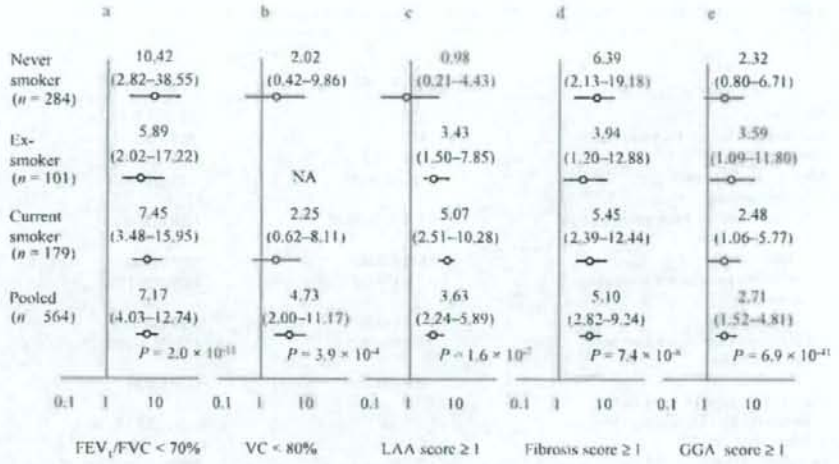

Figure 2 Odds ratios and 95% confidence intervals for lung cancer risk associated with the indicated parameters (bottom), in the gender- and age-matched subpopulations of the lung cancer and control groups. Odds ratios were calculated by the Mantel–Haenszel projection method, with stratification according to smoking status (never smoker, ex-smoker or current smoker), and are presented with the pooled estimates for odds ratios. The pooled odds ratios were significantly high for every parameter. Among these parameters, VC < 80% was shown to be a consequence of lung cancer and should not be considered as a risk factor for lung cancer (see text). Data for VC < 80% was not available for ex-smokers as the number of subjects was too small. LAA, low attenuation area; GGA, ground glass attenuation.

subpopulations, the higher prevalence of FEV_1/FVC < 70% in the lung cancer group in comparison to the control group cannot be attributed to impaired pulmonary function due to lung cancer. Therefore, reduced FEV_1/FVC was most likely a risk factor contributing to lung cancer. Third, only three CT slices were reviewed for evaluation of LAA, GGA and fibrosis. Although the scoring methods used have been published and widely recognized, more sophisticated methods, for example computer assisted quantitative diagnosis, should be developed. Finally, other biases may have existed between the two groups. For example, individuals who participate in a cancer screening programme may be more health conscious than others. It is likely, therefore, that individuals in the control group had healthier lifestyles, besides smoking habit, than those in the lung cancer group.

There are several reports on the association between COPD or airflow limitation and lung cancer risk,[19–21] with odds ratios or relative risks ranging from 2 to 6. ILD has also been suggested to predispose to lung cancer, with a relative risk of 8.25[14] or 14.[13] Although these case–control studies[13,14,20,21,25] were carefully controlled for gender, age and smoking status, over-diagnosis bias could not be excluded, as patients with COPD or ILD receive medical check-ups more frequently than control subjects. Other studies that have attempted to evaluate odds ratios for lung cancer by comparing the prevalence of COPD in lung cancer and control groups[10,23,26] seemed to be deficient in assessing COPD diagnosis among control subjects, because this was based on interviews or questionnaires. In contrast, the present study is the first to evaluate semi-quantitative CT findings and spirometric data in lung cancer and control groups, in order to minimize these biases and deficiencies.

Of particular importance is the fact that CT and spirometric abnormalities were risk factors for lung cancer even after adjusting for smoking status. This suggests two possibilities, namely, that these risk factors are not related to smoking, or that there is inter-individual variability in the sensitivity to smoking, and persons who are sensitive to smoking are predisposed to multiple smoking-related pulmonary diseases, including COPD, ILD and lung cancer. In conclusion, patients with lung cancer had a higher prevalence of LAA, fibrosis and GGA, as assessed by CT, and reduced FEV_1/FVC compared with control subjects, even after adjusting for gender, age and smoking status.

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REFERENCES

1 American Thoracic Society. Cigarette smoking and health. *Am. J. Respir. Crit. Care Med.* 1996; **153**: 861–5.
2 Rabe KF, Hurd S, Anzueto A, Barnes PJ, Buist SA *et al.* Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am. J. Respir. Crit. Care Med.* 2007; **176**: 532–55.
3 Iwai K, Mori T, Yamada N, Yamaguchi M, Hosoda Y. Idiopathic pulmonary fibrosis. Epidemiologic approaches to occupational exposure. *Am. J. Respir. Crit. Care Med.* 1994; **150**: 670–5.
4 Hubbard R, Lewis S, Richards K, Johnston I, Britton J. Occupational exposure to metal or wood dust and aetiology of cryptogenic fibrosing alveolitis. *Lancet* 1996; **347**: 284–9.
5 Baumgartner KB, Samet JM, Stidley CA, Colby TV, Waldron JA. Cigarette smoking: a risk factor for idiopathic pulmonary fibrosis. *Am. J. Respir. Crit. Care Med.* 1997; **155**: 242–8.
6 American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). *Am. J. Respir. Crit. Care Med.* 2000; **161**: 646–64.
7 Kurishima K, Satoh H, Ishikawa H, Yamashita YT, Homma T *et al.* Lung cancer patients with chronic obstructive pulmonary disease. *Oncol. Rep.* 2001; **8**: 63–5.
8 Kumar P, Goldstraw P, Yamada K, Nicholson AG, Wells AU *et al.* Pulmonary fibrosis and lung cancer: risk and benefit analysis of pulmonary resection. *J. Thorac. Cardiovasc. Surg.* 2003; **125**: 1321–7.
9 Loganathan RS, Stover DE, Shi W, Venkatraman E. Prevalence of COPD in women compared to men around the time of diagnosis of primary lung cancer. *Chest* 2006; **129**: 1305–12.
10 Ramanakumar AV, Parent M-E, Menzies D, Siemiatycki J. Risk of lung cancer following nonmalignant respiratory conditions: evidence from two case–control studies in Montreal, Canada. *Lung Cancer* 2006; **53**: 5–12.
11 Niho S, Goto K, Yoh K, Kim YH, Ohmatsu H *et al.* Interstitial shadow on chest CT is associated with the onset of interstitial lung disease caused by chemotherapeutic drugs. *Jpn J. Clin. Oncol.* 2006; **36**: 269–73.
12 Mohan A, Mohan C, Pathak AK, Pandey RM, Guleria R. Impact of chronic obstructive pulmonary disease on respiratory status and quality of life in newly diagnosed patients with lung cancer. *Respirology* 2007; **12**: 240–7.
13 Turner-Warwick M, Lebowitz M, Burrows B, Johnson A. Cryptogenic fibrosing alveolitis and lung cancer. *Thorax* 1980; **35**: 496–9.
14 Hubbard R, Venn A, Lewis S, Britton J. Lung cancer and cryptogenic fibrosing alveolitis. A population-based cohort study. *Am. J. Respir. Crit. Care Med.* 2000; **161**: 5–8.
15 Samet JM. Does idiopathic pulmonary fibrosis increase lung cancer risk? *Am. J. Respir. Crit. Care Med.* 2000; **161**: 1–2.
16 de Rijke JM, Schouten LJ, ten Velde GPM, Wanders SL, Bollen ECM *et al.* Influence of age, comorbidity and performance status on the choice of treatment for patients with non-small cell lung cancer; results of a population-based study. *Lung Cancer* 2004; **46**: 233–45.
17 Goddard PR, Nicholson EM, Laszlo G, Watt I. Computed tomography in pulmonary emphysema. *Clin. Radiol.* 1982; **33**: 379–87.
18 Kazerooni EA, Martinez FJ, Flint A, Jamadar DA, Gross BH *et al.* Thin-section CT obtained at 10-mm increments versus limited three-level thin-section CT for idiopathic pulmonary fibrosis: correlation with pathologic scoring. *Am. J. Roentgenol.* 1997; **169**: 977–83.
19 Davis AL. Bronchogenic carcinoma in chronic obstructive pulmonary disease. *JAMA* 1976; **235**: 621–2.
20 Skillrud DM, Offord KP, Miller RD. Higher risk of lung cancer in chronic obstructive pulmonary disease. A prospective, matched, controlled study. *Ann. Intern. Med.* 1986; **105**: 503–7.
21 Tockman MS, Anthonisen NR, Wright EC, Donithan MG. Airways obstruction and the risk for lung cancer. *Ann. Intern. Med.* 1987; **106**: 512–18.
22 Kuller LH, Ockene J, Meilahn E, Svendsen KH. Relation of forced expiratory volume in one second (FEV1) to lung cancer mortality in the Multiple Risk Factor Intervention Trial (MRFIT). *Am. J. Epidemiol.* 1990; **132**: 265–74.
23 Nomura A, Stemmermann GN, Chyou PH, Marcus EB, Buist AS. Prospective study of pulmonary function and lung cancer. *Am. Rev. Respir. Dis.* 1991; **144**: 307–11.
24 Nakayama M, Satoh H, Sekizawa K. Risk of cancers in COPD patients. *Chest* 2003; **123**: 1775–6.
25 Turner MC, Chen Y, Krewski D, Calle EE, Thun MJ. Chronic obstructive pulmonary disease is associated with lung cancer mortality in a prospective study of never smokers. *Am. J. Respir. Crit. Care Med.* 2007; **176**: 285–90.
26 Samet JM, Humble CG, Pathak DR. Personal and family history of respiratory disease and lung cancer risk. *Am. Rev. Respir. Dis.* 1986; **134**: 466–70.

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Circulating Endothelial Cells in Non-small Cell Lung Cancer Patients Treated with Carboplatin and Paclitaxel

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Introduction: Circulating endothelial cells (CECs) increase in cancer patients and play an important role in tumor neovascularization.
Methods: This study was designed to investigate the role of CEC as a marker for predicting the effectiveness of a carboplatin plus paclitaxel based first line chemotherapy in advanced non-small cell lung cancer (NSCLC).
Results: The CEC count in 4 ml of peripheral blood before starting chemotherapy (baseline value) was significantly higher in NSCLC patients, ranging from 32 to 4501/4 ml (n = 31, mean ± SD = 595 ± 832), than in healthy volunteers (n = 53, 46.2 ± 86.3). We did not detect a significant correlation between the CEC count and estimated tumor volume. CECs were significantly decreased by chemotherapy as compared with pretreatment values (175.6 ± 24 and 173.0 ± 24, day +8, +22, respectively). We investigated the correlation between baseline CEC and the clinical effectiveness of chemotherapy. CEC values are significantly higher in patients with clinical benefit (partial response and stable disease, 516 ± 458, 870.8 ± 1215, respectively) than in progressive disease patients (211 ± 150). Furthermore, a statistically significant decrease in CECs, on day 22, was observed only in patients with partial response. Patients who had a baseline CEC count greater than 400/4 ml showed a longer progression-free survival (>400, 271 days [range: 181–361] versus <400, 34 [range: 81–186], p = 0.019).
Conclusion: CEC is suggested to be a promising predictive marker of the clinical efficacy of the CBDCA plus paclitaxel regimen in patients with NSCLC.

Key Words: Circulating endothelial cell, NSCLC, Chemotherapy.

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Angiogenesis plays a critical role in the growth and metastasis of solid tumors.[1] The clinical importance of angiogenesis in human tumors has been demonstrated by several reports indicating a positive relationship between the blood vessel density in the tumor mass and poor prognosis, i.e., survival, in patients with various types of cancers including non-small cell lung cancer (NSCLC).[2–6] Furthermore, Natsume et al.[7] reported the antitumor activities of anticancer agents to be less active against vascular endothelial growth factor-secreting cells (SBC-3/VEGF), in vivo as compared with its mock transfectant (SBC-3/Neo). In recent years, antiangiogenic agents have also been demonstrated to be active against a variety of malignancies, including lung, colorectal, and renal cancer.[8–10] Thus, angiogenesis is a promising target for cancer treatment and is related to the prognosis and efficacy of these drugs, though the tumor vessel biomarkers which predict the effectiveness of antiangiogenic agents and other anticancer agents are not always useful and have not become well-established.

Circulating endothelial cells (CECs) have been recognized as a useful biomarker for vascular damage. CECs are increased in cardiovascular disease, vasculitis, infectious disease, and various cancers.[11–14] Recently, CECs were found to be more numerous and viable in cancer patients than in healthy subjects.[14,15] Furthermore, elevated CECs in cancer patients were found to be nearly normalized when the tumor was removed surgically or with chemotherapy.[15] Therefore, most CECs are considered to be disseminated tissue endothelial cells in the tumors and the CEC number may reflect the extent of tumor angiogenesis. Indeed, the CEC level has been demonstrated to correlate with the plasma level of VEGF, one of the pivotal factors promoting tumor angiogenesis.[15] Mancuso et al. reported that CEC kinetics and viability are promising predictors of the response to chemotherapy with antiangiogenic activity in patients with advanced breast cancer.[16] Thus, CEC is likely to be a useful marker for predicting the effectiveness of chemotherapy as a noninvasive angiogenesis marker.

NSCLC is the leading cause of cancer-related death worldwide. NSCLC accounts for approximately 50% of patients presenting with unresectable advanced stage,[17] and platinum-based chemotherapy offers only a small improve-

ment in survival with advanced NSCLC.[18,19] Over the past decade, several new agents against NSCLC have become available, including the taxanes, gemcitabine, vinorelbine, and irinotecan. The combination of platinum and these new agents has resulted in a high response rate and prolonged survival compared with older chemotherapy regimens (e.g., vindesine, mitomycin, ifosfamide, with cisplatin). Therefore, these regimens are considered standard chemotherapy for advanced NSCLC.[20–26] Although new agents have different mechanisms of action, these combination regimens have not been administered based on the biologic characteristics of each tumor.

Paclitaxel inhibits several endothelial cell functions in vitro such as proliferation, migration, morphogenesis, and metalloprotease production.[27–29] These activities result in antiangiogenic activity in in vivo xenograft models.[27,30] Interestingly, human endothelial cells are more sensitive to paclitaxel than other cellular types.[29] We hypothesized that the CEC value is associated with tumor neovascularization, which is one of the targets of paclitaxel. In the present study, we investigated whether the CEC count at baseline is associated with the effectiveness of the CDDP plus paclitaxel regimen in patients with advanced-stage NSCLC.

MATERIALS AND METHODS

Patients

Patients with histologically or cytologically documented advanced NSCLC were eligible for this study. Each patient was required to meet the following criteria: (1) no prior treatment including chemotherapy, surgery, irradiation, or any fluid drainage; (2) no prior general anesthesia for diagnostic procedures including mediastinoscopy or thoracoscopy; (3) no concomitant diseases including ischemic heart diseases, systemic vasculitis, pulmonary hypertension, or serious complications including infectious disease or diabetes; (4) written informed consent. The trial document was approved by the institutional review board. The clinical characteristics of the patients are shown in Table 1.

Treatment Schedule and Response Evaluation

All patients were treated according to the following chemotherapeutic regimen: paclitaxel at 200 mg/m^2 over a 3-hour period followed by carboplatin at a dose with an area under the curve of 6 on day 1, repeated every 3 weeks. The treatment was repeated for three or more cycles unless the patients met the criteria for progressive disease (PD) or experienced unacceptable toxicity.

The major axis (a) and minor axis (b) of the tumor mass in each patient were measured with computed tomography. Estimated tumor volume (ETV) was calculated using the following formula: ETV = $4/3 \times \pi$ (a/2 × b/2) × (a/2 + b/2)/2. Computed tomography examinations were performed before treatment and with every one or two cycles of chemotherapy. Response was evaluated according to the RECIST, and tumor markers were excluded from the criteria.[31]

Assay for CEC

Blood samples from NSCLC patients and healthy volunteers were drawn into a 10-ml Cellsave Preservative Tube (Immunicon Corp. Huntingdon Valley, PA) for CEC enumeration. The CEC protocol used was approved by the Institutional Review Board and written informed consent was obtained from each subject. Samples from NSCLC were obtained before (baseline) and 8 and 22 days after starting chemotherapy. Samples were kept at room temperature and processed within 42 hours after collection. All evaluations were performed without knowledge of the clinical status of the patients. The CellTracks system (Immunicon Corp) which consists of CellTracks AutoPrep system and the CellSpotter Analyzer system was used for endothelial cell enumeration.[32,33] In this system, CD146+/DAPI+/CD105-PE+/CD45APC- cells are defined as CECs. Briefly, cells which express CD146 were immunomagnetically captured using ferrofluids coated with CD146 antibodies. The enriched cells were then labeled with the nuclear dye 4V,6-diamidino-2-phenylindole (DAPI), CD105 antibodies conjugated to phycoerythrin (CD105-PE), and the pan-leukocyte antibody CD45 conjugated to allophycocyanin (CD45-APC). In this system, the CD146-enriched, fluorescently labeled cells were identified as CECs when the cells exhibited the DAPI+/CD105+/CD45- phenotype. We performed CEC enumeration twice, using the same sample, and calculated the mean value.

TABLE 1. Baseline Characteristics of the Patients

Characteristic	$N = 31$ No. (%)
Gender	
Male	17 (55)
Female	14 (45)
Median age (yr)	60
Range	43–71
ECOG performance status	
0	18 (58)
1	13 (42)
Stage	
IIIA	2 (6)
IIIB	7 (23)
IV	22 (71)
Histology	
Adenocarcinoma	23 (74)
Squamous cell carcinoma	4 (13)
Others	4 (13)

Statistical Analyses

This study was carried out as exploratory research for detecting CECs from NSCLC patients. The number of enrolled patients was therefore not precalculated. Spearman's correlation analysis was performed to investigate the correlation between CEC count and ETV. Between-group comparisons were made using the t test. The association between CEC count and progression free survival (PFS) was estimated using the Kaplan-Meier method. The log-rank test was used to assess the survival difference between strata. Differences were considered statistically significant at $p < 0.05$.

RESULTS

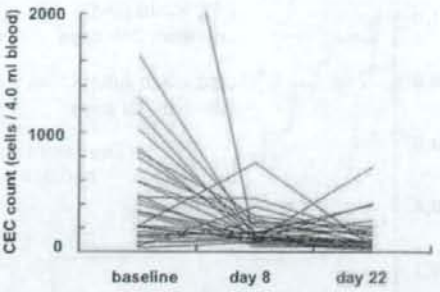
Patient Characteristics

A total of 32 patients were enrolled in the study between August 2005 and March 2006 (Table 1). One patient withdrew consent to participate. Table 1 summarizes the characteristics of the study population. The median age of the patients was 60 years (range, 43–71). The histologic and/or cytologic diagnosis was adenocarcinoma in 23 patients (74.2%), squamous cell carcinoma in 4 (12.9%), and unclassified NSCLC in 4 (12.9%). There were 17 males (54.8%). The clinical stage was IIIA in 2 patients (6.5%), IIIB in 7 (22.6%), and IV in 22 (71.0%).

Ninety-two CEC samples from 31 patients (three samples per patient) were obtained and analyzed. One sample, obtained 22 days after treatment, was not examined because of inadequate collection.

Quantification of CEC

In 31 advanced NSCLC patients, CECs ranged from 32 to 4501 cells/4.0 ml of blood, mean ± SD = 595 ± 832 at baseline. CEC counts were elevated in a large portion of patients with NSCLC as compared with healthy volunteers (n = 53, mean ± SD = 46.2 ± 86.3/4 ml). Case 21 had an exceptionally high CEC count (4501 at baseline). We did not detect a significant correlation between the CEC count and ETV in the 28 assessable patients (p = 0.84, Figure 1). The analysis of CECs during the first course of treatment showed CEC levels to be reduced by CBDCA plus paclitaxel chemotherapy as compared with pretreatment values (176 ± 141 at 8 days and 173 ± 189 at 22 days after treatment) (Figure 2). These reductions were significant (p = 0.011 on day 8 and p = 0.04 on day 22), but there was no significant difference between CEC amounts on day 8 versus day 22 (p = 0.476). There was no difference in the amount of CEC at baseline when patients were subgrouped according to characteristics, such as sex, smoking history, histologic type, and clinical stage. Furthermore, there was no correlation of CEC amounts with the blood examination data (e.g., number of white blood cells, neutrophils, lymphocytes, hemoglobin, platelets, albumin, LDH, CRP, CEA, CYFRA).



	baseline	day 8	day 22
mean ± SD	595 ± 832	176 ± 141 *	173 ± 189 *

FIGURE 2. Circulating endothelial cell (CEC) levels during the first course of CDDP plus paclitaxel chemotherapy. *p < 0.05 versus values at baseline.

CEC Amounts and Objective Tumor Response to Chemotherapy

Thirteen (41.9%) of the 31 patients who received carboplatin and paclitaxel therapy showed a partial response (PR) and 12 (38.7%) showed stable disease (SD). The other 6 patients (19.4%) showed PD. The amounts of CEC at baseline in the patients who showed PR and SD were 516 ± 458/4 ml and 871 ± 1215/4 ml, respectively, and these values were significantly higher than in PD patients (211 ± 150/4 ml, p = 0.023 and p = 0.044, respectively) (Figure 3A). Although CEC decrements during chemotherapy were observed in all three subgroups, the extent of the decrements tended to be greater in

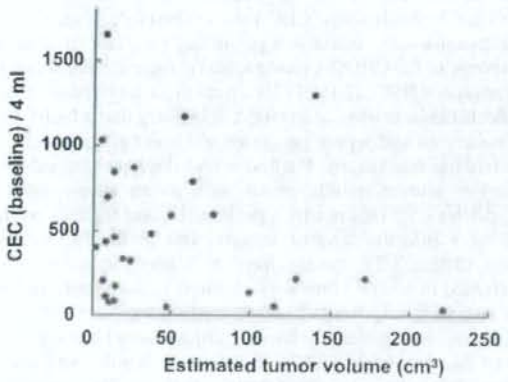


FIGURE 1. Scatter plot analysis to determine the correlation between the number of circulating endothelial cell (CEC) and estimated tumor volume (ETV). ETV is calculated with computed tomography (CT) examination. Case 21 is not included.

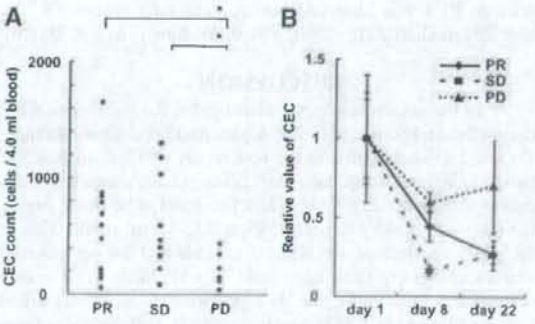


FIGURE 3. A, Comparison of circulating endothelial cell (CEC) amount at baseline in non-small cell lung cancer (NSCLC) patients with different clinical responses to CBDCA plus paclitaxel chemotherapy. *p < 0.05 versus values of patients with progressive disease (PD). Case 21 is not included. B, Relative change in CEC amount in patients with partial response (PR), stable disease (SD), and PD.

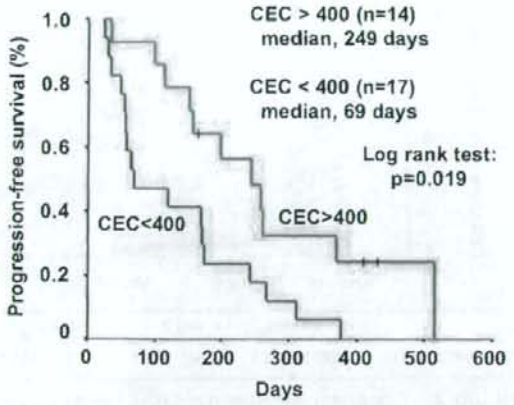


FIGURE 4. Progression-free-survival according to circulating endothelial cell (CEC) count at baseline. The median duration of progression-free survival was greater in patients whose CEC count exceeded 400 (median, 244 days) than in patients whose CEC count was less than 400 (69 days).

patients with PR and SD than in those with PD (Figure 3*B*). In the subgroup analysis, a significant decrease in CECs was observed on day 22 only in PR patients ($p = 0.018$).

CEC Amounts and PFS

For all 31 patients, the median PFS was 154 days (range, 81–361 days). Univariate analysis indicated that patients who had a CEC count of more than 400/4 ml at baseline showed a significantly improved PFS ($n = 14$, median; 244 days) (Log-rank test, $p = 0.019$, Figure 4). A CEC count below 400 at baseline was associated with a poorer PFS ($n = 17$, median; 69 days). The CEC count did not exceed the value of 400/4 ml in any of the healthy volunteers. When we compared the patients whose CEC counts exceeded 200 with those whose counts were less than 200, a consistent difference in PFS was observed between the two groups (>200; $n = 22$, median 227, <200; $n = 9$, median 116, $p < 0.039$).

DISCUSSION

In the present study, we investigated the number of CEC during the first course of CBDCA plus paclitaxel chemotherapy. To our knowledge, this is the first report of CEC in NSCLC patients before treatment. Our findings demonstrated CEC counts in advanced NSCLC at baseline level to be much higher than those in healthy subjects (595 ± 832/4.0 ml versus 32.6 ± 29.5/4.0 ml). Because the NSCLC patients had not yet received anticancer therapy, these increased CECs are likely to be mostly derived from the tumor site. In a previous study, it was found that the amounts of CECs correlate strongly with tumor volume in vivo in an animal model[34]. Nevertheless, we did not find a significant correlation between CECs and ETV. Because the number of CECs could be influenced by many factors related to tumor vasculature, neovascularization, and localization of the tumor, our failure to identify a strong correlation in this study is not surprising. We were also unable to detect a significant direct correlation between CEC amounts and various blood examination data including tumor markers such as CEA and CYFRA. It is unclear at present what biologic characteristics of the tumor or clinical features the CEC number most closely reflects as a biomarker. Mancuso et al. reported that CECs are strongly associated with plasma levels of VCAM-1 and VEGF in breast cancer and lymphoma patients.[15,34] Because VCAM-1 and VEGF are crucial factors for tumor angiogenesis, the variability in CEC values among NSCLC patients might indicate a difference in the neovascularization of each tumor.

We were further able to demonstrate that elevated CECs decreased dramatically after CBDCA plus paclitaxel treatment, but did not reach the level of healthy subjects. Decreased CEC values did not rise again during the first cycle of chemotherapy. Although myelosupression was observed on day 8 and recovered on day 22 in many patients (data not shown), CEC kinetics do not parallel those of WBC, indicating that CEC kinetics might not be influenced by myelopoiesis. Several clinical studies in the field measuring CEC found chemotherapy to be associated with either an increase or a decrease in CECs.[35–39] The different tumor types, stages, prior therapy or not, the anticancer drugs used, measuring points and quantification methods of CEC might have influenced the CEC results after treatment. In the present study, the pretreatment CEC value was much higher than that in lung cancer with metastasis (mean ± SD = 146 ± 270/4 ml), as reported elsewhere.[33] Although the details of the prior therapy in patients with metastatic carcinoma were not provided,[33] chemotherapy can eventually decrease the CEC count.

Schiller et al. compared four standard chemotherapy regimens, cisplatin plus paclitaxel, cisplatin plus gemcitabine, cisplatin plus docetaxel, and carboplatin plus paclitaxel and found no significant difference in survival.[25] Despite the different modes of action of each nonplatinum agent against tumors and different biologic characteristics of each tumor, we could not select the regimen based on these characteristics. In our small study, the patients with PR/SD and longer PFS had higher baseline CEC values. Therefore, it seems that the baseline CEC count is a promising predictor of clinical response to the CBDCA plus paclitaxel regimen and survival in advanced NSCLC. If CEC is a marker for angiogenesis and reflects tumor neovascularization, it is likely that a high CEC is associated with a poor prognosis and lower effectiveness of antiangiogenic therapy. Paclitaxel and docetaxel are categorized as mitotic spindle agents with potent antiangiogenic properties.[27–30] This is why a paclitaxel based regimen might be more effective against tumors with high CEC values. Nevertheless, CEC counts have also been reported to be increased in several clinical syndromes, such as cardiovascular diseases, infectious diseases, and vasculitides.[11–13] The CEC counts in patients with vasculitides have been reported to be dozens of fold higher than those in healthy subjects,[12] therefore, we have to consider the patient condition carefully while interpreting the CEC counts in individual patients, although there were no patients with vasculitis in the present study. Further clinical investigation, with a similar approach, including other nonplatinum anticancer agents, such as

CDDP plus gemcitabine, is essential for the clinical application of CEC for made-to-order chemotherapy in NSCLC.

Antiangiogenic therapy targeting the VEGF pathway such as bevacizumab and VEGFR inhibitors have shown promise in the treatment of solid tumors.[8,39] These agents inhibit endothelial cells through inhibition of the VEGF pathway. It was recently demonstrated that the addition of bevacizumab to CBDCA plus paclitaxel in advanced NSCLC patients produces a significant survival benefit as compared with chemotherapy alone.[40] Considering the outstanding clinical trial and our present study, it would be of great interest to investigate the role of CEC in this regimen.

In conclusion, CECs were measured in NSCLC patients before treatment. Our small clinical study indicates that the CEC count at baseline is a potential biomarker for predicting the response to chemotherapy and PFS, but further clinical evaluation is needed. In the near future, we will start a clinical investigation, using a similar approach, to examine other chemotherapeutic regimens.

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REFERENCES

1. Folkman J. Anti-angiogenesis: new concept for therapy of solid tumors. *Ann Surg* 1972;175:409–416.
2. Gasparini G, Harris AL. Clinical importance of the determination of tumor angiogenesis in breast carcinoma: much more than a new prognostic tool. *J Clin Oncol* 1995;13:765–782.
3. Dickinson AJ, Fox SB, Persad RA, Hollyer J, Sibley GN, Harris AL. Quantification of angiogenesis as an independent predictor of prognosis in invasive bladder carcinomas. *Br J Urol* 1994;74:762–766.
4. Takahashi Y, Kitadai Y, Bucana CD, Cleary KR, Ellis LM. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. *Cancer Res* 1995;55:3964–3968.
5. Williams JK, Carlson GW, Cohen C, Derose PB, Hunter S, Jurkiewicz MJ. Tumor angiogenesis as a prognostic factor in oral cavity tumors. *Am J Surg* 1994;168:373–380.
6. Koukourakis MI, Giatromanolaki A, Thorpe PE, et al. Vascular endothelial growth factor/KDR activated microvessel density versus CD31 standard microvessel density in non-small cell lung cancer. *Cancer Res* 2000;60:3088–3095.
7. Natsume T, Watanabe J, Koh Y, et al. Antitumor activity of TZT-1027 (Soblidotin) against vascular endothelial growth factor-secreting human lung cancer in vivo. *Cancer Sci* 2003;94:826–833.
8. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med* 2004;350:2335–2342.
9. Yang JC, Haworth L, Sherry RM, et al. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 2003;349:427–434.
10. Johnson DH, Fehrenbacher L, Novotny WF, et al. Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. *J Clin Oncol* 2004;22:2184–191.
11. Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood* 1999;93:2951–2958.
12. Woywodt A, Streiber F, De Groot K, Regelsberger H, Haller H, Haubitz M. Circulating endothelial cells as markers for ANCA associated small-vessel vasculitis. *Lancet* 2003;361:206–210.
13. Mutunga M, Fulton B, Bullock R, et al. Circulating endothelial cells in patients with septic shock. *Am J Respir Crit Care Med* 2001;163:195–200.
14. Beerepoot LV, Mehra N, Vermaat JS, Zonnenberg BA, Gebbink MF, Voest EE. Increased levels of viable circulating endothelial cells are an indicator of progressive disease in cancer patients. *Ann Oncol* 2004;15:139–145.
15. Mancuso P, Burlini A, Pruneri G, Goldhirsch A, Martinelli G, Bertolini F. Resting and activated endothelial cells are increased in the peripheral blood of cancer patients. *Blood* 2001;97:3658–3661.
16. Mancuso P, Colleoni M, Calleri A, et al. Circulating endothelial-cell kinetics and viability predict survival in breast cancer patients receiving metronomic chemotherapy. *Blood* 2006;108:452–459.
17. Bülzebruck H, Bopp R, Dringe P, et al. New aspects in the staging of lung cancer. Prospective validation of the International Union Against Cancer TNM classification. *Cancer* 1992;70:1102–1110.
18. Grilli R, Oxman AD, Julian JA. Chemotherapy for advanced non-small-cell lung cancer: how much benefit is enough? *J Clin Oncol* 1993;11:1866–1872.
19. Non-small Cell Lung Cancer Collaborative Group. Chemotherapy in non-small cell lung cancer: a meta-analysis using updated data on individual patients from 52 randomised clinical trials. *BMJ* 1995;311:899–909.
20. Kubota K, Watanabe K, Kunitoh H, et al. Phase III randomized trial of docetaxel plus cisplatin versus vindesine plus cisplatin in patients with stage IV non-small cell lung cancer: the Japanese Taxotere Lung Cancer Study Group. *J Clin Oncol* 2004;22:254–261.
21. Le Chevalier T, Brisgand D, Douillard JY, et al. Randomized study of vinorelbine and cisplatin versus vindesine and cisplatin versus vinorelbine alone in advanced non-small cell lung cancer: results of a European multicenter trial including 612 patients. *J Clin Oncol* 1994;12:360–367.
22. Belani CP, Lee JS, Socinski MA, et al. Randomized phase III trial comparing cisplatin-etoposide to carboplatin-paclitaxel in advanced or metastatic non-small cell lung cancer. *Ann Oncol* 2005;16:1069–1075.
23. Yana T, Takada M, Origasa H, et al. New chemotherapy agent plus platinum for advanced non-small cell lung cancer: a meta-analysis. *Proc Am Soc Clin Oncol* 2002;21:328a.
24. Baggstrom MQ, Stinchcombe TE, Fried DB, Poole C, Hensing TA, Socinski MA. Third-generation chemotherapy agents in the treatment of advanced non-small cell lung cancer: a meta-analysis. *J Thorac Oncol* 2007;2:845–853.
25. Schiller JH, Harrington D, Belani CP, et al; Eastern Cooperative Oncology Group. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. *N Engl J Med* 2002;346:92–98.
26. Ohe Y, Ohashi Y, Kubota K, et al. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan. *Ann Oncol* 2007;18:317–323.
27. Belotti D, Vergani V, Drudis T, et al. The microtubule-affecting drug paclitaxel has antiangiogenic activity. *Clin Cancer Res* 1996;2:1843–1849.
28. Hayot C, Farinelle S, De Decker R, et al. In vitro pharmacological characterizations of the anti-angiogenic and anti-tumor cell migration properties mediated by microtubule-affecting drugs, with special emphasis on the organization of the actin cytoskeleton. *Int J Oncol* 2002;21:417–425.
29. Wang J, Lou P, Lesniewski R, Henkin J. Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly. *Anticancer Drugs* 2003;14:13–19.
30. Vacca A, Ribatti D, Iuliaro M, et al. Docetaxel versus paclitaxel for antiangiogenesis. *J Hematother Stem Cell Res* 2002;11:103–118.
31. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;92:205–216.
32. Smirnov DA, Foulk BW, Doyle GV, Connelly MC, Terstappen LW, O'Hara SM. Global gene expression profiling of circulating endothelial cells in patients with metastatic carcinomas. *Cancer Res* 2006;66:2918–2922.
33. Rowand JL, Martin G, Doyle GV, et al. Endothelial cells in peripheral blood of healthy subjects and patients with metastatic carcinomas. *Cytometry A* 2007;71A:105–114.
34. Mancuso P, Calleri A, Cassi C, et al. Circulating endothelial cells as a novel marker of angiogenesis. *Adv Exp Med Biol* 2003;522:83–97.

35. Beaudry P, Force J, Naumov GN, et al. Differential effects of vascular endothelial growth factor receptor-2 inhibitor ZD6474 on circulating endothelial progenitors and mature circulating endothelial cells: implications for use as a surrogate marker of antiangiogenic activity. *Clin Cancer Res* 2005;11:3514–3522.
36. Fürstenberger G, von Moos R, Lucas R, et al. Circulating endothelial cells and angiogenic serum factors during neoadjuvant chemotherapy of primary breast cancer. *Br J Cancer* 2006;94:524–531.
37. Rademaker-Lakhai JM, Beerepoot LV, Mehra N, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral protein kinase C beta-inhibitor enzastaurin in combination with gemcitabine and cisplatin in patients with advanced cancer. *Clin Cancer Res* 2007;13:4474–4481.
38. McAuliffe JC, Trent JC. Biomarkers in gastrointestinal stromal tumor: should we equate blood-based pharmacodynamics with tumor biology and clinical outcomes? *Clin Cancer Res* 2007;13:2643–2650.
39. Hanrahan EO, Heymach JV. Vascular endothelial growth factor receptor tyrosine kinase inhibitors vandetanib (ZD6474) and AZD2171 in lung cancer. *Clin Cancer Res* 2007;13:S4617–S4622.
40. Sandler A, Gray R, Perry MC, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. *N Engl J Med* 2006;355:2542–2550; 2007;356:318.

Clinical Relevance of Sputum Cytology and Chest X-Ray in Patients with Suspected Lung Tumors

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Clinical Relevance of Sputum Cytology and Chest X-Ray in Patients with Suspected Lung Tumors

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Abstract

Objective To review diagnostic procedures, therapeutic modalities, and follow-up methods in patients with suspected lung tumors.

Methods We retrospectively examined 70 patients who underwent a complete medical checkup because they had been positive for sputum cytology and had presented no chest X-ray findings for the 10-year period between 1994 and 2004. To make a diagnosis, we conducted the first complete medical checkup that included chest X-ray, sputum cytology, chest computed tomography (CT), and bronchoscopy. In the case that no diagnosis could be made, we repeated the chest X-ray and sputum cytology every 3 to 6 months and additionally conducted chest CT and bronchoscopy according to abnormal findings.

Results Among 70 patients, there were 36 and 13 who were diagnosed during the first complete medical checkup and follow-up, respectively, 13 who remained undiagnosed, and eight for whom follow-up was discontinued. Among the 49 diagnosed patients, 40, 8, and 1 patient had lung cancer, upper respiratory tract carcinoma (URTC), and esophageal carcinoma (EC), respectively. Among the 40 patients with lung cancer, 34 had a stage 0 or I tumor and 15 were radically treatable by photodynamic therapy and endobronchial irradiation. Nine among 11 patients whose lung cancer was detected during follow-up had a stage 0 or IA tumor.

Conclusion Not only lung cancer but also URTC and EC were successfully detected in patients who were positive for sputum cytology and presented negative chest X-ray. Radical treatment was possible in 38 (76%) of 50 diagnosed patients, thus indicating the importance of follow-up through these procedures.

Key words: sputum cytology, roentgenographically occult lung cancer, photodynamic therapy, endobronchial irradiation

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Introduction

Lung cancer is a disorder with a poor prognosis because it is rarely detected in a condition where radical treatment can be initiated. Therefore, sorts of efforts are made for its early detection. Among patients whose lung cancer was detected during mass screening only by chest X-ray, two-thirds of them present an advanced or metastatic lesion (1). Hence, mass screening by chest X-ray alone is not considered sufficient for the early detection of lung cancer. Large-scale randomized clinical trials in Europe and the United States (2-6) have not revealed the lung cancer mortality-reducing effect of mass screening for lung cancer that combine chest X-ray to detect peripheral lung cancer with sputum cytology to detect central lung cancer. Therefore, medical institutions in Europe and the United States do not officially recommend the screening. However, case-control studies in Japan (7-10) have demonstrated the lung cancer mortality-decreasing effect of screening, warranting its conduct in Japan.

Screening based on the Elderly Health Law in Japan include chest X-ray for males and females aged 40 years or over, as well as mass screening for lung cancer consisting of sputum cytology for individuals aged 50 years or over who

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Table 1. Patient Characteristics (n=70)

Gender	
Male/female gender, No.	66/4
Age (median), yr	26-81 (69)
Smoking history	
Smoker, No.	55
Ex-smoker, No.	12
Nonsmoker, No.	3
Brinkmann Index (median)	2.25-175 (50) packs/year
≥ 30 packs/year	63
< 30 packs/year	4
Motive of detection	
Mass screening for lung cancer, No.	34
Subjective symptoms, No.	28
Follow-up of another disorder, No.	8
Sputum cytology	
Squamous cell carcinoma, No.	60
Adenocarcinoma, No.	10

Brinkmann index: (Number of cigarette packs per day)×(Number of years the subject has smoked)

have a Brinkmann index of ≥30 packs/year and for symptomatic individuals presenting with bloody sputum (11). Sputum cytology is considered an important screening procedure for lung cancer in heavy smokers. A few study reports are available that have described the follow-up of patients who were positive for sputum cytology and presented no chest X-ray findings (12-14).

Materials and Methods

Diagnostic procedures, therapeutic modalities, and follow-up methods were reviewed retrospectively in 70 patients who provided positive results in sputum cytology or screening for lung cancer; due to the lack of chest X-ray findings, they underwent a medical examination in the Department of Respiratory Medicine at Osaka City General Hospital for the last 10 years between 1994 and 2004. Among 70 patients, there were 65 males and 5 females aged 26 to 81 years (median: 69). Regarding smoking history, there were 55 smokers, 12 ex-smokers, and three nonsmokers. The Brinkmann index was 2.25 to 175 packs/year (median: 50) (Table 1).

To make a diagnosis, the first complete medical checkup (X-ray according to the direct method, repeated sputum cytology using pooled sputum, chest CT, and bronchoscopy) was conducted in the above patents. In the case that no diagnosis could be made, chest X-ray and sputum cytology using pooled sputum were repeated every 3 to 6 months. Chest computed tomography (CT) and bronchoscopy were conducted additionally according to abnormal findings. Chest X-ray and sputum cytology were continued wherever possible until making a diagnosis. Furthermore, the clinical stages were determined in accordance with the NCCN guideline (15). A lesion, about which carcinoma *in situ* was considered and whose diagnosis was made not by resected specimen examination but by bronchoscopic biopsy only, could not be diagnosed as a stage 0 tumor unless the biopsy specimen successfully verified the invasion of the basal membrane. Therefore, the lesion was staged 0-IA.

Sputum cytology

Sputum cytology was conducted according not to Saccomanno's method but to the sputum pooling method using sputum that had been pooled for 3 days and the pooling solution that had been improved by the addition of mucosa-dissolving agent and other compounds. The Japan Lung Cancer Society has established the evaluation criteria and guidance for sputum cytology in mass screening for lung cancer according to the pooling method (11). In the present study, cells were assessed in accordance with the criteria, and two professionals (one cytotechnologist and one pathologist) made a diagnosis.

Chest X-ray

Chest X-ray (posteroanterior and lateral) according to the direct method was conducted. Roentgenograms were read by the pneumologist. Chest roentgenograms indicating old lung tuberculosis in fixed foci, pneumoconiosis, lung asbestosis, and pulmonary fibrosis were considered not to show abnormal findings.

Chest CT

CT using the 1-cm slice low-frequency algorithm was conducted to image the region from the upper portion of the clavicle to the level immediately above the diaphragm. One radiologist and one pneumologist read the lung fields and mediastinum. Chest tomograms indicating old lung tuberculosis in fixed foci, pneumoconiosis, lung asbestosis, and pulmonary fibrosis were considered not to show abnormal findings.

Bronchoscopy

After the intramuscular injection of opium alkaloid hydro-

chloride and atropine sulfate, 4% lidocaine hydrochloride was injected to anesthetize the pharynx. Subsequently, the bronchoscopist used a bronchoscope (Olympus BF-200, Tokyo, Japan) to observe the oral cavity, larynx, pharynx, vocal cord, trachea, and at least the third-order bronchi. Abnormal findings were assessed according to the Japanese rule (16). When an abnormality of the bronchial mucosa was found in the region visible to the bronchoscope, bronchoscopic biopsy was conducted actively. When the bronchoscopy was difficult to conduct despite its location in the visible field, the lesion was scraped with a brush. When tests were completed without finding any abnormalities, the sputum collected after the completion of bronchoscopy was subjected to cytology.

Results

The motives for the detection of positive findings in sputum cytology were as follows: mass screening for lung cancer in 34 patients; subjective symptoms in 28 patients; and screening for another disorder in eight patients. Regarding the histological types of the tumor in sputum cytology, there were 60 cases of squamous cell carcinoma (SCC) and 10 cases of adenocarcinoma (AC).

Among 70 patients, there were 36 and 13 who were diagnosed during the first complete medical checkup and follow-up, respectively, 13 who remained undiagnosed, and eight patients for whom follow-up was discontinued, i.e., seven patients at their discretion and one patient who showed deteriorating symptoms of another disease at 3 months (Fig. 1).

Thirty-one patients, who were diagnosed during the first complete medical checkup, had a lesion that was located in the region visible to the bronchoscope: 24 with smoking history had lung cancer and seven had upper respiratory tract carcinoma (URTC) (Table 2). The lesion was located in the subsegmental branches inclusive as follows: 0-order bronchi, three patients; first-order bronchi, six patients; second-order bronchi, 10 patients; and third-order bronchi, five patients. Bronchoscopic findings: hypertrophy, 14 patients; nodule, six patients; and polyp, four patients. Regarding histopathology, all but one case of AC were SCC. Simultaneously, there was one patient with double carcinoma.

There were five patients in whom chest CT revealed an abnormality despite the lack of abnormal findings on chest X-ray during the first complete medical checkup. Among them, three patients had lung field mass shadow, one patient had lung field stripes, and one patient had mediastinal lymphadenopathy without lung field lesion. The mass was located at a site that was difficult to discriminate unless conducting chest CT.

Regarding clinical stages and treatment of 24 patients with lung cancer in whom the first complete medical checkup led to a diagnosis under TBB: 13, 9, one, and one had a tumor staged 0-IA, IA, IIIA, and IIIB, respectively. Treatment was photodynamic therapy (PDT) in seven among 13 patients with a stage 0-IA tumor and was endobronchial irradiation in five among them. Another remaining patient

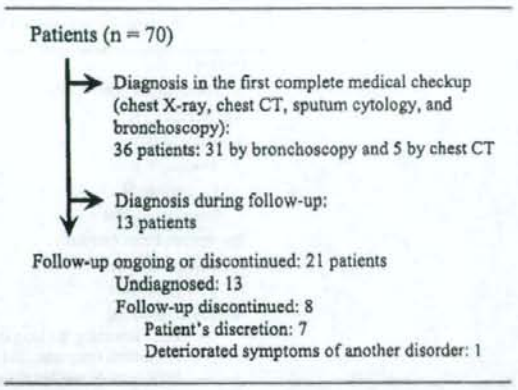

Figure 1. Clinical course until making a definite diagnosis. To make a definite diagnosis in 70 patients who provided positive results in sputum cytology and presented no chest x-ray findings, posteroanterior and lateral x-rays of the chest, sputum cytology using pooled sputum, computed tomography of the chest, and bronchoscopy were conducted as the first complete medical checkup. When no definite diagnosis was obtained, chest x-ray and sputum cytology (pooled sputum method) were repeated every 3 to 6 months.

survived for 5 years and 6 months by external irradiation (EI). We conducted PDT for one SCC and then EI to another SCC in a patient with double cancer, and two sites of them had a complete response. All nine patients with a stage IA tumor underwent surgery, with five assigned to lobectomy, two to segmentectomy, and two to sleeve lobectomy (Table 2).

Five patients with lung cancer in whom chest CT revealed abnormal shadows had tumors categorized to the following clinical stages: IA, one patient; IB, two patients; IIIB, one patient; and IV, one patient. A patient with a stage IA tumor and the patients with a stage IB tumor underwent surgery. Treatment was restricted only to EI toward the mediastinum in the patient with a stage IIIB tumor because of the previous follow-up for chronic disseminated intravascular coagulation (DIC) in Department of Hematology. The patient with a stage IV tumor developed cardiac tamponade due to cancerous pericarditis before receiving anticancer agents and underwent palliative care (Table 3).

EI alone was conducted in five of eight patients with URTC, and all of them had a complete response. In one patient disease recurred after surgery for URTC and was transferred to the hospital where the surgery had been conducted. Another remaining patient underwent tumor resection after preoperative chemotherapy and presented short survival despite subsequent radiation therapy (Tables 2, 4).

Among patients in whom the first complete medical checkup did not lead to a diagnosis, a diagnosis was made subsequently in 13 patients during follow-up, the breakdown of whom was as follows: 11 patients with lung cancer; one patient with esophageal carcinoma (EC); and one patient

Table 2. Direct Views Available by Bronchoscopy in the First Complete Medical Checkup (n=31)

Lung cancer	24
Orders of branching	
Zero-order: 3; first-order: 6; second-order: 10; third-order: 5	
Bronchoscopic findings	
hypertrophy: 14; node: 6; polyp: 4	
Histopathology	
SCC: 22; adenocarcinoma: 1; double carcinoma (SCC at two sites): 1	
Clinical stages of disease	
0-IA: 13; IA: 9; IIIA: 1; IIIB: 1	
Upper respiratory tract carcinoma	7

SCC: squamous cell carcinoma

Table 3. Patients with Abnormal Chest CT Findings in the First Complete Medical Checkup (n=5)

CT Findings	Histopathology	Diagnostic Method	Clinical Stage	Treatment
Lung field mass shadow in rt-S^1	SCC	TBB	IB	S (lobectomy)
Lung field mass shadow in rt-S^2	SCC	CT-guide	IA	S (lobectomy)
Lung field mass shadow in lt-S^6	SCC	TBB	IB	S (lobectomy)
Lung field stripes	AC	Brushing	IV	BSC
Mediastinum lymphadenopathy	AC	TBAC	IIIB	EI

rt: right, lt: left, SCC: squamous cell carcinoma, AC: adenocarcinoma, TBB: transbronchial biopsy, CT-guide: Computed tomography-guided lung biopsy, TBAC: transbronchial aspiration cytology, S: surgery, BSC: best supportive care, EI: external irradiation

with URTC. In all the patients who had smoking history, the histopathological types of the tumors were identical between the first and second sputum cytologies. After conducting the first medical checkup, patients were followed by sputum cytology and chest X-ray. Bronchoscopy revealed abnormal findings in six patients. In six patients, chest CT indicated mass shadows. In one patient, a diagnosis was made under gastroendoscopy to closely examine hematemesis of unknown etiology. Regarding bronchoscopic findings in lung cancer, four and one had nodular and polyp tumors, respectively. The period until making a diagnosis was 2 to 47 months (median, 12). In 10 among 13 patients, a diagnosis was made within 1 year. Among 11 lung cancer patients in whom a diagnosis was made during follow-up, five and four had a stage 0-IA tumor and a stage IA tumor, respectively. In eight patients of the 11 lung cancer patients [PDT, four patients; and surgery, four patients (lobectomy: three patients and segmentectomy: one patient)], radical treatment was conducted successfully. Active treatment was not desired for one patient because of symptoms of dementia, and thus palliative care was provided (Table 4).

Currently, 13 patients are under follow-up because of failure to make a diagnosis. The median value for observation period is 28 months. The histopathological types of their tumors in sputum cytology were SCC in 10 patients and AC in three patients. Sputum cytology during follow-up was negative in most patients. However, the procedure was positive once in two patients during follow-up but was not positive thereafter. In one patient, furthermore, the procedure revealed suspicious findings only once. Among eight patients about whom follow-up was discontinued, the clinical course was difficult to investigate in two patients who failed to visit the hospital in order to undergo a medical examination immediately after the first complete medical checkup. However, the remaining six patients were followed for 3, 9, 17, 24, 61, and 83 months.

The contents of the examinations during follow-up were compared between 13 patients in whom a diagnosis was made during follow-up and 19 undiagnosed patients (13 patients under follow-up and six patients for whom follow-up was discontinued, except for two patients who failed to undergo a medical examination after the first complete medical

Table 4. Clinical Profiles of Patients Who Were Diagnosed during Follow-Up (n=13)

Diagnosis	Method	Period (month)	Order of Bronchus	Bronchoscopic Finding	Tumor Diameter	Clinical Stage	Treatment
Lung cancer (SCC)	Direct view	7	I	Node	-	0-IA	PDT
Lung cancer (SCC)	Direct view	8	III	Node	-	0-IA	PDT
Lung cancer (SCC)	Direct view	9	III	Node	-	0-IA	PDT
Lung cancer (SCC)	Direct view	27	IV	Polyp	-	0-IA	PDT
Lung cancer (SCC)	Direct view	47	I	Node	-	0-IA	BSC
Lung cancer (AC)	CT	3	-	-	28 mm	IA	S
Lung cancer (AC)	CT	4	-	-	20 mm	IA	S
Lung cancer (AC)	CT	6	-	-	12 mm	IA	S
Lung cancer (SCC)	CT	12	-	-	10 mm	Unknown	BSC
Lung cancer (AC)	CT	20	-	-	25 mm	IV	BSC
Lung cancer (SCC)	CT	33	-	-	15 mm	IA	S
URTC	Direct view	2	-	-	-	II	S + EI
EC	Gastroscopy	8	-	-	-	IIb	S

SCC: squamous cell carcinoma, AC: adenocarcinoma, CT: computed tomography, PDT: photodynamic therapy, S: surgery, EI: external irradiation, BSC: best supportive care, URTC: upper respiratory tract carcinoma, EC: esophageal carcinoma

Table 5. Follow-Up Period and Medical Examination of Patients under Follow-Up

	Diagnosis (n = 13)	Undiagnosis (n = 19)
Follow-up period (median), mth	2-47 (12)	3-83 (28)
Smoker, No.	10	12
Ex-smoker, No.	3	3
Nonsmoker, No.	0	3
Examination contents		
Chest X-ray (median), No.	0-8 (4)	1-11 (5)
Sputum cytology (median), No.	1-12 (3)	1-14 (4)
Sputum cytology-positive patients, %	20	4.7
Chest CT (median), No.	0-3 (1)	0-5 (1)
Bronchoscopy (median), No.	0-6 (2)	0-4 (1)

Chest CT: chest computed tomography

checkup). Consequently, a trend for difference was found only in the positivity rate of sputum cytology (Table 5). The clinical stages and treatment of 50 patients in whom a diagnosis could be made are shown in Table 6.

Discussion

In the course of mass screening for lung cancer, many patients are referred to a hospital for the purpose of undergoing a complete medical checkup because they were positive for sputum cytology and presented no chest X-ray findings. However, a definite diagnosis is difficult to make in such patients, they are frequently difficult to follow up, and there are a limited number of papers of reference (12-14).

We conducted relatively inexpensive sputum cytology and chest X-ray as procedures for follow-up at 3- to 6-month intervals and performed bronchoscopy as required when sputum cytology was positive. Among 11 patients whose lung cancer was detected during follow-up at these 3- to 6-month intervals, five and four had stage 0-IA and IA tumors, respectively. The conceivable reasons why the first complete medical checkup failed to determine the presence of tumors

Table 6. Clinical Stages and Treatments at the Time of Diagnosis (n=49)

Lung cancer		
0-IA (n = 17)	Bronchoscopic treatment (PDT: 10; endobronchial irradiation: 5)	15
	External irradiation	1
	BSC	1
IA (n = 14)	Surgery (lobectomy: 9; segmentectomy: 3 sleeve lobectomy: 2)	14
IB (n = 2)	Surgery (lobectomy: 2)	2
IIIA (n = 1)	Chemotherapy + surgery	1
IIIB (n = 2)	Chemotherapy + external irradiation	1
	External irradiation	1
IV (n = 2)	BSC	2
Double cancer	PDT + external irradiation (0-IA at two sites)	1
Unknown	BSC	1
Upper respiratory tract carcinoma (n = 8)		8
Esophageal cancer (n = 1)		1

PDT: photodynamic therapy, BSC: best supportive care

are as follows: central lung cancers were possibly detected in a super early detection stage where they were unverifiable under bronchoscopy; and peripheral lung cancers were difficult to identify when present in a mixture of old tuberculosis of lung, pneumoconiosis, asbestosis, pulmonary fibrosis, and other disorders.

The NCCN guideline also recommends the repetition of bronchoscopy at 3-month intervals in patients who underwent surgery for lung cancer, for whom its recurrence is considered based on positive findings in sputum cytology and on negative chest X-ray and chest CT (17). Therefore, we deemed that follow-up at 3-month intervals during the clinical course observation period involved no concerns.

There are 21 patients whose follow-up is ongoing or discontinued. In advanced SCC, many cancer cells appear in specimens; therefore, cytodiagnosis is easy to make because these cancer cells present strong atypia. However, the number of appearing cancer cells is small in early SCC. Furthermore, these cancer cells present poor atypia and it may be difficult to differentiate them from atypical metaplasia of squamous cells attributable to the inflammation of bronchial epithelium or from atypical cells originating from atypical epithelium and other tissues (17, 26). Furthermore, a patient with adenocarcinoma of the lung who experienced pulmonary infarction provided false-positive results in sputum cytology (27). Two of three patients with undiagnosed adenocarcinoma in the present study had a history of chest pain, leading us to presume that they possibly had false-positive results in sputum cytology. Therefore, patients with concerns about the precision of sputum cytology are possibly included in 13 patients under follow-up. This possibility seems to denote the limitation of sputum cytology that makes a diagnosis by taking cell degeneration into consideration.

One issue regarding the patients for whom follow-up was discontinued is that they easily forgot to undergo a regular medical examination because they had no symptoms despite providing positive results in sputum cytology. When no definite diagnosis was obtained in the first complete medical checkup, we noted that they tended not to undergo a medical examination again unless receiving a full explanation about the need for regular follow-up.

In recent years, marked progress in medical devices has been noted. There are reports which have described that ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) is effective for the early detection of lesions in the lower respiratory tract (18, 19). However, FDG-PET is difficult to include in routine medical tests due to its high costs. As compared with FDG-PET, sputum cytology and chest X-ray remain attractive because they are inexpensive.

Regarding therapeutic outcomes in the present study, 33 patients with lung cancer [0-IA + IA+ IB, excluding patients receiving best supportive care (BSC)] eventually underwent radical treatment; 15 of them, in whom early lung cancer had been detected bronchoscopically, could be radically treated by endoscopic procedures (PDT and endobronchial irradiation). Fujimura et al recommended the adoption of their strategies for patients with roentgenographically occult lung cancer and obtained favorable results (12, 20-22). Unlike surgical therapy, radical treatment without provoking a decrease in pulmonary function will be achieved as described above if treating eligible patients for whom PDT and endobronchial irradiation are expected to provide radical treatment (23-25).

Therefore, endoscopic treatment constitutes the best therapeutic modality. However, we believe that active search combining sputum cytology suitable for central lung cancer with chest X-ray suitable for peripheral lung cancer is important in such patients because they may include patients with a radically treatable tumor. In addition, we consider that sputum cytology serves not only for the early detection of central lung cancer—the original objective of sputum cy-

tology, but also for the detection of cancers of other organs (especially URTC) and of peripheral lung cancer.

Conclusion

Not only lung cancer but also URTC and EC were successfully detected in patients who showed positive results in sputum cytology and presented a negative chest X-ray. Radical treatment was possible in 38 (76%) of 50 diagnosed patients, thus indicating the importance of follow-up through these procedures.

References

1. Silverberg E, Boring CC, Squires TS. Cancer statistics, 1990. CA Cancer J Clin **40**: 9-26, 1990.
2. Frost JK, Ball WC Jr, Levin ML, et al. Early lung cancer detection: Results of the initial (prevalence) radiologic and cytologic screening in the John Hopkins study. Am Rev Respir Dis **130**: 549-554, 1984.
3. Flehinger BJ, Melamed MR, Zaman MB, Heelan RT, Perchick WB, Martini N. Early lung cancer detection: results of the initial (prevalence) radiologic and cytologic screening in the Memorial Sloan-Kettering study. Am Rev Respir Dis **130**: 555-560, 1984.
4. Fontana RS, Sanderson DR, Woolner LB, et al. Screening for lung cancer: a critique of the Mayo Lung Project. Cancer **67**: 1155-1164, 1991.
5. Kubik A, Polak J. Lung cancer detection results of a randomized prospective study in Czechoslovakia. Cancer **57**: 2427-2437, 1986.
6. Marcus PM, Bergstralh EJ, Fagerstrom RM, et al. Lung cancer mortality in the Mayo Lung Project. J Natl Cancer Inst **92**: 1308-1316, 2000.
7. Okamoto N, Suzuki T, Hasegawa H, et al. Evaluation of a clinic-based screening program for lung cancer with a case-control design in Kanagawa, Japan. Lung Cancer **25**: 77-85, 1999.
8. Tsukada H, Kurita Y, Yokoyama A, et al. An evaluation of screening for lung cancer in Niigata Prefecture, Japan: a population-based case-control study. Br J Cancer **85**: 1326-1331, 2001.
9. Sagawa M, Tsubono Y, Saito Y, et al. A case-control study for evaluating the efficacy of mass screening program for lung cancer in Miyagi Prefecture, Japan. Cancer **92**: 588-594, 2001.
10. Nishii K, Ueoka H, Kiura K, et al. A case-control study of lung cancer screening in Okayama Prefecture, Japan. Lung Cancer **34**: 325-332, 2001.
11. The Japan Lung Cancer Society. General Rule for Clinical and Pathological Record of Lung Cancer. 2nd ed. Kanehara & Co., Ltd., Tokyo, 2003: 177-186.
12. Fujimura S, Sakurada A, Sagawa M, et al. A therapeutic approach to roentgenographically occult squamous cell carcinoma of the lung. Cancer **89**: 2445-2448, 2000.
13. Bechtel JJ, Petty TL, Saccomanno G. Five year survival and later outcome of patients with X-ray occult lung cancer detected by sputum cytology. Lung Cancer **30**: 1-7, 2000.
14. Bechtel JJ, Kelly WR, Petty TL, Patz DS, Saccomanno G. Outcome of 51 patients with roentgenographically occult lung cancer detected by sputum cytologic testing: a community hospital program. Arch Intern Med **154**: 975-980, 1994.
15. NCCN Practice Guidelines in Oncology-v.2. 2008. (Non-Small Cell Lung Cancer) http://www.nccn.org/professionals/physician_gls/PDF/nscl.pdf Accessed: 1 October 2007.
16. Ono R. Indications for bronchoscopic brachytherapy and conditions for irradiation. Ono R. Brachytherapy. Nakayama-Shoten Co., Ltd., Tokyo, 1995: 65-78.
17. Saccomanno G. Diagnostic Pulmonary Cytology. 2nd ed. American College of Clinical Pathologists, Chicago, IL, 1986.
18. Watanabe S, Tanaka D, Nakamura Y, et al. Occult cancer detected by positron emission tomography/computed tomography image fusion. Anticancer Res **25**: 459-461, 2005.
19. Pasic A, Brokx HA, Comans EF, et al. Detection and staging of preinvasive lesions and occult lung cancer in the central airways with 18F-fluorodeoxyglucose positron emission tomography: a pilot study. Clin Cancer Res **11**: 6186-6189, 2005.
20. Endo C, Sagawa M, Sato M, et al. What kind of hilar lung cancer can be a candidate for segmentectomy with curative intent?: Retrospective clinicopathological study of completely resected roentgenographically occult bronchogenic squamous cell carcinoma. Lung Cancer **21**: 93-97, 1998.
21. Sagawa M, Koike T, Sato M, et al. Segmentectomy for roentgenographically occult bronchogenic squamous cell carcinoma. Ann Thorac Surg **71**: 1100-1104, 2001.
22. Saito Y, Nagamoto N, Ota S, et al. Results of surgical treatment for roentgenographically occult bronchogenic squamous cell carcinoma. J Thorac Cardiovasc Surg **104**: 401-407, 1992.
23. Furuse K, Furoka M, Kato H, et al. A prospective phase II study on photodynamic therapy with Photofrin II for centrally located early-stage lung cancer. The Japan Lung Cancer Photodynamic Therapy Study Group. J Clin Oncol **11**: 1852-1857, 1993.
24. Sutedja G, Baris G, van Zandwijk N, Postmus PE. High-dose rate brachytherapy has a curative potential in patients with intraluminal squamous cell lung cancer. Respiration **61**: 167-168, 1994.
25. Edell ES, Cortese DA. Photodynamic therapy in the management of early superficial squamous cell carcinoma as an alternative to surgical resection. Chest **102**: 319-322, 1992.
26. Breuer RH, Pasic A, Smit EF, et al. The natural course of preneoplastic lesions in bronchial epithelium. Clin Cancer Res **11**: 537-543, 2005.
27. Kaminsky DA, Leiman G. False-positive sputum cytology in a case of pulmonary infarction. Respir Care **49**: 186-188, 2004.

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Research Paper

Decreased Biosynthesis of Lung Surfactant Constituent Phosphatidylcholine Due to Inhibition of Choline Transporter by Gefitinib in Lung Alveolar Cells

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Purpose. We investigated whether gefitinib, an anticancer agent, inhibits phosphatidylcholine (PC) biosynthesis and choline uptake by alveolar epithelial type II cells.
Materials and Methods. Uptake of choline and PC biosynthesis were examined *in vitro*, using human alveolar epithelia-derived cell line A549 and rat alveolar type (AT) II cells as models.
Results. Gefitinib reduced the incorporation of [^{3}H]choline into PC in A549 and rat ATII cells. The uptake of [^{3}H]choline by A549 and rat ATII cells was concentration-dependent, and the Km values were 15.0 and 10–100 μM, respectively. The uptake of [^{3}H]choline by A549 and rat ATII cells was weakly Na^+-dependent, and inhibited by hemicholinium-3. RT-PCR revealed expression of choline transporter-like protein (CTL)1 and organic cation transporter (OCT)3 mRNAs in both cells. The choline uptake by A549 and rat ATII cells was strongly inhibited by gefitinib with the IC_{50} value of 6.77 μM and 10.5 μM, respectively.
Conclusions. Our results demonstrate that gefitinib reduces PC biosynthesis via inhibition of cellular choline uptake by A549 and rat ATII cells, which is mainly mediated by CTL1, resulting in abnormality of lung surfactant that can be one of mechanisms of the interstitial lung disease associated with gefitinib.

KEY WORDS: alveolar type II; choline; gefitinib; lung toxicity; phosphatidylcholine.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths worldwide. Non-small cell lung cancer accounts for 80% of lung cancer, and is usually associated with a poor prognosis (1). Gefitinib is an oral selective inhibitor of epidermal growth factor receptor tyrosine kinase (EGFR-TK), and has received approval in the US, Japan and a number of other countries for the treatment of advanced non-small cell lung cancer. The most commonly reported toxicities associated with gefitinib are mild and self-limiting, such as rash, skin toxicities and diarrhea. Although gefitinib was recognized as a relatively safe oral anticancer agent, accumulating evidence has demonstrated that gefitinib has been associated with severe interstitial lung disease (ILD) (2,3). On the other hand, in the Iressa Survival Evaluation in Lung cancer (ISEL) study, events of the ILD were found, but there was no significant difference between the gefitinib and placebo groups (4). Accordingly, the involvement of gefitinib in ILD that occurs during gefitinib treatment is still unclear. The median time to onset of ILD associated with gefitinib was 24 days in Japan. In addition to normal onset of ILD, sudden onset of ILD only 2 or 4 days after exposure to gefitinib has been reported in elderly patients (78 or 75 years old) with lung cancer (2,5). The mechanism(s) of sudden onset of ILD associated with gefitinib treatment is unknown.

Phosphatidylcholine (PC) is synthesized within alveolar type (AT) II epithelia and secreted into the alveolus as the major phospholipid component of lung surfactant, which is essential to establish normal breathing. Interference with surfactant synthesis is recognized as a hallmark of the neonatal respiratory distress syndrome, and is to some degree a component of acute and chronic lung disease (6,7). PC synthesis in all eukaryotic cells occurs predominantly through the cytidine 5′-diphosphocholine (CDP-choline) pathway in which three enzymes, choline kinase, phosphocholine cytidilyltransferase and cholinephosphotransferase, are involved. However, the uptake mechanism of choline by ATII cells, which could be considered the first step of the CDP-choline pathway, has not been elucidated yet.

Choline transport is classified into two major categories based on the degree of affinity for choline and the Na^+-dependence (8). Na^+-dependent high-affinity choline transport is localized at cholinergic nerve terminals, where it contributes to the synthesis of acetylcholine, and is antagonized by low

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ABBREVIATIONS: ATII, alveolar type II; CDP, cytidine 5′-diphosphocholine; CTL, choline transporter-like protein; EGFR-TK, epidermal growth factor receptor tyrosine kinase; HC-3, hemicholinium-3; ILD, interstitial lung disease; MPP^+, 1-methyl-4-phenylpyridinium; NMG, N-methylglucamine chloride; OCT, organic cation transporter; PC, phosphatidylcholine; siRNA, small interfering RNA.

concentrations of a choline analogue, hemicholinium-3 (HC-3). A low-affinity and Na^+-independent system is found widely in various tissues, and this system primarily supplies choline for the synthesis of PC and other phospholipids in the cellular membrane (8). The organic cation transporter (OCT)2 has been proposed as a low-affinity choline transporter across the ventricular membrane of the choroid plexus (9), and two major higher-affinity choline transporters belonging to different protein families have been cloned. The first, CHT1, exhibits all the characteristics of the canonical high-affinity choline transporter associated with cholinergic nerve terminals (10,11). The second, CTL1, belongs to a new family of choline transporter-like (CTL) proteins. CTL1-mediated transport exhibits only a weak sodium dependence (12) and CTL1 is expressed in several neuronal populations, including motorneurones and oligodendrocytes (13).

In order to elucidate the mechanism of ILD associated with gefitinib completely, a step-by-step approach is required. Thus, we focused on the lung surfactant as one of factors. We hypothesized that gefitinib inhibits the uptake of choline by human ATII cells, and that the resultant reduction of PC synthesis causes a lung surfactant disorder, because PC is a major constituent of the surfactant. In the present study, we examined whether gefitinib inhibits PC biosynthesis and choline transport in primary-cultured rat ATII cells and human alveolar epithelia-derived cell line A549, which has the characteristics of type II cells, including production of surfactant protein and lipids (14,15). Furthermore, we characterized the mechanism of choline transport in A549 and primary-cultured rat ATII cells.

MATERIALS AND METHODS

Chemicals

Gefitinib (ZD1839) (Fig. 1) was provided by AstraZeneca (Macclesfield, UK). Stock solutions were prepared in dimethyl sulfoxide and stored at −20°C. [Methyl-^{3}H]choline chloride was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). HC-3, 1-methyl-4-phenylpyridinium (MPP^+), histamine, corticosterone, and dipalmitoyl-D-α-phosphatidylcholine were purchased from Sigma-Aldrich (St. Louis, MO). Choline chloride was obtained from Wako Pure Chemicals (Osaka, Japan). All other chemicals and reagents were commercial products of reagent grade.

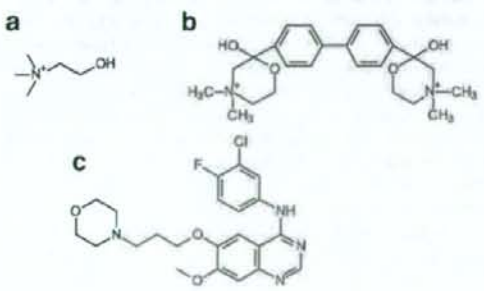

Fig. 1. Chemical structures of choline (**a**), hemicholinium-3 (**b**) and gefitinib (**c**).

Isolation of Rat ATII Cells

ATII cells were isolated from adult Wistar rat lung according to the procedure of Dobbs *et al.* (16). Briefly, lungs of anesthetized rats were cleared of blood by perfusion and removed from the thorax. After lavage, the lungs were instilled with 15 ml of elastase (4.3 U/ml, Wako Pure Chemicals), incubated at 37°C for 20 min and minced with scissors in a mixture of 5 ml of fetal bovine serum (FBS) and 4 ml of 250 μg/ml deoxyribonuclease (Sigma-Aldrich). The cell suspension was sequentially filtered through nylon gauze (150-, 41- and 15-μm mesh size) and centrifuged at 130 g for 10 min. The resultant cell pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) and panned on an IgG-coated bacteriological plastic dish at 37°C for 1 h. The unattached cells were centrifuged and suspended in DMEM supplemented with 10% FBS, 139 μg/ml streptomycin, 70 μg/ml penicillin and 10 μg/ml gentamycin. The type II cells were more than 80% of total cells by evaluation with the modified Papanicolaou stain, throughout the experiment (17).

Cell Culture

Human lung adenocarcinoma A549 cells (ATCC-CCL185) were routinely grown at 37°C in F12-K medium (Dainippon Pharmaceutical, Osaka, Japan) supplemented with 10% FBS in a 5% CO_2 humidified incubator.

Uptake of Choline

A549 cells (5.0×10^4 cells/well) and rat ATII cells (4.0×10^5 cells/well) were seeded into normal and fibronectin coated 24-well plates (BD Biosciences, Bedford, MA) and grown for 72 and 20 h, respectively. Uptake was initiated by adding 0.25 ml of transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM D-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM Hepes, adjusted to pH 7.4) containing radiolabeled and unlabeled substrates after the cells had been washed twice and preincubated with transport buffer alone for 15 min. To investigate the effect of preincubation with gefitinib, a preincubation time of 60 min with transport buffer alone or containing gefitinib was used. The solvent concentration in the final transport solution did not exceed 0.5% (v/v). For the evaluation of membrane potential dependence, uptake of [^{3}H]choline was measured in the presence of increasing and decreasing concentrations of KCl (6 to130 mM) and NaCl (125 to 1 mM), respectively, at pH 7.4 for designated times. To measure the effect of Na^+, sodium chloride in the incubation buffer was replaced with N-methylglucamine (NMG) and lithium chloride. In case of the replacement from Na^+ to NMG^+, chloride concentration was adjusted by hydrochloric acid to afford same chloride concentration as that in the normal transport buffer. The pH of the transport buffer was varied by adjusting the concentrations of Mes, Hepes and Tris to obtain the desired pH. The uptake was terminated at designated times by adding 0.5 ml of ice-cold transport buffer after removal of the incubation buffer. The cells were washed twice with 0.5 ml of ice-cold transport buffer, solubilized in 1 N NaOH, and kept for 1 h at 37°C. Aliquots were transferred to scintillation vials after neutralization with HCl. The radioactivity associated with the cells and incubation

buffer was measured in a liquid scintillation counter (TRI-CARB 2500TR, Perkin Elmer, Boston, MA or LSC-5100, Aloka, Tokyo, Japan) after adding 2 ml of scintillation fluid. The remaining 25 μl of cell lysate was used to determine the protein concentration by the method of Lowry or Bradford with bovine serum albumin as a standard.

Biosynthesis of Phosphatidylcholine

The incorporation of [^{3}H]choline into PC was carried out as described previously (15). A549 cells (2.0×10^5 cells/well) and rat ATII cells (7.0×10^6 cells/well) were seeded into normal and fibronectin-coated 6-well plates (BD Biosciences) and grown for 72 and 20 h, respectively. Cells in each well were incubated with 3–5 μCi/ml [methyl-^{3}H]choline chloride in transport buffer after they had been washed twice and preincubated with transport buffer alone or containing gefitinib at 37°C for 60 min. The solvent concentration in the final transport solution did not exceed 0.5% (v/v). They were washed three times with ice-cold transport buffer, scraped off the plate into CH_3OH/H_2O (2/1, v/v), and briefly sonicated for the determination of apparent uptake of [^{3}H]choline. Cellular lipids were extracted according to Bligh and Dyer (18). The chloroform phase was dried under nitrogen, and PC was dissolved in the development solvent of $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (100/50/14/7) and separated on silica gel chromatographic plates (Kieselgel 60 F_{254}, Merck) with the same solvent used for the determination of the incorporation of [^{3}H]choline into PC. The Rf values of PC were determined from the locations of spots of authentic PC visualized with iodine vapor.

RNA Extraction and RT-PCR

Cultured A549, primary-cultured rat ATII cells and Calu-3 (a human bronchial epithelial cell line) cells, rat liver, and rat kidney were washed with sterile PBS and total RNA was extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA from human tissues of lung (64092-1), trachea (636541), liver (64099-1) and kidney (64097-1) were purchased from Clontech (Palo Alto, CA). After deoxyribonuclease treatment, total RNA of A549, Calu-3, and tissues was reverse-transcribed using an RNA PCR kit (Takara Shuzo Co., Ltd., Kyoto, Japan) according to the manufacturer's instructions. For conversion of total RNA to cDNA, a reaction mixture (10 μl) was prepared containing 2.5 U of reverse transcriptase avian myeloblastosis virus (Takara Shuzo Co., Ltd., Kyoto, Japan), 1 x RT-PCR buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl], 5 mM $MgCl_2$, 1 mM deoxynucleotide triphosphates, 25 pmol of random 9-mer primers, 10 U of ribonuclease inhibitor, and 1 μg of total RNA. The reaction was carried out at 55°C for 60 min. RT (reverse transcription) was terminated by heating the reaction mixture to 99°C for 5 min, followed by rapid chilling at 4°C. RT reaction mixtures, including cDNA products, were stored at −20°C until used. A single cDNA produced from total RNA was amplified by PCR with primers for OCT1, OCT2, OCT3, CTL1, CTL2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rOct1, rOct2, rOct3, rCTL1 and rGAPDH. Specific oligonucleotide primer pairs for human OCT1, OCT2, OCT3, GAPDH, rOct1, rOct2, rOct3, rCTL1, and rGAPDH were synthesized as listed in Table I. Specific oligonucleotide primer pairs for CTL1 (QT00075838) and CTL2 (QT00081214) were purchased from Qiagen (Basel, Switzerland). The expected product sizes from OCT1, OCT2, OCT3, CTL1, CTL2, GAPDH, rOct1, rOct2, rOct3, rCTL1 and rGAPDH specific primers were 197, 599, 455, 138, 95, 676, 481, 246, 266, 678, and 985 bp, respectively. For PCR amplification of cDNA, the PCR was allowed to proceed for 25 to 35 cycles in 25-μl aliquots of reaction mixture containing 1×polymerase reaction buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$), 0.2 mM deoxynucleotide triphosphates, 0.625 U of EX *Taq* DNA polymerase (Applied Biosystems, Foster City, CA), 50 and 20 ng of human and rat cDNA, respectively, and 5 pmol of the specific primers. The following temperature program was used: initial denaturation (94°C) for 2 min, 94°C for 30 s, 52°C/human, 58°C/rat for 30 s, 72°C for 30 s for 25–35 cycles, and a final extension at 72°C for 10 min. A two-step temperature program was applied for the amplification of rGAPDH: initial denaturation (94°C) for 2 min, 94°C for 30 s, 68°C for 2 min for 25 cycles, and a final extension at 68°C for 7 min. Aliquots (10 μl) of amplified cDNA products were separated by electrophoresis using 1–1.5% agarose gels. Gels were stained with ethidium bromide and visualized under UV light.

CTL1 Small Interfering RNA (siRNA) and siRNA Transfection

Three kinds of siRNA (SI00342468, SI00342475 and SI00342482) targeted to different regions of hCTL1 gene and negative control siRNA AlexaFluor 488 used as negative control (1022563) were purchased from Qiagen (Basal, Switzerland). A549 cells (5.0×10^4 cells/well) were seeded

Table I. Specific Oligonucleotide Primer Pairs

Transporter	Forward (5'–3')	Reverse(5'–3')	Ref
OCT1	CGATCATGTACCAGATGGCC	TCTTCATCCCTCCAACACGAC	(36)
OCT2	GATTTCTTCTACTCTGCCCTGGTT	GGATTTCTACTTTTGGTCTTGCTG	(37)
OCT3	GACAAGAGAAGCCCCCAACCTGAT	CACTAAAGGAGAGCCAAAAATGTC	(37)
GAPDH	ACTGGCGTCTTCACCACCAT	TCCACCACCCTGTTGCTGTA	(38)
rOct1	GATCTTTATCCCGCATGAGC	CTTCTGGGAATCCTCCAAGT	
rOct2	CATCGAGGATGCCGAGAA	ACAGACCGTGCAAGCTAC	
rOct3	TCAGAGTTGTACCCAACGACATT	TCTGCCACACTGATGCAACT	
rCTL1	GGCAGTGCTGTTCAGAATGA	ACAGGAAGCAATGAGCGACT	(39)
rGAPDH	TGAAGGTCGGTGTGAACGGATTTGGC	CATGTAGGCCATGAGGTCCACCAC	