

## Recent Development of Molecular-Targeted Drugs in Lung Cancer

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### Abstract

Numerous molecular target drugs have been introduced for the treatment of advanced malignancies. In the treatment of lung cancer, epidermoid growth factor receptor-tyrosine kinase inhibitors (EGFR-TKI) demonstrate striking antitumor activity in selected EGFR mutation positive patients. Patient selection by biomarker is extremely important to obtain successful results. The anti-vascular endothelial growth factor (VEGF) antibody, bevacizumab, shows a markedly increased response rate, progression free survival of advanced non-squamous cell lung cancer when combined with cytotoxic drugs. The classification of lung cancer is rapidly changing based on the advances in molecular biology. Here, the recent development of new molecular target drugs against lung cancer is thoroughly reviewed in addition to EGFR-TKIs and bevacizumab with special emphasis on the clinical application.

**Key words:** molecular target drugs, lung cancer, EGFR-TKI, VEGF, angiogenesis

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### Introduction

In recent years, the understanding of cancer at the molecular level has progressed, and numerous genes and proteins which play important roles in the growth, invasion and metastasis of tumors have been identified. Furthermore, by setting these genes and proteins as the targets, small molecular weight drugs called signal transduction inhibitors (e.g., tyrosine kinase inhibitors), monoclonal antibodies, etc., have been developed for the treatment of cancer(s). Numerous molecular-targeted drugs have been developed, including epidermal growth factor receptor (EGFR) inhibitors, vascular endothelial growth factor (VEGF) antibodies, etc, have also been developed for the treatment of non-small cell lung cancer (NSCLC), and a number of clinical studies on these new drugs have been conducted towards the goal of their clinical application.

#### 1. Treatment targeted at EGFR

EGFR is a transmembrane-type receptor protein composed of an extracellular ligand-binding domain, a transmembrane domain, and an intracellular tyrosine kinase do-

main. When a growth factor binds to this receptor, a dimer is formed and the downstream signal transduction system is activated, resulting in cancer cell proliferation, metastasis, vascularization and apoptosis, etc (1-3).

Excessive EGFR expression has been reported to be detected in 32-81% of all cases of NSCLC (4-6). Two therapeutic strategies designed to inhibit the EGFR signal transduction system have been developed. One is to use EGFR tyrosine kinase inhibitors (EGFR-TKIs) which are low molecular weight compounds that bind to the ATP-binding site of intracellular tyrosine kinase, inhibiting the self-phosphorylation of EGFR. The other strategy is to use monoclonal antibodies that bind specifically to the extracellular domain of EGFR, thereby inhibiting ligand binding to EGFR.

#### 1) EGFR tyrosine kinase inhibitors

##### a. Gefitinib

The results of randomized phase II clinical studies of gefitinib in previously treated cases of NSCLC were reported in 2002. In the Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL)-1 study, carried out primarily in Europe and Japan, the response rate was 18.4% in the 250 mg/day

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group and 19.0% in the 500 mg/day group (7). Also in IDEAL-2, carried out in the USA, the response rates were almost the same between the 250 mg/day group (11.8%) and the 500 mg/day group (8.8%), and there was no difference in the survival period between the two dose groups (8). Toxicity was lower in the 250 mg/day group than in the 500 mg/day group, and the dose level of 250 mg/day was adopted as the recommended dose level. In a subgroup analysis, the response rate was significantly higher in females, patients with adenocarcinoma, and Japanese patients. On the basis of these results, the Japanese regulatory authority approved the use of gefitinib in 2002, earlier than in other countries around the world.

The Iressa Survival Evaluation in Advanced Lung Cancer (ISEL) was a large-scale phase III clinical study in which 1,692 previously treated patients with NSCLC were randomly allocated to the gefitinib and the placebo group. The results revealed that the response rate was significantly higher in the gefitinib group than in the placebo group (8% vs. 1%,  $p < 0.0001$ ). Of the primary endpoints, the median survival time (MST) and one-year survival rate were 5.1 months and 21%, respectively, in the placebo group and 5.6 months and 27% in the gefitinib group, respectively, with no significant difference between the two groups ( $p = 0.087$ ) (9). In the subgroup analysis, however, gefitinib was shown to extend the survival in non-smokers (MST: 8.9 months vs. 6.1 months,  $p = 0.012$ ) and Asian patients (MST: 9.5 months vs. 5.5 months,  $p = 0.01$ ). A randomized phase III clinical study (V-15-32) aimed at confirming the non-inferiority of gefitinib to docetaxel (DOC) was carried out in Japan, involving 490 previously treated patients with NSCLC. The response rate was significantly higher in the gefitinib group (22.5%) than in the DOC group (12.8%) ( $p = 0.009$ ). The median progression-free survival (mPFS) was 2.0 months in both groups. The MST (a primary endpoint) was 14.0 and 11.5 months in the two groups, respectively. The hazard ratio (HR) was 1.12 (95% confidence interval [CI]: 0.89-1.40). Thus, the study did not demonstrate non-inferiority of gefitinib to DOC (10). In addition, a report was published of a randomized phase III clinical study (INTEREST) carried out in 24 countries (Europe, USA and Asia), comparing gefitinib with DOC in 1,433 previously treated patients with NSCLC. In that study, the response rate did not differ significantly between the gefitinib group (9.1%) and the DOC group (7.6%) ( $p = 0.33$ ), and there was no significant difference in the mPFS either between the gefitinib group (2.2 months) and the DOC group (2.7 months) ( $p = 0.47$ ). In the analysis of the overall survival period, the primary endpoint, the HR was 1.020 (95%CI: 0.905-1.150) and did not exceed the preset upper limit (1.154), thus endorsing the non-inferiority of gefitinib to DOC (11). In the evaluation of toxicity, the gefitinib group most frequently developed skin eruptions and diarrhea, while the DOC group most frequently developed decreased blood neutrophil count, myasthenia, and alopecia. In 2008, interesting results were reported from a phase III clinical study (Iressa Pan Asia

Study: IPASS) comparing gefitinib therapy with carboplatin (CBDCA) + paclitaxel (PTX) therapy, each administered as the initial therapy (to be described in detail later) (12). Furthermore, a randomized phase III clinical study (WJTOG 0203) was carried out in Japan, comparing platinum-based chemotherapy (3-6 cycles) with platinum-based chemotherapy (3 cycles) + sequential gefitinib therapy in 598 previously untreated patients with NSCLC. In that study, mPFS was significantly longer in the sequential therapy group (4.60 months) than in the platinum-based chemotherapy alone group (4.27 months) ( $p < 0.001$ ), while the overall survival period (a primary endpoint) did not differ significantly between the two groups (MST: 12.89 months vs. 13.68 months,  $p = 0.10$ ). In a subset analysis, the overall survival period of adenocarcinoma patients was extended by the sequential therapy (MST: 14.33 months vs. 15.42 months,  $p = 0.03$ ) (13). In a randomized phase II clinical study in which 97 previously untreated patients with NSCLC were divided into two groups, one group receiving oral gefitinib therapy after 4 cycles of CBDCA + PTX therapy until exacerbation of the condition and the other receiving oral gefitinib therapy (until exacerbation of the condition) followed by 4 subsequent cycles of CBDCA + PTX therapy, the overall survival period (a primary endpoint) differed little between the two groups (MST: 18.8 months vs. 17.2 months) (14).

#### b. Erlotinib

In a phase II clinical study of erlotinib monotherapy involving 57 previously treated patients of NSCLC showing positive immunostaining of the tumor cells for EGFR, the response rate was 12.3% and the MST was 8.4 months (5). In this study, the results suggested that the overall survival period was probably correlated with the incidence and severity of skin eruptions (15).

In sharp contrast to the findings of the above-mentioned studies on gefitinib were the results obtained in a phase III clinical study comparing erlotinib with BSC. In this phase III comparative study (BR.21) carried out by the National Cancer Institute of Canada Clinical Trial Group (NCIC), 731 previously treated patients with NSCLC were allocated randomly to the erlotinib group and the placebo group at a ratio of 2:1. In analysis of the primary endpoints, erlotinib was significantly superior in terms of both the overall survival (MST: 6.7 months in the erlotinib group vs. 4.7 months in the placebo group,  $p < 0.001$ ) and the progression-free survival (2.2 months in the erlotinib group vs. 1.8 months in the placebo group,  $p < 0.001$ ) (16). On the basis of the results of this study, erlotinib was adopted as one of the standard therapies for previously treated cases of NSCLC. Following publication of the results of this study, erlotinib was approved in 2004 in the USA and in 2007 in Japan. Regarding the discrepancy of the results between ISEL and BR.21, the influence of pharmacological differences has been pointed out, such as the difference in the dose level (erlotinib dose level equal to the MTD and gefitinib dose level equivalent to about 1/3 of the MTD) and the difference in the affinity for EGFR (17). In addition, a phase IV clini-

**Table 1. Phase II Study of EGFR- TKI in EGFR Mutation (+) Patients**

	n	EGFR-TKI	RR (%)	mPFS (M)	MST (M)
Morita S (I-CAMP)	148	gefitinib	76.4	9.7	24.3
Sirera R	193	erlotinib	70.8	12.0	22.0
Sequist LV	34	gefitinib	55	9.2	17.5

EGFR-TKI: Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor

RR: Response Rate

mPFS: median Progression-Free Survival

MST : Median Survival Time

M: Month

cal study (TRUST) was carried out on erlotinib monotherapy for NSCLC. In an analysis of the interim results of this study covering 6,809 patients, the response rate was 13% and mPFS was 3.5 months, close to the results obtained in the BR.21 study (18). At a meeting of ASCO in 2009, the results of a phase III clinical study (SATURN) comparing maintenance erlotinib therapy with placebo were reported, demonstrating the superiority of erlotinib in terms of the PFS as a primary endpoint (19). At present, clinical studies such as a phase III clinical study of erlotinib with chemotherapy of pemetrexed (PEM) or DOC as secondary chemotherapy and a phase III clinical study (RADIANT) comparing erlotinib with placebo as postoperative adjuvant therapy are ongoing. Interesting results from these studies are expected.

## 2) Predictors of responses to EGFR-TKI

EGFR gene mutations are reported as the most important factor predictive of the responses of NSCLC to EGFR-TKI (20-22). More than forty mutations of the EGFR gene in exon 18-21 of the tyrosine kinase domain have been reported. Among others, deletion of 5 amino acids in exon 19 and the L858R point mutation of exon 21 are reported to account for more than 80% of all mutations of the EGFR gene (3, 23). EGFR gene mutations have also been reported to be correlated with clinical factors associated with a high sensitivity to EGFR-TKI, such as adenocarcinoma, female gender, non-smoker and Asian race (20, 24, 25). In addition, the results of a phase II clinical study of EGFR-TKI in patients carrying EGFR gene mutations have been reported (Table 1) (26-28). In 2008, the results of an integrated analysis of the results of 7 Japanese phase II clinical studies of gefitinib (I-CAMP) were reported. In that analysis, EGFR-TKI therapy yielded excellent outcomes in 148 patients carrying gene mutations, with a response rate of 76.4%, mPFS of 9.7 months, and MST of 24.3 months (26). The responses of the gene mutation-positive cases to this therapy were also favorable in other studies, suggesting that the presence of EGFR gene mutation serves not only as a predictor of the response to treatment, but also as a prognostic factor (29).

There are also reports on the usefulness of the number of EGFR gene copies, evaluated by fluorescence *in situ* hy-

bridization (FISH), as a predictor of the response to treatment (30-32). In an evaluation of patients registered with the BR.21 study, amplification of gene copies was significantly correlated with the response rate to erlotinib, whereas the presence of gene mutation was not correlated with the response rate (33). In a similar analysis of cases registered with the ISEL study, patients with gene copy amplification tended to have a longer survival period following gefitinib therapy, although the difference was not statistically significant ( $p=0.07$ ). In that analysis, it was not possible to evaluate the correlation of the presence of gene mutations with survival, because the number of gene mutation-positive cases was not sufficiently large (34). In Western countries, the number of gene copies is often used as a predictor of response to treatment, because the frequency of gene mutations is low.

KRAS gene mutation is seen in 20-40% of cases of NSCLC and has been reported to serve as a predictor of a poor response to EGFR-TKI and chemotherapy (35, 36) but to date there is not sufficient evidence.

## 3) EGFR-TKI as a means of primary treatment

### a. EGFR-TKI monotherapy

The results of a phase II clinical study on gefitinib conducted on previously untreated patients with NSCLC in National Cancer Center Hospital East have been reported. Of the 40 patients eligible for the study, 40% were female, 75% had adenocarcinoma and 20% were non-smokers. The response rate was 30%, MST was 13.9 months, and the one-year survival rate was 55%. However, death from acute lung disorders as an adverse event occurred in 10% of all patients (37). EGFR-TKI monotherapy also did not yield promising results in other phase II studies which did not incorporate careful patient selection (38). In addition, the results of phase II studies of the efficacy of initial treatment with EGFR-TKIs incorporating patient selection have also been reported. In a phase II study of gefitinib in 36 non-smokers with adenocarcinoma, the response rate was as high as 69%. The mPFS was 8.3 months and the estimated one-year survival rate was 73%, representing more favorable results as compared to the results of previously reported studies on standard chemotherapy (39). Furthermore, a phase II study on gefitinib as the initial chemotherapy was carried

**Table 2. Randomized Controlled Trial of EGFR-TKI vs Platinum Doublet in EGFR Mutation(+) Patients**

Trial	n	EGFR-TKI	Chemotherapy	Primary Endpoint	Results
NEJ002	320	gefitinib	CBDCA + PTX	PFS	Positive
WJOG3405	200	gefitinib	CDDP + DOC	PFS	Positive
EURTAC	146	erlotinib	platinum-doublet*	PFS	On going
ML20981	150	erlotinib	CBDCA + GEM	PFS	On going

\* GEM + CDDP, DOC + CDDP, GEM + CBDCA, DOC + CBDCA

EGFR-TKI: Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor

PFS: Progression-Free Survival

CBDCA: Carboplatin

PTX: Paclitaxel

CDDP: Cisplatin

DOC: Docetaxel

GEM: Gemcitabine

out in Japan, in 30 patients with NSCLC satisfying one of the following requirements: 1) EGFR gene mutation-positive elderly patients; and 2) patients with poor performance status (PS) who were not candidates for standard chemotherapy. In that study, the outcome was excellent, with a response rate of 66% and MST of 17.8 months, and number of treatment-associated deaths was zero (40).

A phase III study (Iressa Pan Asia Study: IPASS) was carried out in 10 East Asian countries including Japan to compare gefitinib therapy with carboplatin (CBDCA) + paclitaxel (PTX) therapy as the first-line treatment in patients with clinical factors (adenocarcinoma; non-smoker or light smoker) possibly associated with a high sensitivity to EGFR-TKIs. In this study, 1,217 patients were allocated randomly into two groups, and the response rate was significantly higher in the gefitinib group (43.0%) than in the CBDCA + PTX group (32.2%) ( $p=0.0001$ ). In the analysis of the PFS (the primary endpoint), the HR was 0.741 (95% CI: 0.651-0.845,  $p<0.0001$ ) and the outcome was significantly better in the gefitinib group. However, since the survival curves for the two groups crossed each other, the interpretation of the data was controversial. When the patients of this study were divided according to the presence/absence of EGFR gene mutation, the crossing of the survival curves disappeared, and the PFS was significantly longer in the gene mutation-positive group. Data on the patient overall survival in this study have not yet been reported because they are still premature (12). At present, four phase III studies comparing EGFR-TKIs with standard chemotherapy in EGFR gene mutation-positive patients are under way and preliminary results of two studies have been reported (Table 2) (41, 42). EGFR-TKIs are now viewed as useful alternatives for first-line chemotherapy in EGFR gene mutation-positive patients with NSCLC, although they have not been demonstrated to be superior to platinum-based therapy in overall survival.

#### b. Combined EGFR-TKI + chemotherapy

In regard to studies conducted to evaluate the significance

of combining EGFR-TKI with chemotherapy, the Iressa NSCLC Trial Assessing Combination Therapy (INTACT)-1 (43) and INTACT-2 (44) have been carried out using gefitinib as the EGFR-TKI. These studies, however, failed to endorse the significance of administering gefitinib in combination with chemotherapy. Similarly, phase III studies of erlotinib administered in combination with chemotherapy have been carried out, however, no enhancement of the efficacy with the use of erlotinib in combination with chemotherapy was demonstrated in either the Tarceva Lung Cancer Investigation (TALENT) study (45) or the Tarceva Responses in Conjunction with Taxol and Carboplatin (TRIBUTE) study (46) (Table 3). However, subgroup analysis of the data from the TRIBUTE study revealed a significant extension of the survival period in non-smokers following the addition of erlotinib to the chemotherapeutic regimen (MST: 22.5 months vs. 10.1 months,  $p=0.01$ ). Therefore, it would seem valuable to conduct similar studies on appropriately selected patients for further evaluation.

#### 4) Toxicity of EGFR-TKIs

The major toxicities of EGFR-TKIs are skin disorders (eruption, dry skin, pruritus, etc.), diarrhea, and liver dysfunction. Interstitial lung disease (ILD) is a toxicity that needs the greatest attention. The incidence of this adverse reaction is reported to be about 3.5-5% and some of the risk factors for its onset are advanced age, male gender, poor PS, positive smoking history and the presence of underlying interstitial disease (47, 48). When EGFR-TKIs are used, it is essential to take into account the risk of onset of ILD.

#### 5) Second-generation EGFR-TKIs

Recurrence of disease occasionally takes place within about 12 months after successful treatment with EGFR-TKIs (gefitinib, erlotinib, etc.) even in gene mutation-positive cases (49). This has been explained by the development of tumor resistance to EGFR-TKIs through the development of secondary EGFR gene mutations such as mutation of T790

**Table 3. Randomized Phase III Trial of Platinum Doublet ± EGFR-TKI**

Trials	n		Response Rat (%)	TTP (M)	MST (M)	p Value
INTACT-1	1,093	GP + gefitinib (500mg)	49.7	5.5	9.9	NS
		GP + gefitinib (250mg)	50.3	5.8	9.9	
		GP + placebo	44.8	6.0	10.9	
INTACT-2	1,037	PC + gefitinib (500mg)	30.0	4.6	8.7	NS
		PC + gefitinib (250mg)	30.4	5.3	9.8	
		PC + placebo	28.7	5.0	9.9	
TRIBUTE	1,059	PC + erlotinib (150mg)	21.5	5.1	10.6	NS
		PC + placebo	19.3	4.9	10.5	
TALENT	1,172	GP + erlotinib (150mg)	31.5	5.9	10.8	NS
		GP + placebo	29.9	6.2	11.0	

GP : GEM + CDDP, PC : PTX + CBDCA

EGFR-TKI: Epidermal Growth Factor Receptor-Tyrosine Kinase Inhibitor

TTP: Time To Progression

MST: Median Survival Time

M: Month

NS: Not Significant

GEM: Gemcitabine

CDDP: Cisplatin

PTX: Paclitaxel

CBDCA: Carboplatin

M (50, 51). For the treatment of such resistant cases, an irreversible EGFR inhibitor has been developed and clinical trials are now under way (52).

## 6) Anti-EGFR antibodies

Antibodies directed against EGFR that are used for therapy include cetuximab (a chimeric IgG1 antibody), matuzumab (a humanized IgG1 antibody), panitumumab (a completely humanized IgG2 antibody), etc. Clinical trials of these agents are now under way in patients with various cancers.

### a. Cetuximab

A phase II clinical study of cetuximab monotherapy in previously treated cases of NSCLC yielded a response rate of 4.5%. Toxicity was mild, but skin eruptions were seen in about 90% of all patients (grade 3/4 in 6.1%) (53).

A randomized phase II clinical study designed to evaluate the effects of the addition of cetuximab to CDDP + vinorelbine (VNR) therapy in 86 previously treated cases of NSCLC has been reported. The response rate, mPFS and MST were 35%, 5.0 months and 8.3 months, respectively, in the CDDP + VNR + cetuximab group, while they are 28%, 4.6 months and 7.3 months, respectively, in the CDDP + VNR group (54). In another randomized phase II clinical study (SWOG 0342) comparing a synchronous combined therapy (4 cycles of CBDCA + PTX therapy and simultaneously started cetuximab therapy for one-year) with a sequential combined therapy group (start of cetuximab therapy after completion of 4 cycles of CBDCA + PTX therapy), the response rate tended to be higher in the synchronous com-

bined therapy group (34% vs. 31%), while a PFS of 4 months and MST of 11 months was obtained in both groups (55).

A randomized phase III clinical study (BMS 099) was carried out to compare CBDCA + taxane (PTX or DOC) therapy with CBDCA + taxane + cetuximab therapy in 676 previously untreated patients with NSCLC. The response rate was 17.2% in the CBDCA + taxane group and 25.7% in the CBDCA + taxane + cetuximab group, while no significant difference was noted in the primary endpoint, that is, PFS between the two groups (4.24 months vs. 4.40 months,  $p=0.2358$ ) (56). Furthermore, a randomized phase III clinical study (FLEX) was carried out to compare CDDP + VNR therapy with CDDP + VNR + cetuximab therapy in 1,125 previously untreated patients with NSCLC showing positive EGFR expression. The response rate was significantly higher in the CDDP + VNR + cetuximab group (36%) than in the CDDP + VNR group (29%) ( $p=0.010$ ). No significant difference in the PFS was observed between the two groups (mPFS: 4.8 months in both groups), however, the MST (a primary endpoint) was extended in the group additionally receiving cetuximab (10.1 months vs. 11.3 months,  $p=0.044$ ) (57). In a subgroup analysis in the same study, the survival of Asian patients was poorer in the CDDP + VNR + cetuximab group (20.4 months vs. 17.6 months), probably because of the influence of EGFR-TKIs used for second-line and subsequent treatment.

Higher efficacy was obtained when cetuximab, an anti-EGFR antibody, was used in combination with chemotherapy than when it was used alone, unlike the findings ob-

**Table 4. Clinical Trials of Cetuximab**

Investigator	No of cases	Regimen	Response Rate (%)	PFS (M)	MST (M)	IHC
Rosell	44	CDDP + VNR	28	4.2	7.0	+
	43	CDDP + VNR + C225	35	4.8	8.3	+
Butts	66	Plat + GEM	18	4.2	9.3	—
	65	Plat + GEM + C225	28	5.1	12.0	—
Herbst	106	CBDCA + PTX + C225	34	4.0	11.0	—
	117	CBDCA + PTX ⇒ C225	31	4.0	11.0	—
Lynch	338	CBDCA + taxane	17	4.2	—	—
	338	CBDCA + taxane + C225	26	4.4	—	—
Pirker	568	CDDP + VNR	29	4.8	10.1	+
	557	CDDP + VNR + C225	36	4.8	11.3	+

PFS: Progression-Free Survival

MST: Median Survival Time

IHC : Immunohistochemistry

M : Month

CDDP: Cisplatin

PTX: Paclitaxel

tained for EGFR-TKIs (Table 4). A phase III clinical study designed to evaluate the effects of the addition of cetuximab to second-line chemotherapy (PEM or DOC) is now underway (58).

## 2. Anti-VEGF antibodies

### 1) Bevacizumab

Bevacizumab is a humanized monoclonal antibody directed against vascular endothelial growth factor (VEGF). VEGF is not only involved in neovascularization, but also enhances the vascular permeability (59). Bevacizumab binds to VEGF to inhibit the binding of VEGF to VEGF-R, thereby also inhibiting vascularization. In addition, this drug reduces the interstitial pressure within tumor cells through normalizing tumor vessels, possibly leading to improved delivery of cytotoxic anticancer agents to tumor cells and manifestation of synergistic effects when this antibody is used in combination with chemotherapy (60).

The Eastern Cooperative Oncology Group (ECOG) carried out a randomized phase II clinical study to compare three groups of patients with advanced or recurrent NSCLC, i.e., the CBDCA + PTX (CP) therapy group (control arm), the CBDCA + PTX + bevacizumab 7.5 mg/kg group (CPB 7.5 group) and the CBDCA + PTX + bevacizumab 15 mg/kg group (CPB 15 group). The primary endpoint of progression-free survival was significantly longer in the CPB 15 group.\* p=0.023 (control vs. CPB 7.5 group vs. CPB 15 group: 4.2 months\*\* vs. 4.3 months vs. 7.4 months\*), and the best response rate (18.8% vs. 28.1% vs. 31.5%) and overall survival (14.9 months vs. 11.6 months vs. 17.7 months) were also obtained in the CPB 15 group (61). However, severe hemoptysis was seen in 6 patients (9%) and 4 patients died following combined use of

bevacizumab with chemotherapy. Squamous cell carcinoma, presence of tumor necrosis and a central location of the tumor were identified as the factors associated with severe hemoptysis. In a subsequent randomized phase III clinical study (ECOG 4599) designed to compare CBDCA + PTX (CP group) with CBDCA + PTX + bevacizumab 15 mg/kg (CPB group), patients with squamous cell carcinoma and patients who had hemoptysis or brain metastasis were excluded from the subject population to reduce the incidence of severe adverse events. In this study, significantly better outcomes were obtained in the CPB group in terms of the response rate (15% vs. 35%, p<0.001), PFS (mPFS: 4.5 months vs. 6.2 months, p<0.001) and overall survival (MST: 10.3 months vs. 12.3 months, p=0.003) (62). This was the first study to demonstrate prolongation of the survival period in patients with NSCLC following administration of a molecule-targeted drug in combination with chemotherapy. On the basis of the results of this study, CPB therapy was adopted by ECOG as the new standard therapy for non-squamous cell carcinoma. However, despite exclusion of patients who were at a high risk for hemoptysis from the subject population, the incidence of grade 3 or more severe bleeding was still significantly higher in the CPB group (0.7% vs. 4.4%, p<0.001) and 7 patients from the CPB group died of bleeding (5 deaths from hemoptysis and 2 deaths from gastrointestinal bleeding). The non-hematological toxicities (grade 3/4) observed at a high incidence were hypertension (5.6%), proteinuria (4.2%), malaise (5.1%), and dyspnea (5.6%).

The results of a randomized phase III clinical study (AVAiL) in which 1,043 patients with NSCLC (excluding squamous cell carcinoma) with no prior history of chemotherapy were divided into three treatment groups, i.e., the GEM + CDDP therapy group (GC group), GEM + CDDP +

bevacizumab 7.5 mg/kg therapy group (GCB 7.5 mg/kg group), and the GEM + CDDP + bevacizumab 15 mg/kg therapy group (GCB 15 mg/kg group). The primary endpoint, PFS, was extended significantly by the addition of bevacizumab to the therapy (mPFS: 6.2 months vs. 6.8 months vs. 6.6 months,  $p=0.0003^*$ ) and the response rate was also higher in the GCB groups (20.1% vs. 34.1% vs. 30.4%) (63). However, the MST did not differ significantly between any two of the three groups (13.1 months vs. 13.6 months vs. 13.4 months,  $p=0.42^*$ ) (\*comparison between the GC group and GCB 7.5 mg/kg group) (64). The absence of significant inter-group differences in the overall survival period despite the finding of significant inter-group differences in the PFS was considered to be attributable to the chemotherapy administered for the second/subsequent-line treatment. During the ASCO meeting in 2009, the results of a randomized phase II clinical study in Japanese patients were reported. This study, which was designed to evaluate the safety and efficacy of two regimens (CPB vs. CP), similar to the evaluation in ECOG 4599, revealed favorable outcomes of CPB (65).

In addition, a study to evaluate the safety of initial treatment with a combination of standard chemotherapy and bevacizumab (7.5 or 15 mg/kg) was carried out (SAiL study) in 2,240 patients with advanced NSCLC. The incidence of severe adverse events associated with bevacizumab was 23.5%, however, the incidence of grade 3-5 bleeding in the central nervous system was 0.4% and that of hypertension was 0.7%. Thus, the therapy could be administered relatively safely (66).

A randomized phase II clinical study was carried out in patients with recurrent or therapy-resistant NSCLC (other than squamous cell carcinoma) allocated to one of the three following treatment arms, Arm 1: chemotherapy (DOC or pemetrexed [PEM]) + placebo, Arm 2: chemotherapy (DOC or PEM) + bevacizumab, and Arm 3: erlotinib + bevacizumab. The response rate, mPFS and MST were 12.2%, 3.0 months and 8.6 months, respectively, in Arm 1, 12.5%, 4.8 months and 12.6 months, respectively, in Arm 2, and 17.9%, 4.4 months and 13.7 months, respectively, in Arm 3. Thus, the regimens containing bevacizumab tended to yield better outcomes. The incidence of severe toxicities was the lowest in the erlotinib + bevacizumab group (67). On the basis of these results, a randomized phase III clinical study (BETA Lung) was carried out, comparing erlotinib + placebo therapy (E+P group) with erlotinib + bevacizumab therapy (E+B group) in 636 patients with recurrent NSCLC. The response rate (6.2% vs. 12.6%,  $p=0.006$ ) and PFS (mPFS: 1.7 months vs. 3.4 months,  $p<0.0001$ ) were significantly better in the E+B group, while the overall survival period (MST: 9.2 months vs. 9.3 months,  $p=0.7583$ ) did not differ significantly between the two groups. As for the reason why the significant inter-group difference in the PFS was not reflected in the overall survival period, it was pointed out that tertiary treatment had been administered to 60% or more of all patients in each group and quaternary and subsequent

treatment had also been administered in a considerable number of the patients. In the analysis of toxicity, the incidence of skin eruptions and thrombosis was higher in the E+B group, but it did not differ from the previously reported rate (68).

At the ASCO meeting in 2009, the results of a phase III clinical study (ATLAS) designed to compare bevacizumab monotherapy with bevacizumab doublet + erlotinib therapy, both administered as maintenance therapy after platinum + bevacizumab therapy in previously untreated cases of NSCLC (other than squamous cell carcinoma), were reported. In terms of the primary endpoint of PFS, the results in the combined therapy were superior to those in the monotherapy group (69). Some of the studies now under way include a phase III clinical study (ECOG 1505) designed to evaluate the effect of addition of bevacizumab to postoperative adjuvant therapy in completely resected cases of NSCLC and a phase III clinical study (SABRE-L) designed to evaluate the effect of the addition of sunitinib to CBDCA + PTX + bevacizumab therapy administered as initial chemotherapy.

### 3. VEGFR tyrosine kinase inhibitors

#### 1) Vandetanib (ZD6474)

Vandetanib is a multi-targeted tyrosine kinase inhibitor capable of inhibiting both VEGFR and EGFR at the same time. In a randomized phase II clinical study comparing DOC monotherapy with DOC + vandetanib therapy (100 or 300 mg/day) in previously treated cases of NSCLC, significant prolongation of the PFS was observed in the combined treatment group given vandetanib (100 mg/day) (mPFS: 12 weeks in the DOC monotherapy group vs. 18.7 weeks in the DOC + vandetanib 100 mg/day group,  $p=0.037$ , one-tailed; vs. 17.0 weeks in the DOC + vandetanib 300 mg/day group,  $p=0.231$ , one-tailed) (70). On the basis of these results, a phase III clinical study (ZODIAC) comparing DOC + placebo therapy with DOC + vandetanib (100 mg/day) therapy was carried out, and a significant difference in the PFS (primary endpoint) between the two groups was reported during the ASCO meeting in 2009 (71). In a phase II study comparing vandetanib with gefitinib, the PFS (primary endpoint) was significantly longer in the vandetanib group (11.0 weeks vs. 8.1 weeks,  $p=0.025$ ) (72). In an early phase II dose-determination study conducted in Japan, in which the drug was administered at three dose levels (100, 200 and 300 mg/day), the response rates in the three dosage groups were 17.6%, 5.6% and 16.7%, respectively (73). In a randomized phase II clinical study comparing CBDCA + PTX therapy (PC group) with CBDCA + PTX + vandetanib therapy (VPC group), with both administered as the initial chemotherapy, the response rate and mPFS were 25% and 23 weeks, in the PC group, and 32% and 24 weeks, respectively, in the VPC group (74).

During the meeting of ASCO in 2009, the results of a phase III clinical study (ZEAL) comparing vandetanib with

erlotinib administered as the second-line chemotherapy (75) and a phase III clinical study of PEM ± vandetanib were reported. Neither of these studies revealed superiority of vandetanib over the reference drug in terms of the PFS (primary endpoint) (76). A phase III clinical study (ZEPHYR) comparing this drug with placebo in patients with NSCLC treated previously with EGFR inhibitors also showed negative results.

## 2) Sorafenib

Sorafenib serves as a tyrosine kinase inhibitor for Raf-kinase, VEGFR-2, VEGFR-3, PDGFR-B, Flt-3 and c-kit (77). A phase II clinical study of sorafenib monotherapy in previously untreated cases of NSCLC was started, but it was discontinued when only 25 cases had been registered, because of the poor responses (78). In this study, the response rate, mPFS and MST were 12%, 2.9 months and 8.8 months, respectively. A phase II clinical study of sorafenib monotherapy was also performed in 52 cases of recurrent NSCLC, which yielded tumor reduction in 29% of all cases, and the mPFS and MST of 11.9 weeks and 29.3 weeks, respectively. As grade 3 or more severe toxicities, the hand-foot syndrome (10%) and hypertension (4%) were noted (79). A randomized phase II clinical study was carried out in 83 patients with NSCLC with a history of having received two or more regimens of chemotherapy before. These 83 patients were initially treated with sorafenib and later with either with placebo (placebo group) or sorafenib (continued sorafenib therapy group). The percentage of patients rated as showing SD or a better outcome at 2 months (primary endpoint) was 19% in the placebo group and 47% in the continued sorafenib therapy group, indicating the significantly better outcome in the continued sorafenib therapy group ( $p=0.01$ ). Significant difference in the PFS was also found (mPFS: 2.0 months vs. 3.6 months,  $p=0.009$ ), however, there was no significant difference in the overall survival between the two groups (MST: 9.0 months vs. 11.9 months,  $p=0.18$ ) (80).

In addition, a phase III clinical study (ESCAPE) comparing PTX + CBDCA + placebo therapy with PTX + CBDCA + sorafenib therapy in 926 previously untreated cases of NSCLC was carried out, which yielded no significant intergroup difference in the response rate (23% vs. 25%), PFS (mPFS: 4.8 months vs. 4.8 months,  $p=0.92$ ) or overall survival (MST: 10.6 months vs. 10.7 months,  $p=0.93$ ) between the two treatment groups (81). At present, a phase III clinical study designed to evaluate the effect of addition of sorafenib to GEM + CDDP therapy is underway.

## 3) Sunitinib

Sunitinib is a multi-targeted tyrosine kinase inhibitor for VEGFR-1, -2 and -3, PDGFR- $\alpha$  and - $\beta$ , KIT, RET, CSF-1R and Flt-3. A phase II clinical study of sunitinib 50 mg/day (oral treatment for 4 weeks, followed by drug cessation for 2 weeks) was carried out in 63 previously treated cases of NSCLC. In this study, 22% of all patients required dose re-

duction. The major toxicities observed were malaise, myalgia, nausea and hypertension. The response rate, median time to progression (mTTP) and MST were 11.1%, 12.0 weeks and 23.4 weeks, respectively (82). On the basis of these results, a phase II clinical study was conducted to evaluate the effect of daily treatment with sunitinib (37.5 mg/day) in 47 previously treated cases of NSCLC. In this study, dose reduction was needed in 29.8% of all the patients. The response rate, mPFS and MST were 2.1%, 12.3 weeks and 37.1 weeks, respectively. These results suggest that this drug may be a promising agent for the treatment of recurrent NSCLC. The major toxicities were malaise, dyspnea and hypertension. Grade 3/4 hemoptysis was noted in 2% of all the patients (83).

In addition, several clinical studies designed to evaluate the efficacy of sunitinib combined with other therapies (chemotherapy including platinum preparations, single chemotherapy or EGFR-TKI) have been carried out. A phase III clinical study comparing erlotinib monotherapy with erlotinib + sunitinib therapy in previously treated cases of NSCLC is now underway (84).

## 4) Cediranib (AZD2171)

Cediranib is a tyrosine kinase inhibitor for VEGFR. A randomized double-blind phase II/III study (BR.24) was carried out, comparing CBDCA + PTX + cediranib therapy with CBDCA + PTX + placebo therapy in patients with advanced NSCLC. In this study, 150 patients were allocated to the CBDCA + PTX + placebo group (CPP group) or the CBDCA + PTX + cediranib 30 mg/day group (CPC group). The response rate was significantly higher in the CPC group (38%) than in the CPP group (16%) ( $p<0.001$ ), but the PFS did not differ significantly between the two groups (mPFS: 5.0 months vs. 5.6 months,  $p=0.13$ ). In the analysis of toxicity, the incidence of diarrhea, dehydration, mucositis, hand-foot syndrome, hypertension and decreased blood neutrophil count was higher in the CPC group. A clinical study on cediranib administered at a lower dose level (20 mg) is now planned (85).

## 4. Other molecule-targeted drugs

### 1) Bexarotene

Retinoids play an important role in the growth, division and differentiation of cells and the activation of cell apoptosis. Bexarotene is considered to exert antitumor activity through its selective actions on the retinoid X receptor (86).

A phase I/II clinical study on bexarotene combined with VNR + CDDP as the initial chemotherapy for NSCLC was carried out. In this study, the maximum-tolerated dose (MTD) was 400 mg/m<sup>2</sup>/day. In the phase II trial, the response rate and MST were 25% and 14 months, respectively (87). In a phase II clinical study of bexarotene + GEM + CBDCA in 47 previously untreated cases of NSCLC, the response rate, MST and one-year survival rate were 25%, 12.7 months and 53%, respectively. In the analy-



sis of toxicity in the same study, all of the adverse reactions other than hypertriglyceridemia were tolerable (88). On the basis of these results, two randomized phase III studies were carried out in previously untreated cases of NSCLC to evaluate the effect of the addition of bexarotene to platinum-based chemotherapy. In one of these studies (SPIRIT I), 623 patients were allocated to either the VNR + CDDP group (VP group) or the VNR + CDDP + bexarotene group (VPB group). The response rate was significantly higher in the VP group (24.4% vs. 16.7%,  $p=0.0224$ ), and the VP group also tended to have a better outcome in terms of the PFS (mPFS: 5.0 months vs. 4.3 months,  $p=0.095$ ) and overall survival (MST: 9.9 months vs. 8.7 months,  $p=0.3$ ). In the analysis of toxicity, the incidences of hypertriglyceridemia and hypothyroidism were higher in the group treated with bexarotene (89). In a second study (SPIRIT II), 612 patients were allocated to either the CBDCA + PTX group (CP group) or the CBDCA + PTX + bexarotene group (CPB group). The outcomes tended to be better in the CP group, in terms of the response rate (23.5% vs. 19.3%,  $p=0.24$ ), PFS (mPFS: 4.9 months vs. 4.1 months,  $p=0.061$ ) and the overall survival (MST: 9.2 months vs. 8.5 months,  $p=0.2$ ) (90). Neither of the two studies demonstrated that the use of bexarotene in combination with platinum-based chemotherapy augmented the effects of platinum-based chemotherapy.

## 2) Figitumumab (CP-751,871)

Figitumumab is a completely humanized IgG2 type monoclonal antibody directed against insulin-like growth factor I (IGF-I) receptor. A randomized phase II clinical study was carried out comparing CBDCA + PTX therapy (TC group) with figitumumab + CBDCA + PTX therapy (TCI group) in 150 previously untreated cases of advanced NSCLC. The response rate was 41% in the TC group and 54% in the TCI group. The response rate was higher among patients with squamous cell carcinoma (46% vs. 78%), suggesting that the addition of anti-IGF-IR antibody is likely effective in patients with squamous cell carcinoma (91). In the analysis of toxicity, the incidences of hyperglycemia and dehydration were higher in the TCI group. Figitumumab may thus be a promising agent for the treatment of squamous cell carcinoma of the lung.

A randomized phase III clinical study comparing TC with TCI in previously untreated cases of NSCLC and a randomized phase III clinical study comparing figitumumab therapy with erlotinib + figitumumab therapy for recurrent NSCLC are now underway. There are also ongoing clinical studies on several other products of anti-IGF-IR antibody, such as R 1507, and the results of these studies are awaited.

## 3) ASA404

ASA404 is an agent causing vascular destruction and has been reported to induce irreversible tumor vessel destruction, hemorrhagic necrosis at the center of the tumor, and the production of cytokines. This drug is considered to induce tumor necrosis through its actions on existing blood vessels

rather than on the newly formed blood vessels (92-95). Its target molecules remain unidentified. A randomized phase II clinical study was carried out in 76 previously untreated cases of advanced NSCLC, comparing ASA404 + CBDCA + PTX therapy (ASA404-CP group) with CBDCA + PTX therapy (CP group). The outcomes were better in the ASA404-CP group in terms of the response rate (31% vs. 22%), mTTP (5.4 months vs. 4.4 months) and MST (14.0 vs. 8.8 months). In the analysis of toxicity, no differences were noted between the two groups (95). On the basis of these results, a randomized phase III clinical study is now underway.

## Conclusion

In recent years, the development of molecular-targeted drugs has progressed remarkably, and numerous clinical studies have been carried out on molecular-targeted drugs for the treatment of NSCLC. In this paper, the results obtained to date have been presented, focusing on drugs for which phase III clinical studies have been carried out. In parallel with clinical studies, studies exploring biomarkers have also been carried out. It is essential to develop biomarkers to serve as predictors of the responses to treatment with molecular-targeted drugs, like EGFR gene mutations serving as a predictor of the response to EGFR tyrosine kinase inhibitors. The outcomes of NSCLC treatment will improve if the appropriate therapeutic strategies are applied to appropriately selected patients on the basis of clinical factors (histological type, etc.) and biomarkers found in the tumor tissues and serum.

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# Hypertrophic Pulmonary Osteoarthropathy as a Paraneoplastic Manifestation of Lung Cancer

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**Introduction:** This study examined the frequency of hypertrophic pulmonary osteoarthropathy (HPO) and the clinical characteristics of lung cancer with HPO.

**Methods:** The results of 2625 lung cancer patients who underwent bone scintigraphy were reviewed to identify patients with HPO, which was diagnosed when the bone scintigram showed a diffuse, symmetric pattern of bilateral increased uptake in the long tubular bones. Clinical characteristics were investigated based on the clinical and pathologic records.

**Results:** Nineteen patients (0.72%) were found to have HPO: 17 were men, 17 were heavy smokers, and 13 had clinical stage IIIB or IV disease. Ten patients complained of pain or edema in the extremities, and seven of them had stage IIIB or IV disease. In four patients with clinical stage IIIB or IV disease, HPO was not detected at the first presentation, and the diagnosis was made after disease progression. The symptoms of HPO improved in two patients who underwent surgical resection but in only three of five patients who received chemotherapy. The HPO findings on the bone scintigram improved in 2 of 3 patients who underwent surgical resection and 5 of 11 patients who received chemotherapy.

**Conclusions:** Less than 1% of the lung cancer patients developed HPO as a paraneoplastic manifestation. Males, heavy smokers, and advanced disease predominated in lung cancer patients with HPO. The symptoms and bone scintigram findings of HPO improved in half of the patients on treating the lung cancer.

**Key Words:** Hypertrophic pulmonary osteoarthropathy, Lung cancer, Bone scintigraphy, Surgical resection, Chemotherapy.

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Hypertrophic osteoarthropathy (HOA)<sup>1</sup> is manifest clinically by clubbed fingers, periostitis of the long tubular bones, and arthritis. It is classified into primary and secondary forms, with the latter accounting for 95% of cases. In 1889, Bamberger described two bronchiectasis patients with HOA, and the following year, Marie reported a case of pulmonary tuberculosis with HOA and coined the term “hypertrophic pulmonary osteoarthropathy” (HPO). HPO is now defined as HOA secondary to pulmonary disease (e.g., primary lung cancer). In approximately 90% of cases, HPO is associated with intrathoracic neoplasm and 80% of these are lung cancers.<sup>2</sup> According to various reports, 0.2 to 17% of lung cancer patients develop HPO<sup>2–10</sup>; however, because of its low incidence, the clinicopathologic characteristics of lung cancer with HPO have not been examined.

Classically, HPO is diagnosed based on clinical symptoms (unremitting pain, edema, and erythema in the extremities) and radiographic findings. Periostitis is the hallmark of HPO, and bone radiography reveals periosteal membrane thickening and periosteal new bone formation. Bone scintigraphy is a useful, widespread medical imaging procedure that enables the detection of HPO. The scintigrams show bracelet-like or diffusely increased uptake throughout most of the skeleton.

This study determined the incidence of HPO and clinical characteristics of lung cancer patients with HPO based on a review of a large population of lung cancer patients.

## PATIENTS AND METHODS

Between November 2000 and April 2006, 2625 lung cancer patients underwent bone scintigraphy at the National Cancer Center Hospital East, Chiba, Japan; 652 patients (25%) were females and 1973 (75%) were males. Histopathologically, the cancer was adenocarcinoma in 1328 patients (50.6%), squamous cell carcinoma in 590 patients (22.5%), small cell carcinoma in 363 patients (13.8%), large cell carcinoma in 64 patients (2.4%), pleomorphic carcinoma in 25 patients (0.95%), adenosquamous carcinoma in 11 patients (0.4%), carcinoid tumor in 8 patients (0.3%), adenoid cystic carcinoma in 1 patient, and mucoepidermoid carcinoma in 1 patient. Non-small cell lung carcinoma was diagnosed in 234 patients (9.2%) when there was no evidence of small cell carcinoma or specific evidence of differentiation into adenocarcinoma or squamous cell carcinoma in biopsy specimens.

All the scintigrams were reviewed to identify cases complicated by HPO. All of the bone scintigrams had been performed with 740 mBq Tc-99m-hydroxymethylene diphosphate, and static imaging was performed at 3 to 6 hours. HPO was diagnosed by experienced radiologists based on a diffuse, symmetric pattern of bilateral increased uptake in the long tubular bones on the bone scintigrams. The clinical characteristics were investigated based on available clinical and pathologic records. For patients receiving chemotherapy, the efficacy was evaluated based on the "Response Evaluation Criteria in Solid Tumors" (RECIST) guidelines.

Survival rates were calculated using the Kaplan-Meier method using the SPSS software for Windows (SPSS Inc., Chicago, IL).

## RESULTS

HPO was identified in 19 (0.72%) of the primary lung cancer patients; their clinical characteristics are summarized in Table 1. Their median age was 63 years (range, 46–76 years); there were 17 men and 2 women. Eighteen patients had a smoking history and 17 were heavy smokers (>30 pack-years). Two patients had performance status of 0 and 17 had performance status of 1.

Histopathologically, for HPO secondary to lung cancer, the cancer was adenocarcinoma in 10 patients, squamous cell carcinoma in 4 patients, adenosquamous carcinoma in 1

patient, large cell carcinoma in 1 patient, and 2 patients were diagnosed with non-small cell lung carcinoma. Only one patient had small cell carcinoma.

The clinical stage was IIIB in five patients and IV in eight patients.

Ten patients complained of pain or edema of the extremities in their clinical course, and seven of them had clinical stage IIIB or IV disease. HPO was diagnosed based on bone scintigraphy findings at the time of diagnosis of the lung cancer in 15 patients, whereas in 4 patients with clinical stage IIIB or IV, evidence of HPO emerged during disease progression.

Five patients underwent complete surgical resection of their lung cancer, and two of them had complained of edema or pain of extremities caused by HPO before surgery. Postoperatively, their symptoms resolved completely. In three patients, bone scintigraphy was repeated postoperatively, and the HPO findings improved in two of them (Figure 1).

Thirteen patients received systemic chemotherapy for their lung cancer. Five of them complained of HPO symptoms before chemotherapy, and remission of the symptoms was achieved in three (60%) of them. Regarding tumor shrinkage, according to the RECIST criteria, a partial response was achieved in four patients (31%). Eleven patients underwent bone scintigraphy after systemic chemotherapy, and the HPO findings on the bone scan improved in five (45%). Only one patient with a partial response simultaneously showed improvement of the bone scintigraphy findings and HPO (Figure 2).

The median follow-up time of the surgically resected group and other patients was 1462 and 332 days, respectively. No cancer death was observed among the surgically resected group at that time. The 1-year overall survival rate of the nonresected patients was 71% and their median survival time was 554 days.

## DISCUSSION

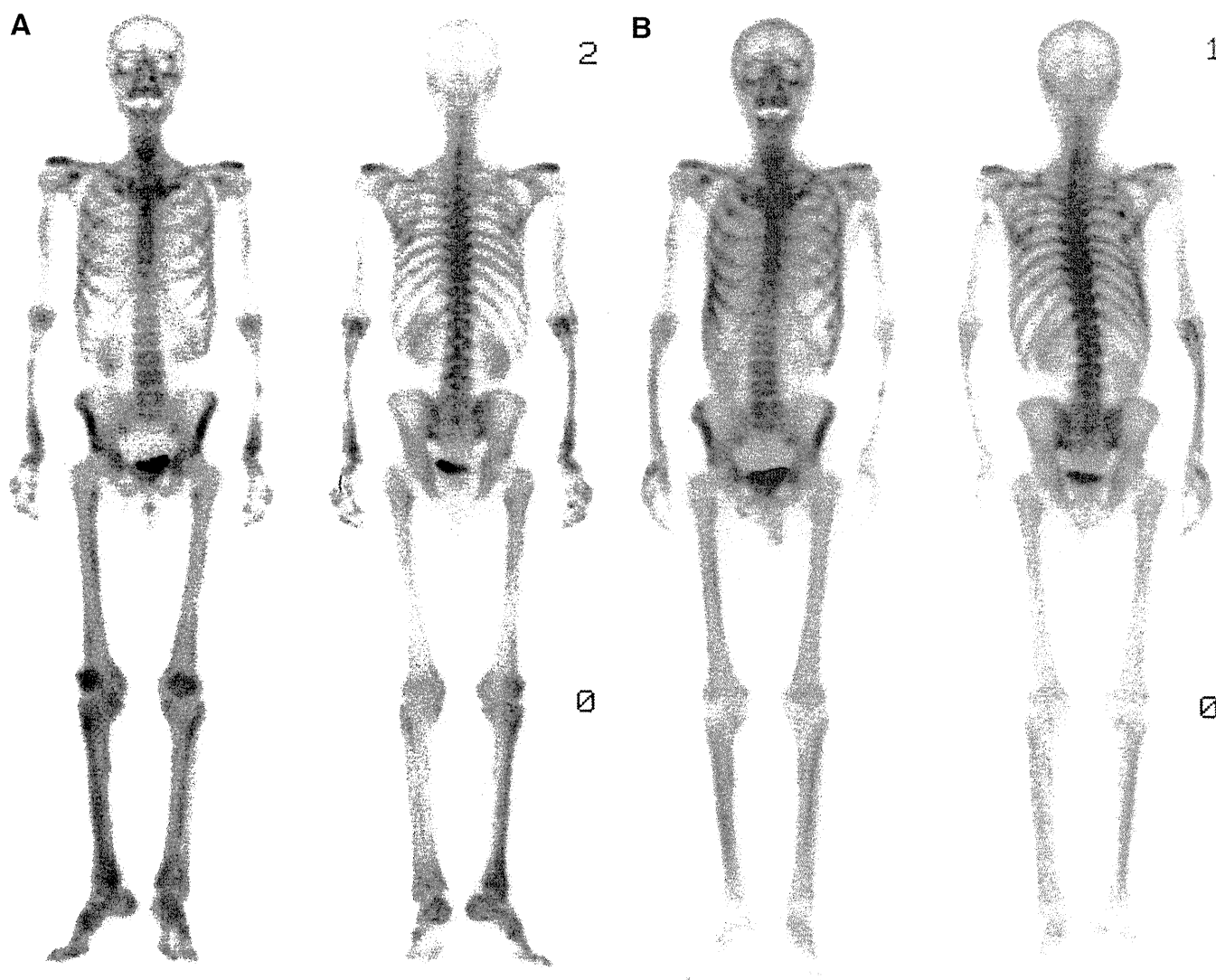
To our knowledge, this is the largest reported study of HPO secondary to lung cancer since the introduction of the clinical use of bone scintigraphy. Incidences of HPO of 0.2 to 17%<sup>2–10</sup> in lung cancer patients have been reported (Table 2). In the two studies that investigated more than 1000 cases of lung cancer, the incidence was less than 1%, similar to the rate of this complication in our study.

Most of the patients with HPO in our study were men (89%), consistent with the results of previous studies (67–79%; Table 2), and the proportions of current or ex-smokers (95%) and heavy smokers (89%) were very high. Segal and Mackenzie<sup>5</sup> described the smoking history of lung cancer patients with HPO and reported that 10 patients (63%) in their series were heavy smokers (Table 2), similar to our findings. Histopathologically, adenocarcinoma predominated (10 patients, 0.75% of all adenocarcinoma patients, and 53% of all lung cancer patients with HPO), squamous cell carcinoma was second (4 patients, 0.67% of all squamous cell carcinoma patients, and 21% of all lung cancer patients with HPO), and there was only one case of small cell carcinoma (0.27% of all

**TABLE 1.** Clinical Characteristics of Lung Cancer Patients with HPO

Total	19
Age	
Median (range)	63 (46–76)
Gender	
Male	17
Female	2
Smoking history	
Current or ex-smoker	18
Never smoker	1
ECOG performance status	
0	2
1	17
Histology	
Adenocarcinoma	10
Squamous cell carcinoma	4
Adenosquamous carcinoma	1
Large cell carcinoma	1
Non-small cell carcinoma	2
Small cell carcinoma	1
Stage	
IA	1
IB	3
IIB	1
IIIA	1
IIIB	5
IV	8

HPO, hypertrophic pulmonary osteoarthropathy; ECOG, Eastern Cooperative Oncology Group.



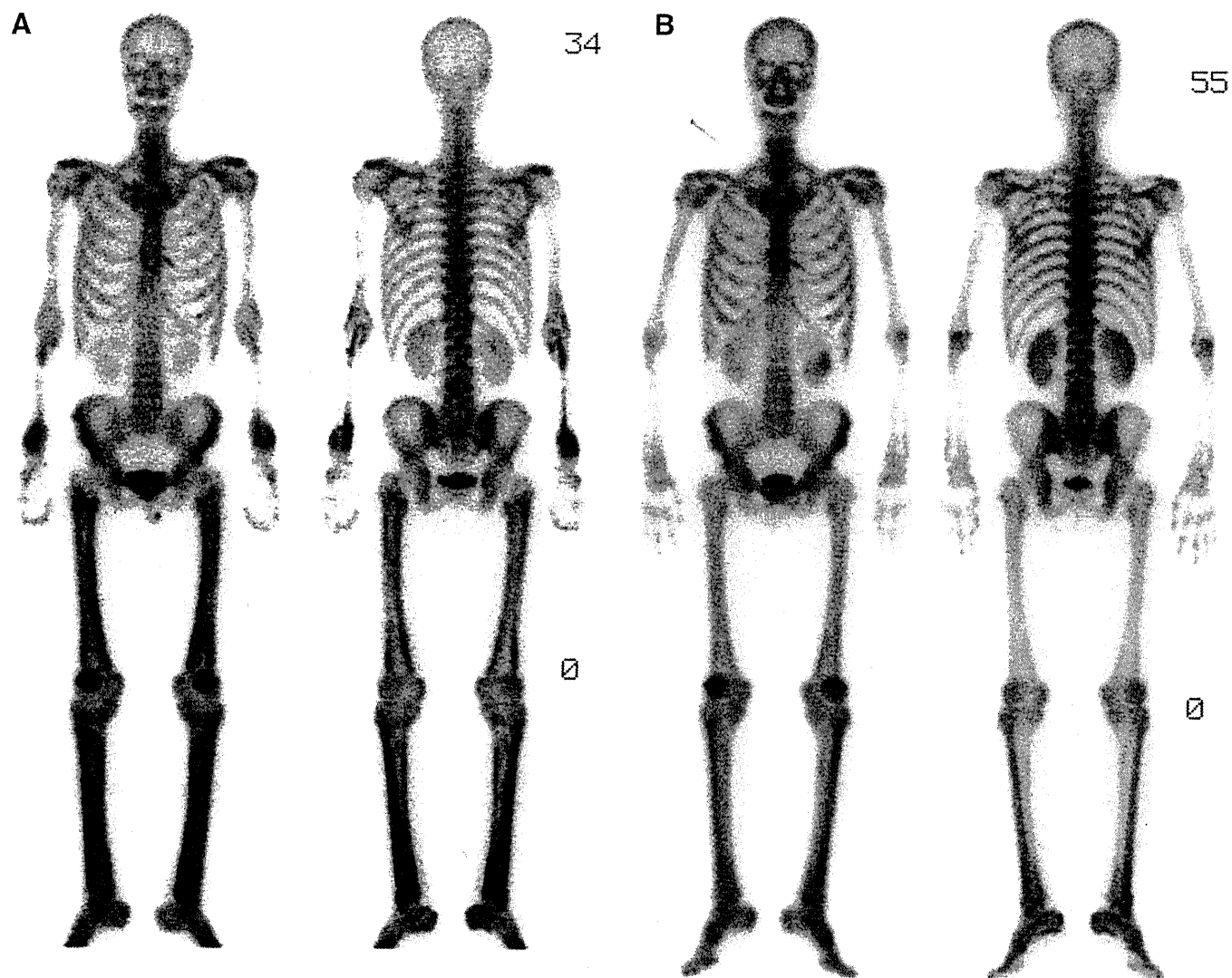
**FIGURE 1.** Bone scan of a lung cancer patient with HPO who underwent complete surgical resection of the lung cancer. The pathologic stage of the resected tumor was T2aN0M0, stage IB. *A*, Scan before the operation, performed on August 27, 2002. Note the bilateral localized uptake of the radionuclide at the wrist, femur, tibia, fibula, knee, and ankle. Abnormal uptake is observed along the cortical margins of the femur or tibia. *B*, Scan after surgery, performed on February 2, 2004. The abnormal uptake decreased, compared with the preoperative scan. The operation was performed on November 11, 2002.

small cell carcinoma patients and 5.2% of all lung cancer patients with HPO). Regarding adenocarcinoma and squamous cell carcinoma, the prevalence of secondary HPO was similar in our study. In published reports, adenocarcinoma accounted for 11 to 50% of lung cancer patients with HPO (Table 2), whereas squamous cell carcinoma accounted for 25 to 50%, a higher rate than in our series. The percentages of patients with small cell carcinoma were very low (0–14%), consistent with our results. Patients with clinical stage IIIIB or IV (68%) disease predominated in our study, and the patients treated surgically accounted for only a small proportion of our series (Table 2).

In four patients in our study, the HPO manifested as the lung cancer progressed, and two case reports described a similar phenomenon.<sup>8,11</sup> In our series, the HPO symptoms

improved promptly after surgical resection of the lung cancer, and many reports have described that surgical resection frequently leads to immediate marked remission of joint symptoms and abnormal bone scintigram findings of HPO secondary to lung cancer. Kroon and Pauwels<sup>6</sup> found that the symptoms and findings of HPO on the bone scintigram improved in parallel in a patient in whom bone scintigraphy was performed before and after surgical resection of the lung cancer.

Similar to surgical treatment, systemic chemotherapy improved both the symptoms and bone scintigram findings of HPO. Kroon and Pauwels,<sup>6</sup> Uchisako et al.,<sup>12</sup> and Albrecht and Keller<sup>13</sup> described improvement in the HPO after chemotherapy or chemoradiotherapy, and our results were consistent with their observations.



**FIGURE 2.** Bone scan of a lung cancer patient with HPO who underwent chemotherapy. The clinical stage was T2bN2M1b, stage IV. The clinical response to treatment with chemotherapy was stable disease. *A*, Scan before chemotherapy, performed on January 5, 2006. Marked abnormal diffuse, strong uptake is seen in the lower extremities. Abnormal uptake is seen in both elbows and wrists. *B*, Scan after the second course of chemotherapy, performed on May 24, 2006. The abnormal uptake decreased.

**TABLE 2.** Comparison of Characteristics of Lung Cancer Patients with HPO

Author	Incidence HPO/Total (%)	Gender Male (%)	SI (Pack-Years)		Histology				Clinical Stage	
			<30 (%)	>30 (%)	Ad (%)	Sq (%)	Sm (%)	Others (%)	IA–IIIA (%)	IIIB–IV (%)
Ray and Fisher <sup>3</sup>	13/149 (8.7)	NM	NM	NM	3 (23)	8 (62)	0 (0)	3 (21)	NM	NM
Honma <sup>4</sup>	7/128 (5)	NM	NM	NM	NM	NM	NM	NM	NM	NM
Segal and Mackenzie <sup>5</sup>	16/1920 (0.8)	11 (69)	6 (38)	10 (63)	6 (38)	4 (25)	0 (0)	6 (38)	NM	NM
Kroon and Pauwels <sup>6</sup>	4/100 (4)	3 (75)	NM	NM	2 (50)	2 (50)	0 (0)	0 (0)	NM	NM
Katsuyama et al. <sup>7</sup>	3/500 (0.6)	NM	NM	NM	NM	NM	NM	NM	NM	NM
Hirakata and Kitamura <sup>8</sup>	3/1364 (0.2)	2 (67)	NM	NM	1 (33)	1 (33)	0 (0)	1 (33)	1 (33)	2 (67)
Morgan et al. <sup>9</sup>	28/164 (17)	22 (79)	NM	NM	3 (11)	12 (43)	4 (14)	9 (32)	NM	NM
Sato et al. <sup>10</sup>	2/274 (0.7)	NM	NM	NM	NM	NM	NM	NM	NM	NM
This study	19/2625 (0.7)	17 (89)	1 (5)	17 (89)	10 (53)	4 (21)	1 (5)	4 (21)	6 (32)	13 (68)

HPO, hypertrophic pulmonary osteoarthropathy; SI, smoking index; Ad, adenocarcinoma; Sq, squamous cell carcinoma; Sm, small cell carcinoma; NM, not mentioned.



The pathogenesis of HPO is not fully understood. There are three major hypotheses for the pathogenesis of HPO secondary to lung cancer: the mechanical, biochemical, and neurogenic hypotheses. The mechanical hypothesis is based on pulmonary arteriovenous shunting within the tumor. When arteriovenous shunting is established in the lung, vasoactive compounds in the pulmonary circulation are not deactivated, and megakaryocytes escape the filtering effect of the pulmonary capillaries and stimulate the production of platelet-derived growth factor or tissue growth factor by the endothelial cells of the systemic peripheral capillaries. These substances induce a periosteal reaction. The only experimental model of HOA in the dog was established by creating a pulmonary venoarterial shunt.<sup>14</sup> The biochemical hypothesis states that biochemical compounds are produced and released by the lung cancer itself, and elevated serum growth hormone, growth hormone releasing hormone, vascular endothelial growth factor, and gonadotropins levels have been reported in some lung cancer patients with HPO.<sup>11,12,15-17</sup> The neurogenic hypothesis is based on the experimental and clinical observation that unilateral vagotomy improves HPO symptoms.<sup>14</sup> Further study is needed to clarify the pathogenesis of HPO secondary to lung cancer. A proteomics analysis of lung cancer patients with HPO is in progress at the National Cancer Center Hospital East.

In conclusion, HPO is a rare paraneoplastic manifestation of lung cancer, occurring in less than 1% of lung cancer patients. Males, heavy smokers, adenocarcinomas, and advanced disease predominated in lung cancer patients complicated by HPO. In half of the patients, the symptoms and bone scintigram findings of HPO improved on treating the lung cancer. Occasionally, patients develop manifestations of HPO after progression of the lung cancer.

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## Relationship of mRNA expressions of RanBP2 and topoisomerase II isoforms to cytotoxicity of amrubicin in human lung cancer cell lines

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### Abstract

**Purpose** RanBP2 is a small ubiquitin-like modifier ligase for DNA topoisomerase II (TopoII) and plays a role in maintaining chromosome stability by recruiting TopoII to centromeres during mitosis. Engineered-mice with low amounts of RanBP2 have been reported to form lung adenocarcinomas. Furthermore, in the murine embryonic fibroblasts, formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by chemical inhibition of TopoII, has been reported. In this study, we tested whether the association between mRNA expression of the RanBP2 gene and chemosensitivity of a TopoII inhibitor, amrubicin could be seen.

**Methods** Using a panel of 20 lung cancer cell lines, the mRNA expression levels of the RanBP2, TopoII-alpha and TopoII-beta genes were examined by quantitative real-time reverse transcription PCR. The in vitro cytotoxicity of amrubicin was assessed using a tetrazolium-based colorimetric assay (MTT assay).

**Results** Although RanBP2 mRNA expression was infrequently downregulated in human lung cancer cell lines, significantly higher RanBP2 transcripts were observed in small cell lung cancer than non-small cell lung cancer. There were no correlations between chemosensitivity of amrubicin and mRNA expression levels of the RanBP2, TopoII-alpha and TopoII-beta genes.

**Conclusions** Our in vitro results suggest that mRNA expressions of RanBP2 and TopoII isoforms are unlikely to be a predictive biomarker for the sensitivity to amrubicin.

**Keywords** SUMO ligase · Topoisomerase II inhibitor · Predictive biomarker · Chromosomal instability · Lung cancer

### Introduction

Lung cancer is a leading cause of cancer mortality in the United States and in Japan [13, 29]. Lung cancer has two main types: small cell lung cancer (SCLC) and non-SCLC (NSCLC). The major histological subtypes of NSCLC include adenocarcinoma, squamous carcinoma and large cell carcinoma. About 15% of lung cancers are SCLC. SCLC spreads rapidly and widely forming additional large tumors in lymph nodes, bones, adrenal glands, liver and brain. Because of its aggressive nature the overall survival of SCLC is worse than that of NSCLC and only 5–10% at 5 years. Survival of patients with either SCLC or NSCLC is strongly correlated with the stage of disease. For patients with advanced tumors, the prognosis is dismal because the available treatment regimens such as chemotherapy and radiation therapy are essentially palliative and primarily serve only to prolong survival. In fact, combination chemotherapy with etoposide plus cisplatin or irinotecan plus cisplatin for extensive-stage (ES) SCLC as well as the common first-line platinum-based combination regimens for advanced NSCLC only produced a median survival time of about 1 year [18, 19, 23]. Thus, new treatment approaches are clearly required.

Amrubicin, developed and approved in Japan for the treatment of SCLC and NSCLC, is a totally synthetic

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anthracycline anticancer agent and a potent TopoII inhibitor [14]. Amrubicin monotherapy with 45 mg/(m<sup>2</sup> day) for 3 consecutive days by intravenous administration produced response rates of 75.8 and 27.9% for previously untreated patients with ES-SCLC and advanced NSCLC, respectively. A phase II study of the combination of 60 mg/m<sup>2</sup> cisplatin and 40 mg/(m<sup>2</sup> day) amrubicin for 3 days has been reported to show response rate of 87.8%, the MST of 13.6 months and 1-year survival rate of 56.1% against ES-SCLC. Based on this result, Japan Clinical Oncology Group (JCOG) is conducting a randomized phase III study to compare the combinations of cisplatin plus amrubicin and cisplatin plus irinotecan for previously untreated ES-SCLC.

To improve clinical outcomes in advanced lung cancer, clinical integration of molecular biomarkers that predict responses to chemotherapeutic agents may be indispensable [16]. Recently, RanBP2 has been reported to act as a small ubiquitin-like modifier (SUMO) ligase for DNA TopoII and play an important role in targeting TopoII to centromeres during mitosis and in maintaining chromosome stability [5]. Embryonic fibroblasts derived from the engineered mutant mice with low expression of RanBP2 have been reported to show formation of chromatin bridges in anaphase, a distinctive feature of cells with impaired DNA decatenation by chemical inhibition of TopoII [4], suggesting that low expression of RanBP2 may have an analogous effect of TopoII inhibitors. In addition, RanBP2 has a tumor suppressor function since these mutant mice succumbed to a range of cancers, primarily lung carcinomas, and were also susceptible to chemically-induced tumorigenesis. Based on these observations, we hypothesized that RanBP2 expression might be involved in chemosensitivity of a TopoII inhibitor, amrubicin.

The identification of molecular biomarkers with the potential to predict treatment outcomes is essential for individualizing the most beneficial chemotherapy. As one of the multiple approaches to establishing predictive biomarkers, we evaluated whether there would be associations between mRNA expression of the RanBP2 gene as well as the TopoII-alpha and beta genes and chemosensitivity to amrubicin using human lung cancer cell lines.

## Materials and methods

### Cell lines and drug

Fifteen NSCLC and five SCLC cell lines used were described previously [24]. These cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Amrubicin was kindly provided by Sumitomo pharmaceuticals Company, Osaka, Japan.

### Cytotoxicity assay

Cytotoxicity was evaluated using an MTT assay as described previously [11]. Suspensions of exponentially growing cells were dispensed into wells of 96-well tissue-culture plates. After incubation at 37°C for 24 h, the solutions of amrubicin at various concentrations were added, and then incubated for 3 days. The effects of treatment were expressed as percent growth inhibition using untreated cells as the uninhibited control and assessed by IC<sub>50</sub> (drug concentrations inducing a 50% reduction of cell survival) which was calculated from dose–response curves.

### RNA preparation and RT-PCR amplification

Total RNA was extracted and further purified as described previously [24]. The RNAs were stored at –80°C until use. Total RNA (50 ng) extracted from each cell line was subjected to one-step real-time reverse transcriptase-PCR (RT-PCR) for absolute quantitating mRNA levels of the RanBP2, TopoII-alpha, TopoII-beta and beta-actin genes as described previously. The PCR primers used were as follows.

RanBP2-S: 5'-CAATGGAAATGGGGAAGACTTT-3'  
 -AS: 5'-CATCACTTCAGTCCCACCTGTA-3'  
 TopoII-alpha-S: 5'-GGTGTGGAAGTAGAAGGCCTAA-3'  
 -AS: 5'-TGAATCAGACCAGGGATTCTC-3'  
 TopoII-beta-S: 5'-TTTTTCACCATCATTGGTCTG-3'  
 -AS: 5'-GGGCTTAGGGACTGTATCTGAA-3'  
 Beta actin-S: 5'-TTCTACAATGAGCTGCGTGTG-3'  
 -AS: 5'-CAGCCTGGATAGCAACGTACA-3'

Linear regression analysis of standard-curves demonstrated a strong correlation for all the genes ( $R^2 > 0.99$ ). The relative gene expression levels were normalized with a house keeping gene, beta-actin.

### Western blot analysis

Western blot analysis was done as described previously [11], using the following primary antibodies: anti-RanBP2 (ab2938, Abcam), anti-TopoII-alpha (ab45175, Abcam), anti-TopoII-beta (ab58442, Abcam) and anti-actin (A2268, Sigma-Aldrich) antibodies.

### Statistical analyses

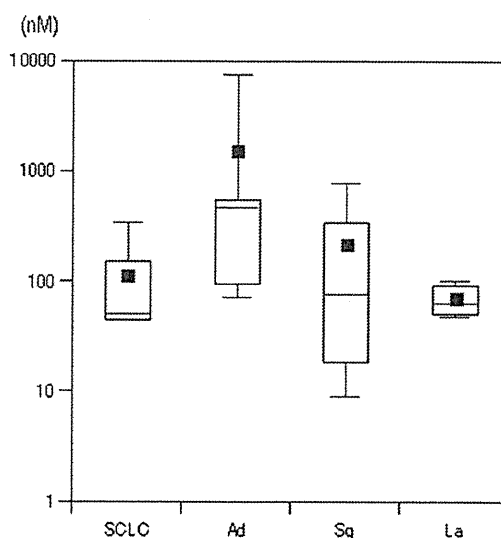
The strength of the association between the expression levels of RanBP2, TopoII-alpha and TopoII-beta and chemosensitivity data was calculated by either Pearson's correlation coefficient or linear regression analysis. Correlations were considered significant at  $P < 0.05$ . For comparison of IC<sub>50</sub> values of amrubicin and each gene expression level among histological subtypes, we employed one-way

analysis of variance (ANOVA) followed by Bonferroni post-test. All analysis was performed with the use of Stat View software version 5.0.

## Results

Chemosensitivity of amrubicin was examined using 20 human lung cancer cell lines including 15 NSCLC cells and 5 SCLC cells. Cytotoxicity following a 72 h continuous exposure of amrubicin was measured by MTT assay. The IC<sub>50</sub> value of amrubicin in SK-LC-3 was about 9  $\mu$ M, while the IC<sub>50</sub> values in the other cell lines were less than 1  $\mu$ M as shown in Table 1. There was no significant difference between histological types (Fig. 1).

The mRNA quantifications of the RanBP2, TopoII-alpha, TopoII-beta genes were carried out in real-time PCR and the expression levels were normalized with beta-actin as an internal control (Table 1). Among 20 cell lines tested, the level of RanBP2 mRNA expression in an H460 cell line was about 20-fold lower than those in non-tumorous lung tissues obtained from two patients with lung cancer. There were statistically significant differences in the RanBP2 expression between SCLC and the other histological subtypes ( $p < 0.05$ ) (Fig. 2a). We checked RanBP2 protein expression in two lung cancer cell lines, SK-LC-2 and H460, representing high and low expression of the RanBP2



**Fig. 1** IC<sub>50</sub> values of Amrubicin in lung cancer cell lines. Box plots show relationships between IC<sub>50</sub> values of Amrubicin and the four histological subtypes of lung cancer. The horizontal line within each box represents the median value and the closed box shows the mean value, respectively

gene, and found similar mRNA and protein expression patterns (Fig. 2d). We also found statistically higher expression levels of TopoII-alpha in SCLC and adenocarcinoma cell lines compared with those in normal lung tissues,

**Table 1** IC<sub>50</sub> values for amrubicin and relative mRNA expression for RanBP2, TopoII alpha and TopoII beta in lung cancer cell lines

Cell line	Histology	Amrubicin ( $\mu$ M)	RanBP2	TopoIIa	TopoIIb
ACC-LC-94	Ad	0.0668	0.621	1.324	1.174
ACC-LC-319	Ad	0.579	1.108	1.682	1.437
SK-LC-3	Ad	8.99	2.634	2.009	1.899
A549	Ad	0.131	1.191	1.553	1.665
SK-LU-1	Ad	0.492	1.307	2.930	1.479
VMRC-LCD	Ad	0.0835	4.134	2.660	2.942
RERF-LC-MT	Ad	0.469	0.661	0.8719	0.753
Calu1	Sq	0.203	1.280	2.173	1.750
SK-MES-1	Sq	0.0768	1.160	0.883	0.807
PC-1	Sq	0.009	1.937	1.739	2.888
RERF-LC-A	Sq	0.0222	0.717	1.454	1.036
PC-10	Sq	0.77	0.713	1.049	1.170
NCI-H460	La	0.101	0.043	1.518	2.098
Calu6	La	0.0469	1.467	1.116	1.828
SK-LC-6	La	0.0632	2.362	2.508	4.383
ACC-LC-48	SCLC	0.0512	1.957	1.672	2.044
ACC-LC-49	SCLC	0.0866	2.592	1.975	3.523
ACC-LC-80	SCLC	0.0459	3.953	1.361	1.993
ACC-LC-172	SCLC	0.0439	2.387	4.450	4.207
SK-LC-2	SCLC	0.337	3.662	3.510	4.264
NL 1	Normal lung	NA	1.006	0.158	2.447
NL 2	Normal lung	NA	0.913	0.179	1.937

NL 1 and NL 2: non-tumorous lung tissues obtained from two patients with lung cancer.

Ad adenocarcinoma,

La large cell carcinoma,

SCLC small cell lung cancer,

Sq squamous cell carcinoma.

NA not available