in this group. There may be some differences in population-based pharmacogenomics. Grade 3/4 neuropathy, conversely, was more frequent in the SWOG 0003 trial due to differences in the cumulative paclitaxel dose because of the higher absolute dose and higher median numbers of treatment courses. The response rates were exactly the same, but 1 year survival was better in the FACS trial. These results suggest that future joint Japan–USA clinical trials should consider possible pharmacogenomic differences in drug disposition between Japanese and American populations.[5]

Molecular target-based drugs in advanced recurrent NSCLC

Numerous molecular target-based drugs have been introduced for the treatment of NSCLC, but can they replace current therapy? Can they be used as an adjuvant to current therapy? Can they be combined with other chemotherapeutic agents, radiotherapy and/or surgery?

We hypothesize that incorporation of novel molecular target-based therapies into current treatment paradigms will improve outcomes. However, carefully designed clinical trials and translational science will be required to identify subsets of patients who will benefit.

If we are to use them, we must first answer the following critical questions. Is the target required for a response? Whether or not we know a real and correct molecular target is still questionable. Is the presence of the target sufficient for a response, and can we measure the target in a biologically relevant and/or technologically valid way? Does the agent inhibit the proposed target at the dose and schedule used? Is the target a critical driving force for cell growth in the tumor type in question? The answers to these questions are crucial to treatment with molecular target-based drugs.

Various molecular target-based drugs for advanced NSCLC have been evaluated in randomized controlled trials, but the majority, including a matrix metalloprotease inhibitor, a protein kinase C inhibitor and trastsuzumab, have yielded negative results.[6–8] Gefitinib is an orally available selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor that exhibits antitumor activity in patients with previously treated advanced NSCLC.

Clinical trial of gefitinib and erlotinib

Four open-label multicenter phase I studies have identified diarrhea, skin rash/acne and nausea as common adverse events.[9,10] Two large-scale, multicenter randomized controlled phase II trials, IDEAL 1 and 2, have demonstrated clinically significant antitumor activity of gefitinib monotherapy, and erlotinib has also shown promising antitumor activity.[11] Neither drug showed any additive and/or synergistic effect when combined with platinum-based chemotherapy as a first-line treatment for NSCLC.[12,13]

On December 17, 2004, AstraZeneca announced the preliminary results of their Iressa Survival Evaluation in Lung Cancer (ISEL) study. The study had accrued 1692 patients with advanced recurrent/refractory NSCLC. Unfortunately, Iressa failed to significantly prolong survival compared with a placebo (HR = 0.89, $P = 0.087$) in the overall patient population or among patients with adenocarcinoma (HR = 0.83, $P = 0.089$), although a tendency toward a survival benefit was observed in the gefitinib group.[14] The less than 10% response rate did not result in an overall prolongation of survival. A retrospective analysis of patients treated with gefitinib in clinical practice showed that tumor response predictors included 'adenocarcinoma', 'no history of smoking', 'women', and 'Japanese'. Survival in the gefitinib group in the ISEL study was significantly higher for non-smokers ($P < 0.01$) and Asians ($P < 0.01$) than in the placebo group. The survival curves of the two treatment groups were the same for non-Asians. The data obtained from the ISEL study were not surprising, although most observers had expected positive overall results.

The results of similar randomized trials of erlotinib (BR21) were presented at the American Society Clinical Oncology (ASCO) meeting in 2004. Erlotinib significantly prolonged survival in patients with advanced, previously treated refractory/recurrent NSCLC.[15] The two studies referred to above differed in several respects. Sample size was larger in the ISEL study than in the BR21 study, and 10% of the patients in the latter study had a performance status (PS) of 3, whereas only PS-2 patients were accrued by the ISEL study. The follow-up period of the ISEL study was also relatively short (4 months). The overall percentage of patients with adenocarcinoma and the percentage of non-smokers was 50% and 20%, respectively, in both studies. Data stratification into Asians and non-Asians was only performed in the ISEL study. The stratified survival data for Asians in the BR21, submitted to the US FDA, showed a tendency that was similar to the stratified data in the ISEL study. The survival of non-smokers in the erlotinib group in the BR21 study was extremely good and contributed to the improvement in overall survival in the erlotinib group. How can we explain the discrepancy of the result from the ISEL and BR21 studies? Part of the explanation is that the dose of gefitinib in the ISEL study was low, while the BR21 study used nearly the maximum tolerated dose. Another hypothesis is that patient populations in the ISEL study were inappropriately selected, for example, subjects with poor prognostic factors. The shapes of the survival curves for the Intact 1 and 2, TALENT and TRIBUTE studies and for the non-Asians in the ISEL study suggest that EGFR-TKI does not prolong the survival of non-Asian patients with NSCLC, with or without prior chemotherapy.[12,13,16,17] The stratified survival data of the Asians in the Intact 1 and 2, TALENT and TRIBUTE studies should be analyzed.

In the SWOG 0023 trial, patients with stage III NSCLC received chemoradiation therapy then three cycles of a single agent, docetaxel, followed by either a placebo or gefitinib as maintenance. This trial was projected to have 80% of the patients receiving either placebo or gefitinib with a drop off of 20% during this part of the therapy. The drop off rate before randomization was a bit larger than the expected rate because of progressive disease or death. Investigators asked the Data Safety Monitoring Committee to look at the data to see if they should actually continue the trial because the results of the ISEL study were negative. This early unplanned analysis showed there was no difference in time to progression in either arm and the P-value for difference was 0.54. Similarly, there was no statistically significant difference in

survival and the *P*-value was 0.09, favoring the placebo group. It was surprising and disappointing that the gefitinib-treated patients were actually experiencing worse survival than the placebo patients. This trial had the power to show a 0.33% advantage for gefitinib and the data were sufficient to state that the likelihood of showing a 33% survival improvement was 0.0015.[18] These data suggested that there is no rationale for using gefitinib in locally advanced NSCLC in the adjuvant setting.

Molecular marker predicting clinical outcome of EGFR-TKI

The activities of epidermal growth factor receptor (EGFR) inhibitors, gefitinib and erlotinib in lung cancer and the correlation of responses to somatic mutations are the focus of translational research performed in 2004 and 2005. This answers the major question; which patients respond and why? We have demonstrated that PC-9 cells with a 15 bp deletion in exon 19 of the EGFR gene are extremely sensitive to EGFR-TKI.[19] In April and May 2004, Paez and Lynch reported that activating mutations in EGFR are present in a subset of NSCLC tumors and that the tumors are highly sensitive to gefitinib and erlotinib.[20,21] EGFR expression levels are not a predictor of response and EGFR amplification may have an impact, but EGFR-TK mutations seem to be better predictors of responsiveness to gefitinib and erlotinib.[22–24] Mutant EGFR are more sensitive to ligand stimulation and are dramatically more sensitive to EGFR-TKIs.[19–21] The incidence of EGFR mutations is reportedly higher in Asians, including Japanese,[25,26] and Mitsudomi has reported cumulative percentages of those with EGFR mutation-positive status in 1104 patients with NSCLC to be 34% among Asians and 8% among non-Asians.[27] Eighty percent of the patients who responded to EGFR-TKI carried an EGFR mutation (non-Asians, 79% [30/35]; Japanese, 81%: [39/48]). Among non-responders, 0% of non-Asians and 21% of Japanese patients carried an EGFR mutation. These data suggest that the presence of an EGFR mutation is a strong predictor of a favorable response to EGFR-TKI. Mutations have been reported to be significantly more frequent in women, in patients with adenocarcinoma, and in never smokers, and these findings are consistent with the clinical predictors of tumor response in patients treated with EGFR-TKI. Mitsudomi recently reported that the del 746–750 mutation might be superior to the L858R mutation for predicting the gefitinib response and those patients with EGFR mutations survived longer after the initiation of gefitinib treatment than those without mutations.

Recently it has been demonstrated that an additional mutation at codon 790 induced resistance to originally sensitive mutant cells.[28,29]

A variety of results were presented at the ASCO 2005 meeting in Orland with regards to molecular analysis of the EGFR gene and protein expression in patients accrued to pivotal studies of EGFR-TKIs.[30] Lynch reported the results of an analytical study using resected specimens and biopsy samples obtained during IDEAL and INTACT studies of gefitinib.[31] Patients with either an EGFR mutation or amplification represented distinct populations. Among cases with mutations, large numbers were female, non-smokers, had adenocarcinoma or bronchioloalveolar carcinoma, were Eastern-Asian and often showed dramatic response rates to gefitinib. Because the number of cases for this analysis was not sufficient, it was impossible to draw any conclusions about the impact of mutation and amplification on survival.

Tsao tried to identify certain relations among the response rate and survival and molecular biological features such as the mutation, protein expression and gene copy numbers in the BR21 study conducted by NCI-Canada clinical trial group, which demonstrated that erlotinib does significantly prolong survival as compared with a placebo. Response rates were higher in patients with EGFR mutations, immunohistochemistry (IHC)-positive tumors and high gene copy numbers, but a statistically significant difference was observed for copy numbers only. Survival benefit was greater in patients who were IHC positive and had high gene copy numbers. However, mutation positive patients did not benefit more than mutation negative patients. From these data, Tsao concluded that mutation analysis is not required for the selection of patients who will receive erlotinib.[32]

There are some controversial data on the relationship between biomarkers and clinical outcome.[33–37] One of the reasons for discrepant data is the validity of techniques including the quality of the samples analyzed. Giaccone conducted a cross validation analysis of EGFR mutations in samples obtained from the Free University (the Netherlands) and the Dana Faber Cancer Institute.[38] The results were discrepant in some samples because of poor quality. Another reason is patient selection because it was impossible to obtain samples from all patients with advanced lung cancer. In the retrospective studies reported to date, only a small proportion of patients have had tumor samples evaluable for each biomarker, making patient selection problematic and prone to the introduction of selection bias. It is therefore extremely important that samples be obtained from all patients in studies evaluating the relationships between clinical outcome and biomarkers such as EGFR expression, amplification and mutation. Of course, the techniques for evaluable biomarkers should be valid. In this regard, the report of Takano is most reliable because they analyzed all the samples from all patients using three techniques: IHC, gene copy number and mutation. There were no problems with patient selection. Because they used surgically resected specimens they were able to obtain adequate specimen amounts. It could be concluded that if the analyses were conducted accurately, EGFR mutational status would be the major predictor of outcome and increased EGFR copy number associated with gefitinib sensitivity would significantly depend on the presence of EGFR mutations.[39] Technical innovations are essential for the reproducible and reliable analysis of samples from advanced disease patients because only small amounts of the specimen could be obtained from inoperable lung cancer patients.

EGFR-TKI seems to be a very promising drug for the treatment of East-Asian patients with NSCLC with and without a history of prior chemotherapy. The response rate has ranged from 20% to 33% clinically, and it was 30% in a prospective phase II trial on 100 previously untreated NSCLC patients. The median survival time of the Japanese population in the IDEAL 1 trial was 13.8 months.[11] To date, no survival

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data from a phase III study of gefitinib and erlotinib in East Asia are available because no phase III study has been conducted. However, a randomized controlled trial comparing gefitinib and docetaxel as a second-line treatment is in progress in Japan. The trial has a non-inferiority design and a definitive conclusion will be difficult to obtain. An erlotinib phase II evaluation has just finished the accrual of patients in Japan, but government approval will require more time.

The frequency of EGFR mutations and response rate are higher in East-Asian populations than in Western countries. A global randomized controlled trial is scheduled for comparison of first-line standard platinum-based chemotherapy versus gefitinib in East Asians, non-smokers versus light smokers, and patients with adenocarcinoma.

Bevacizumab

Vascular endothelial growth factor (VEGF) was originally described as vascular permeability factor. VEGF is involved in the regulation of new vessel growth, promotion of the survival of immature vasculature and binding to one of two receptors such as FLT-1 or KDR.[40]

Bevacizumab is a monoclonal antibody against VEGF. It is 93% human, it recognizes all isoforms of VEGF-A and has a prolonged half life which makes it very convenient to administer on an every 2- or 3-week basis.

The preliminary randomized phase II trial of ECOG using 7.5 mg/kg or 15 mg/kg of bevacizumab every 3 weeks did meet its primary objective of improvement in time to progression on the high dose arm; 7.4 versus 4.2 months. Also, response and survival were numerically better. Problems with hemoptysis or pulmonary hemorrhage occurred in six patients (four squamous cell and two adeno), four of which actually proved to be fatal.[41] Based on these experiences, the ECOG 4599 trial was designed. The primary objective was to compare survival and secondary objectives were to look at the response rate, time to progression and toxicity.

Eligibility criteria included non-squamous cell carcinoma, no history of major hemoptysis and of neither thrombotic nor hemorrhagic disorders, and no central nervous system metastasis. Patients received standard dose carboplatin and paclitaxel with or without high dose bevacizumab 15 mg/kg every 3 weeks. The sample size was calculated to be over 842, providing the investigators with 80% power to detect a 25% improvement in median survival time from the usual 8–10 months. ECOG had two planned interim analyses at 286 and 455 deaths. The study was closed after the second interim analysis. Response rate was significantly higher in the bevacizumab arm (27%) versus the control arm (10%). Progression free survival also favored the bevacizmab arm. Overall survival was highly statistically significant; 12.5 months in the bevacizumab arm and 10.2 months in the control arm. The hazard ratio was 0.77.[42] Hemorrhage was more common in the bevacizumab arm with a 45% incidence compared to less than 1% in the control arm. There were eight treatment-related deaths in the bevacizumab arm and two in the control arm. These data lead to the conclusion that bevacizumab improves survival compared to platinum and paclitaxel in patients with non-squamous NSCLC, although a small increase in severe bleeding can be expected. ECOG considers paclitaxel, carboplatin with bevacizumab to be a standard for the treatment of this NSCLC subgroup. The study group suggested some future plans for combining bevacizumab with chemotherapy, radiotherapy and other targeted agents in neoadjuvant or adjuvant settings. In Europe, a clinical trial of bevacizumab combined with cisplatin + gemcitabine is ongoing. The critical question is whether or not they can obtain reproducible positive data even if the chemotherapy regimen is changed from pacltiaxel + carboplatin to cisplatin + gemcitabine. In Japan, a combination phase I/II study of bevacizumab with 5FU + LV or FOLFOX recently completed the accrual of patients. Combination treatment using bevacizumab with paclitaxel + carboplatin is scheduled. How to manage severe bleeding, even in selected populations, and the extremely high cost of bevacizumab will be major issues.

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Eg5 expression is closely correlated with the response of advanced non-small cell lung cancer to antimitotic agents combined with platinum chemotherapy

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Summary
Background: Eg5 is a microtubule motor protein that functions in bipolar spindle assembly. We investigated the relationship between Eg5 expression and the response to chemotherapy of patients with advanced non-small cell lung cancer (NSCLC).
Patients and methods: Eg5 expression was investigated immunohistochemically in 122 formalin-fixed tumor samples from untreated stage IIIB or IV NSCLC patients. We also investigated cyclin B1 expression, which is involved in the G2/M transition. All patients received antimitotic agents combined with platinum chemotherapy. The response to chemotherapy was compared in relation to Eg5 and cyclin B1 expression and in relation to clinicopathological factors.
Results: The response rate to chemotherapy of patients with Eg5-positive tumors was 37%, as opposed to 10% for patients with Eg5-negative tumors, and Eg5 expression was significantly associated with the response to chemotherapy ($P=0.002$). The response rate of patients with cyclin B1-positive tumors (53%) was higher than that of patients with cyclin B1-negative tumors (23%) ($P=0.009$), and Eg5 expression was significantly correlated with cyclin B1 expression ($P=0.005$). A multivariate analysis confirmed Eg5 status to be an independent variable related to response to chemotherapy ($P=0.008$).
Conclusions: Eg5 expression can predict a response to antimitotic agents combined with platinum chemotherapy among patients with advanced NSCLC.

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1. Introduction

Lung cancer is a major cause of death from cancer worldwide, and non-small cell lung cancer (NSCLC) accounts for ~85% of all cases of lung cancer. More than half of patients with NSCLC have advanced stage IIIB or IV disease at presentation, and patients with advanced NSCLC are candidates for systemic chemotherapy [1]. Meta-analyses have demonstrated that cisplatin-based chemotherapy for metastatic NSCLC statistically improves patient survival, compared with supportive care alone [2]. However, the response rate to chemotherapy has been poor, and very few patients survive for 5 years [3]. During the 1990s, five new drugs became available for the treatment of metastatic NSCLC: paclitaxel, docetaxel, vinorelbine, gemcitabine, and irinotecan. Each of these drugs has since been evaluated in combination regimens with cisplatin or carboplatin and has produced responses in 20–30% of patients [1]. Unfortunately, despite the increasing number of active chemotherapeutic agents, none of these chemotherapeutic regimens has offered a significant advantage over the others in the treatment of advanced NSCLC in randomized studies [4,5], and advanced NSCLC patients still have a median survival time of <1 year. Several reasons have been offered to explain the response to chemotherapy, such as the presence of drug-resistant tumor cells [6] and the redistribution of tumor cells within the cell cycle after chemotherapy. However, the molecular basis of the response to chemotherapy remains to be explored.

A network of microtrabecular filaments forms the cytoplasmic matrix, giving rise to the concept of the cytoskeleton, which comprises microtubules, actin, and intermediate filaments. Microtubules display a remarkable versatility of function and are involved in multiple biologic phenomena, including mitosis, cell shape determination, cell locomotion, and the movement of intracellular organelles [7]. Microtubule-polymerizing agents, including paclitaxel and docetaxel, and microtubule-depolymerizing agents, including vinorelbine, target preliminary tubulin and can induce disrupting kinetic stabilization of microtubules' polymerization–depolymerization, thus blocking the cell cycle in the mitotic phase [8].

Microtubule motors bind to and move unidirectionally on microtubules, and they have been proposed to generate the force required for spindle assembly and maintenance, attachment of the chromosomes to the spindle, and movement of chromosomes toward opposite poles. The microtubule motor proteins, which are members of the kinesin, dynein, or myosin families, can account for many of the movements of the spindle and chromosomes in dividing cells. Kinesin motors have been shown to be necessary to establish spindle bipolarity, position chromosomes on the metaphase plate, and maintain forces in the spindle [9]. Evidence that kinesin motors facilitate microtubule depolymerization also exists, raising the possibility that the motors modulate microtubule dynamics during mitosis. Eg5, which is a part of the kinesin-5 molecule (a member of the kinesin superfamily), is a microtubule motor protein. Eg5 accounts for many of the movements of the spindle and chromosomes in dividing cells and localizes to the spindle in mitotically dividing cells. It has been implicated in spindle function by both its cellular localization and the effects of mutations. Eg5 function in centrosome or spindle pole body separation is necessary for bipolar spindle assembly [10]. The latest antimitotic agent, named monastrol, is an inhibitor of mitotic kinesin Eg5 [11,12]. Monastrol arrests mitosis by reversibly inhibiting mitotic kinesin Eg5 and impairing bipolar mitotic spindle formation. Prolonged mitotic arrest leads to apoptosis in tumor cells and to senescence or apoptosis in primary cells, and the inhibition of mitotic kinesin Eg5 results in the formation of monoaster spindles leading to mitotic arrest [13].

Cyclin and cyclin-dependent kinase complexes play an important role in the control of the cell cycle [14], and the cyclin B1/cdc2 complex has a role as a maturation/mitosis-promoting factor in the G2–M phase transition during the cell cycle [15]. Thus, lack of regulation of cyclin B1 expression may be involved in uncontrolled cell growth and malignant transformation. Overexpression of cyclin B1 has been reported in various malignant tumors and has been shown to predict a poor outcome in NSCLC, esophageal carcinoma, and head and neck cancer [16–18].

In this retrospective study, we investigated the level of expression of Eg5, in addition to cyclin B1—a molecule involved in the G2/M transition, in clinical samples from patients with advanced NSCLC who were subsequently treated with antimitotic agents and investigated whether its expression predicts response to chemotherapy and outcome.

2. Materials and methods

2.1. Subjects

A total of 122 stage IIIB or IV NSCLC patients received platinum-based combination chemotherapy combined with docetaxel, paclitaxel or vinorelbine at the National Cancer Center Hospital East between August 1997 and July 2004 because of PS 0 or 1 on the Eastern Cooperative Oncology Group scale. Adequate tumor biopsy specimens were obtained from all 122 of these patients before chemotherapy and were analyzed in this study. All of the tumor specimens were obtained before chemotherapy, by bronchoscopy in 83 patients, by percutaneous needle biopsy in 31 patients, by thoracotomy in five patients, and by mediastinoscopy in three patients. The histological classification was based on the third edition of the WHO classification. Clinical staging was based on an initial evaluation consisting of a clinical assessment, chest radiography, computed tomography of the chest and abdomen, computed tomography or magnetic resonance imaging of the brain, and bone scintigraphy. The current international staging system was used for clinical disease staging [19]. The clinicopathological characteristics of all the patients are listed in Table 1. Their median age at diagnosis was 62 years (range, 42–78 years). Seven of the 43 stage IIIB patients were women, and 32 of the 79 stage IV patients were women. All of the patients were treated with antimitotic agents combined with platinum chemotherapeutic regimens in what were considered standard regimens for patients with metastatic NSCLC [20]. Nine of the 43 stage IIIB patients received thoracic radiotherapy after the completion of chemotherapy; three of these patients were women. The median follow-up time of the 122 patients was 26 months (range, 18–54 months).

Table 1 Characteristics of 122 patients with advanced NSCLC

Characteristics	No. of patients
Total no. of patients	122
Gender	
Male	83
Female	39
Age (years)	
Median	62
Range	42–78
Histology	
Adenocarcinoma	80
Squamous cell carcinoma	28
Large cell carcinoma	13
Others	1
Stage	
IIIB	43
IV	79
Performance status	
0	32
1	90
Chemotherapeutic regimen	
Cisplatin + vinorelbine	76
Cisplatin + docetaxel	20
Carboplatin + paclitaxel	26
Smoking history	
Positive	91
Negative	31

NSCLC: non-small cell lung cancer.

After obtaining informed consent in accordance with our institution's guidelines, all of the patients underwent a tumor biopsy and chemotherapy.

2.2. Chemotherapy

The platinum-based regimens were vinorelbine (25 mg/m^2) on days 1 and 8 plus cisplatin (80 mg/m^2) on day 1 of a 21-day cycle (76 patients), docetaxel (60 mg/m^2) on day 1 plus cisplatin (80 mg/m^2) on day 1 of a 21-day cycle (20 patients), and paclitaxel (200 mg/m^2 administered over 3 h) on day 1 plus carboplatin (dosed with an area under the curve of 6) on day 1 of a 21-day cycle (26 patients). All of the patients received two or more courses of chemotherapy before the appearance of progressive disease. We used the RECIST guidelines [21] to evaluate the response to chemotherapy. A complete response was defined as the disappearance of all clinically detectable lesions for at least 4 weeks. A partial response required a minimum of a 30% reduction in the greatest diameter of all of the measurable lesions for a minimum of 4 weeks. Progressive disease was defined as the appearance of new lesions or an increase in disease of >20% measured in the same manner as for partial response. All other results were classified as "no change". The response rate was defined as the total of the complete response cases and partial response cases expressed as a percentage of all cases. PFS (progression-free survival) was measured from the start of chemotherapy until the documentation of progressive disease or death.

2.3. Immunohistochemistry

Immunostaining was performed on 4-μm formalin-fixed, paraffin-embedded tissue sections. The slides were deparaffinized in xylene and dehydrated in a graded ethanol series. For antigen retrieval, the slides for cyclin B1 were immersed in 10 mM citric buffer solution (pH 6.0) and the slides for Eg5 were immersed in 1 mM EDTA retrieval fluid (pH 8.0). All of the slides were heated to 95 °C by exposure to microwave irradiation for 20 min. The slides were then cooled for 1 h at room temperature and washed in water and PBS. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 15 min. Non-specific binding was blocked by preincubation with 2% BSA plus 0.1% NaN_3 for 30 min; after draining off the blocking serum, the slides were incubated overnight at 4 °C with anti-Eg5 monoclonal antibody (Clone, 20; Dilution, 1:50; BD Biosciences, NJ, USA) or with anti-cyclin B1 monoclonal antibody (Clone, 7A9; Dilution, 1:20; Novocastra Laboratories, Newcastle upon Tyne, UK). The slides were then washed three times in PBS and incubated with a labeled polymer Envision+ (DAKO, Glostup, Denmark) for 60 min. The chromogen used was 2% 3,3′-diaminobenzidine in 50 mM Tris buffer (pH 7.6) containing 0.3% hydrogen. Slides were counterstained with hematoxylin [22,23]. Normal human lung tissue was used as a positive control.

Eg5 staining was considered positive if the cytoplasm of >10% of the tumor cells stained positive. Cyclin B1 staining was considered positive if the nuclei of >10% of the tumor cells stained positive, because the cyclin B1/cdc2 complex translocates from the cytoplasm into the nucleus during the G2/M transition [24–26]. Thus, the criteria for cyclin B1 positivity used in the present report differed from those used in other reports on non-small cell lung cancer, esophageal carcinoma and head and neck cancer. All of the slides were examined and scored independently by two observers (T.S. and G.I.) who had no knowledge of the patients' clinical data. When the antibody evaluations differed between the observers, the observers discussed the results, with or without re-evaluating the slides, until an agreement was reached.

2.4. Statistical analysis

The correlations between immunohistochemical expression and the clinical variables and response to chemotherapy were evaluated by the χ^2-test or Fisher exact test, as appropriate. PFS was used as a clinical marker for duration of response to chemotherapy. Overall survival was measured from the start of chemotherapy to the date of death from any cause or the date the patient was last known to be alive. Survival curves were estimated using the Kaplan–Meier method, and any differences in PFS and survival between the subgroups were compared by using the log-rank test. The Cox proportional hazards model was used for a multivariate analysis. A multivariate analysis examining the correlation between variables and response to chemotherapy was performed by using logistic regression. P values <0.05 were

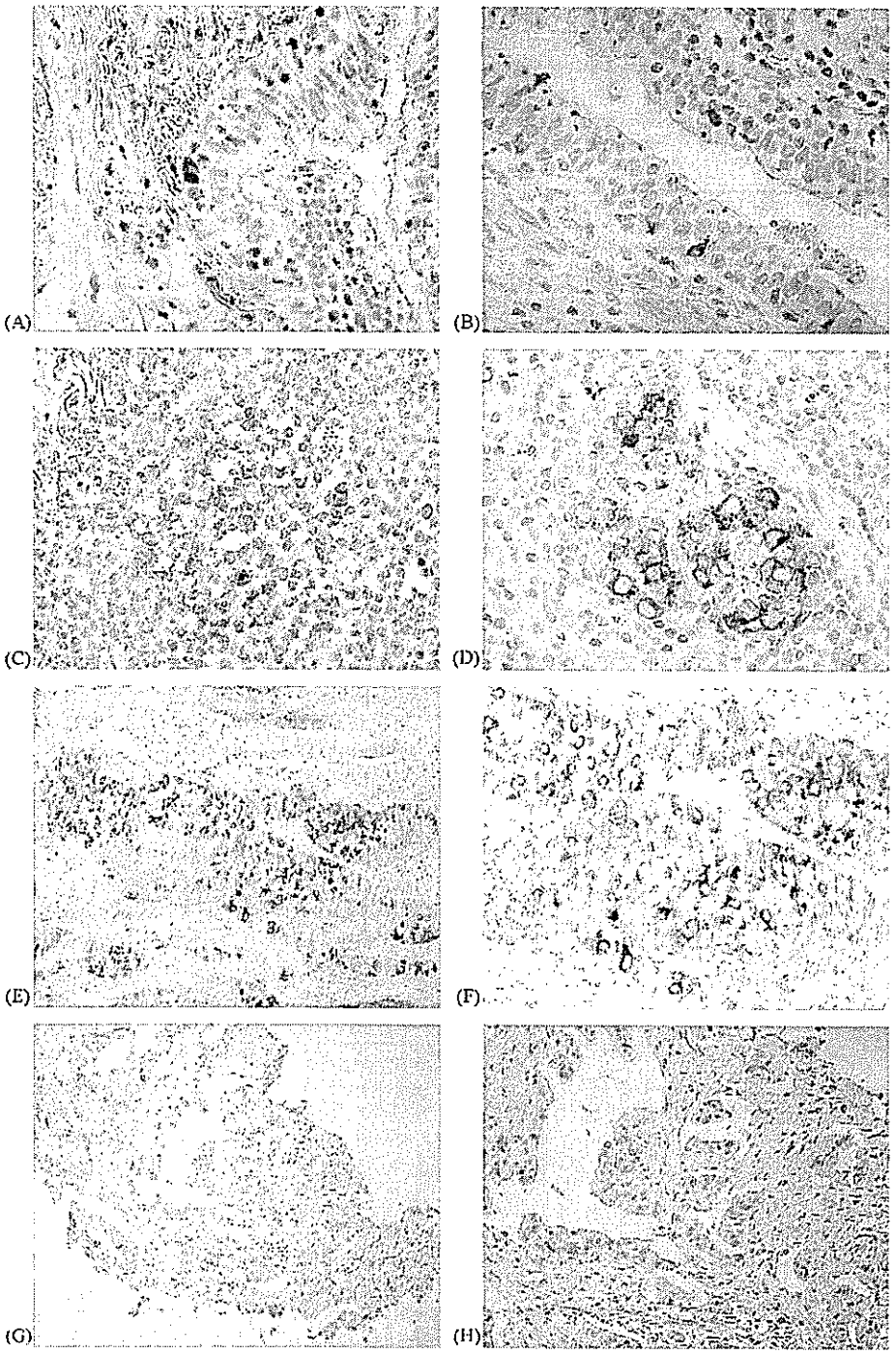

Fig. 1 (A–D) Immunohistochemical staining of Eg5 in normal lung tissue (A), Eg5 is present in part of the basal layer of the bronchial epithelium in this frozen section of normal lung tissue (400×). (B) Eg5 is also present in parts of the basal layer of the bronchial epithelium in this formalin-fixed, paraffin-embedded section of normal lung tissue (400×). (C) Eg5 expression is visible in germinal center lymphocytes giving rise to follicular hyperplasia in this frozen section of normal lung tissue (400×). (D) Eg5 expression is also visible in germinal center lymphocytes giving rise to follicular hyperplasia in this formalin-fixed, paraffin-embedded section of normal lung tissue (400×). (E–H) Immunohistochemical staining of Eg5 in NSCLC (E), low magnification (100×) of squamous cell carcinoma of the lung showing Eg5 immunoreactivity (F), high magnification (200×) of squamous cell carcinoma of the lung showing Eg5 immunoreactivity (G), Eg5 staining was considered to be negative in this adenocarcinoma of the lung: the cytoplasm of <10% of the tumor cells were stained (low magnification; 100×). (H) Eg5 staining was considered to be negative in this adenocarcinoma of the lung: the cytoplasm of <10% of the tumor cells were stained (high magnification; 200×).

considered significant. Two-sided statistical tests were used in all of the analyses. Statistical analysis software (StatView-J Ver. 5.0, Windows) was used for the analyses.

3. Results

3.1. Expression of Eg5 in normal lung tissue

To investigate the validation of immunostaining in the present experiment, we first evaluated Eg5 immunostaining in frozen sections and paraffin-embedded tissue sections of surgical specimens and confirmed that the staining intensity and specificity in the paraffin-embedded tissue sections were almost the same as in the frozen sections. Next, to choose the criteria for immunohistochemical positivity, normal lung tissue was used for Eg5 immunohistochemical staining. Representative immunohistochemical Eg5 staining in normal lung tissue is shown in Fig. 1A–D. In normal lung tissue, Eg5 expression was observed in some of the cells in the basal layer of the bronchial epithelium (Fig. 1A and B) and in germinal center lymphocytes exhibiting follicular hyperplasia (Fig. 1C and D). The frequency of positivity for bronchial epithelial cells and lymphoid germinal center lymphocytes were roughly more than 50% and 90%, respectively. We used these tissues as positive controls. Eg5 immunoreactivity was not detected in the pulmonary parenchyma.

3.2. Expression of Eg5 in NSCLC

The tumors of 82 (67%) of the 122 patients were Eg5 positive. Cytoplasmic staining was observed in most of the Eg5-positive tumors, but some tumors also showed nuclear staining. The median of the percentage staining of the lung cancer cells for Eg5 was 35% (range, 0–100%). Representative immunohistochemical Eg5 staining in NSCLC is shown in Fig. 1E–H. Fig. 1E and F shows the staining results for an Eg5-positive squamous cell carcinoma of the lung. The cytoplasm of almost 80% of the cancer cells stained positive for Eg5. Fig. 1G and H shows an Eg5-negative adenocarcinoma of the lung; this adenocarcinoma of the lung was judged to be negative for Eg5 because the cytoplasm of <10% of the tumor cells showed evidence of staining.

The relationships between the expression of Eg5 and clinical variables are shown in Table 2. Eg5 expression was significantly higher in males than in females ($P=0.03$), in squamous cell carcinoma than in non-squamous cell carcinoma ($P=0.02$), and in current and former smokers than in non-smokers ($P=0.03$).

The tumors of 18 (95%) of the 19 patients with cyclin B1-positive tumors were Eg5 positive, and the tumors of 39 (98%) of the 40 patients with Eg5-negative tumors were cyclin B1-negative (data not shown). Eg5 expression was significantly correlated with cyclin B1 expression ($P=0.005$; data not shown).

3.3. Expression of Eg5 and clinical outcome

All 122 patients were assessed for response to chemotherapy and survival. The relationships between clinical variables, Eg5 expression, and cyclin B1 expression, and the response to chemotherapy and survival in this study are shown in Table 3.

The chemotherapy response rate of patients with Eg5-positive tumors was 37%, as opposed to 10% for patients with Eg5-negative tumors. Eg5 expression was significantly associated with response to chemotherapy ($P=0.002$). The chemotherapy response rate of patients with cyclin B1-positive tumors was 53%, as opposed to 23% for patients

Table 2 Relationship between clinical variables and expression of primary antibodies

	n	Eg5-positive (%) patients	Cyclin B1-positive (%) patients
Total	122	82 (67)	19 (16)
Gender			
Male	83	61 (73)*	15 (18)
Female	39	21 (54)	4 (10)
Histology			
Sq	28	24 (86)**	6 (21)
Non-sq	94	58 (62)	13 (14)
Stage			
IIIB	43	30 (70)	8 19)
IV	79	52 (66)	11 (14)
PS			
0	32	20 (63)	1 (3)
1	90	62 (69)	18 (20)**
Smoking history			
Positive	91	66 (73)*	17 (19)
Negative	31	16 (52)	2 (6)

Sq: squamous; PS: performance status.
* $P=0.03$.
** $P=0.02$.

Table 3 Summary of the relationships between clinical variables and response to chemotherapy and survival

	n	Response rate (%)	*P*	PFS (months)	*P*	MST (months)	*P*
Total	122	28		5.0		12.0	
Gender							
Male	83	28	0.95	5.0	0.43	10.0	0.046
Female	39	28		7.0		15.0	
Histology							
Sq	28	32	0.57	5.0	0.72	9.0	0.64
Non-sq	94	27		5.0		13.0	
Stage							
IIIB	43	33	0.39	6.0	0.01	17.0	0.07
IV	79	25		5.0		11.0	
PS							
0	32	25	0.67	5.0	0.21	14.0	0.16
1	90	29		5.0		10.0	
Smoking history							
Positive	91	27	0.87	5.0	0.23	10.0	0.035
Negative	31	29		6.0		15.0	
Eg5							
Positive	82	37	0.002	5.0	0.08	10.0	0.006
Negative	40	10		6.0		13.0	
Cyclin B1							
Positive	19	53	0.009	5.0	0.77	8.0	0.31
Negative	103	23		5.0		13.0	

PFS: progression-free survival; MST: median survival time.

with cyclin B1-negative tumors, and cyclin B1 expression was also significantly associated with response to chemotherapy ($P=0.009$).

The each of PFS and overall survival curves calculated using the Kaplan–Meier method according to Eg5 expression was shown in Fig. 2. The median PFS time for the Eg5-negative group was 6.0 months, as opposed to 5.0 months for the Eg5-positive group (Fig. 2A). The median survival time for the Eg5-negative group was 13.0 months, as opposed to 10.0 months for the Eg5-positive group (Fig. 2B). According to the overall survival data, the Eg5-positive group had a significantly poorer outcome than the Eg5-negative group ($P=0.006$).

The median PFS time in both the cyclin B1-negative and the cyclin B1-positive group was 5.0 months (Fig. 2C). The median survival time in the cyclin B1-negative group was 13.0 months, as opposed to 8.0 months in the cyclin B1-positive group (Fig. 2D). Cyclin B1 expression was not associated with PFS or overall survival. Among the clinical variables, gender and smoking history were significantly associated with overall survival, and disease stage was significantly associated with PFS, also.

3.4. Multivariate analysis for response to chemotherapy, PFS, and overall survival

Following the univariate analyses for response to chemotherapy, PFS, and overall survival, we performed multivariate analyses. Table 4 shows the results of the multivariate analysis for response to chemotherapy, PFS, and overall survival. The multivariate analysis for response to chemotherapy was performed using logistic regression to determine the prognostic value of Eg5 when other prognostic factors were considered. A multivariate analysis that included gender, histology, stage, PS, smoking history, Eg5 expression and cyclin B1 expression, showed that Eg5 expression was the only significant independent variable correlated with response to chemotherapy ($P=0.008$).

A multivariate analysis using the Cox proportional hazards model for PFS and overall survival was performed, using gender, histology, stage, PS, smoking history, Eg5 expression and cyclin B1 expression, as variables. No correlation between variables and PFS was found in the multivariate analysis. Stage was the only independent variable significantly correlated with overall survival ($P=0.036$).

4. Discussion

This is the study to investigate the relationship between the level of expression of Eg5 and the clinical response to chemotherapy and outcome of previously untreated patients with advanced NSCLC. Eg5, a kinesin motor, accounts for many of the movements of the spindle and chromosomes in dividing cells. It localizes to the spindle in mitotically dividing cells and has been implicated in spindle function by both its cellular localization and the effects of mutations.

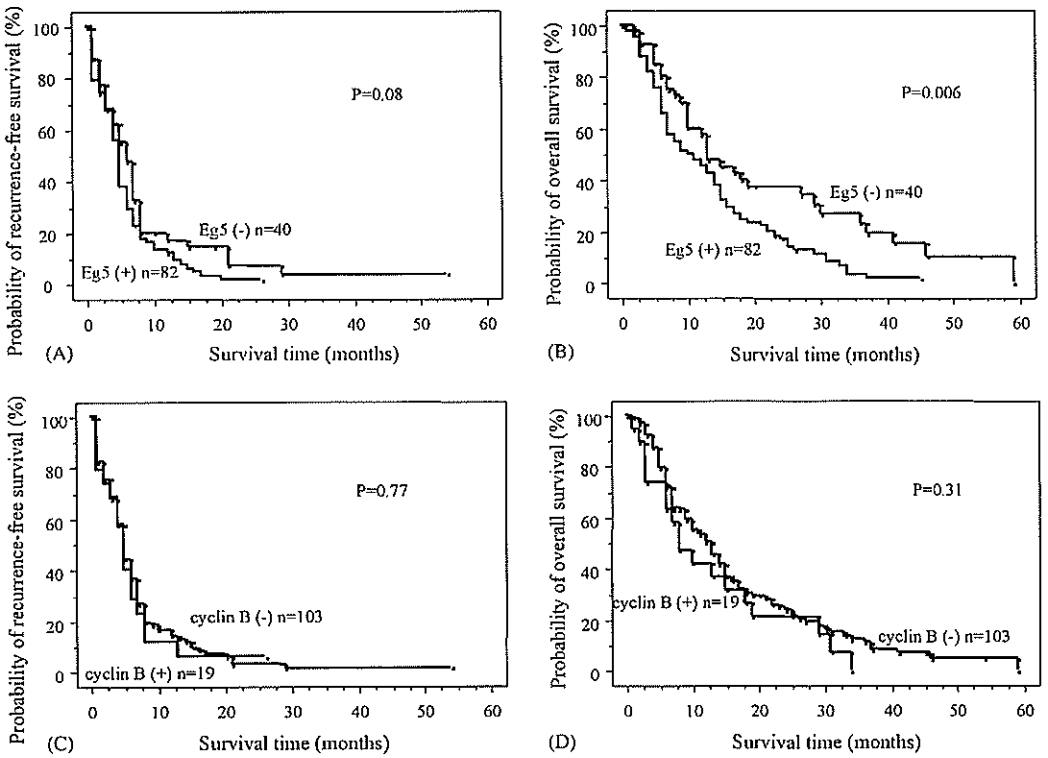

Fig. 2 (A) Progression-free survival curves of 122 patients with advanced non-small cell lung cancer, according to Eg5 expression. The median progression-free survival periods of Eg5-negative and -positive patients were 6.0 and 5.0 months, respectively. (B) Overall survival curves for 122 patients with advanced non-small cell lung cancer, according to Eg5 expression. The median survival periods for Eg5-negative and -positive patients were 13.0 and 10.0 months, respectively. (C) Progression-free survival curves of 122 patients with advanced non-small cell lung cancer, according to cyclin B expression. The median progression-free survival periods of Eg5-negative and -positive patients were 5.0 and 5.0 months, respectively. (D) Overall survival curves for 122 patients with advanced non-small cell lung cancer, according to cyclin B1 expression. The median survival periods for cyclin B1-negative and -positive patients were 13.0 and 8.0 months, respectively.

Eg5 function in centrosome or spindle pole body separation is necessary for bipolar spindle assembly [10].

In normal lung tissue, Eg5 expression was found to be present in some of the cells in the basal bronchial layer of the bronchial epithelium, but its expression in this region was not as strong as in lung cancer tissue. The overexpression of cyclin B1 has been reported in various malignant tumors and has been shown to predict a poor outcome in patients with NSCLC, esophageal carcinoma, and head and neck cancer [16–18]. It has been postulated that the overexpression of cyclin B1 is involved in uncontrolled cell growth and the malignant potential of carcinoma cells. Since the expression of Eg5 in lung cancer tissue has been found to be correlated with the expression of cyclin B1, lung cancer tissue that overexpresses Eg5 in comparison with normal lung tissue is assumed to have greater malignant potential than lung cancer tissue that does not.

Eg5 expression before chemotherapy was correlated with response to chemotherapy and Eg5 status was found to be an independent prognostic factor of response to chemotherapy in a multivariate analysis. Further investigation showed that Eg5 expression was correlated with the response to each type of regimen: the taxan regimens (CDDP + docetaxel: $n = 20$; CBDCA + paclitaxel: $n = 26$; $P = 0.046$), and the vinca alcaroid regimen (CDDP + vinorelbine: $n = 76$; $P = 0.02$) (data not shown). The mechanisms by which Eg5 overexpression affects chemotherapy have not been fully elucidated; nevertheless, Marcus et al. [27] recently reported that mitotic kinesin Eg5 inhibitors induce mitotic arrest and cell death in both paclitaxel-resistant and paclitaxel-sensitive cancer cells and that Eg5 was required for paclitaxel-induced microtubule aster formation (multi-polar spindle configuration) in an *in vitro* assay. They suggested that Eg5 functionality is necessary for paclitaxel-induced mitotic arrest and cell death. These findings may explain our result that Eg5 overexpression before chemotherapy was significantly correlated with response to chemotherapy. The results for docetaxel can be explained in the same manner as for paclitaxel because their modes of action are the same. On the other hand, vinorelbine inhibits the polymerization of tubulin. We suspect that some unknown interaction between tubulin and Eg5 may be modified by vinca alcaloids.

Although Eg5 expression was significantly correlated with response to chemotherapy, the Eg5-positive cases tended to have a poorer outcome in terms of overall survival than the Eg5-negative cases. The reason why the Eg5-positive cases had a poorer outcome remains unclear; despite their higher response to antimitotic agents, Eg5-positive cells may have

Table 4 Multivariate analysis

Variables	Category	Risk ratio	95% CI	P
Multivariate analysis for response of advanced NSCLC patients				
Gender	Male vs. female	0.77	0.245–2.42	0.66
Histology	Sq vs. non-sq	0.89	0.31–2.57	0.83
Stage	IIIB vs. IV	0.64	0.25–1.65	0.35
PS	0 vs. 1	0.98	0.34–2.82	0.97
Smoking history	(−) vs. (+)	0.59	0.18–1.95	0.39
Eg5	(−) vs. (+)	5.16	1.54–17.29	0.008
Cyclin B1	(−) vs. (+)	2.82	0.94–8.45	0.06
Multivariate analysis for PFS of advanced NSCLC patients				
Gender	Male vs. female	0.90	0.56–1.45	0.67
Histology	Sq vs. non-sq	0.89	0.55–1.43	0.63
Stage	IIIB vs. IV	0.60	0.39–0.93	0.02
PS	0 vs. 1	0.92	0.59–1.45	0.72
Smoking history	(−) vs. (+)	0.84	0.51–1.39	0.50
Eg5	(−) vs. (+)	0.77	0.50–1.19	0.24
Cyclin B1	(−) vs. (+)	1.09	0.62–1.89	0.77
Multivariate analysis for OS of advanced NSCLC patients				
Gender	Male vs. female	0.74	0.44–1.26	0.27
Histology	Sq vs. non-sq	1.03	0.63–1.67	0.92
Stage	IIIB vs. IV	0.63	0.41–0.98	0.04
PS	0 vs. 1	0.76	0.47–1.22	0.25
Smoking history	(−) vs. (+)	0.74	0.43–1.30	0.30
Eg5	(−) vs. (+)	0.62	0.39–0.97	0.04
Cyclin B1	(−) vs. (+)	1.03	0.59–1.78	0.93

PFS: progression-free survival; NSCLC: non-small cell lung cancer; PS: performance status; CI: confidence interval; OS: overall survival.

a higher malignant potential, contributing to a poor clinical outcome. This appears to be consistent with the expression of Eg5 being significantly correlated with the expression of cyclin B1, which may be involved in uncontrolled cell growth and the malignant potential of cancer cells.

The inhibition of Eg5 has recently been exploited as an aid to cancer treatment [12–14,27–32], and small cell-permeable molecules that inhibit mitotic kinesin Eg5 and do not target tubulin arrest cells in mitosis with monoastral spindles. Chromosomes in Eg5 inhibitor-treated cells frequently have both sister kinetochores attached to microtubules extending to the center of the monoaster. The mitotic kinesin Eg5 inhibitor also induces apoptosis and is effective in inhibiting the proliferation of cancer cells through mitotic arrest. The first small molecule inhibitor of Eg5 was monastrol [11,12], and second-generation Eg5 inhibitors like CK0106023 [29] and HR22C16 [27], which are specific allosteric inhibitors of Eg5 and exhibit antitumor activity *in vivo* or *in vitro*, have been discovered by drug screens. Therapeutic intervention with Eg5-specific inhibitors has also been reported, and SB-715992 has been shown to be a potent inhibitor of mitotic kinesin Eg5. Eg5 inhibitors may be used as new antimitotic agents to treat advanced NSCLC in the future.

In conclusion, our findings indicated that the expression of the mitotic kinesin Eg5 can predict a response to antimitotic agents combined with platinum chemotherapy among patients with advanced NSCLC. Our results have important implications for the treatment of NSCLC because Eg5 inhibitors, which cause tumor cell apoptosis, may be effective in patients with advanced NSCLC.

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Molecular Biology, Genomics, and Proteomics in Bronchioloalveolar Carcinoma

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Abstract: The charge of the Molecular Biology, Genomics, and Proteomics in Bronchioloalveolar Carcinoma Committee was to evaluate the molecular biology, genomic changes, and proteomic findings in patients with bronchioloalveolar carcinoma compared with other types of lung cancer. The literature was reviewed and unpublished information was presented by the committee members at the session. The molecular biology studies have included findings on epidermal growth factor receptor (*EGFR*) mutations, p53 mutations, K-*ras* mutations, and loss of heterozygosity. The genomic changes have mostly focused on the mRNA expression arrays as well as protein studies. The current state of knowledge was reviewed, the missing information was acknowledged, and proposals for future research were identified.

Key Words: Lung neoplasm, Adenocarcinoma, Bronchioloalveolar, Adenocarcinoma, Carcinoma, Non-small cell lung cancer.

(*J Thorac Oncol.* 2006;1: S8–S12)

Little information is available about p53 mutations and p53 protein overexpression detected by immunohistochemistry, microsatellite loss of heterozygosity (LOH), and K-*ras* mutations in adenocarcinoma of the bronchioloalveolar subtype, according to the last World Health Organization (WHO) pathological classification proposed in 1999. However, the frequency of these molecular abnormalities seems to increase during the multistep process of carcinogenesis of peripheral adenocarcinoma going from atypical alveolar hyperplasia adenocarcinoma to bronchioloalveolar carcinoma (BAC) and to invasive adenocarcinoma.

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ATYPICAL ADENOMATOUS HYPERPLASIA

There is an increasing body of evidence to support the concept of atypical adenomatous hyperplasia (AAH) as the precursor of at least a subset of adenocarcinomas.[1] AAH is most frequently detected in lungs from patients bearing lung cancers (9–20%), especially adenocarcinomas (up to 40%) compared with squamous cell carcinomas (11%).[2] Several molecular changes frequently present in lung adenocarcinomas are also present in AAH lesions, and there is further evidence that AAH may represent true preneoplastic lesions.[1] The most important findings are the presence in AAHs of K-*ras* (codon 12) mutations (40%),[3] loss of *LKB1* function (20%),[4] allelic losses in chromosomes 3p (20%), 9p ($p16^{INK4a}$, 10%), 9q (50%), 17q, and 17p (*TP53*, 5%),[5,6] and overexpression of cyclin D1 (70%), p53 (ranging from 10 to 60%),[7] and survivin (50%).[8] Despite the evidence that AAH is a precursor lesion for a subset of lung adenocarcinomas, there is general consensus that the pathogenesis of most adenocarcinomas is still unknown. The findings of relatively infrequent tyrosine kinase domain epidermal growth factor receptor (*EGFR*) mutations in AAH lesions (three out of 40 examined)[9,10] and no *EGFR* mutation[11,12] or relatively low frequency in true BACs of the lung[9] support the concept that genetic abnormalities of *EGFR* are not relevant in the pathogenesis of alveolar types of lung neoplasia. In addition, Tang et al.[13] recently reported that *EGFR* mutation is an early event in the pathogenesis of lung cancer, being identified in histologically normal epithelium of small bronchi and bronchioles adjacent to *EGFR* mutant lung adenocarcinomas in nine out of 21 (43%) patients examined, but in none of the patients without mutation in the tumor. These data further support the notion than AAH lesions are not involved in the pathogenesis of *EGFR* mutant lung adenocarcinomas.

BAC, ADENOCARCINOMA WITH BRONCHIOLOALVEOLAR FEATURES, AND ADENOCARCINOMA OF THE LUNG

The frequency of *EGFR* mutations has also been studied in patients with BAC, adenocarcinoma with BAC features, and adenocarcinomas of the lung. Although responses to EGFR tyrosine kinase inhibitors have been reported to be higher[14] and EGFR mutations were preferentially observed in tumors having BAC features,[12,15] we did not find association with the BAC subtype of adenocarcinoma in 97 cases from

the United States[11] using the criteria stated by the 1999 WHO classification of lung tumors.[16,17]

In addition to the WHO system, Noguchi et al.[18,19] have classified adenocarcinomas into different categories that have different frequencies of genetic changes. Koga et al.[20] reported that p53 mutations were present in approximately 0% of 17 pure BAC, 11% of 27 mixed adenocarcinoma with BAC features, and 48% of 101 invasive adenocarcinomas. Similar to the frequency of mutations, the frequency of p53 protein overexpression detected by immunohistochemistry increased from 6% (2/32 tumors) in pure BAC to 28% (27/133) in BAC with foci of active fibroblastic proliferation (Noguchi type C) and to 40% (14/35) in adenocarcinoma.[21] p53 mutation and protein overexpression were also correlated with the size and invasive component of small peripheral adenocarcinomas (≥5 mm: 41%; <5 mm: 20%).[22,23]

The frequency of allelic losses also increased significantly during malignant progression. According to Noguchi's classification,[18,19] frequencies of allelic losses at chromosomal loci 3p, 17p, 18q, and 22q were significantly lower in BAC with or without alveolar collapse (Noguchi types A and B, respectively) than in BAC with active fibroblastic proliferation (Noguchi type C) in a series of 66 small peripheral adenocarcinomas.[24]

The frequency and type of K-*ras* mutation in BAC are related to the cytological features (mucinous versus nonmucinous). This raises the question of whether the mucinous form might represent a biological entity separate from the nonmucinous form. Small series of tumors (all <50) from patients with adenocarcinoma of the lung show that the K-*ras* mutation is present in 73 to 100% of the mucinous types and that the type of the mutation was usually G to A (codon 12), whereas it was seen in 10 to 43% in the nonmucinous types, usually in G to T transversions.[25–27] Mutations at codon 12 of the K-*ras* oncogene were found in 39% of 41 AAH, 42% of 18 adenocarcinomas, and none of five lung neoplasms that were not adenocarcinomas. Of the patients with both an AAH and a synchronous adenocarcinoma, more than half did not have the mutation in both the AAH and the synchronous lung adenocarcinoma, suggesting that peripheral adenocarcinomas arise not always from AAH but sometimes directly from a background of field cancerization.[27]

Adenocarcinomas with BAC features are also characterized by an intense inflammatory reaction especially containing alveolar neutrophils and macrophages. Increased numbers of tumor-infiltrating neutrophils are linked to poorer outcomes in these patients.[28] Tumor environment drives local neutrophil recruitment and activation via C-X-C chemokine release such as interleukin-8 and epithelial cell–derived neutrophil activating protein 78 but also prolongs alveolar neutrophil survival through the production of soluble antiapoptotic factors (granulocyte–macrophage colony–stimulating factor and granulocyte colony–stimulating factor).[29,30] The mechanisms by which neutrophils influence the prognosis of adenocarcinoma with BAC features could be multiple. It has been postulated that the persistence of neutrophil alveolitis would result in persistent release of proinflammatory mediators such as cytokines, proteases, and reactive oxygen and nitrogen species that can damage DNA and activate oncogenes.[31,32] Among these factors released by neutrophils, hepatocyte growth factor seems to be particularly involved in the progression of these types of tumors, especially through its mitogenic and scattering properties, favoring c-Met expressing tumor-cell migration along the alveolar basal membrane.[33] Lastly, neutrophils might be involved in luminal tumor spread by promoting tumor-cell shedding (M. Wislez, AACR 2004), described pathologically as the presence of micropapillary clusters that are also involved in the mechanism of aerogenous progression.[34]

GENOMIC AND PROTEOMIC STUDIES OF BAC

As mentioned before, BAC is thought to arise from AAH and is potentially an intermediate to invasive adenocarcinoma. Extensive analyses of BAC using gene-expression profiling and proteomic-based studies have not yet been performed and are only available for limited numbers of these cancers. These types of studies may have the potential to define similarity or differences in the observed types of adenocarcinoma of the lung. Of particular interest is the potential regulatory pathway involved in the lepidic growth patterns of BAC, which is different from most other adenocarcinomas of the lung. The observation that some adenocarcinomas can exhibit regions of BAC provides complexity and has resulted in multiple pathological-based classifications.[14,16–19] Genomic studies have the potential to define the similarities as well as key differences between BAC, adenocarcinomas with BAC features, and adenocarcinomas of the lung.

Recent studies examining individual genes have hinted at differences between BAC and adenocarcinomas. The tumor suppressor in the lung cancer-1 gene encodes an adhesion molecule and is frequently associated with LOH at that locus in non–small-cell lung cancer. Both normal lung cells and BAC retain expression of tumor suppressor in lung cancer-1, whereas 63% of adenocarcinomas demonstrated decreased expression detected by immunohistochemistry.[35] BACs have very low p53 DNA mutation frequencies compared with adenocarcinomas of the lung.[20] LOH at the 3p FHIT loci was observed in 43% of BAC, and 12th codon K-*ras* mutations are detected in the mucinous form of BAC.[36] A comparative LOH study between 14 BAC and 20 stage I lung adenocarcinomas using nine chromosomal regions revealed that the most frequently affected chromosomal regions in BAC were 8q and 17p.[37] In adenocarcinomas of the lung, LOH at 1p, 3p, 7q, and 18q was more frequent than in BAC, and fractional allele loss was greater in adenocarcinomas of the lung than BAC.

Using immunocytochemistry to examine protein expression, detection of the thyroid transcription factor-1 (TTF-1), cytokeratin 7, and cytokeratin 20 were measured in both mucinous and nonmucinous BAC.[38] TTF-1 was detected in 17% of mucinous and 94% of nonmucinous BAC, cytokeratin 7 was detected in 100% of mucinous and 23% of nonmucinous BAC, and cytokeratin 20 was detected in 60% of mucinous and 0% of nonmucinous BAC.[38] In a study that examined MUC protein expression in AAH, BAC, and adenocarcinomas with BAC features, MUC1 decreased from

AAH to BAC and from BAC to adenocarcinoma, whereas MUC2, MUC5AC, MUC6, and depolarized MUC6 increased.[39] Alterations in p53 and the increased expression of MUC1, MUC5AC, and MUC6 were noted.

ADDITIONAL GENOMIC AND PROTEOMIC STUDIES

A comparison of normal lung tissue and BAC using oligonucleotide arrays was reported by Goodwin et al.[40] and identified 12 up-regulated and six down-regulated genes in the BAC tumors. Although this analysis provides some information, a comparison of BAC and adenocarcinomas was not included, which may be most relevant in defining critical genes involved in the development of these cancers. We used oligonucleotide arrays to examine gene expression in 14 BAC and 73 adenocarcinomas.[41] The most highly expressed genes that were significantly different between the BAC tumors and adenocarcinomas and higher in BAC included the surfactant pulmonary-associated proteins A1, A2, C and D, MUC1, TTF-1 and TTF-3, villin 2, and prostaglandin D2 synthetase. Interestingly, higher mRNA expression for both fos and jun B were detected in BAC, which may reflect an elevated AP-1 activity and upstream signaling events in these tumors. The higher level of expression of surfactant genes is consistent with the well-differentiated phenotypic characteristics of BAC. TTF-1 was the most differentially expressed gene between BAC and adenocarcinomas, consistent with the high TTF-1 protein expression reported in BAC.[38] Because of the small numbers of tumors for our analyses, it was not possible to divide the BAC tumors into separate categories such as mucinous, nonmucinous, and mixed histology. Although we found MUC1 mRNA present in both BAC and adenocarcinomas of the lung, the significantly increased expression in BAC is consistent with the higher MUC1 protein levels that have been reported in these tumors.[39]

Analysis of survival-related genes revealed prostaglandin D2 synthetase and neutrophil elastase 2 to be more highly expressed in BAC than the other adenocarcinomas. In contrast, much lower levels of vascular endothelial growth factor were detected in the BAC, possibly reflecting a lesser level of angiogenesis and hypoxia in these tumors relative to the adenocarcinomas. Adenocarcinomas also expressed increased levels of metallothionein 2A and thioredoxin reductase mRNA. We speculate that these genes may correspond to smoking-related alterations because these genes may change in response to reactive oxygen species originating from tobacco smoking or in response to inflammatory cells. Alternately, the expression of thioredoxin reductase and metallothionein 2 may reflect the higher rates of cell proliferation in the lung adenocarcinomas relative to BAC.

Few, if any, large-scale proteomic analyses of BAC have been reported. We examined the same BAC and lung adenocarcinomas for mRNA using oligonucleotide arrays and also at the protein level with two-dimensional gel electrophoresis and mass spectrometry.[42] A total of 682 protein spots were quantified, and 75 proteins were found to differ significantly ($p < 0.05$) between BAC and lung adenocarcinomas. Thirty-eight protein spots were successfully identified using mass spectometry. Of interest were the relatively higher expression of the ras-related protein RAB-14, glutathione-s-transferase-pi, cytokeratin 7, and three isoforms of the selenium-binding protein 1 in BAC compared with adenocarcinomas of the lung. Adenocarcinomas expressed higher levels of phosphoglycerate kinase 1, pyruvate kinase M1/M2, and stathmin (OP-18) compared with BACs. Increased phosphoglycerate kinase 1 is consistent with higher hypoxia-induced glycolysis in the adenocarcinomas of the lung relative to BAC.[42]

Future studies that include sufficient numbers of the various histological subtypes of BAC are needed to provide insight into the similarities and differences among these tumors and as compared with lung adenocarcinomas. The NCI Director's Challenge: Validation Study of Lung Adenocarcinomas will examine gene expression using Affymetrix 133A oligonucleotide arrays among approximately 500 tumors. Thus, a relatively large number of BACs will be included in this study, allowing potential gene pathways to be defined that may be relevant to our understanding of the growth- and cell-signaling systems in BAC. These analyses will also incorporate detailed pathologic assessment of each tumor so that the subtypes of each BAC can be compared. It is expected that these data, made available to the research community, will then stimulate further research into potential new markers for early diagnosis and possible therapeutic intervention strategies that may be effective for BAC.

FUTURE DIRECTIONS

The Committee responsible for Molecular Biology, Genomics, and Proteomics in Bronchioloalveolar Carcinoma outlined studies that will provide further insights into BAC. The most important part of the meeting was partial agreement and understanding about the interpretation of the pathological classification. The participants in the meeting agreed on a common set of descriptors for the pathological interpretation of BAC that will be used more consistently in the future.

TABLE 1. Different Biological Properties in Atypical Adenomatous Hyperplasia, Pure Bronchioloalveolar Cancer, Adenocarcinoma with Bronchioloalveolar Cancer Features, and Adenocarcinoma

	Atypical Adenomatous Hyperplasia	Bronchioloalveolar Carcinoma	Adenocarcinoma with Bronchioloalveolar Carcinoma Features	Adenocarcinoma of the Lung
EGFR mutation	↓ <5%	10%		↑ 40%
TP53 mutations	Not reported	↓ 0%	↓ 10%	↑ 50%
p53 by immunohistochemistry	Not reported	↓ 5%	↑ 30%	↑ 50%

Upcoming technological improvements will provide additional insights into the biology of BAC. These will include the increasing ability to detect genetic changes in BAC and adenocarcinomas including, but not be limited to, *EGFR*, HER-2/neu, B-raf, K-*ras*, and *TP53*. In addition, there is the ability to detect genetic loss in the whole genome using studies with single-polynucleotide polymorphisms or array chromosomal genomic hybridization. There is increasing ability to use small and smaller amounts of DNA and DNA from paraffin-embedded tissues. Future studies will provide information on the degree of genetic changes seen in early lesions (<1cm) that are being detected more often as computerized tomographic scanning of the chest is becoming more widely used. These findings can be compared with the more advanced lesions. The genetic changes can also provide insights into the clonality of the BACs to determine whether the multiple lesions in the lungs arise from single or multiple clones. Table 1

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Common arm analysis: One approach to develop the basis for global standardization in clinical trials of non-small cell lung cancer

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Summary The global development of new anticancer treatments is desirable. However, whether results of clinical trials performed in one population can be fully extrapolated to another population remains in question. We retrospectively compared "common arms" of platinum-based doublet phase III trials among Japanese, European, and American patients with non-small cell lung cancer to develop the basis for global standardization in clinical trials. Patient demographics were very similar through all studies, indicating that extrinsic ethnic factors including socioeconomic factors, medical service background, and patient selection process for clinical trials may be consistent between geographically different oncology groups. The doses of docetaxel, gemcitabine, and vinorelbine were lower in Japanese studies. The toxicity profile was generally acceptable and similar among many studies. Thus, the dose and schedule of anticancer agents established in prior phase I and II studies conducted in each country were appropriate and applicable to large patient populations in these countries. Response rates seemed to be distributed randomly from one study to another, whereas patient survival might be better in Japanese studies. In conclusion, geographical differences in the dose of anticancer agents, response, survival and toxicity of lung cancer chemotherapy were actually observed. However, extrapolation of clinical data obtained in one country to another population and global clinical trials were considered possible with adequate dose adjustment based on dose finding studies using a carefully projected protocol.

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1. Introduction

Lung cancer is one of the most common malignancies and the leading cause of cancer-related deaths in many countries. In the year 2000, the annual number of deaths from lung cancer was estimated to be 1.1 million worldwide, and the incidence lung caner is increasing globally at a rate of 0.5% per year [1]. Lung cancer currently claims more than 55 000 lives annually in Japan, and this figure is projected to double during the next three decades due to the aging of the Japanese population [2]. Non-small cell lung cancer (NSCLC) comprises 80% of all lung cancers, and more than half of the patients with this disease are found to have developed distant metastases or pleural effusion at the time of the initial diagnosis. These patients can be treated with systemic chemotherapy, but the efficacy of currently available anticancer agents is limited to the extent that patients with advanced disease rarely livelong [3].

The development of new anticancer agents and chemotherapeutic regimens are among the urgent tasks for medical oncologists who are involved in the treatment of lung cancer. Since it is time- and money-consuming work, the development of new agents and regimens is desirable on a global scale. Under the present situation in Japan, in that we are considerably behind with the development of new anticancer agents, it is worth evaluating the possibility that the results of clinical trials held outside Japan could be used for approval of these agents by the Japanese authorities. However, whether the results of clinical trials performed in one population can be fully extrapolated to another population remains in question due to the potential differences in trial designs, study-specific criteria, patient demographics, and population-related pharmacogenomics. According to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Guideline E5, Ethnic Factors in the Acceptability of Foreign Clinical Data, the impact of genetic and physiologic (intrinsic) factors and cultural and environmental (extrinsic) factors upon the efficacy and safety of anticancer agents at a particular dosage and dose regimen must be assessed for the application of new agent approval [4].

One approach to develop the basis for global standardization in clinical trials of anti-NSCLC agents is a planned comparative analysis of a "common arm" with similar eligibility, staging, response and toxicity criteria of prospectively designed and conducted separate phase III trials for the treatment of advanced NSCLC, although this approach may have potential limitation in comparability [5]. In this review we retrospectively compared the outcome of phase III trials conducted in Japan, Europe, and USA for chemotherapy doublet regimens using a platinum and a third-generation cytotoxic agent, including paclitaxel, docetaxel, gemcitabine, and vinorelbine.

2. Methods

Combinations of paclitaxel and carboplatin, docetaxel and cisplatin, gemcitabine and cisplatin, and vinorelbine and cisplatin were evaluated in patients with advanced NSCLC as the post-marketing sponsored phase III trials in Japan [6,7]. Phase III trials evaluating these regimens conducted outside Japan were identified by Medline searches. The selection criteria of phase III trials for this analysis were (1) first-line treatment for stage IIIB or IV NSCLC; (2) not intended for a special cohort of patients such as the elderly or those with poor performance status; (3) each arm included more than 120 patients; (4) tumor response was evaluated according to the World Health Organization (WHO) criteria, modified WHO criteria such as Eastern Cooperative Oncology Group (ECOG) criteria and Southwest Oncology Group (SWOG) criteria, or response evaluation criteria in solid tumors (RECIST) criteria; (5) toxicity was evaluated according to the WHO criteria or the National Cancer Institute-Common Toxicity Criteria (NCI-CTC). The dose and schedule of anticancer agents, patient demographics, treatment delivery, tumor response, patient survival, and toxicity were compared between common arms in separate phase III trials. To assess the influence of demographic variables on tumor response and survival, multiple linear regression analysis was performed as previously described [8].

3. Results

3.1. Taxane and platinum

The schedule was identical between the studies in both paclitaxel and carboplatin, and docetaxel and cisplatin combinations (Tables 1 and 2). The dose of paclitaxel ranged from 175 to 225 mg/m^2 without ethnic tendency. The dose of docetaxel was set to be 20% lower in a Japanese study [7] than that of USA studies [9,10]. This difference was mainly attributable to differences in the criteria of the maximum tolerated dose in phase I studies of docetaxel between Japan and the USA. Patient demographics were very similar among these studies. Response rates (RRs) in the combination of paclitaxel and carboplatin varied widely from 17% to 46%, and median survival time (MST) from 7.8 to 12.3 months. The RR and MST in Japanese and Greek studies appeared to be better than those in ECOG study, but did not differ from those in other American studies. A multiple linear regression analysis failed to show correlation between demographic variables and the RR or MST. In the docetaxel and cisplatin combination, the RR and survival in the Japanese study appeared to be better that those in the ECOG study [9], but similar to those in the other USA study [10].

Among paclitaxel and carboplatin studies, the incidence of grade 3-4 neutropenia and febrile neutropenia was higher in the Japanese study than in the other studies. The toxicity profile of the docetaxel and cisplatin combination was identical among all studies.

3.2. Gemcitabine and cisplatin

The dose of gemcitabine per one course was smaller in the Japanese study than in other studies outside Japan (Table 3). The RR in ECOG study was lower than that in European studies, while the MST of 14.8 months and 1-year survival rate of 60% in the Japanese study seemed higher than those in the other studies [6]. There was no correlation between demographic variables and the RR or MST in a multiple linear regression analysis.