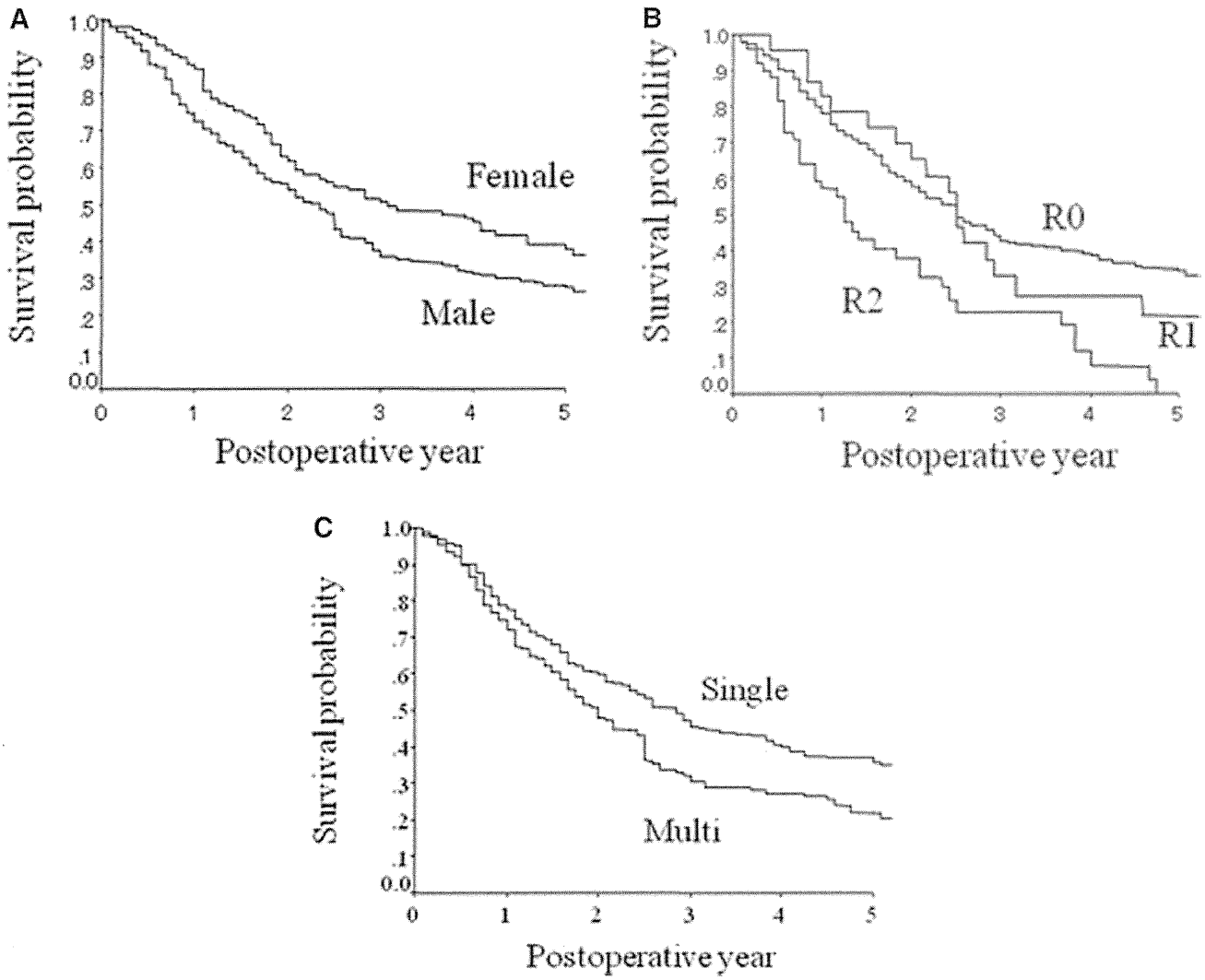

FIGURE 2. Survival curves for subpopulations of c-stage N2/pN2 non–small-cell lung cancer patients from the 2004 registry. *A*, Male and female survival curves. The 5-year survival rates were 27.5% in 324 males and 37.8% in 104 females; this difference was statistically significant ($p = 0.0245$). *B*, Subpopulations categorized by level of residual disease. The 5-year survival rates were 33.4% in 353 R0 patients, 21.7% in 24 R1 patients, and 0.0% in 51 R2 patients; the differences between R1 patients and R2 ($p = 0.0098$) and between R0 and R2 patients ($p < 0.0001$) were statistically significant, whereas no significant difference was observed between R0 and R1 patients ($p = 0.6423$). *C*, Single- and multistations N2. The 5-year survival rates were 35.8% in 235 patients with single-station N2 and 22.0% in 151 with multistation N2; this difference was statistically significant ($p = 0.0053$).

pneumonectomy. In 2004, 10.4% of the patients underwent pneumonectomy, compared to 14.0% in 1999 and 20.0% in 1994; thus, it seems that even among cN2/pN2 cases, less advanced cases were selected for surgery. The decreased rate of pneumonectomy may result in decreased hospital mortality. Actually, the series of the Japanese nationwide registry clearly revealed a time trend for improved survival of the stage IIIA-cN2/pN2 disease (Fig. 1). Several types of N2 cases, such as single-station or single-node N2 cases, have experienced a good prognosis after surgery.[20,21] The Japan Clinical Oncology Group conducted a questionnaire study regarding outcomes in stage IIIA-pN2 patients who underwent complete resection from 1992 to 1993.[21] Five-year survival rates were 31% for all pN2 cases, 27% for cN2 cases, and 43% for single-station N2 cases. In our series, the 5-year survival rate of single-station pN2 was also significantly higher than that of multistation pN2, and a proportion of single-station pN2 was 61 % in this study that was relatively higher than 52 % of the Japan Clinical Oncology Group study, which suggests that such a single-station N2 was likely to be selected for surgery in Japan of 2004.

Increase of adenocarcinoma may be another reason for the surgical results because the histology is associated with favorable prognosis.[13,14] Thus, recent cN2/pN2 NSCLC

TABLE 2. Historical Profile of Surgical Results for cN2/pN2 NSCLC

Report	Dates	Combined Modality	Number of Patients	Rate of R0 (%)	Rate of 5-year Survival (%)
Pearson et al.[1]	1964–1980	Induction R.	79	65	9
Martini and Flehinger[2]	1974–1981	Adjuvant R	179 (only CR)	—	18
Funatsu et al.[4]	1970–1989	S alone	91[a]	14	6
Watanabe et al.[3]	1980–1990	S alone	106[a]	50	16
Roth et al.[5]	1987–1993	Induction C	28	61	56 (at 3 years)
		S alone	30	66	19 (at 3 years)
Rosell et al.[6]	1989–1991	Induction C	30	85	25 (at 2 years)
		Adjuvant R	30	90	0 (at 2 years)
Choi et al.[8]	1988–1995	Induction CR (R: twice daily)	42	81	37
Ichinose[21]	1992–1993	S alone or S first	164 (only CR)	—	27
Albain et al.[9]	1994–2001	Induction CR	202	71	27
		Definite CR	194		20
Uy et al.[25]	1997–2004	Induction CR	40	93	52 (at 3 years)
Present	2004	Various (all)	436	83	30
			Single 235		36
			Multi151		22
		Adjuvant C	151		28
		Induction C/CR	108		28
		S alone	137		34

[a]Cases with exploratory thoracotomy were excluded from the study.
R, Radiation; C, chemotherapy; S, surgery; CR, chemoradiation; Single, single-station N2; Multi, multistation N2; NSCLC, non–small-cell lung cancer.

patients who undergo surgery are distinct from the cN2/pN2 NSCLC population of previous decades. Often, improvements in diagnostic facilities outpace changes in treatment outcomes, and such a transition of the medical environment may always influence the changes in patient selection and characteristics.

With respect to surgery alone, the present data are much valuable because cN2/pN2 is now usually contraindicated for surgery alone and the surgical outcome of modern series has been rarely presented. In our study, data of 137 patients with stage IIIA-cN2/pN2 patients treated by surgery alone in the particular period (2004) were retrospectively collected from the large-scale registry, and the relatively favorable outcome was revealed. Although the detailed reasons for surgical indication was unknown, they might be highly selected or might have unusual surgical indication because these cases only represented 1.2% of all resected NSCLC cases; therefore, surgery alone cannot yet be recommended as a treatment option in practice.

Although the prognosis of patients in the present study was superior to those previously reported, it remains unsatisfactory, especially considering that the majority of the patients underwent perioperative therapies. Whether or not induction therapy followed by surgery provides a survival benefit for resectable cN2/pN2 NSCLC patients has been the focus of much attention. Two meta-analyses of induction chemotherapy reported[22,23] demonstrated significance or tendency of favor of induction chemotherapy for stage III NSCLC; however, those analyses included two controversial studies. In the randomized trials conducted in the 1990s,[5,6] there was significant efficacy of neoadjuvant platinum-based chemotherapy in this patient population (Table 2); however, the results have not been widely accepted because of far lower survival of patients in the surgery-alone groups. Concurrent chemoradiotherapy as induction has been expected to be a more promising strategy for fit cases[7,8,24] (Table 2). Compared to chemotherapy, chemoradiotherapy results in better local control and a higher incidence of downstaging, which is a strong indicator of efficacy. In the present series, induction therapy was administered to 108 patients (24.8%), 84 of whom received chemotherapy and 23 received chemoradiotherapy; however, survival of these patients was equivalent to that of patients who underwent surgery alone. These results may be explained by the fact that the downstaged cases were automatically excluded from the present cohort through the retrospective selection of cN2/pN2 cases, and may also show that survival benefit of induction therapies was hardly recognized in non-downstaged cases. Taking into account that the indication of induction treatments could not be clarified in this retrospective study, no conclusion can be drawn for this issue.

The North America Intergroup Trial 0139, which compared concurrent chemoradiation followed by surgery (trimodal therapy) versus definitive chemoradiation (bimodal therapy) for resectable c-stage IIIA-N2 cases,[9] importantly revealed that no difference in overall survival occurred between the two treatment arms, although patients in the trimodal-therapy arm experienced superior recurrence-free survival. However, in a retrospective matched-cohort analysis, trimodal-therapy patients who underwent lobectomy experienced significantly better survival than bimodal-therapy patients who were selected by matching age, sex, performance status, and cT factor; thus, trimodal therapy was suggested to be effective for fit patients. Uy et al.[25] reported that in a study in which 40 out of 550 c-stage IIIA-N2 referrals received trimodal therapy in a community practice using the same regimen as that used in the North America Intergroup Trial 0139 (cisplatin/etoposide/45 Gy), the R0 resection rate was 92.5% and the 3-year overall

and disease-free survival rates both exceeded 50%. The above results indicate that induction treatments with chemoradiation could enhance the role of surgery for the disease if patients are properly selected.

Recent clinical trials have revealed that adjuvant cisplatin doublets increase postoperative 5-year survival rates by 15% in postoperative stage IIIA-N2 NSCLC cases[10,11]; however, no information regarding the c-stage of such cases was reported. In the present study, 151 patients who received adjuvant chemotherapy experienced a survival rate similar to the 137 patients who underwent surgery alone. In this retrospective study, however, indication of adjuvant therapy was not clarified for each case; hence, no conclusions about the efficacy of adjuvant therapy for c-stage IIIA-cN2/pN2 were determined.

Despite several limitations, this large nationwide database study has demonstrated the finding of a modern surgical outcome for selected patients with stage IIIA-cN2/pN2 NSCLC, and that the postoperative survival was favorable in comparison with those previously reported.

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ORIGINAL ARTICLE

The value of pleural lavage cytology examined during surgery for primary lung cancer

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Abstract

OBJECTIVES: The pleural invasion (PL) score is a useful prognostic indicator in lung cancer. However, in many cases, the cancer may exfoliate itself into the pleural cavity and may progress to a malignant pleural effusion without invading the parietal pleura. This stage is not currently evaluated, but it is detectable by means of the pleural lavage cytology (PLC). However, PLC's contribution to TNM staging has not yet been clarified. The purpose of this investigation was to demonstrate the usefulness of PLC in the precise staging of patients with such an occult pleural dissemination.

METHODS: A total of 3231 patients who were included in a multi-institutional database were studied retrospectively. PLC was performed by washing the thoracic cavity with a small amount of physiological saline immediately after opening the thoracic cavity during lung cancer surgery.

RESULTS: The incidence of positive PLC findings was 4.58%. In comparison with the negative group, the survival curves were significantly worse ($P < 0.001$) and the incidence of recurrence with pleuritis carcinomatosa was significantly higher ($P < 0.001$). According to the subset analysis, the survival difference was prominent in earlier stage groups and lower PL score groups. The positive findings were confirmed to be a significantly poor prognostic indicator ($P = 0.016$) by multivariate analysis using the Cox proportional hazard model (Cox analysis). However, integration of the positive findings with the PL score was attempted for the convenience of TNM staging. To find the accurate PL score for positive PLC findings, the Cox analysis was re-estimated using the PL score upgraded stepwise. The most reliable model with the highest score for the likelihood ratio χ^2 statistic was obtained by scoring positive findings as PL3. So, it was considered to be the most reliable conversion.

CONCLUSIONS: Examining PLC in clinical practice is useful for detecting occult pleural dissemination before the appearance of a malignant pleural effusion. Evidence of positive findings should be treated as supplemental information to the precise diagnosis of TNM staging. Scoring positive PLC findings as PL3 (=T3) was appropriate.

Keywords: Pleural lavage cytology • Cytological examination of pleural lavage fluid • Occult pleural dissemination • TNM staging lung cancer • Non-small-cell lung cancer

THORACIC

INTRODUCTION

The peripheral lung cancer tends to grow by invading the visceral pleura and then progressing to the parietal pleura. This progression is expressed by a pleural invasion (PL) score from PL0 to PL3, which is considered to be useful for predicting prognosis by providing supplemental information to TNM staging [1]. However, it is evident that lung cancer may progress via another route. After reaching the surface of the visceral pleura, cancer cells may exfoliate themselves into the pleural cavity and potentially progress to a malignant pleural effusion. Although this type of progression is not currently considered for staging purposes, it is detectable by the cytological examination of the pleural cavity, such as via pleural lavage cytology (PLC). Several reports have suggested that PLC findings obtained during surgery are an important prognostic indicator [2–14]. However, PLC's contribution to TNM staging has not yet been clarified. The purpose of this investigation was to demonstrate the usefulness of PLC in the precise staging of patients with such an occult pleural dissemination.

MATERIALS AND METHODS

Patients

A multi-institutional retrospective database analysis was performed to identify patients with lung cancer who underwent

operation between 2000 and 2007. Patients with obvious malignant pleural effusion or with Stage IV disease were excluded before the registration. A total of 3493 patients were registered from 12 institutes in which PLC had been routinely examined. After excluding the patients against eligible criteria (small cell carcinoma, 40; low-grade malignancy, 4; multiple primary lung cancer or pulmonary metastasis, 20; M1a or M1b, 62; incomplete data, 136), a total of 3231 patients were included in the study.

Methods

PLC was performed by washing the thoracic cavity with 20–500 ml of physiological saline immediately after opening the thoracic cavity during surgery; a 10–20 ml of specimen was collected for cytological examination. Actually, in most institutes, physiological saline of ≤100 ml was used for lavage fluid. Washing with 500 ml, which was used in two institutes, may increase the false-negative findings due to the over-dilution. However, according to the result of preliminary analysis that incidence of positive PLC findings per each institutes had no statistical difference (P = 0.208), we accepted the registration from the 500 ml institutes. We recommend the amount of lavage fluid not to exceed 100 ml. In this study, a routine radical operation for lung cancer, with mediastinal lymph node dissection conforming to the *General Rule for Clinical and Pathological Record of Lung Cancer* (6th edition) by the Japanese Lung Cancer Society [15], was performed in all patients irrespective of their PLC results. In cases where parietal pleural invasion was identified, combined resection of the pleura and chest wall, if necessary, was performed. Postoperative pathological evaluation was performed by each institute's pathologist to determine the histology, tumour size and pathological TNM. pleural invasion was also evaluated by the pathologist as a PL score ranging from PL0 to PL3 as follows: PL0, tumour within the subpleural lung parenchyma; PL1, invasion beyond the elastic layer; PL2, invasion to the pleural surface; PL3, invasion to the parietal pleura [1]. Data were collected from databases, including the result of PLC, age, gender, survival time, dead or alive (all death or censored), operative procedure, actual disease-free time, site of recurrence and information about adjuvant chemotherapy. The pathological T (pT) and pathological N (pN) scores were converted to the new 7th Edition TNM Classification [16, 17], but some stage migration of the N score could not be avoided because of the discontinuity between the Naruke map and the Rusch–Asamura map [18].

Statistical analyses

In a background analysis, age, gender, histology, pathological stage (p-Stage), pT, pN and PL scores were compared between the PLC-positive (PLC^+) group and the PLC-negative (PLC^-) group. Differences were assessed statistically using a t-test for the numerical variables and a χ^2 test for the categorical variables. A P-value of <0.05 was considered to be statistically significant. Survival analysis was performed first with the entire cohort; next, subset analyses were performed on the histology (adenocarcinoma, squamous cell carcinoma and others), p-Stage, pT, pN and PL scores. Survival curves were generated via the Kaplan–Meier method, and statistical differences between the PLC^+ group and PLC^- group were evaluated by the logrank test. A multivariate analysis using a Cox proportional hazard model (Cox analysis) was also performed to evaluate the significance of prognostic factors (PLC, age, gender, tumour size, pN and PL scores), and the hazard ratio, likelihood ratio χ^2 statistic (χ^2) and P-value (probability > χ^2) were estimated. All statistical analyses were performed using StatMate IV software (ATMS, Tokyo, Japan) or JMP 8.0 software (SAS Institute Japan, Tokyo, Japan).

Integration of the pleural lavage cytology-positive findings with the existing staging factors

After the evaluation of the six prognostic factors, integration of the PLC^+ findings with the existing staging factors was attempted for convenience of TNM staging. According to the results of the subset analysis and theoretical considerations, integration of the PLC^+ findings with the PL score was considered to be most reasonable. Seeking the appropriate PL score matching to the PLC^+ findings, the Cox analysis was re-estimated using a corrected PL score by replacing the score of underestimated cases with a higher score in a gradual manner (PL1, PL2 and then PL3). The

Table 1: Patient characteristics of studied groups

	PLC^+ group	PLC^- group	P-value
Age [mean (SD)]	66.9 (10.2)	65.6 (9.9)	0.118
Gender[a]			
Male	81 (54.7%)	1929 (62.6%)	0.054
Female	67 (45.3%)	1154 (37.4%)	
Histology[a]			
Adenocarcinoma	111 (75.0%)	2137 (69.3%)	0.015[b]
Adenosquamous cell carcinoma	6 (4.1%)	83 (2.7%)	
Squamous cell carcinoma	22 (14.9%)	752 (24.4%)	
Large cell carcinoma	5 (3.4%)	88 (2.9%)	
LCNEC	2 (1.4%)	9 (0.3%)	
Others	2 (1.4%)	14 (0.5%)	
Pathological stage[a]			
IA	24 (16.2%)	1114 (36.1%)	<0.001[b]
IB	51 (34.5%)	924 (30.0%)	
IIA	16 (10.8%)	343 (11.1%)	
IIB	11 (7.4%)	184 (6.0%)	
IIIA	43 (29.1%)	492 (16.0%)	
IIIB	3 (2.0%)	26 (0.8%)	
Pathological T score[a]			
T1a	15 (10.1%)	772 (25.0%)	<0.001[b]
T1b	13 (8.8%)	494 (16.0%)	
T2a	86 (58.1%)	1350 (43.8%)	
T2b	9 (6.1%)	147 (4.8%)	
T3	20 (13.5%)	273 (8.9%)	
T4	5 (3.4%)	47 (1.5%)	
Pathological N score[a]			
N0	91 (61.5%)	2298 (74.5%)	<0.001[b]
N1	15 (10.1%)	351 (11.4%)	
N2	41 (27.7%)	422 (13.7%)	
N3	1 (0.7%)	12 (0.4%)	
Pathological PL score[a]			
PL0	44 (29.7%)	1739 (56.4%)	<0.001[b]
PL1	45 (30.4%)	917 (29.7%)	
PL2	43 (29.1%)	246 (8.0%)	
PL3	16 (10.8%)	181 (5.9%)	

PLC: pleural lavage cytology; PLC^+: PLC-positive group; PLC^-: PLC-negative group; LCNEC: large cell neuroendocrine carcinoma.
[a]Expressed by the number of the cases with its ratio.
[b]Statistical difference was confirmed with $P < 0.05$.

reliability of each Cox proportional hazard model was evaluated by the χ^2 and *P*-value with regard to the whole model and to the PL score. Since the *P*-values were too small to compare with each other, χ^2 was used in this instance. The model with the largest χ^2 has the smallest *P*-value and, therefore, is the most reliable model.

RESULTS

The incidence of PLC$^+$ findings was 4.58% (148/3231). In a background analysis, histology, pathological stage (p-Stage), pT, pN and PL scores had significant differences between the groups (Table 1). It was suspected that the PLC$^+$ group consisted of patients whose cancer had advanced to a particular stage. Regarding to the higher incidence of N2 disease in the PLC$^+$ group, cancer may migrate the lymphatic channels of the pleura and may cause the lymph node metastasis. However, the recurrence rate associated with the mediastinal–supraclavicular lymph node enlargement had no statistical difference ($P = 0.450$) between the PLC$^+$ group and the PLC$^-$ group, which was estimated to be 8.8 and 6.6%, respectively. The survival curve of the PLC$^+$ group was significantly worse than that of the PLC$^-$ group in terms of both the overall survival (OS) and disease-free survival (DFS) (Fig. 1). Differences in the subset analysis are shown in Table 2, and DFS curves for each p-Stage and each PL score are shown in Figs 2 and 3, respectively. In Stages IA and IB, the survival curves of the PLC$^+$ group were significantly worse than those of the PLC$^-$ group. As for the PL score, the survival curves of the PLC$^+$ group were also worse in the PL0 and PL1 groups. However, differences were not observed in the PL2 and PL3 groups. These findings suggested that the PLC$^+$ patients should not be included in these earlier stages.

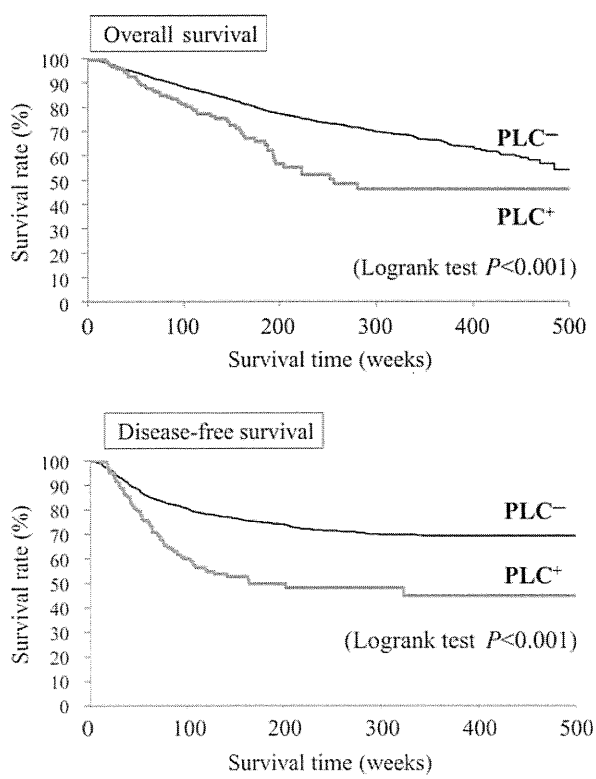

Figure 1: Comparison of survival curves by PLC status. Overall survival curves are shown in the top panel and DFS curves in the bottom panel. Statistical differences (*P*-values) were calculated by the logrank test. PLC: pleural lavage cytology; PLC$^+$: PLC-positive group; PLC$^-$: PLC-negative group.

Table 2: Differences in survival between the PLC$^+$ group and the PLC$^-$ group

	Overall survival	Disease-free survival
Histology		
Adenocarcinoma	$P < 0.001$*	$P < 0.001$*
Squamous cell carcinoma	$P = 0.496$	$P = 0.188$
Others	$P = 0.877$	$P = 0.837$
Pathological stage		
IA	$P = 0.045$*	$P < 0.001$*
IB	$P = 0.010$*	$P < 0.001$*
IIA	$P = 0.821$	$P = 0.270$
IIB	$P = 0.004$*	$P = 0.003$*
IIIA	$P = 0.984$	$P = 0.993$
IIIB	$P = 0.984$	$P = 0.149$
Pathological T score		
T1a	$P = 0.928$	$P = 0.025$*
T1b	$P = 0.094$	$P = 0.009$*
T2a	$P = 0.023$*	$P < 0.001$*
T2b	$P = 0.668$	$P = 0.923$
T3	$P = 0.273$	$P = 0.151$
T4	$P = 0.204$	$P = 0.783$
Pathological N score		
N0	$P < 0.001$*	$P < 0.001$*
N1	$P = 0.281$	$P = 0.023$*
N2+3	$P = 0.472$	$P = 0.351$
Pathological PL score		
PL0	$P = 0.129$	$P = 0.013$*
PL1	$P = 0.026$*	$P < 0.001$*
PL2	$P = 0.184$	$P = 0.079$
PL3	$P = 0.948$	$P = 0.875$

Expressed by *P*-values of the logrank test.
PLC: pleural lavage cytology; PLC$^+$: PLC-positive group; PLC$^-$: PLC-negative group.
*Statistical difference was confirmed with $P < 0.05$.

In an analysis of recurrent cases, the incidence of a malignant pleural effusion or obvious pleural dissemination (pleuritis carcinomatosa) was 17.6% (26/148) in the PLC$^+$ group, compared with 2.8% (86/3083) in the PLC$^-$ group, a significant difference ($P < 0.001$). However, no difference was apparent with regard to sites of distant metastasis. For this reason, it was concluded that PLC$^+$ findings was a preliminary stage of a malignant pleural effusion.

Among the six variables analysed by Cox analysis, all were statistically significant in terms of OS and DFS (Table 3). PLC$^+$ findings were confirmed as a significantly poor prognostic factor in both OS ($P = 0.016$) and DFS ($P = 0.026$). However, it would be more convenient if the PLC$^+$ findings were integrated with one of the existing TNM staging factors. A total of 89 cases (60.1%) with PLC$^+$ findings had been diagnosed as either PL1 or PL0, which the subset analysis showed to be underestimations of the disease stage. To find the accurate PL score for positive PLC findings, the Cox analysis was re-estimated using the PL score upgraded stepwise. The χ^2 regarding to the whole model reached its maximum value by a correction to PL3 in both OS (uncorrected, PL2, PL3; 654.67, 658.99, 659.04) and DFS

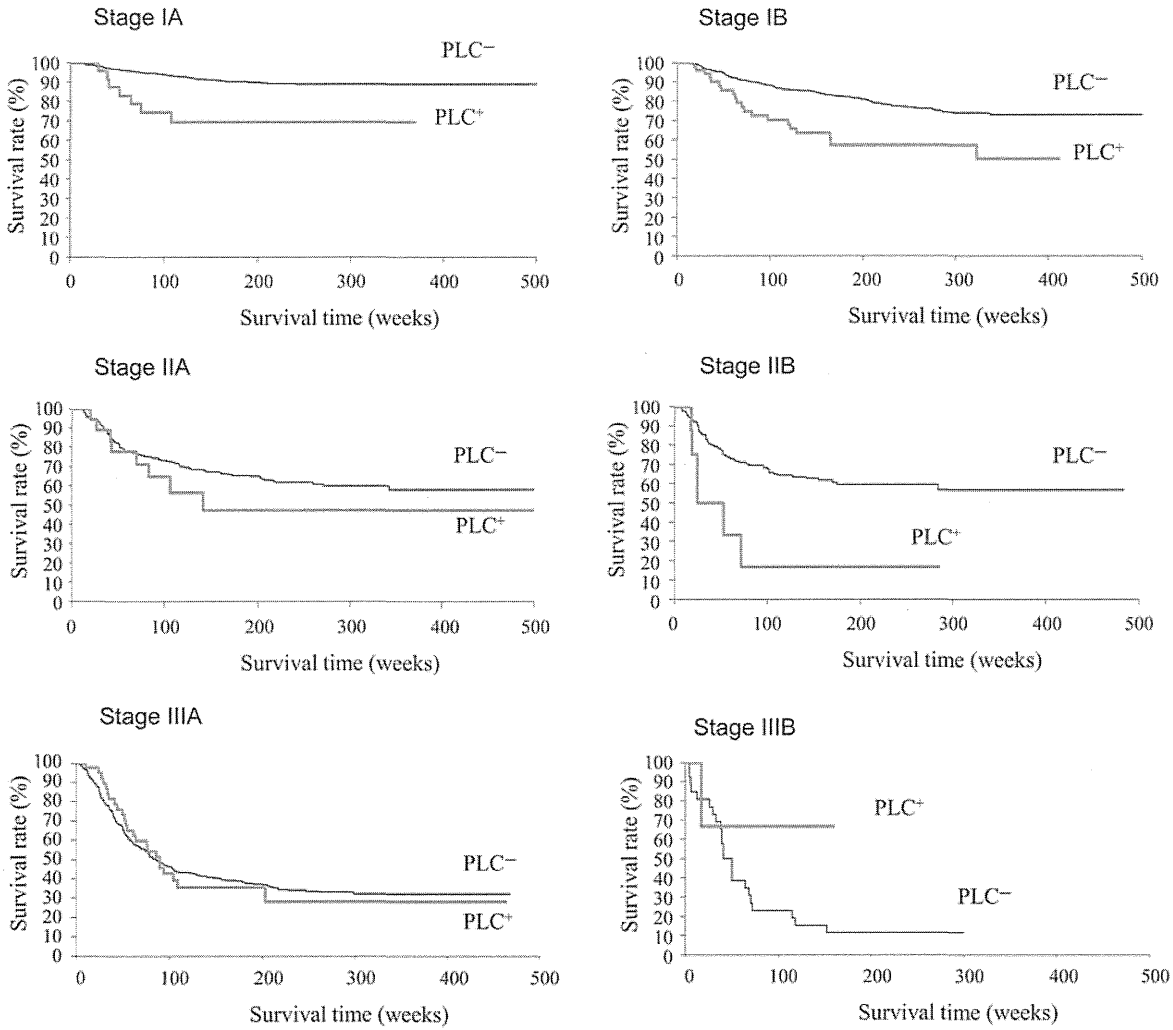

Figure 2: Comparison of DFS curves by pathological stage. Statistical differences (*P*-values) were calculated by the logrank test. PLC: pleural lavage cytology; PLC^+: PLC-positive group; PLC^-: PLC-negative group.

(uncorrected, PL2, PL3; 600.56, 609.28, 609.84). Conversion of the PLC^+ findings to PL3 (=T3) was, therefore, considered to be most appropriate. DFS curves that were re-estimated using the corrected PL score are shown in Fig. 4 to demonstrate the efficacy of correction.

DISCUSSION

The previously reported incidence of PLC^+ findings ranges from 2.7 to 41.7% [2–14]. However, restricting to the papers of large series, the incidence of PLC^+ findings was found to be within the range of 3–6%. PLC^+ findings were reasonably estimated to be 4.58% in our study. Although the survival differences between the PLC^+ and PLC^- groups are obvious, these differences may not have been due only to the sequelae of PLC^+ findings, because many of the other patient characteristics were also significantly different. For this reason, a Cox analysis was performed. All of the six variables analysed were statistically significant and PLC^+ findings were confirmed as a significantly poor prognostic factor. As for the results of the Cox analysis, many investigators [2, 4, 7, 8, 10, 12, 14] have reported that PLC^+ findings are an independent prognostic factor in lung cancer. However, their analysed explanatory variables are inconsistent. Above all, pN, which is widely believed to be the most important prognostic factor, is not included in many studies [2, 4, 7, 8, 10]. In some study, it is converted to a much rougher score, such as 'N0 vs. N1–3' [12]. In our study, the explanatory variables were simplified into two categories, one concerning the life expectancy (age and gender) and the other concerning the tumour growth (tumour size, pN and PL score); p-Stage and pT were not included because these factors may depend on other factors. We used the raw values of pN and PL score. If either of these scores was excluded from the explanatory variables, PLC^+ findings acquire a much smaller *P*-value ($P = 0.001$/OS without pN, $P < 0.001$/OS without PL score, $P < 0.001$/DFS without pN, $P < 0.001$/DFS without PL score) and will be regarded as a much more important prognostic factor. However, this is nothing more than a statistical artefact. The impact of PLC^+ findings should not be overstated. We were simply analysing a particular stage of cancer progression.

The extent of pleural invasion is expressed by a pleural invasion score ranging from PL0 to PL3 and is considered to be useful in

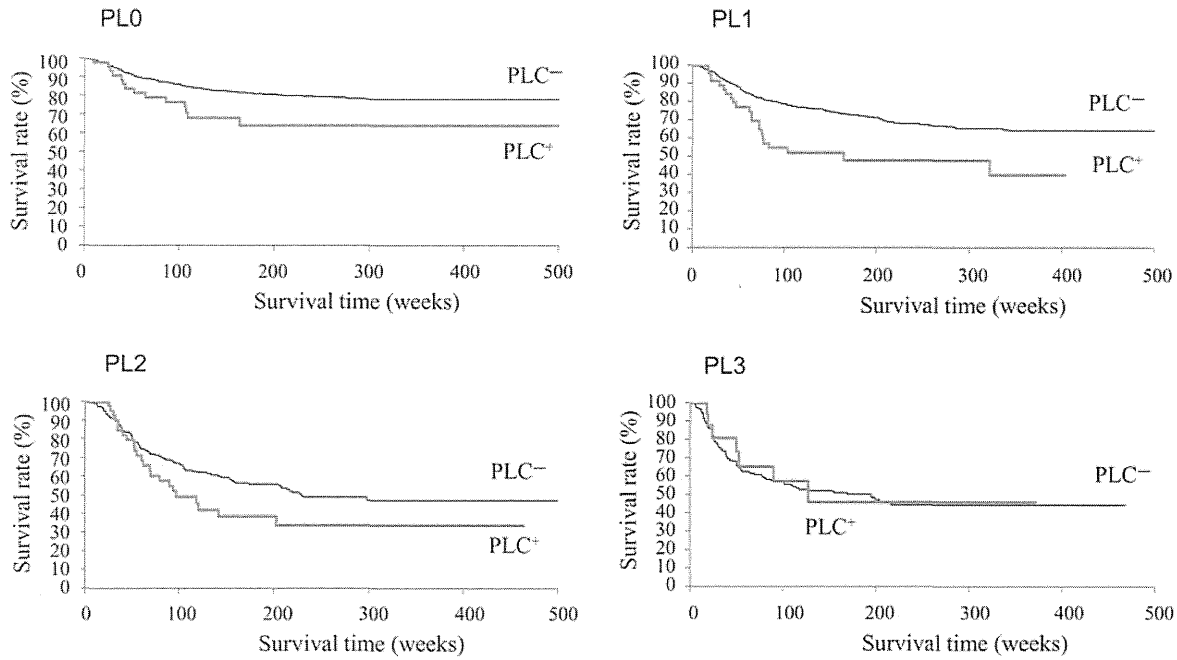

Figure 3: Comparison of DFS curves by the PL score. Statistical differences (*P*-values) were calculated by the logrank test. PLC: pleural lavage cytology; PLC⁺: PLC-positive group; PLC⁻: PLC-negative group.

Table 3: Results of the Cox analysis for overall survival and DFS

Factors	Hazard ratio	Likelihood ratio, χ^2	*P*-value
PLC⁺		5.848 (4.930)	0.016 (0.026)
PLC⁺/PLC⁻	1.436 (1.361)		
Age	1.037 (1.008)	83.419 (4.653)	<0.001 (0.031)
Gender		79.221 (9.458)	<0.001 (0.002)
Male/Female	2.107 (1.265)		
Tumour size	1.014 (1.012)	47.090 (34.032)	<0.001 (<0.001)
N score		227.301 (326.769)	<0.001 (<0.001)
N1/N0	2.036 (2.333)		
N2/N0	3.591 (4.546)		
N3/N0	7.253 (6.579)		
PL score		46.667 (46.140)	<0.001 (<0.001)
PL1/PL0	1.116 (1.343)		
PL2/PL0	1.695 (1.810)		
PL3/PL0	2.079 (2.001)		

Results for DFS data are shown in parentheses.
Cox analysis: multivariate analysis using the Cox proportional hazard model; PLC: pleural lavage cytology; PLC⁺: PLC-positive group; PLC⁻: PLC-negative group.

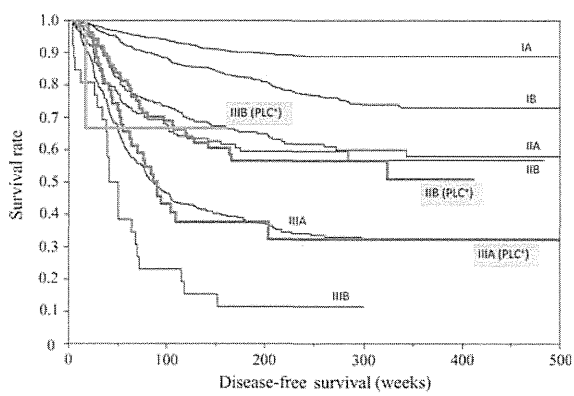

Figure 4: DFS curves after re-staging of the PLC⁺ patients, shown in comparison with that of the PLC⁻ patients. PLC: pleural lavage cytology; PLC⁺: PLC-positive; PLC⁻: PLC-negative; IIB (PLC⁺): Stage IIB in the PLC-positive group; IIIA (PLC⁺): Stage IIIA in the PLC-positive group; IIIB (PLC⁺): Stage IIIB in the PLC-positive group.

predicting prognosis [1]. PL3 is classified as T3 in the TNM classification; recently, PL1 and PL2 were classified as T2a or T2b (depending on tumour size) in the 7th Edition TNM classification [16, 17]. Moreover, in the 7th Edition TNM classification system, the classification of a malignant pleural effusion (pleuritis carcinomatosa) increased from T4 to M1a [16, 17] because of its vicious prognosis. Before the appearance of a pleural effusion, occult (microscopic) dissemination must occur. Although this stage is not currently evaluated, it is detectable by the cytological examination of the pleural cavity, such as via PLC. Theoretically, patients with PLC⁺ findings must be given a score of PL2 or higher because the cancer cells were exfoliated from the lung surface. However, 60.1% of the cases had been diagnosed as either PL1 or PL0 in our study. There is a discrepancy between the theory and clinical data. To evaluate the reliability of the staging, subset analysis was performed. In Stages IA and IB, the survival curves of the PLC⁺ group were significantly worse than those of the PLC⁻ group. As for the PL score, the survival curves of the PLC⁺ group were also worse in the PL0 and PL1 groups. These findings suggested that the PLC⁺ patients should not be included in these stages; instead, they should be classified in more advanced stages. As for the cause of discrepancy in the PL score, two possible explanations are conceivable: (i) cancer cells in the pleural cavity came from another origin, for example, exudation from the

lymphatic channels or nodes; (ii) diagnosis of PL0 or PL1 was made using inappropriate section of histopathological specimen, for example, in the case with deep pleural indentation. The former is a most likely explanation, but it cannot be a single credible cause, because the ratio of N1–2 patients per PL0–1 patients in the PLC^+ group was only 31% in our data. We cannot get farther information because of the limitation of retrospective study. Although cancer cells in the pleural cavity do not always originate from the lung surface, microscopic dissemination should be recognized as a preliminary stage of the malignant pleural effusion. This is the reason why we proposed the re-staging by PLC^+ findings.

Although the PLC^+ findings were confirmed as a significantly poor prognostic factor in the Cox analysis, it would be more convenient if the PLC^+ findings were integrated with one of the existing TNM staging factors. Integration of PLC^+ findings into the PL score may positively contribute to the precise diagnosis of cancer advancement and, therefore, will be useful in evaluating its prognosis. Scoring PLC^+ findings as PL3 (=T3) should be a reasonable method to express the stage between PL2 (=T2a–b) and T4 (=M1a).

Standard operation for lung cancer should not be given up because of the positive findings of PLC. The DFS of the PLC^+ patients, whose stages were re-staged to be either IIB (T3N0) or IIIA (T3N1 and T3N2), were almost equal with that of the ordinary (PLC^- group) Stage IIB or IIIA patients. Their survival is much better than that of the patients with malignant pleural effusion. Although we could not prove the efficacy of adjuvant therapy, due to the retrospective clinical data analysis, adjuvant chemotherapy will be indispensable. Intra-operative intra-pleural administration of hypotonic cysplatin [19] is a procedure of great interest. But farther investigations will be necessary to establish its efficacy.

CONCLUSION

Examining PLC in clinical practice is useful for detecting occult pleural dissemination before the appearance of a malignant pleural effusion. Evidence of PLC^+ findings should be treated as supplemental information to the precise diagnosis of PL score. Scoring PLC^+ findings as PL3 (=T3) was appropriate. However, standard operation should not be given up because of the positive PLC findings. The corrected survival curves of the PLC^+ group were almost equal with that of the ordinary stage IIB or IIIA patients.

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Conflict of interest: none declared.

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Serum Hepatocyte Growth Factor and Interleukin-6 Are Effective Prognostic Markers for Non-small Cell Lung Cancer

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Abstract. *Aim: We surveyed prognostic biomarkers for resectable non-small cell lung cancer (NSCLC). Patients and Methods: We obtained preoperative serum from 109 patients, and measured the levels of hepatocyte growth factor (HGF), interleukin-6 (IL-6), and nicotinamide N-methytransferase (NNMT) in the sera. Results: The median HGF and IL-6 contents were 860 pg/ml and 2.7 pg/ml, respectively. Analysis of survival curves indicated that an HGF or IL-6 level higher than the median was associated with poor overall survival (HGF, p=0.019; IL-6, p=0.002). In addition, we analyzed stage III lung cancer alone. Higher HGF and IL-6 levels were associated with poor overall survival (HGF, p=0.016; IL-6, p=0.013). Disease-free survival was not statistically significantly affected by these cytokine contents. The tumor status (pT factor) and nodal status (pN factor) were not associated with the survival of stage III patients. Conclusion: The levels of HGF and IL-6 in serum could be useful prognostic indicators of the survival of patients with stage III NSCLC undergoing surgery and chemotherapy.*

Lung cancer is a fatal malignant tumor that develops at high frequency in most countries at present. There are no tumor markers that are sufficiently useful for detecting lung cancer at a stage where the patients can be cured completely. We previously examined whether or not nicotinamide *N*-methytransferase (NNMT) is a potential biomarker of non-small cell lung cancer (NSCLC) (1, 2). The serum levels of NNMT were significantly higher in patients with lung cancer than in healthy donors and in patients with non-neoplastic disease.

In the present study, we analyzed prognostic factors in 109 cancer patients. Surgical resection of the tumor is the principle form of treatment for patients with stage I or stage II lung cancer. However, treatment of patients with stage III disease is not as simple. Some patients with stage III disease undergo surgery for tumor resection. Another treatment option is preoperative chemoradiotherapy and, if a response is seen, application of follow-up resectioning of any remaining tumor. The remaining patients are not surgical candidates. Therefore, predictors of the prognosis in stage III NSCLC would be useful for selection of the most appropriate treatment (3).

Hepatocyte growth factor (HGF) was originally found as a blood-derived factor released during regeneration of the liver (4, 5). At present, it is recognized that this factor is involved in the development of various organs during embryogenesis and tissue regeneration. Although HGF is produced mainly by mesenchymal cells, it acts on epidermal- and endothelial-derived cells (6, 7). It is also involved in cancer growth and metastasis by enhancing the motility of cancer cells and by stimulating angiogenesis (8, 9). The hepatocyte growth factor receptor (*c-MET*), proto-oncogene product, is expressed on most epidermal cells and a wide variety of cancer cells (7). Clinical studies have shown an association between the concentration of HGF in serum or cancer tissue and the progression of the disease in various cancer types, including breast (10, 11), gastric (12), bladder (13), colorectal (14), and small cell lung (15) cancer, myeloma (16, 17), and synovial sarcoma (18). For NSCLC, the intratumoral HGF level was reported to be a prognostic indicator (19-21), but the significance of the serum HGF level was not reported until fairly recently (22, 23). HGF is mainly produced by stromal fibroblasts (24). We previously showed that the *HGF* gene in cancer cells is transcriptionally activated by leukemia inhibitory factor (LIF) through the signal transducers and activators of transcription 3 (STAT3) (25). Furthermore, the expression of

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Key Words: Hepatocyte growth factor, HGF, interleukin-6, IL-6, non-small cell lung cancer, NSCLC, prognostic markers, epidermal growth factor receptor, EGFR, c-Met, hepatocyte growth factor receptor.

HGF in human lung fibroblasts and MRC-5 cells, derived from lung fibroblasts is correlated with that of IL-6. Expression of the *HGF* gene in MRC-5 cells was suppressed by treatment with curcumin, an inhibitor of STAT3 (unpublished results). Therefore, cytokines, such as LIF and IL-6, that activate STAT3 might stimulate stromal cells to produce HGF.

The inflammatory cytokine IL-6 exhibits multiple functions and stimulates the progression of various kinds of cancers (17, 26, 27). Constitutive activation of the STAT3 pathway in alveolar epithelial cells induces inflammation and adenocarcinomas in mouse lungs (28). In human lung adenocarcinoma, mutant epidermal growth factor receptor (EGFR) activates the STAT3 pathway through IL-6 up-regulation (29). It has been reported that increased serum IL-6 levels were associated with poor survival in patients with NSCLC (30, 31).

In this study, we analyzed the relationship between the levels of HGF, IL-6 and NNMT in sera, and the survival of patients with NSCLC.

Patients and Methods

Serum samples. This study and the use of blood samples collected after obtaining informed consent, were approved by the Ethical Committee of the Saitama Cancer Center (1). As preoperative blood samples, serum was collected from 109 patients undergoing radical pulmonary resection at the Department of Thoracic Surgery of Saitama Cancer Center Hospital during the period November 2006 to November 2007. The blood was maintained at room temperature for 20 min before centrifugation. The serum was separated, and then frozen at –70°C.

The 109 lung cancer patients included 79 with adenocarcinoma, 26 with squamous cell carcinoma (SCC), and 4 with other non-small cell lung cancer (Table I). For the examined tumors, the pathological stage was determined based on the standard criteria, UICC 7th edition (32).

Enzyme linked immunosorbent assay (ELISA) of cytokines and NNMT. ELISA assays for HGF and IL-6 were performed using a Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The levels of NNMT were measured by a method developed by Tomida *et al.* 2009 (1). The limit of detection for this method is 30 pg NNMT. The levels of carcinoembryonic antigen (CEA) were measured by routine examination at our hospital.

Statistical analysis. The relationships between the clinical outcome, and the HGF and IL-6 contents were analyzed by means of the Mann Whitney or Kruskal Wallis test. The correlation was examined by means of Spearman's rank correlation test. Regarding the total survival period and disease-free period, we prepared a Kaplan Meier survival curve (33). Univariate analysis of prognostic factors was performed by means of the log-rank test. Multivariate analysis was performed using the Cox proportional-hazards model (34). Statistical calculations were performed using the SPSS software (ver.17.0) (IBM, Chicago, IL, USA). When the two-sided *p*-value was lower than 0.05, statistical significance was considered.

Table I. *Clinical profile of 109 patients, and serum levels of hepatocyte growth factor (HGF) and interleukin-6 (IL-6).*

Factor	n	HGF (pg/ml)		IL-6 (pg/ml)	
		Median (range)	*p*-Value	Median (range)	*p*-Value
Age, years					
>66	53	895 (360-2000)	0.109	3.7 (1.1-62)	0.001
≤66	56	753 (215-1625)		2.0 (0-24)	
Gender					
Male	67	895 (215-2000)	0.103	2.7 (0-26)	0.278
Female	42	700 (230-1500)		2.6 (0-62)	
Stage					
I-II	67	775 (215-2000)	0.584	2.4 (0-62)	0.115
III	38	875 (230-1575)		2.7 (1.5-23)	
IV	2	795 (440-1150)		0.9 (0-1.7)	
Histology					
ADC	79	730 (215-1625)	0.086	2.4 (0-26)	0.001
SCC	26	1000 (360-2000)		4.2 (1.5-62)	
Other	4	1088 (675-1525)		4.6 (3.9-5.7)	
CEA (ng/ml)					
≥4.6	34	875 (360-2000)	0.631	3.4 (1.6-62)	0.088
<4.6	75	775 (215-1575)		2.3 (0-26)	
NNMT (pg/ml)					
≥710	27	875 (230-2000)	0.352	2.7 (0-62)	0.136
<710	82	730 (215-1625)		2.4 (0-24)	
pN factor					
N (–)	72	820 (215-2000)	0.645	2.4 (0-62)	0.796
N (+)	31	912 (295-1625)		2.7 (1.6-10.3)	
pT factor					
T1-2	77	820 (215-2000)	0.381	2.3 (0-62)	0.103
T3-4	28	830 (230-1550)		3.4 (1.5-24)	

ADC: Adenocarcinoma; SCC: squamous cell carcinoma; CEA: carcinoembryonic antigen; NNMT: nicotinamide *N*-methytransferase.

Results

Table I shows the clinical profiles of the 109 patients, and the HGF and IL-6 levels in each group. The levels of HGF detected in serum samples of various patients were in the range of 215-2,000 pg/ml. The median HGF level in the 109 patients was 860 pg/ml, with the average being 875 pg/ml. The HGF levels were not significantly different between the groups.

The IL-6 levels detected in the serum samples from the 109 patients were in the range of 0-62 pg/ml. The median IL-6 level was 2.7 pg/ml, with the average being 5.0 pg/ml. The IL-6 concentrations were significantly higher in older patients and in patients with SCC.

The relationship between the HGF and IL-6 levels in the sera from the patients was examined. As shown in Figure 1, the HGF levels were correlated with the IL-6 concentrations (Spearman's correlation coefficient by rank test: r=0.435; $p<0.0001$).

For the analysis of overall survival of the 109 patients, the median HGF value (860 pg/ml) was used as the cut-off. As shown in Figure 2a, patients with low HGF levels had a

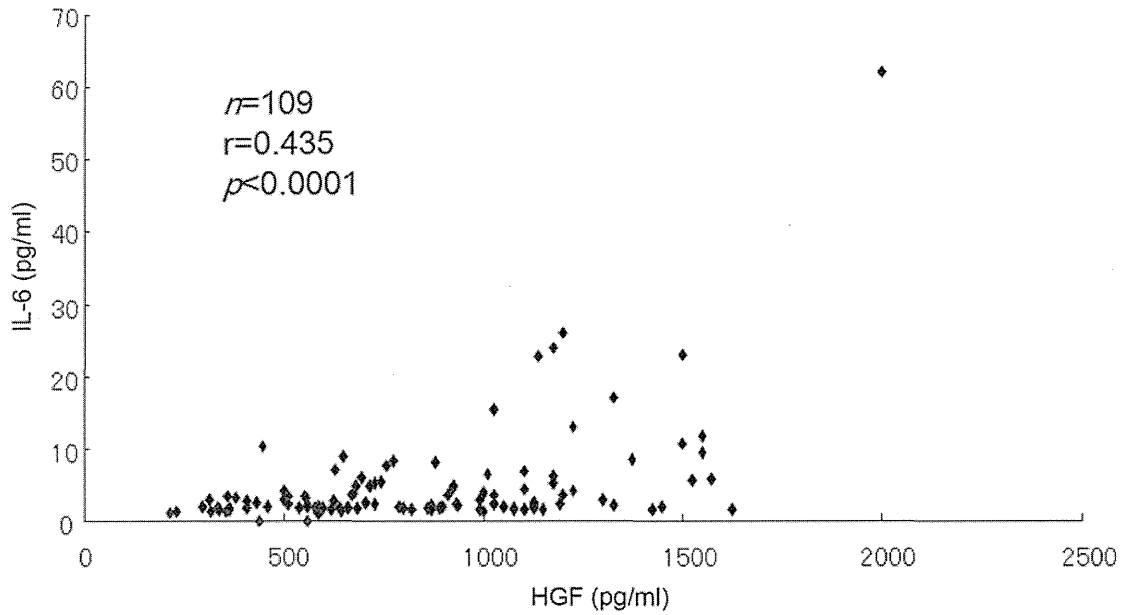

Figure 1. *The serum levels of hepatocyte growth factor (HGF) in patients with lung cancer were correlated with those of interleukin-6 (IL-6). The correlation was examined by means of Spearman's rank-correlation test.*

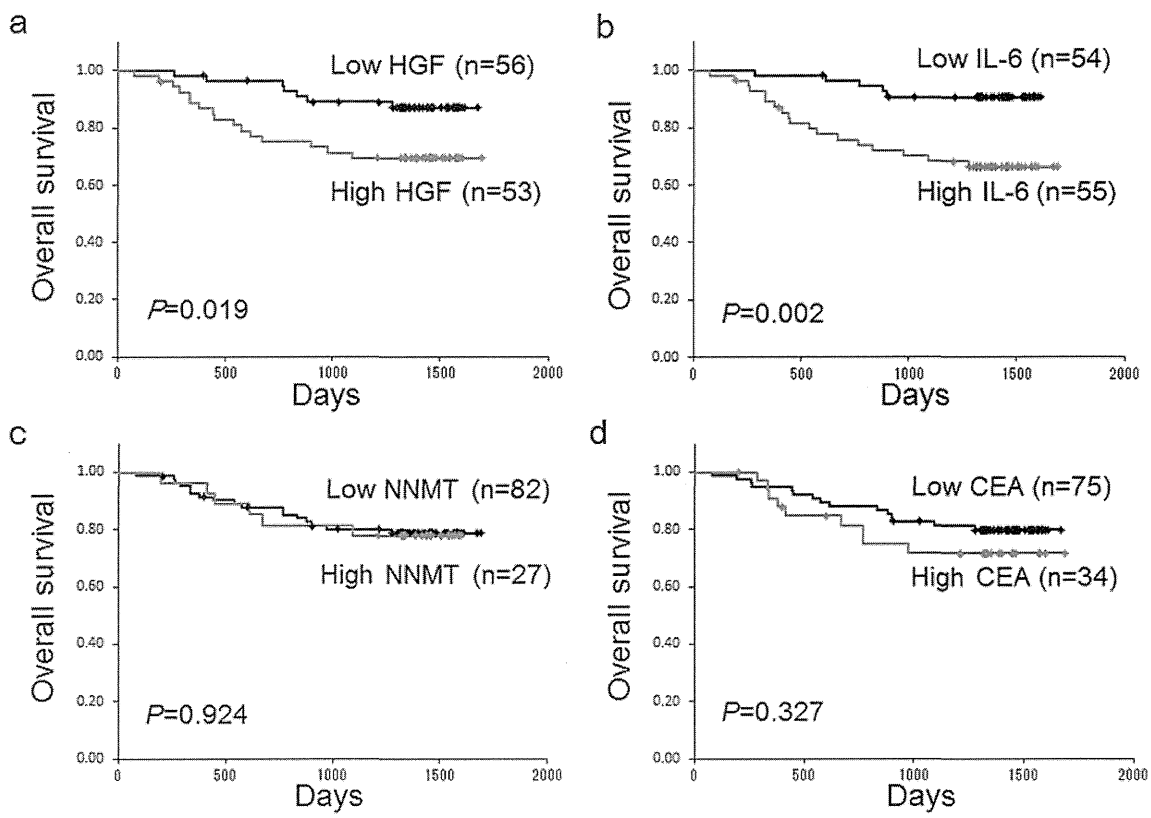

Figure 2. *Overall survival curves for 109 lung cancer patients, according to the hepatocyte growth factor (HGF) (a), interleukin-6 (IL-6) (b), nicotinamide N–methytransferase (NNMT) (c), and carcinoembryonic antigen (CEA) (d) levels. The cut-off values are shown in Table II.*

Table II. *Univariate and multivariate analyses of overall and disease-free survival of 109 patients.*

Factor	n	Overall survival			Disease-free survival		
		Univariate	Multivariate (Cox regression model)		Univariate	Multivariate (Cox regression model)	
		p-Value	Hazard ratio (95% CI)	*p*-Value	*p*-Value	Hazard ratio (95% CI)	*p*-Value
Age, years							
>66	53	0.057			0.519		
≤66	56						
Gender							
Male	67	0.023			0.806		
Female	42						
Stage							
I-II	67	<0.001			<0.001	2.67 (0.96-7.46)	0.061
III	38						
Histology							
ADC	79	0.007	2.35 (0.94-5.84)	0.067	0.035	2.40 (1.15-5.02)	0.02
Other	30						
CEA (ng/ml)							
≥4.6	34	0.327			0.765		
<4.6	75						
NNMT (pg/ml)							
≥710	27	0.924			0.956		
<710	82						
HGF (pg/ml)							
≥860	53	0.019			0.439		
<860	56						
IL-6 (pg/ml)							
≥2.7	55	0.002	3.46 (1.10-10.8)	0.034	0.052		
<2.7	54						
pN factor							
N0	72	0.026	3.71 (1.51-9.12)	0.004	0.014	2.41 (1.06-5.54)	0.036
N1-2	31						
pT factor							
T1-2	77	<0.001	4.41 (1.80-10.9)	0.001	<0.001	2.35 (0.85-6.49)	0.099
T3-4	28						

ADC: Adenocarcinoma; CEA: carcinoembryonic antigen; NNMT: nicotinamide *N*-methytransferase; IL-6: interleukin-6; HGF: hepatocyte growth factor; CI: confidence interval.

significantly better overall survival than ones with elevated HGF levels (p=0.019). For the patients who died during the follow-up observation period, the median HGF level was 1,025 pg/ml. On the other hand, the median HGF level in the surviving patients was 738 pg/ml, with there being a significant difference (p=0.036).

The IL-6 level was also associated with overall survival when the median IL-6 level (2.7 pg/ml) was used as the cut-off value (p=0.002) (Figure 2b). The median IL-6 level, 4.0 pg/ml, in the patients who died was higher than that of 2.3 pg/ml in the survivors (p=0.027).

Tumor marker CEA was reported to be a prognostic factor for patients with surgically resected NSCLC (3). We analyzed CEA and NNMT, which we reported as candidate tumor markers for NSCLC (1). However, the CEA and NNMT levels were not associated with the overall survival rate (Figure 2c and d).

When examining the overall survival rate of patients at individual disease stages, a difference was confirmed in the survival rate between patients with disease at stages I-II and III (p=0.0005). The analyzed patients included 50 patients at stage I, 17 at stage II, and 38 at stage III. Since the number of patients with disease at stage II was small, and they principally underwent the same surgery as in patients at stage I, the patients at stages I and II were analyzed together (Table I). The T status (pT factor) was also a strong prognostic factor.

In this study, the prognosis of patients with adenocarcinoma was better than that of patients with other types of NSCLC (p=0.007). The prognosis of female patients was also better than that of male ones (p=0.023). On univariate analysis, it was found that gender, stage, histological type, HGF level, IL-6 level, pT, and pN contributed to the overall survival rate (Table II).

On multivariate analysis of the overall survival rate, IL-6 (p=0.034, hazard ratio HR=3.46), pN factor (p=0.004,

Table III. *Univariate and multivariate analyses of overall survival of patients with stage III disease.*

Factor	n	Univariate	Multivariate (Cox regression model)	
		p-Value	Hazard ratio (95% CI)	*p*-Value
Age				
>66	18	0.255		
≤66	20			
Gender				
Male	26	0.12	2.89 (0.63-13.3)[a]	0.172
Female	12		3.17 (0.70-14.3)[b]	0.133
Histology				
ADC	29	0.007	4.07 (1.32-12.5)[a]	0.015
Other	9		2.73 (0.92-8.16)[b]	0.072
CEA (ng/ml)				
≥4.6	15	0.249		
<4.6	23			
NNMT (pg/ml)				
≥710	11	0.517		
<710	27			
HGF (pg/ml)				
≥860	20	0.016	3.97 (1.08-14.6)[a]	0.038
<860	18			
IL-6 (pg/ml)				
≥2.7	23	0.013	4.76 (1.03-21.9)[b]	0.045
<2.7	15			
pN factor				
N (–)	14	0.455		
N (+)	21			

ADC: Adenocarcinoma; CEA: carcinoembryonic antigen; NNMT: nicotinamide *N*-methytransferase; HGF: hepatocyte growth factor; IL-6: interleukin-6; CI: confidence interval. [a]IL-6 as a factor was excluded from the variables; [b]HGF as a factor was excluded from the variables.

HR=3.71), and pT factor (p=0.001, HR=4.41) were statistically significant (Table II).

On analysis of the disease-free survival period, histological type, stage, pN factor and pT factor were found to be significant on univariate analysis, and histological type (p=0.02, HR=2.40) and pN factor (p=0.036, HR 2.41) to be significant on multivariate analysis (Table II).

Next, we analyzed the patients with stage III disease separately (Table III). Elevated HGF and IL-6 levels were associated with poor overall survival (Figure 3b and d). Disease-free survival was also affected by these cytokines, but not statistically significantly (Figure 3a and c). The pT and pN factors were not related to overall or disease-free survival of patients with stage III disease.

The results of multivariate analysis of overall survival of stage III patients are shown in Table III. Because there is a correlation between the IL-6 and HGF levels, each HR is significant only when the other factor is omitted from the analysis. A poorer prognosis was associated with higher HGF levels (p=0.038, HR=3.97) and higher IL-6 levels (p=0.045 HR=4.76). The prognosis of patients with NSCLC other than adenocarcinoma was poorer than that of those with adenocarcinoma, but not statistically significantly when IL-6 was included in the analysis, since the histological types affected IL-6 level (Table I).

On the other hand, the HGF and IL-6 levels were not associated with overall or disease-free survival of patients with stage I-II disease (Figure 3e and f).

Furthermore, we analyzed the overall survival of 109 patients based on combinations of the HGF and IL-6 levels (Figure 4). In patients with low HGF and low IL-6 levels, prognosis was favorable and the survival rate was 93%. On the other hand, the survival rate in patients with high levels of both cytokines, who had the poorest prognosis, was 56%. The survival rates in patients with high HGF and low IL-6 levels, and those with low HGF and high IL-6 levels, were 86% and 78%, respectively. Therefore, it would be effective in regard to prediction of the survival rate to use a combination of two biomarkers (p=0.0011).

Discussion

The results found in this study suggest that the HGF and IL-6 levels in blood are useful as predictors of the aggressive characteristics of stage III NSCLC. Since it is known that HGF is a stimulating factor for infiltration, and that it induces cell division and angiogenesis, a high HGF level in blood may be a marker suggesting latent metastasis. It is also suggested that cytokines, such as IL-6, released from a tumor and inflammatory cells stimulate fibroblasts in the lungs to produce HGF (24, 25). In addition, IL-6 directly stimulates the progression of lung tumor (29). It was reported that the IL-6 level would increase with increasing NSCLC stage, suggesting a correlation between high efficacy of chemotherapy and low IL-6 level in blood (34, 35). If tumor cells move from the primary tumor site and then induce local release of IL-6 and HGF, this will contribute to aggravation of the disease. Our study results showed a correlation between HGF and IL-6 levels, suggesting that IL-6 levels would reflect the HGF levels to a certain extent.

Disease-free survival was affected by these cytokines, but the correlation between relapse and these cytokines was not statistically significant. Chemotherapy may affect overall survival. Recently, the serum HGF levels in patients with advanced NSCLC were analyzed (22, 23). An association between HGF and gefitinib resistance was found in accordance with previous studies showing that the HGF/MET pathway played a role in the development of gefitinib resistance in NSCLC with an *EGFR* gene mutation (37, 38). It has also been reported that IL-6 is a biomarker of resistance to multitargeted receptor tyrosine kinase inhibitors in prostate cancer (39). In our study, patients with resectable stage III cancer were subjected to adjuvant chemotherapy using paclitaxel and carboplatin, but not gefitinib. Our results suggest that HGF and

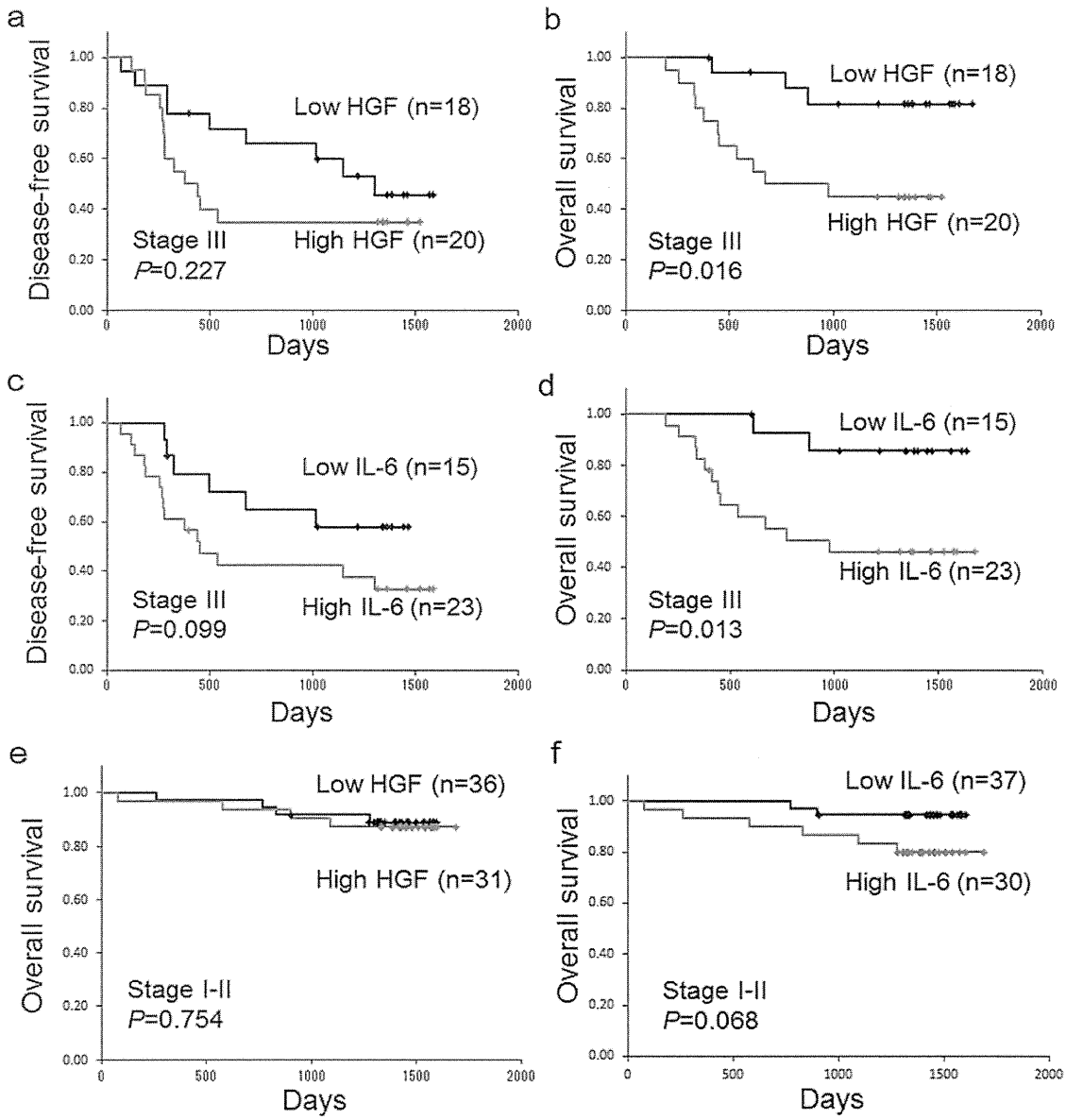

Figure 3. *Disease-free and overall survival curves for patienrs with stage III (a-d) and stage I-II (e-f) disease, according to the hepatocyte growth factor (HGF) (a, b, e) and interleukin-6 (IL-6) (c, d, f) levels.*

IL-6 generally stimulate resistance to chemotherapy. It has been reported that IL-6 reduces the sensitivity of cancer cells to chemotherapeutic agents, such as paclitaxel and cisplatin, by activating the PI3K/AKT and STAT3 pathways in cells (40); HGF has similar actions (41).

All these studies show that HGF and IL-6 are good molecular targets for cancer therapy (42-44). Our findings suggest that patients with stage III NSCLC who have low levels of HGF and IL-6 should be considered to be surgical candidates.

Disclosure Statement

The Authors have no conflict of interest.

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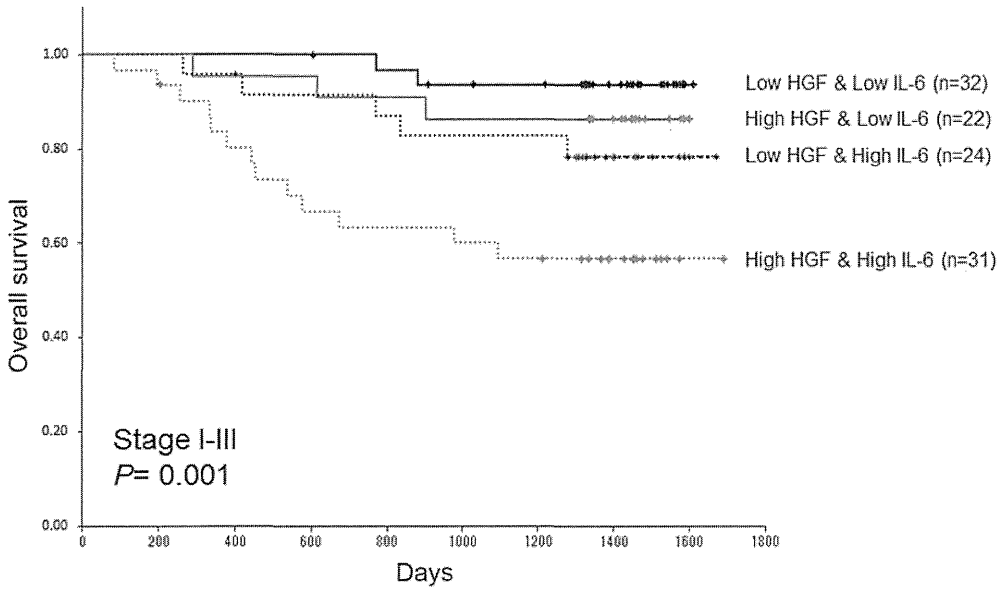

Figure 4. *Overall survival curves for 109 lung cancer patients, according to the combination of hepatocyte growth factor (HGF) and interleukin-6 (IL-6) levels.*

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Antiproliferative action of metformin in human lung cancer cell lines

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Abstract. The oral antidiabetic agent metformin has anticancer properties, probably via adenosine monophosphate-activated protein kinase activation. In the present study, growth inhibition was assessed by a clonogenic and by a cell survival assay, apoptosis induction was assessed by Hoechst staining and caspase activities and cell cycle alteration after exposure to metformin, and the interaction of metformin with cisplatin *in vitro* were elucidated in four human lung cancer cell lines representing squamous, adeno-, large cell and small cell carcinoma. Clonogenicity and cell proliferation were inhibited by metformin in all the cell lines examined. This inhibitory effect was not specific to cancer cells because it was also observed in a non-transformed human mesothelial cell line and in mouse fibroblast cell lines. Inhibition of clonogenicity was observed only when the cells were exposed to metformin for a long period, (10 days) and the surviving fraction, obtained after inhibiting proliferation by increasing the dose, reached a plateau at approximately 0.1-0.3, indicating the cytostatic characteristics of metformin. Metformin induced significant apoptosis only in the small cell carcinoma cell line. A tendency of cell cycle accumulation at the G0/G1 phase was observed in all four cell lines. Cisplatin, in a dose-dependent manner, severely antagonized the growth inhibitory effect of metformin, and even reversed the effect in three cell lines but not in the adenocarcinoma cell line. The present data obtained using various histological types of human lung cancer cell lines *in vitro* illustrate the cytostatic nature of metformin and its cytoprotective properties against cisplatin.

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Key words: metformin, lung cancer, cisplatin, apoptosis, cell line, cell cycle

Introduction

Metformin is an oral biguanide agent used worldwide to treat non-insulin-dependent diabetes mellitus. The initial reports related to the anticancer effects of metformin were epidemiological studies demonstrating a lower incidence of the occurrence and death of cancer in patients with diabetes mellitus treated with metformin compared to those treated with other antidiabetic agents (1,2). Consequently, these reports have triggered several clinical observational studies. Jiralerspong *et al* reported a significantly increased pathologically complete response rate in induction chemotherapy for breast cancer in diabetes patients receiving metformin compared to those not receiving metformin (3). Mazzone *et al* reported that diabetes patients with lung cancer who were previously treated with metformin or thiazolidinediones had a lower incidence of metastatic disease at the time of diagnosis and a reduced risk of death compared to those who did not receive the same treatment (4). Thereafter, the antiproliferative action of metformin was confirmed via *in vivo* and *in vitro* experiments in various cancer cell lines including breast (5-8), prostate (9), pancreas (10), and ovarian cancer (11-13) as well as lung adenocarcinoma (8).

Metformin is considered to exert anticancer effects via inhibition of insulin and the mammalian target of rapamycin (mTOR) pathways. Since insulin is a growth-promoting hormone with a mitogenic effect (14), metformin could indirectly inhibit tumor growth by ameliorating hyperinsulinemia, which is frequently observed in patients with non-insulin-dependent diabetes mellitus (15). In fact, Algire *et al* reported that metformin inhibited mouse lung tumor growth under specific conditions in which the animals were bred on a high-calorie diet (16). Although this would explain the effect of the agent *in vivo*, its *in vitro* effects (5-12) cannot be explained by this anti-insulin action. It has been reported that metformin inhibits complex-I of the respiratory chain in mitochondria, leading to increased AMP expression and liver kinase B1 (LKB1)-mediated activation of AMP kinase, finally inhibiting the mTOR downstream (15). Despite these findings, the precise mechanisms of the metformin-induced effects are not fully understood. In particular, controversy remains about

whether metformin is apoptotic (6, 10) or just cytostatic (5, 9) and whether it kills cancer cells synergistically with cytotoxic agents including cisplatin (11,13), paclitaxel (8), and doxorubicin (17), or if it is antagonistic to cisplatin (18,19).

In the present study, the cytotoxic effects of metformin were elucidated in various types of human lung cancer cell lines including squamous, adeno-, large, and small cell carcinoma, together with non-transformed cell lines. The drug-drug interaction between metformin and cisplatin was also investigated.

Materials and methods

Reagents. Metformin (1,1-dimethylbiguanide hydrochloride, #D150959-5G; Sigma-Aldrich Co., St. Louis, MO, USA) was diluted in distilled water. Cisplatin solution at a concentration of 0.5 mg/ml (pH 2.5–5.5) was purchased from Nippon Kayaku Co. (Tokyo, Japan).

Cells and cell culture. The human lung cancer cell lines RERF-LC-AI (#RCB0444) and A549 (#RCB0098) were purchased from the Riken Cell Bank (Tsukuba, Japan), while IA-5 (#RCB0548) and WA-hT (#RCB2279) (20) were established and maintained in our laboratory. The established mouse fibroblast cell line Balb/3T3 clone A31 (A31, #RCB0005) was purchased from the Riken Cell Bank. A nontumorigenic human mesothelial cell line derived from pleural effusion and immortalized by the pRSV-T plasmid Met5A (#CRL-9444) was purchased from the American Type Culture Collection (Manassas, VA, USA). The histological cell line types included RERF-LC-AI, squamous cell carcinoma; A549, adenocarcinoma; IA-5, large cell carcinoma; and WA-hT, small cell carcinoma. They were cultured as a monolayer in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ ml streptomycin in a 37˚C humidified atmosphere containing 5% CO_2.

Clonogenic assay. For a clonogenic assay involving a 10-day exposure to metformin, subconfluent cultured cells were trypsinized to obtain cell suspensions. Subsequently, a varied number of cells, such that the resulting colony number per plate would be approximately 20-50, were immediately replated onto 6-cm culture dishes in triplicate, cultured for 24 h in the complete medium until administration of various concentrations of metformin, and further cultured for 10 days without changing the medium. In the clonogenic assay involving 1- and 24-h exposure to the agents, subconfluent cultured cells were treated with various concentrations of agents for 1 or 24 h and then trypsinized, washed twice with the agent-free complete medium, replated on culture dishes as in the 10-day exposure method, and further cultured for 10 days. In each case, the obtained colonies were counted under a dissecting microscope after a 1% crystal violet staining.

Cell survival assay. In the cell survival assay, cells were plated onto 6-cm culture dishes in triplicate at a cell concentration of $1x10^5$/plate in complete medium. The cells were cultured for 24 h, and metformin or cisplatin at various concentrations were added to the medium and cultured for an additional 4 days. Viable cells negatively stained with 0.4% trypan blue were then counted. In the cell survival assays using combined treatment with metformin and cisplatin, doses of cisplatin that reduced the surviving cells to 50% (IC_{50}) and 10% (IC_{90}) with single-agent administration in each cell line were admixed with various concentrations of metformin, with other methods being similar to the methods of the single-agent experiments.

Apoptosis assay. Apoptosis was evaluated using morphological and enzymatic assays, that is, with Hoechst staining and by assessing caspase 3, 8 and 9 activities. For Hoechst staining, trypsinized cells together with floating cells were harvested, fixed with 1% glutaraldehyde, and stained with 1 mM bisbenzimide H 33248 fluorochrome trihydrochloride (Hoechst 33248; Ana Spec, Inc., Fremont, CA, USA). The cells were examined under fluorescence microscopy. Aggregating cells and cells with fragmented chromatin were considered apoptotic. More than 500 cells were evaluated, and the apoptotic cell ratio was recorded in each experiment. The activities of caspases 3, 8 and 9 were evaluated with the synthetic substrates DEVD-, IETD-, and LEHD-pNA, respectively, with the colorimetric assay kits APOPCYTO (Medical & Biological Laboratories Co., Nagoya, Japan) by monitoring the absorbance at a wavelength of 405 nm to measure p-nitroanilide (pNA) cleaved from synthetic substrates with cell extracts.

Cell cycle analysis. Cell cycle distribution was determined by the propidium iodide single-color method using a flow cytometer (FACSCanto II; BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. In brief, cells were trypsinized, fixed with 70% ethanol, washed with PBS(-), and treated with PI/RNase Staining buffer (BD Biosciences) at a concentration of $2x10^6$ cells/ml. The data were analyzed using the BD FACSDiva software (BD Biosciences).

Results

Colony formation and cell proliferation. Metformin exerted inhibitory effects on the clonogenicity of the 4 human lung cancer cell lines as well as that of non-transformed human mesothelial cell line and a mouse fibroblast cell line in a dose-dependent fashion when they were exposed to metformin for 10 days. On the other hand, a 1-h exposure to metformin did not show any significant inhibitory effect on the clonogenicity of any cell line, whereas a 24-h exposure showed slight suppression of clonogenicity in the A549, IA-5, and Met5A lines (Fig. 1). According to the cell survival assay, inhibition of cell proliferation was observed in the 4 human lung cancer cell lines when they were exposed to metformin for 4 days (Fig. 2). Cell proliferation inhibitory effects on the 4 cell lines exposed to cisplatin for 4 days are shown in Fig. 3.

Apoptosis. Apoptosis was assessed by Hoechst staining and by determining the activities of caspases 3, 8 and 9. The effects of metformin at IC_{30} and IC_{70} were examined in each cell line. Experiments with cisplatin at IC_{70} were conducted for comparison with metformin, and those with cisplatin at a higher dose were conducted for assay control. Apoptosis assessed by Hoechst staining failed to show significant apoptosis in all lung cancer cell lines except WA-hT, which had a significantly higher ratio of apoptosis compared to the non-treated cells