

Serum levels of surfactant protein D predict the anti-tumor activity of gefitinib in patients with advanced non-small cell lung cancer

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Abstract

Purpose Gefitinib is an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) that has dramatic effects in selective patients with non-small cell lung cancer (NSCLC). A simple non-invasive method for predicting the efficacy of gefitinib is preferable in clinical settings. In this study, we evaluated prospectively whether surfactant protein-A (SP-A) and -D (SP-D) may be new conventional predictors of the efficacy of gefitinib treatment.

Methods We measured serum SP-A and SP-D levels on days 0 and 29 in 40 patients with advanced NSCLC treated with 250 mg gefitinib daily. Eligibility criteria included performance status ≤ 3 , age ≤ 80 years, and stage IIIB–IV

disease. In addition, *EGFR* mutations were analyzed in 24 patients.

Results Multivariate analysis showed that favorable progression-free survival (PFS) after gefitinib treatment was associated with adenocarcinoma and high serum SP-D levels before treatment. *EGFR* mutation analysis of 24 patients showed that 16 patients had exon 19 deletion and/or exon 21 point mutations. *EGFR* mutations were significantly correlated with response to gefitinib and serum SP-D levels before treatment was significantly high in patients with the *EGFR* mutations. Serum SP-A levels were not associated with PFS.

Conclusions The present study showed that measurement of serum SP-D levels before treatment in patients with NSCLC may be a new surrogate marker for predicting the response to gefitinib.

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Keywords Non-small cell lung cancer · Gefitinib · SP-A · SP-D

Introduction

Gefitinib is an epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) that shows anti-tumor activity for patients with advanced non-small cell lung cancer (NSCLC). Several clinical patient factors, such as being East Asian, female, and non-smoker, and having adenocarcinoma are closely associated with the anti-tumor activity of gefitinib [1–3]. Recently, *EGFR* gene mutations were also found to predict response and survival in patients treated with gefitinib [4–7] and these mutations were more frequently seen in patients with the above-described clinical factors [8]. Thus, *EGFR* mutations in tumor cells have become important information for predicting the efficacy of

gefitinib treatment. High-sensitivity detection methods to detect *EGFR* mutations have recently been developed [9–12]. However, it is sometimes difficult to obtain adequate tumor specimens to analyze *EGFR* mutations, because lung adenocarcinoma often occurs in peripheral areas of the lung.

Surfactant proteins A (SP-A) and D (SP-D) are lung-specific glycoproteins that are produced and secreted by normal alveolar type II cells and Clara cells [13, 14]. A positive response to gefitinib is more frequently observed in adenocarcinomas expressing thyroid transcription factor 1 (TTF-1), which is a transcription factor of surfactant proteins [15]. In addition, EGF is reported to induce the production of SP-A in fetal normal lung tissue and antisense *EGFR* mRNA or an *EGFR*-TKI is reported to inhibit the production of SP-A [16, 17]. In contrast, SP-D, which is well known as a marker of activity of interstitial lung diseases [18, 19], has also been reported to be produced and secreted by lung cancers [20–22] and serum SP-D levels are correlated with the volume of lung cancer [23]. Considering these findings, we hypothesized that these serum surfactant proteins are regulated through the EGF signaling pathway in lung cancer and that gefitinib inhibits the production of surfactant proteins as well as the proliferation of cancer cells. The present study is designed to evaluate whether serum levels of surfactant proteins may be new and convenient surrogate biomarkers for predicting the efficacy of gefitinib treatment in patients with advanced NSCLC.

Patients and methods

Patient selection and treatment

Eligibility criteria were as follows: histologically or cytologically confirmed stage IIIA or IV NSCLC; age ≤ 80 years; and Eastern Cooperative Oncology Group (ECOG) performance status (PS) 0 to 3. The major exclusion criteria were as follows: interstitial pneumonia or pulmonary fibrosis; active concomitant or recurrent history of any malignancy; uncontrolled angina pectoris, myocardial infarction less than 3 months before the enrollment, or congestive heart failure; uncontrolled diabetes mellitus or hypertension; severe infection; intestinal paralysis or obstruction; any women with pregnancy or lactation; and other serious medical conditions. Prior radiation therapy and chemotherapy were to be completed at least 4 weeks before enrollment.

Patients received gefitinib at a dose of 250 mg/day. Treatment continued until disease progression or intolerable toxicities became apparent or the patient refused further treatment. All patients gave written informed consent. This study protocol was approved by the ethics committee of

Nagasaki University School of Medicine and each institution, and performed in accordance with the Declaration of Helsinki (1995, revised in Edinburgh 2000).

Measurement of SP-A and SP-D levels

Serum SP-A and SP-D levels were measured immediately before and 4 weeks after the start of treatment (days 0 and 29). The serum samples were stored at -80°C until assaying. Levels were measured using a commercially available enzyme-linked immunosorbent assay kit (Yamasa Co., Choshi, Japan) according to the manufacturer's protocol. Clinical cut-off levels for serum SP-A (43.8 ng/ml) and SP-D (110 ng/ml) in interstitial lung disease were used for categorizing the groups in the survival curves [24].

Evaluation of tumor response and toxicities

The response to gefitinib treatment was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) [25]. Briefly, complete response was defined as the disappearance of all known disease. Partial response (PR) was defined as a 30% reduction from baseline in the sum of the longest diameters of the target lesions and a lack of disease progression in non-target lesions. Progressive disease (PD) was defined as the development of any new lesions or an increase of 20% in the sum of the longest diameters of the target lesions. Patients with stable disease (SD) did not meet the criteria for PR or PD. We evaluated the best response in each patient within 6 weeks from the start of treatment. Toxicities were assessed according to the United States National Cancer Institute Common Toxicity Criteria (NCI-CTC, version 2) [26].

Genetic analyses of *EGFR*

After additional approval for *EGFR* mutation analysis by the Committee for Ethical Issues in conjunction with the institutional review board at each institution, written informed consent was obtained from each patient. Genomic DNA was extracted from the paraffin-embedded specimens using DEXPATTM reagent (Takara Bio, Inc., Shiga, Japan) from frozen tissue samples that were retrieved from trans-bronchial biopsies or surgically resected, bronchial lavages, and pleural effusions using QuickGene DNA tissue kit S (Fujifilm, Tokyo, Japan) according to the manufacturer's protocol. Subsequently, the deletion in exon 19 and point mutation of L858R in exon 21 in *EGFR* were retrospectively analyzed, using the mutant-enriched polymerase chain reaction (PCR) to increase the sensitivity of these mutations [11, 27]. The deletion region in exon 19 was amplified by PCR with a GeneAmp PCR System 9700

thermal cycler (Applied Biosystems, Foster City, CA, USA) using 20 ng of genomic DNA in a 25- μ l reaction mixture containing 1 $\times$ GoTaq Green master mix (Promega, Madison, WI, USA) and 15 pmol each of forward primer: 5'-ATCCCAGAAGGTGAGAAAGATAAAATTC-3' and reverse primer: 5'-CCTGAGGTTTCAGAGCCATGGA-3'. The amplification protocol consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 60°C, and extension at 72°C for 30 s, and final extension at 72°C for 5 min. The 138-bp PCR products were digested with *Mse*I (New England BioLabs, Inc., Beverly, MA, USA). After the digest was amplified under similar conditions (the number of cycles was changed from 35 to 15), the second PCR products were separated by 6% polyacrylamide gel electrophoresis (PAGE) (Nacalai Tesque, Kyoto, Japan) and visualized with an ultraviolet transilluminator (Alpha Innotech Co., San Leandro, CA, USA) after ethidium bromide (Nacalai Tesque, Kyoto, Japan) staining. Separately, the point mutation region in exon 21 was amplified by PCR using 15 pmol each of forward primer: 5'-CAGCCAGGAACGTACTGGTGA-3' and reverse primer: 5'-TCCTGGTGT CAGGAAAATGCT-3'. The other contents of the PCR mixture and the amplification protocol were the same as described above. The 130-bp PCR products were digested with *Msc*I (New England BioLabs, Inc.). After the digest was amplified under similar conditions (forward primer changed to 5'-CGCAGCATGTCAAGATCACAGAT-3'), the second PCR products were digested with *Asu*I (Fermentas International, Inc., Ontario, Canada). The digests were then subjected to separation by 8% PAGE and visualized by ethidium bromide staining.

Statistical analysis

The primary endpoint of the present study was progression-free survival (PFS), which was defined as the time from the date of beginning the treatment to the date of disease progression or death, or the last follow-up. Secondary endpoints were overall survival (OS) and tumor response. The survival curves were plotted by the Kaplan–Meier method and their difference was determined by the log-rank test. The PFS was expressed as median survival time, one-year survival rate, and 95% confidence interval (95% CI). The prognostic values to survival were analyzed by univariate or stepwise multivariate Cox regression analysis. A two-tailed $P < 0.05$ was considered statistically significant. All analyses were performed using the SPSS statistical software package (version 11.0 for Macintosh; SPSS, Inc., Chicago, IL, USA).

The SP-D levels were expressed as the median, 25th percentile, and 75th percentile values. The difference in SP-D levels was determined by the Mann–Whitney U test for

comparisons between two groups and by the Steel–Dwass test for comparisons among more than two groups.

Results

Anti-tumor activity and toxicities

From February 2003 to March 2006, 40 patients with advanced NSCLC were enrolled in the present study. The patient characteristics at baseline are listed in Table 1. Of these 40 patients, 45.0% were women, 35.0% had never smoked, and 87.5% had adenocarcinoma. Following gefitinib treatment, no patients showed CR, 8 patients (20.0%) showed PR, 18 patients (45%) showed SD, and 14 patients (35.0%) showed PD. The median PFS was 87 days (95% CI 44–130 days) and one-year PFS was 17.5% (95% CI 5.7–29.3%) (Fig. 1a).

No severe toxicities were observed and no treatment-related death occurred in the present study. Three patients (7.9%) experienced grade 3 interstitial pneumonitis. In these patients, pneumonitis occurred after day 29 and SP-A and SP-D levels were not increased on day 29 compared to day 0. Of these three patients, one had a grade 3 skin eruption.

Table 1 Baseline characteristics of patients with non-small cell lung cancer treated with gefitinib

	No. of patients ($n = 40$)	%
Sex		
Male	22	55.0
Female	18	45.0
Age, median years (range)	69 (47–84)	
<70	21	52.5
≥ 70	19	47.5
Stage		
IIIB	9	22.5
IV	31	77.5
ECOG performance status		
0–1	28	70
2–3	12	30
Histology		
Adenocarcinoma	35	87.5
Non-adenocarcinoma	5	12.5
Prior chemotherapy		
0–1	17	42.5
≥ 2	23	57.5
Smoking history		
Never-smoker	24	60.0
Current- or ex-smoker	16	40.0

Fig. 1 **a** Kaplan–Meier plots of progression-free survival curves of all patients ($n = 40$) and stratified by **b** histological type, **c** surfactant protein D levels before treatment, and **d** smoking status (* P by log-rank test)

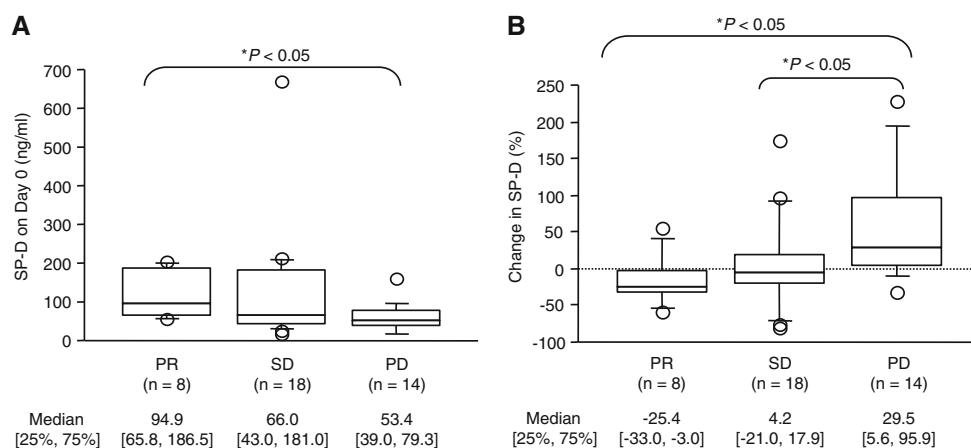
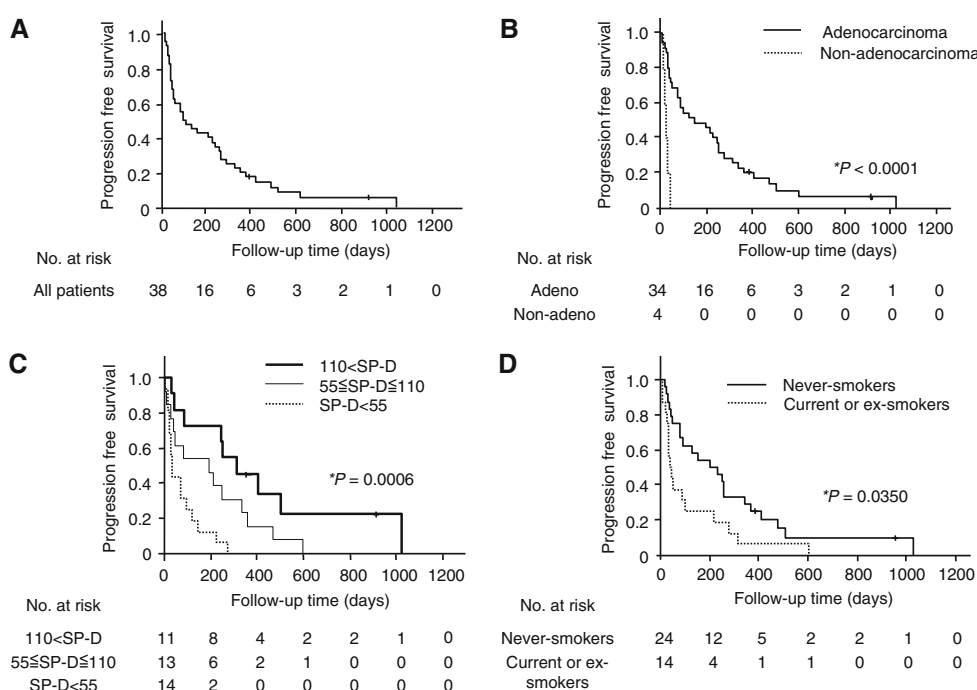


Fig. 2 **a** Relationship between serum surfactant protein D levels before treatment and response to gefitinib in 40 patients. **b** Relationship between the change in surfactant protein D during 28 days of treatment with gefitinib and response in 38 patients; two patients died on days 24

and 29 from disease progression and these patients were excluded from the analysis. The *bottom* and *top* edges of the *box* are the first and third quartiles. The center *horizontal line* is the median value. *Steel–Dwass test for multiple comparisons was used in the analysis

tion, one had grade 3 liver dysfunction, and one had grade 3 ileus. Skin eruptions less than grade 2 were observed in 12 patients. Diarrhea less than grade 2 was observed in 6 patients. There were no relationships between toxicities and surfactant proteins levels (data not shown).

Response and serum surfactant protein levels

We evaluated the relationship between the response to gefitinib and serum SP-A and SP-D levels before treatment. The patients with PR had higher serum SP-D levels before treatment compared to those with PD (median value, 94.9 ng/ml vs. 53.4 ng/ml; $P < 0.05$) and the patients with

SD showed no significant difference in serum SP-D levels before treatment compared to those with PR (Fig. 2a). In contrast, serum SP-A levels were not related with response (data not shown).

Next, we evaluated the change in serum SP-D levels between immediately before and 28 days after gefitinib treatment in 38 patients (Fig. 2b). Two patients died on days 24 and 29 from disease progression, so these patients were excluded from analysis. The decrease in serum SP-D levels was more frequently seen in the patients with PR and SD compared to those with PD (median values, -25.4 and 4.2% vs. 29.5% ; $P < 0.05$, each). There was no significant difference in change in SP-D levels between the patients

with PR and those with SD. In contrast, the change in serum SP-A levels was not related with response (data not shown).

Progression-free survival and serum SP-D levels

In the univariate Cox's hazard analysis of PFS, the patients with adenocarcinoma, those with high serum SP-D levels before treatment, and those who had never smoked had favorable survival (Table 2). The hazard ratio for PFS was 0.145 for adenocarcinoma (95% CI 0.048–0.439), 0.953 for an increase by 10 ng/ml in SP-D levels before treatment (95% CI 0.910–0.997), and 0.497 for never-smokers (95% CI 0.257–0.965). The sex of the patient did not show a significant difference on survival.

Progression-free survival curves were stratified by histology (Fig. 1b), serum SP-D levels before treatment (Fig. 1c), and smoking (Fig. 1d). The grouping of serum SP-D levels was based on clinical cut-off levels for serum SP-D (110 ng/ml) in interstitial lung disease and patients

were divided into three groups. Better PFS was seen in patients with adenocarcinoma ($P < 0.0001$), those with high serum SP-D levels before treatment ($P = 0.0006$), and those who never smoked ($P = 0.0350$). In multivariate Cox's hazard analysis of PFS using the stepwise method, the patients with adenocarcinoma and high serum SP-D levels before treatment had a decreased risk of disease progression. The hazard ratio for PFS was 0.102 for adenocarcinoma (95% CI, 0.031–0.334; $P = 0.0002$) and 0.939 for serum SP-D levels increased by 10 ng/ml (95% CI, 0.892–0.989; $P = 0.0170$).

On the other hand, the serum SP-A and SP-D levels were not related with overall survival (data not shown).

Extension study of EGFR mutations

Because many investigators have reported after we began the study that *EGFR* mutations are closely associated with the anti-tumor activity of gefitinib, we performed an extension study to examine the relationship between serum SP-D levels and *EGFR* mutations in 24 patients, 9 men and 15 women with a median age of 64 years (range 40–77 years). The smoking status was 13 never-smokers and 11 current- or ex-smokers. All of the patients had adenocarcinomas. Following gefitinib treatment, 8 patients showed PR, 9 patients showed SD, and 7 patients showed PD.

In the extension study as well as in the original study, the patients with PR had higher serum SP-D levels before treatment compared to those with PD (Fig. 3a; median value, 83.4 ng/ml vs. 39.0 ng/ml; $P < 0.05$). The decrease in serum SP-D levels 28 days after treatment was more frequently seen in the patients with PR and SD compared to those with PD (Fig. 3b; median values, −31.0 and −4.4% vs. 33.3%; $P < 0.05$, each).

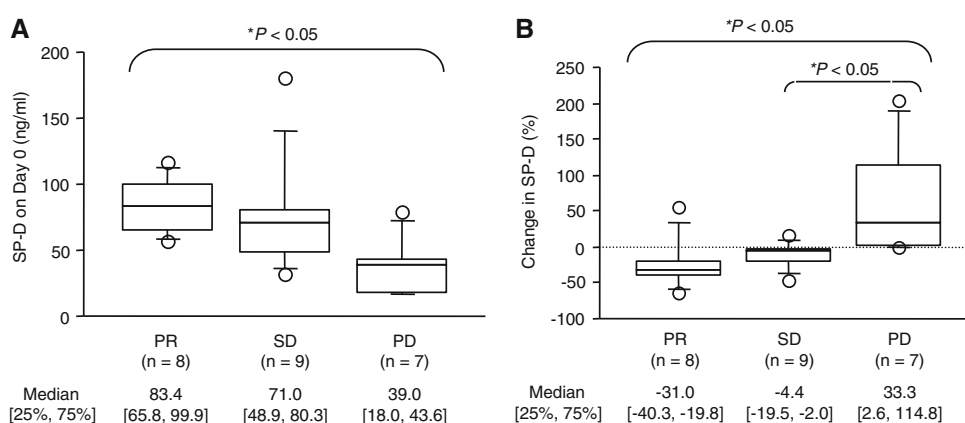
EGFR mutations were detected in 16 out of the 24 patients (66.7%): L858R in 8 patients, exon 19 deletion in 4 patients, and both L858R and exon 19 deletion in 4 patients. The patients with *EGFR* mutations were 15 women and 1 man, and 7 never-smokers and 9 current- or ex-smokers. Of

Table 2 Univariate Cox's regression analysis of progression-free survival after gefitinib treatment in patients with non-small cell lung cancer

Parameter	Hazard ratio	95% CI	P
Histology, adenocarcinoma	0.145	0.048–0.439	0.0006
SP-D (day 0), increased by 10 ng/ml	0.953	0.910–0.997	0.0360
SP-A (day 0), increased by 10 ng/ml	0.938	0.865–1.018	0.1239
Smoking, never-smoker	0.497	0.257–0.965	0.0387
Sex, female	0.678	0.350–1.313	0.2485
Age, <70 years	1.657	0.851–3.229	0.1377
Stage, IIIB	0.872	0.397–1.917	0.7341
PS, 0–1	0.709	0.348–1.444	0.3427
Previous therapy, 0–1	0.933	0.483–1.804	0.8376

SP-D surfactant protein D, SP-A surfactant protein A

Fig. 3 **a** Relationship between serum surfactant protein D levels before treatment and response to gefitinib in the extension study. **b** Relationship between the change in surfactant protein D during 28 days of treatment with gefitinib and response in the extension study. The bottom and top edges of the box are the first and third quartiles. The center horizontal line is the median value



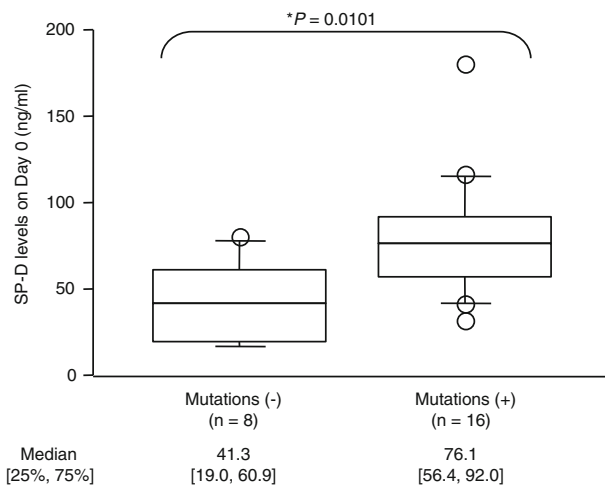


Fig. 4 Relationship between serum surfactant protein D levels before treatment and *EGFR* mutation status. *EGFR* mutations studied were deletion in exon 19 and L858R. The bottom and top edges of the box are the first and third quartiles. The center horizontal line is the median value. *Mann–Whitney *U* test was used

the patients with *EGFR* mutations, 8 showed PR, 5 showed SD, and 3 showed PD, whereas of those with wild-type *EGFR*, none showed PR, 4 showed SD, and 4 showed PD. *EGFR* mutations were significantly associated with response to gefitinib ($P = 0.0192$). The serum SP-D levels before treatment were significantly higher in those with *EGFR* mutations than those with wild-type genes (Fig. 4; median value, 76.1 ng/ml vs. 41.3 ng/ml; $P = 0.0101$). Of the patients with *EGFR* mutations, median PFS was 246 days and one-year PFS was 25.0%, whereas of those with wild-type *EGFR*, median PFS was 42 days and one-year PFS was 0% ($P = 0.0284$). The serum SP-D level and mutation status were not related with overall survival in the extension study (data not shown).

Discussion

In the present study, we demonstrated that serum SP-D levels may predict the response to gefitinib treatment. The patients who showed high serum SP-D levels before treatment achieved better responses and PFS times. In addition, high serum SP-D levels were closely associated with *EGFR* mutations.

Surfactant protein-D (SP-D) is the specific glycoprotein produced and secreted by normal alveolar type II cells and Clara cells, and it is responsible for the production of pulmonary surfactant protein [13, 14]. Most SP-D can be both broken down by pulmonary macrophages and reabsorbed into the lamellar structures of type II cells as the pulmonary surfactant protein, while the remainder of it is transferred into the circulating blood; serum SP-D is well known as a marker of activity of interstitial lung diseases [18, 19].

The concentration of SP-D in pleural effusion from patients with pulmonary adenocarcinoma has been reported to be extremely high and cancer cells isolated from the same patients expressed mRNA for SP-D [20]. Betz et al. [21] stated that reverse transcriptase-polymerase chain reaction analysis (RT–PCR) of surfactant proteins including SP-D was useful for detecting lymph node micrometastasis of pulmonary adenocarcinoma. Moreover, murine pulmonary tumor cells expressed SP-D mRNA and SP-D could be detected by immunostaining [22]. Thus, SP-D is produced and secreted not only by alveolar type II cells and Clara cells, but also by lung cancer cells. Ohyanagi et al. [28] reported that serum SP-D decreased in patients with response to gefitinib treatment; they consider that SP-D may be produced by NSCLC. In the present study, as the tumor size decreased during gefitinib treatment, serum SP-D level was reduced. A significant relationship between decreasing tumor size and serum SP-D levels also suggested that lung tumor cells might produce SP-D.

The efficacy of gefitinib was observed to be significantly more frequent in patients with high serum SP-D levels before the treatment and that serum SP-D levels before treatment in the patients with *EGFR* mutations were significantly higher than that in patients without mutations. Many reports have been published during the present study revealing that the sensitivity to gefitinib treatment is closely related to *EGFR* gene mutations [4–7]. About 90% of the *EGFR* mutations are either the in-frame deletion in exon 19 or mutational L858R in exon 21 [29]. *EGFR* mutations are now considered to be the most important predictive factor of the efficacy of gefitinib treatment. Most adenocarcinomas with the *EGFR* mutations were categorized as terminal respiratory unit (TRU)-type adenocarcinomas, which Yatabe et al. [15] have noted previously. In addition, they reported that the majority of adenocarcinomas with TRU morphology showed TTF-1 positive staining and TTF-1 is a transcription factor that regulates surfactant proteins. Considering these findings, we supposed that SP-D might be produced by TRU-type lung cancer cells. This hypothesis might be supported by the fact that serum SP-D levels before treatment were higher in the patients with *EGFR* mutations.

Several studies have evaluated the relationship between surfactant proteins and the *EGFR* signaling pathway and/or gefitinib. In these reports, epidermal growth factor might induce the production of SP-A in normal fetal lung tissue through ligand binding to the *EGFR* and antisense *EGFR* mRNA or an *EGFR* tyrosine kinase inhibitor was reported to inhibit the production of SP-A [16, 17]. We previously reported that gefitinib suppresses MUC5AC protein synthesis through the epidermal growth factor signaling pathway [30]. MUC5AC is a glycoprotein that is secreted into airways similarly to surfactant proteins and is derived from

goblet cells. It is one of the principal gel-forming mucins shown to be induced by the epidermal growth factor family [31]. Considering the possibility of the EGF system regulating the production of those surfactant proteins and mucins, SP-D synthesis may be regulated through the EGFR signaling pathway and gefitinib could inhibit the production of SP-D. Further studies are needed to investigate the relationship between them.

Measurement of serum SP-D level is convenient compared to the detection of *EGFR* mutations. Various kinds of biopsy specimens have been used for detecting *EGFR* mutations. Recently, high-sensitivity methods to detect *EGFR* mutations have been commonly used with cytological specimens, such as sputum, bronchial lavage fluid, and pleural effusion [9]. However, it is sometimes difficult to obtain adequate tumor specimens for analyzing *EGFR* mutations because most lung adenocarcinomas occur in the peripheral lung field. Recently, Kimura et al. [32] revealed the detection of *EGFR* mutations with the use of a high-sensitivity method called the Scorpion Amplification Refractory Mutation System (SARMS) assay in free plasma DNA from patients with metastatic NSCLC. On the other hand, Maheswaran et al. [33] reported the analysis of *EGFR* mutations by the SARMS assay targeting the circulating tumor cells collected from the peripheral blood samples of patients. They concluded that this assay is more sensitive than using free plasma DNA. However, these analyses are not convenient methods in clinical practice and cost-effectiveness remains unclear. Thus, we consider that serum SP-D might be a relatively convenient surrogate marker to predict the efficacy of gefitinib.

In conclusion, serum SP-D appears to be a surrogate predictive marker of the efficacy of gefitinib in patients with NSCLC. Further investigations are needed to clarify the relationship between the production of SP-D and the EGFR signaling pathways.

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Conflict of interest statement The authors declare no conflict of interest.

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