European study [6] observed a shorter MST (9.5–10.0 months) among patients given three third-generation regimens. MST appears to be longer in Japanese patients with advanced NSCLC than in other populations.

Recently, studies of maintenance therapy [7–9] and molecular targeted therapy [10–12] have reported prolonged progression-free survival (PFS) and overall survival (OS) in patients with advanced NSCLC.

MST in maintenance chemotherapy with gemcitabine, pemetrexed, and docetaxel was 13.0, 13.4, and 12.3, respectively. In the IRESSA® Pan-Asia Study (IPASS), never or light smokers with advanced lung adenocarcinoma had long median OS with carboplatin plus paclitaxel or gefitinib (17.4 vs. 18.8 months, respectively) [10]. Furthermore, in patients with drug-sensitive EGFR mutations receiving the above regimens, median OS was further increased (21.9 months vs. 21.6 months, respectively). In two prospective Japanese trials (WJTOG3405 and NEJ004) that identified NSCLC patients with drug-sensitive EGFR mutations, patients treated with gefitinib had significantly better PFS than did those treated with platinum doublets (9.2 months vs. 6.3 months in WJOTG3405 and 317 days vs. 166 days in NEJ004) [11, 12]. Regarding OS, NEJ004 noted an excellent median OS of 30.5 months in the gefitinib group versus 23.6 months in the comparison chemotherapy group.

Retrospective studies showed that improvements in lung cancer care routinely allow a significant proportion of patients to be considered for third-line treatments or more, a benefit that may be applicable to advanced NSCLC as well [13–15]. In a retrospective study by Massarelli et al. [15], 43 patients with advanced NSCLC who had received third- or fourth-line chemotherapy after two prior chemotherapy regimens had a median survival of 16.9 months. Thus, recent patients with advanced NSCLC tend to receive multiple regimens and are therefore likely to survive longer.

It is not clear why Japanese patients with advanced NSCLC survive longer than those in other countries [5–9]. Ease of access to providers, evaluation, and treatment is facilitated by the Japanese medical insurance system, which was established in 1961[16], and may play an important role in the discrepancy. In 2002, the Japanese Ministry of Health, Labour and Welfare adopted a policy promoting outpatient cancer care, and many patients with malignancies opted to receive outpatient chemotherapy. Subsequently, for more than half a decade, patients have been able to receive long-term chemotherapy with relative ease. In September 2002, the EGFR-TKI, gefitinib was approved for use in Japan. In June 2007, EGFR-genetic testing was approved under the national health insurance system for any patients with NSCLC, thereby further facilitating appropriate treatment with EGFR-TKIs. Thus, in Japan, many patients with advanced NSCLC have received long-term chemotherapy, and this may have contributed to their much longer survival as compared with the previous decade.

In a preliminary study [17], we examined 22 long-term survivors from a group of patients with advanced NSCLC who had received outpatient chemotherapy between September 2004 and May 2009. At the time, we concluded that outpatient chemotherapy at our institution extended the duration of chemotherapy, which may have increased survival among the NSCLC patients. However, that preliminary study was insufficient due to deficiencies in the database. As a result, we were not able to definitively examine survival in patients with advanced NSCLC. The present study utilizes a greatly improved database to retrospectively examine the associations between OS and a number of clinical variables.

Materials and methods

Criteria

This retrospective study used data from advanced NSCLC patients who started first-line chemotherapy between January 1, 2004 and December 31, 2007. The patients were identified using the pharmacy database and records of cancer conferences at our institution. Inclusion criteria were as follows: (a) histopathologically proven primary NSCLC (adenocarcinoma, squamous cell carcinoma, large cell carcinoma, or unclassified NSCLC); (b) advanced stage including stage III (unsuitable for radical treatment) and stage IV at time of diagnosis [18], relapse after chemoradiation, relapse after thoracic radiation alone, and recurrence after surgery; and (c) treatment with systemic antineoplastic drugs (cytotoxic chemotherapy or EGFR-TKIs).

Perioperative chemotherapy or radical chemoradiation therapy were excluded from first-line chemotherapy.

Of the patients who met the initial criteria, the associations between OS and a number of variables were examined among those who received at least one dose or course of outpatient chemotherapy. In contrast, only patient characteristics and OS were investigated in patients who received inpatient chemotherapy.

Clinical review

A retrospective review of the clinical history of eligible patients was performed. Because this study was a retrospective analysis, the approval of an institutional review board was not required. Baseline demographics information including sex, age, performance status (PS), histology, stage, and smoking status at the beginning of first-line chemotherapy were obtained for each patient. A complete history of chemotherapy for each patient was recorded, including regimen and number of cycles, dose, and the start and stop dates of each regimens. PS was rated using ECOG criteria. At the time of first-line chemotherapy, the following data were available for all patients: a complete history and physical examination, surgical reports, mediastinoscopy, fiberoptic bronchoscopy, thoracoscopy, imaging investigations (chest radiography and computed tomography [CT], brain CT or magnetic resonance imaging [MRI], abdominal CT, and bone scintigraphy or positron emission tomography [PET]), pathologic reports, and blood test results.

Best response to first-line chemotherapy

We applied corresponding RECIST criteria [19] to evaluate response to treatment which was discussed at weekly or urgent cancer conferences at our institution. Best response to first-line chemotherapy in this study was collected from records of these meeting and from physicians' summaries.

EGFR-genetic testing

In June 2007, Japanese medical insurance system began reimbursing for EGFR mutation analyses of NSCLC patients. Tissue samples containing NSCLC from surviving patients were sent for EGFR-genetic testing via peptide nucleic acid-locked nucleic acid PCR clamp (Mitsubishi Chemical Mediencie Corp., Tokyo, Japan) between June 1, 2007 and December 31, 2009. Furthermore, as per the protocol of the previous WJOG 3405 [11] and 0403 [20], clinical trials at our institution in which, drug-sensitive EGFR mutations were screened for, EGFR data were available for an additional 12 patients.

Treatment

At our institution, first-line chemotherapy of 4–6 cycles of platinum doublets and non-platinum monotherapy is considered the standard of care in patients with advanced NSCLC. Elderly and high-risk patients received only non-platinum monotherapy for first-line treatment, as did patients who relapsed after first-line therapy. However, if responders to a particular regimen had stable disease (SD) or better, with acceptable toxicity, and wished to continue the regimen, it was continued until progressive disease (PD) was observed. Some patients were treated with 80% or less of the theoretical dose, with correspondingly longer intervals. Outpatient chemotherapy in this study included not only intravenous anti-cancer agents, but also oral agents such as EGFR-TKIs (including gefitinib and erlotinib), or S-1 [21], which had been excluded from outpatient chemotherapy in the preliminary study [17]. Pemetrexed was approved for NSCLC by Japanese Ministry of Health, Labour and Welfare in May 2009. Before 2009, patients had received pemetrexed in clinical trials.

In the present analysis, when a chemotherapy regimen was repeated after the patient had received another, interposing, regimen, the repeat regimen was considered different.

Continuation and discontinuation of chemotherapy

The decision to continue or discontinue chemotherapy was made when patients were re-staged by CT, MRI, or bone scintigraphy/PET. We evaluated response to chemotherapy at weekly or urgent cancer conferences. We consulted with patients as to whether chemotherapy should be continued, based on their wishes and response to treatment. If the patient had PD, chemotherapy was discontinued and the patient was recommended to begin a different regimen or receive palliative therapy alone. If the patient achieved a CR, PR, or SD, we recommend continuation of chemotherapy. If the patient sought to discontinue chemotherapy, we recommended changing to a different regimen—even in cases in which the original regimen had achieved a good response—or to palliative therapy alone. The platinum doublets regimen was continued for a maximum of 6 cycles. In contrast, non-platinum monotherapy or EGFR-TKI was continued until PD if toxicity was acceptable and the patient desired treatment continuation.

Statistical analysis

The chi-square test was used to compare the proportions of several categories of patient characteristics. Survival time was defined as the time from the initiation date of first-line chemotherapy to the date of death or last follow-up. Survival time data were updated on December 31, 2009. Survival rates were estimated by the Kaplan–Meier method [22]. Differences among survival curves were assessed using the log-rank tests. The statistical software package R was used for the multivariate analysis (Cox proportional hazards model) [23]. A two-sided P value of 0.05 was considered to indicate statistical significance.

Results

Patients

A total of 360 patients with advanced NSCLC received first-line chemotherapy between January 1, 2004 and December 31, 2007 at our institution. Of those, 185 received at least one dose or one course of outpatient chemotherapy, and 175 received inpatient chemotherapy only (Table 1). Most patients who received outpatient chemotherapy achieved partial response (PR) or stable disease (SD) with first-line chemotherapy, without severe toxicity. The percentage of patients receiving outpatient chemotherapy (outpatient/total) was 33.3% (27/81) in 2004, 53.8% (50/93) in 2005, 64.6% (53/82) in 2006, and 52.9% (55/104) in 2007.

Table 1 Patient characteristics

Clinical variables	Outpatient chemotherapy (≥1 dose/course)	Inpatient chemotherapy only
Number of patients	185	175
Sex		
Male/female	137/48	131/44
Median age, year (range)	65 (33–86)	66 (31–85)
Performance status		
0/1/2/3–4	38/121/22/4	17/122/28/8
Smoking status		
Never/ever	55/130	39/136
Histology		
Adenocarcinoma	150	115
Squamous cell carcinoma	28	42*
Large cell carcinoma	5	10
Other	2	8
Stage		
IIIA [OP(−), TRT(−)]	10	17
IIIB [OP(−), TRT(−)]	50	43
IV	74	88**
Relapse after CTRT	15	7
Relapse after TRT	2	0
Postoperative recurrence	34	20
Best response to first-line treatment		
PR	78^{+}	37
SD	58	45
PD	25	66^{++}
NE	24	27
EGFR mutation status		
Drug-sensitive mutations	22	0
Wild-type or other mutations	28	7
Unknown	135	168

Drug-sensitive mutations: exon 19 deletion and L858R

OP thoracic surgery, *TRT* radical thoracic radiation therapy, *CTRT* chemoradiation therapy, *PR* partial response, *SD* stable disease, *PD* progressive disease, *NE* not evaluable, *EGFR* epidermal growth factor receptor

* $P = 0.0337$; ** $P = 0.0499$; $^{+}$ $P<0.0001$; $^{++}$ $P < 0.0001$

Table 1 shows a comparison of the characteristics of patients who received outpatient chemotherapy and those who received inpatient chemotherapy only. Patients with squamous cell carcinoma and those with stage IV disease were significantly more likely to receive inpatient chemotherapy only ($P = 0.0337$ and $P = 0.0499$, respectively). Regarding first-line treatment, 78 of the 185 patients who received outpatient chemotherapy had partial response (PR), 58 had stable disease (SD), and 25 had progressive disease (PD). Twenty-four patients were not evaluable (NE), 6 of whom had no measurable target lesions, and 18 of whom discontinued first-line chemotherapy due to treatment toxicity. Among the 175 patients who received inpatient chemotherapy only, 37 had PR, 45 had SD, 66 had PD, and 27 were not evaluable.

Patients receiving outpatient chemotherapy had significantly more PR than did those receiving inpatient chemotherapy alone ($P < 0.0001$), and patients receiving inpatient chemotherapy only had significantly more PD than did those receiving outpatient chemotherapy ($P < 0.0001$).

EGFR mutation status

Fifty patients receiving outpatient chemotherapy underwent EGFR-genetic testing: 16 had an exon 19 deletion, 6 had an L858R, 3 had other mutations, and 25 had wild-type sequences. In contrast, the 7 patients receiving inpatient chemotherapy only who underwent testing all had wild-type sequences.

Treatment

Table 2 shows the seven most frequent chemotherapy regimens in descending order of frequency, categorized by line of treatment. Among the 185 patients, 147 (79.5%) received second-line chemotherapy, 96 (51.9%) third-line chemotherapy, 60 (32.4%) fourth-line chemotherapy, and 67 received fifth to twelfth-line chemotherapy. Of the seven most frequent first-line chemotherapy regimens, 92 patients received platinum doublets such as carboplatin plus paclitaxel ($n = 37$), cisplatin plus gemcitabine ($n = 33$), or carboplatin plus gemcitabine ($n = 22$). Fifty-three patients underwent non-platinum monotherapy with docetaxel ($n = 22$), gemcitabine ($n = 18$), or vinorelbine ($n = 13$). Seventeen patients received an EGFR-TKI (gefitinib/erlotinib: 17/0). Among the top 7 second-line chemotherapy regimens, 95 patients underwent non-platinum monotherapy with docetaxel ($n = 53$), gemcitabine ($n = 20$),

Table 2 Median (range) number of cycles for chemotherapy regimens

Top 7	Line of therapy (*N*)				
	Regimen (*N*)/median cycles or treatment days (range)				
	1st-line (185)	2nd-line (147)	3rd-line (96)	4th-line (60)	≥ 5th-line (67)
1	Cb + PAC(37) 4 (1–15)	DOC (53) 3 (1–32)	EGFR-TKI (23) 120 (6–1593)[a]	EGFR-TKI (13) 64 (2–434)[a]	EGFR-TKI (15) 111 (14–304)[a]
2	Cis + GEM (33) 3 (1–7)	EGFR-TKI (21) 231 (12–1637)[a]	DOC (20) 3 (1–11)	GEM (11) 4 (1–22)	GEM (12) 4 (1–29)
3	Cb + GEM (22) 4 (1–11)	GEM(20) 2 (1–14)	CPT (13) 3 (1–10)	CPT (11) 2 (1–12)	S-1 (9) 4 (1–15)
4	DOC (22) 4 (1–46)	VNR (17) 3 (1–15)	VNR (11) 4 (1–16)	DOC (10) 3 (1–9)	CPT (7) 5 (1–24)
5	GEM (18) 4 (1–21)	Cb + GEM (12) 3 (1–7)	GEM (10) 4 (1–10)	S-1 (5) 3 (2–8)	DOC (6) 2 (2–17)
6	EGFR-TKI (17) 348 (28–1706)[a]	Cb + PAC (7) 4 (1–4)	PL (6) 5 (2–10)	PEM (4) 5 (1–7)	VNR (6) 3 (2–4)
7	VNR (13) 4 (1–17)	PEM (5) 7 (2–20)	S-1 (4) 4 (1–24)	PL (4) 3 (1–10)	PEM (5) 5 (3–9)

Cb carboplatin, *Cis* cisplatin, *PL* platinum doublets, *PAC* paclitaxel, *DOC* docetaxel, *GEM* gemcitabine, *VNR* vinorelbine, *PEM* pemetrexed, *EGFR-TKI* epidermal growth factor receptor tyrosine kinase inhibitor

[a] Median treatment days (range)

vinorelbine ($n = 17$), or pemetrexed ($n = 5$). Nineteen patients received platinum doublets such as cisplatin plus gemcitabine ($n = 12$) and carboplatin plus paclitaxel ($n = 7$). Twenty-one received EGFR-TKIs (gefitinib/erlotinib: 16/5). Among the top 7 third-line treatments, 23 patients received EGFR-TKIs (gefitinib/erlotinib: 18/5). Fifty-eight underwent non-platinum monotherapy with docetaxel ($n = 20$), irinotecan ($n = 13$), vinorelbine ($n = 11$), gemcitabine ($n = 10$), or S-1 ($n = 4$). Six patients received platinum doublets. Among the top 7 fourth-line regimens, 13 patients received EGFR-TKIs (gefitinib/erlotinib: 9/4). Forty-one underwent non-platinum monotherapy with gemcitabine ($n = 11$), irinotecan ($n = 11$), docetaxel ($n = 10$), S-1 ($n = 5$), or pemetrexed ($n = 4$). Four patients received platinum doublets.

For most regimens, the median number of cycles was 3 or 4 cycles; however, some patients underwent multi-cycle chemotherapy (≥20 cycles) during first- through third-line treatment. Some patients received EGFR-TKI for longer than 1,500 days at from first- through third-line treatment.

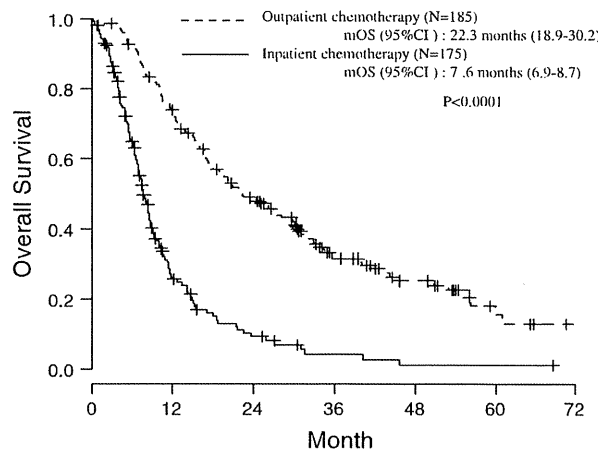

Fig. 1 Kaplan–Meier survival curves of patients who received outpatient and inpatient chemotherapy. Survival was significantly longer in patients receiving outpatient chemotherapy than in those receiving inpatient chemotherapy only (mOS [95% CI]: 22.3 [18.9–30.2] months vs. 7.6 [6.9–8.7] months, $P < 0.0001$). *mOS* median overall survival

Univariate and multivariate analysis of overall survival (OS) according to clinical variables and chemotherapy regimens

OS was significantly longer in patients who received outpatient chemotherapy than in those who received inpatient chemotherapy only (MST [95% confidential interval]: 22.3 [18.9–30.2] months vs. 7.6 [6.9–8.7] months, $P < 0.0001$; Fig. 1). At the time of analysis, 129 of 185 patients who received outpatient chemotherapy had died, 44 were alive, and 12 were lost to follow-up.

Table 3 shows the results of univariate and multivariate analysis of OS among the 185 patients who received outpatient chemotherapy, according to clinical variables, including baseline characteristics and chemotherapy regimens. Female sex, high PS, never smoking, and postoperative recurrence were significantly associated with a better prognostic factors in univariate analysis ($P = 0.0019$, $P = 0.0066$, $P = 0.0001$, and $P = 0.0231$, respectively). Regarding chemotherapy, best response to first-line chemotherapy, use of docetaxel, and EGFR-TKI were significantly correlated with OS ($P = 0.0011$, $P = 0.0250$, and $P = 0.0023$, respectively). In contrast, there was no significant difference in OS in patients treated with or without platinum doublets ($P = 0.941$). No non-platinum monotherapy, except docetaxel, significantly prolonged OS.