Table 3. Data for patients among patients with adenocarcinoma and performance status 1 or less (n = 38) without use of gefitinib

Patient number	Age (years)	Gender	Performance status	Metastasis to appendicular bone	Pathologic fracture	Solitary or multiple	Radiation	Chemotherapy	Outcome	Survival period (days)
1	52	Male	0	–	–	Multiple	+	+	Dead	460
2	61	Male	1	–	–	Multiple	–	–	Alive	48
3	59	Male	0	–	–	Multiple	–	+	Dead	294
4	39	Female	0	–	–	Multiple	+	+	Dead	365
5	28	Female	0	–	–	Multiple	–	+	Dead	336
6	56	Male	0	–	–	Multiple	–	+	Dead	369
7	51	Female	0	–	–	Multiple	+	+	Dead	201
8	61	Male	1	–	–	Solitary	+	–	Dead	188
9	57	Male	1	–	–	Multiple	–	+	Dead	28
10	49	Female	1	–	–	Multiple	+	+	Alive	303
11	49	Male	1	–	–	Multiple	+	+	Dead	243
12	63	Male	1	–	–	Solitary	–	+	Alive	390
13	59	Male	1	–	–	Multiple	–	+	Dead	144
14	54	Female	0	–	–	Multiple	+	+	Alive	285
15	51	Female	1	+	–	Multiple	+	–	Dead	61
16	57	Female	1	–	–	Multiple	+	–	Dead	53
17	63	Female	1	–	+	Multiple	–	+	Dead	244
18	65	Male	1	–	–	Multiple	+	+	Dead	166
19	62	Male	0	–	–	Multiple	–	+	Alive	470
20	55	Female	1	–	–	Solitary	+	+	Alive	207
21	42	Male	0	–	–	Multiple	+	–	Dead	36
22	56	Male	1	+	–	Multiple	+	+	Alive	316
23	28	Female	0	–	–	Multiple	–	+	Alive	308
24	56	Female	1	–	–	Multiple	+	+	Dead	351
25	60	Female	1	+	–	Multiple	+	+	Dead	196
26	63	Female	1	–	–	Multiple	–	–	Dead	68
27	47	Female	1	+	–	Multiple	+	+	Dead	163
28	66	Female	1	–	–	Multiple	–	+	Dead	393
29	45	Female	1	–	–	Multiple	–	+	Dead	345
30	41	Male	1	+	–	Multiple	+	+	Dead	306
31	55	Male	1	–	–	Solitary	–	+	Alive	1619
32	69	Male	1	–	–	Multiple	+	–	Dead	164
33	50	Male	1	–	+	Multiple	–	–	Dead	18
34	57	Female	1	–	–	Multiple	–	+	Alive	855
35	42	Female	1	–	–	Multiple	+	+	Dead	366
36	60	Female	1	–	–	Multiple	–	–	Dead	156
37	51	Male	1	–	–	Multiple	+	+	Dead	387
38	59	Female	1	–	–	Solitary	+	+	Alive	1416

resection with prosthesis implantation and two patients were treated with compound plate osteosynthesis. The remaining 10 patients had spinal compression fractures, of which two patients had complete paralysis of the lower extremities after pathologic fracture.

Regarding treatment of the primary site, radiotherapy was performed in 61 patients and systemic chemotherapy was administered to 67 patients. The administered regimens varied among patients, which included gemcitabine hydrochloride and vinorelbin ditartrate (11 patients), cisplatin and vinorelbin ditartrate (seven patients), carboplatin and vinorelbin ditartrate (six patients), carboplatin and paclitaxel (six patients), carboplatin and etoposide (four patients), carboplatin and etoposide (four patients), cisplatin and paclitaxel (four patients), cisplatin and etoposide (two patients), cisplatin and irinotecan hydrochloride (two

patients), cisplatin and gemcitabine hydrochloride (one patient), carboplatin and docetaxel hydrate (one patient), and unknown (19 patients). Systemic chemotherapy was not given to the remaining 51 patients.

We examined the cumulative survival rate after bone metastasis and prognostic factors for patients with bone metastasis from lung cancer (Table 1) and then calculated overall survival based on absence or presence of an EGFR inhibitor (Tables 2, 3).

Gefitinib (Irresa®; AstraZeneca, Osaka, Japan), an oral selective inhibitor of EGFR, was administered to patients with adenocarcinoma and PS 1 or less. In this study, there were 52 patients with adenocarcinoma and PS 1 or less. Gefitinib was administered to 14 of these patients (seven men, seven women; mean age ± SD, 63.4 ± 8.5 years; range, 42–72 years) (Table 2) and not administered to the remaining 38 patients (18 men, 20 women; mean age ± SD, 53.6 ± 9.5 years; range, 28–69 years) (Table 3).

We estimated patient survival using the Kaplan–Meier survival method, considering the relevant time scale for analysis to begin at the time of bone metastasis. Patients were censored on the basis of whether they were alive. The univariate log rank test was used to evaluate the prognostic importance of age, gender, PS, histologic type, condition of the primary site, number of bone metastases, site of bone metastasis, pathologic fractures, metastasis to the brain or liver, chemotherapy or radiotherapy for the primary site, and use of an EGFR inhibitor (gefitinib). Subsequent multivariate analysis was performed to detect factors independently associated with survival using a Cox proportional hazard survival model [4]. Multivariate regression analysis was performed by including all clinical characteristics that independently predicted 1-year survival. The results are reported as a hazard ratio and 95% confidence interval. As for the influence of gefitinib on survival, we used the Kaplan–Meier curve of overall survival based on absence or presence of gefitinib treatment among patients with adenocarcinoma and PS 1 or less. The log rank test was used to evaluate a difference. For all analyses, a p value of 0.05 or less was considered significant. We used SPSS 11.0 (SPSS Inc, Chicago, IL) software to conduct Kaplan–Meier survival analysis and the Cox proportional hazard survival model.

Results

The overall cumulative survival rate after bone metastasis for all 118 patients was 59.9% for 6-month survival, 31.6% for 1 year, and 11.3% for 2 years. The mean survival period was 9.7 months (SD, 10.3 months; median, 7.2 months; range, 0.1–74.5 months) (Fig. 2). Although the prognosis in patients with bone metastasis was generally poor, seven patients survived for at least 2 years (6%).

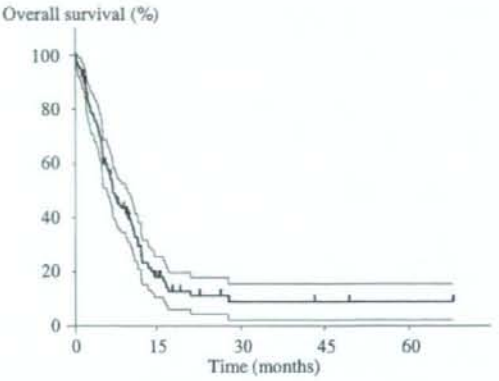

Fig. 2 A Kaplan–Meier curve of overall survival for all patients is illustrated. The dotted lines indicate the 95% confidence interval. The overall cumulative survival rates after bone metastasis for all 118 patients are 59.9% for 6 months, 31.6% for 1 year, and 11.3% for 2 years.

We identified eight prognostic factors: gender, PS, histologic type, number of bone metastases, site of bone metastases (bone metastasis to the appendicular bone), pathologic fracture, systemic chemotherapy, and gefitinib use (Table 4). A favorable prognosis was more likely in women and in patients with PS 1 or less, adenocarcinoma, solitary bone metastasis, no metastases to the appendicular bone, no pathologic fractures, use of systemic chemotherapy, and use of gefitinib.

The presence of adenocarcinoma, evidence of appendicular bone metastases, and use of gefitinib independently predicted survival (Table 5). The prognosis was poorer ($p = 0.03$) in patients with metastasis to the appendicular bone (mean, 6.5 months; range, 0.1–17.7 months) than in patients without metastasis (mean, 10.4 months; range, 0.2–74.5 months) (Fig. 3). The mean survival was longer ($p = 0.005$) in the group treated with gefitinib (17.8 months; range, 8.4–30.1 months) than in the group without gefitinib (10.8 months; range, 0.6–54.0 months) among 52 patients with adenocarcinoma and PS 1 or less (Fig. 4).

Discussion

It is important to know the prognosis after bone metastasis when treating bone metastasis from lung cancer. Primary site, PS, presence or absence of metastasis to organs, and number of bone metastases have been reported as important prognostic indicators in patients with bone metastasis from various cancers [5, 10, 20]. However, we are unaware of any previous reports regarding the prognostic factors of

Table 4. Univariate analysis of 1-year survival rates in patients with skeletal metastases of lung cancer (n = 118)

Prognostic factor	Subgroup	Survival (months)	1-year survival rate (%)	p Value
Age (years)	≥ 60	9.1 (1.3)	27.1 (5.6)	0.38
	< 60	10.4 (1.5)	32.6 (7.2)	
Gender	Female	13 (2.1)	39.3 (8.1)	0.02
	Male	7.9 (0.9)	25.8 (5.2)	
Performance status	0,1	11.6 (1.2)	44.8 (6.4)	< 0.0001
	2, 3, 4	7.1 (1.5)	13.3 (5.0)	
Subtype	Adenocarcinoma	11.3 (1.3)	41.6 (5.7)	< 0.0001
	Nonadenocarcinoma	5.8 (0.8)	8.9 (4.9)	
Surgery for lung cancer	Yes	11.0 (2.0)	27.8 (7.5)	0.89
	No	9.1 (1.1)	32.0 (5.6)	
Number of bone metastases	Solitary	14.0 (3.3)	54.3 (12.2)	0.02
	Multiple	8.9 (0.9)	27.3 (4.7)	
Appendicular bone metastasis	Yes	6.5 (1.1)	12.6 (8.0)	0.03
	No	10.4 (1.1)	35.6 (5.1)	
Pathologic fracture	Yes	6.4 (1.2)	6.7 (6.4)	0.04
	No	10.2 (1.1)	35.7 (5.0)	
Brain metastasis	Yes	9.9 (1.4)	32.7 (7.3)	0.65
	No	9.5 (1.3)	28.9 (5.6)	
Liver metastasis	Yes	7.0 (1.3)	13.4 (8.8)	0.1
	No	10.1 (1.1)	33.3 (4.9)	
Chemotherapy	Yes	11.4 (1.2)	45.3 (6.3)	0.0009
	No	7.5 (1.5)	13.0 (4.9)	
Radiation	Yes	9.7 (1.4)	30.0 (6.2)	0.49
	No	9.6 (1.3)	31.2 (6.5)	
Gefitinib	Yes	17.8 (1.8)	84.6 (10.0)	0.0001
	No	8.6 (1.0)	22.7 (4.4)	

Values are expressed as mean, with standard error in parentheses.

bone metastasis specifically from lung cancer. We examined the survival rates and prognostic indicators after bone metastasis from lung cancer.

The major limitations of our study included the lack of control subjects for comparison. Furthermore, there was a wide range of chemotherapy regimens and a selection bias of gefitinib use among the individual patients. Therefore, we compared the survival based on absence or presence of EGFR inhibitor treatment among patients with adenocarcinoma and PS 1 or less to exclude selection bias. The numbers of patients receiving EGFR was small (14) and therefore the power of the study is limited and must be considered preliminary. However, our study represents the largest followup study of patients with bone metastasis from lung carcinoma at one institution.

Some reports suggest the mean length of survival in patients with Stage IV disease, including distant metastasis, is approximately 6 months [2, 13]. The mean survival period for patients with lung cancer with bone metastasis has been reported as 5 to 6 months [15]. We found a mean survival period after bone metastasis of 9.7 months, with a median of 7.2 months. Approximately 70% of the patients died within 1 year after bone metastasis. Although the prognosis in patients with lung cancer with bone metastasis was extremely poor, seven of the 118 patients (6%)

Table 5. Multivariate analysis of selected clinical factors in patients with skeletal metastasis of lung cancer

Prognostic factor	p Value	Hazard ratio (95% confidence interval)
Positive		
Gender (female)	0.63	1.13 (0.68–1.88)
Performance status (0, 1)	0.09	1.69 (0.93–3.08)
Adenocarcinoma	< 0.01	2.17 (1.30–3.62)
Pathologic fracture	0.33	1.39 (0.71–2.73)
Chemotherapy	0.53	1.20 (0.68–2.11)
Gefitinib	0.03	2.42 (1.09–5.32)
Negative		
Multiple bone metastasis	0.14	1.68 (0.84–3.34)
Appendicular bone metastasis	0.01	2.05 (1.18–3.56)

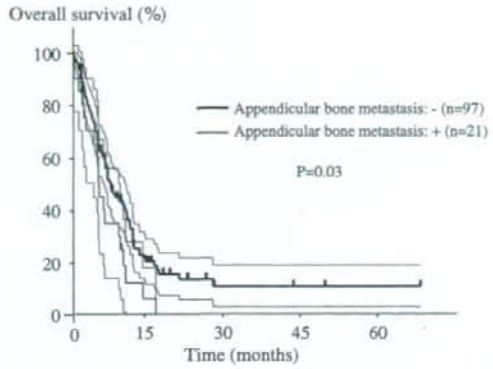

Fig. 3 A Kaplan–Meier curve of overall survival based on absence or presence of metastasis of the appendicular bone is illustrated. The dotted lines indicate the 95% confidence interval. The prognosis is poorer ($p = 0.03$) in patients with metastasis to the appendicular bone than in patients without metastasis.

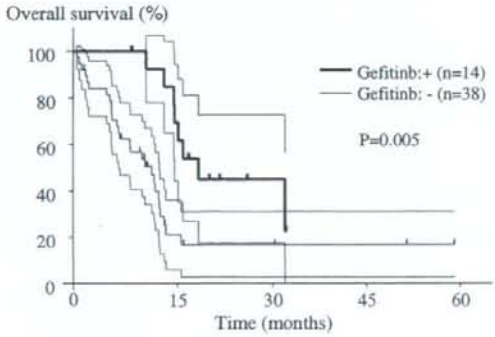

Fig. 4 A Kaplan–Meier curve of overall survival based on absence or presence of gefitinib treatment is illustrated. The dotted lines indicate the 95% confidence interval. The mean survival is longer ($p = 0.005$) in the group treated with gefitinib than in the untreated group among 52 patients with adenocarcinoma and PS 1 or less.

survived for at least 2 years. Hirano et al. [7] reported two patients with a solitary metastasis site who had extended survival by surgical resection of the metastatic site and chemotherapy. Agarwala and Hanna [1] also reported a patient with a solitary bone metastasis had apparently longer survival with aggressive treatment. Ando et al. [2] reported the grade of PS and the number of metastasized organs were important factors in patients with distal metastasis from lung cancer. In our study, the mean length of survival was substantially longer in patients with solitary-site metastasis than in patients with multiple-site metastases, and the survival rate was longer in patients with PS 1 or less than in patients with PS 2 or greater. It is suggested PS and number of bone metastases are associated with survival after bone metastasis [2].

Based on the primary site, Tofe et al. [16] reported a high incidence of metastasis in the lumbar vertebra, femur, and ilium among patients with prostate cancer; in the pelvis, vertebra, femur, and ribs among patients with breast cancer; and in the skull and vertebra among patients with thyroid cancer. We observed a high incidence of bone metastasis from lung cancer in the vertebra, rib, and pelvis, and metastasis to the femur in only 6%. The prognosis was poorer in patients with metastasis to the appendicular bone, such as the femur, than in patients with metastasis only to an axial bone, such as the vertebra, rib, or pelvis. The vertebral vein system is known as a mechanism for spread of axial bone metastasis [3]. In bone metastasis from lung cancer, metastasis may occur easily at an axial bone through the vertebral vein system [3] at an early stage and then at an appendicular bone in more advanced stages of the disease.

Among our study patients, the mean survival period was longer in the group treated with gefitinib than in the untreated group. Gefitinib, an EGFR inhibitor, is a new molecule-targeting treatment for lung cancer. It is reported to have a considerable effect on females and nonsmokers, especially those with adenocarcinoma [6, 11, 12, 21]. Analyses of single and multiple variables indicated better prognoses for patients with adenocarcinoma and patients treated with gefitinib. These findings suggest treatment with gefitinib may improve survival after bone metastasis. However, interstitial pneumonia remains a serious side effect [8]; furthermore, it is reported gefitinib is less effective in patients without the EGFR gene [12]. Therefore, indications for treatment with gefitinib should be considered carefully before improvement in survival can be expected.

We found a favorable prognosis was more likely in women and in patients with PS 1 or less, adenocarcinoma, solitary bone metastasis, no metastasis to the appendicular bone, no pathologic fracture, use of systemic chemotherapy, and use of gefitinib. Histologic subtype, no evidence of appendicular bone metastases, and use of gefitinib independently predicted survival. Our findings suggest treatment with EGFR inhibitor improves survival after bone metastasis. However, further investigations such as controlled clinical trials are needed to verify the usefulness of EGFR inhibitor.

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Efficacy and Safety of Pemetrexed in Combination with Cisplatin for Malignant Pleural Mesothelioma: A Phase I/II Study in Japanese Patients

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Background: Pemetrexed in combination with cisplatin (Pem/Cis) is used globally for the treatment of malignant pleural mesothelioma (MPM). This Phase I/II study was conducted to determine the recommended dose (RD) (Phase I) of Pem/Cis, and evaluate the efficacy and safety (Phase II) in Japanese MPM patients.

Methods: Key eligibility criteria were histologic diagnosis of MPM incurable by surgery, no prior chemotherapy, and a performance status 0–1. Under full vitamin supplementation, pemetrexed was intravenously administered on Day 1 of a 21-day cycle, followed by cisplatin. A cohort of six patients, starting from pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2 (Level 1), were studied in the dose-escalation Phase I (Step 1). The RD determined in Step 1 was carried forward into Phase II (Step 2). Planned number of patients treated with Pem/Cis was 18–38.

Results: In Step 1, 13 patients were enrolled: seven in Level 1 and six in Level −1 (pemetrexed 500 mg/m^2, cisplatin 60 mg/m^2). Two of six evaluable patients had dose-limiting toxicities (pneumonitis and neutropenia) in Level 1, establishing Level 1 as the RD. In Step 2, 12 patients were enrolled, for a total of 19 patients treated at the RD. Seven patients achieved a partial response among these patients, for a response rate of 36.8% (95% confidence interval: 16.3–61.6); overall survival was 7.3 months. One drug-related death occurred due to worsening of a pre-existing pneumonia. Common grade 3/4 toxicities were neutropenia and decreased-hemoglobin.

Conclusion: The Pem/Cis combination provides promising activity and an acceptable safety profile for chemonaive Japanese MPM patients with the same recommend dosage and schedule used in rest of the world.

Key words: cisplatin – mesothelioma – pemetrexed – phase I/II

INTRODUCTION

Malignant pleural mesothelioma (MPM) is a tumor derived from the mesothelium covering the surface of pleural membranes or from undifferentiated mesenchymal cells in connective tissue under the membranes. MPM is a locally invasive and aggressive tumor with a poor prognosis and a median survival time (MST) of ≈9–16 months (1).

MPM is known to be linked to asbestos exposure, and the incidence of this tumor is expected to increase in the next 10–20 years according to an estimation of asbestos consumption in

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the world (9). Recently, the prevalence of MPM in Japan was widely recognized after uncovering the high incidence of MPM and MPM-related deaths in ex-workers of asbestos factories and in residents of the surrounding areas who may have been subject to non-occupational exposure to asbestos fibers.

Surgical resection offers local control of the tumor but its effect on survival remains unclear. In addition, application of radiation therapy is limited because of the diffuse extension of tumor spread. Regimens applied to lung cancer such as platinum-containing chemotherapy have been used for MPM in Japan; however, the efficacy outcomes of these therapies are not satisfactory. Therefore, effective systemic chemotherapy for MPM is clearly needed.

Pemetrexed is a novel antifolate (12) that inhibits three enzymes in folate metabolism: thymidylate synthase, dihydrofolate reductase and glycinamide ribonucleotide formyltransferase (11). Because of the multi-targeted profile of this compound, broad and preferable anti-tumor activity is expected. Pemetrexed has shown clinical activity in various tumors including mesotheliomas (6). A pivotal multicenter, randomized Phase III study of pemetrexed (500 mg/m^2) in combination with cisplatin (75 mg/m^2) versus cisplatin alone (cisplatin 75 mg/m^2) in patients with MPM who had no prior chemotherapy was conducted in 20 countries (not including Japan) (16). A total of 448 patients were randomized and treated in this study (226 treated by pemetrexed/cisplatin (Pem/Cis) and 222 treated by cisplatin). MST in the Pem/Cis arm was 12.1 months compared with 9.3 months in the cisplatin arm ($P = 0.020$, two-sided log rank test). This was the first confirmation of significant prolongation of survival for patients with MPM. On the basis of this evidence, the combination of pemetrexed and cisplatin was approved for the treatment of MPM in the USA in 2004. Since then, the combination therapy has been approved in more than 80 countries and regions for the treatment of MPM, and recognized as a standard care for MPM (8).

In 2005, we initiated a Phase I/II study of Pem/Cis therapy in Japanese patients with MPM who had no prior chemotherapy. The primary objectives of this study were to determine the clinically recommended dose (RD) of Pem/Cis therapy in the Phase I portion of the study (Step 1), and to examine tumor response of the combination therapy in the Phase II portion (Step 2). The secondary objectives included time-to-event efficacy outcomes [the duration of response, progression free survival (PFS), and overall survival time], 1-year survival rate, quality of life (QOL) assessments, pulmonary function tests and safety.

PATIENTS AND METHODS

Patient selection

Chemonaive patients with histological diagnosis of MPM, regardless of clinical stage and who were not candidates for curative surgery, were assessed for eligibility. Eligible patients needed to be 20–74 years old with a life expectancy $\geq$12 weeks and an Eastern Cooperative Oncology Group performance status (PS) 0 or 1. Patients were also required to have adequate organ functions: bone marrow reserve [platelets $\geq 100 \times 10^3/mm^3$, hemoglobin $\geq$9.0 g/dl, and absolute neutrophil count (ANC) $\geq 2.0 \times 10^3/mm^3$], hepatic function [bilirubin $\leq 1.5 \times$ upper limit of normal (ULN), aspartate/alanine transaminase (AST/ALT) $\leq 2.5 \times$ ULN, and serum albumin $\geq$2.5 g/dl], renal function (serum creatinine $\leq$ULN, and calculated creatinine clearance $\geq$45 ml/min using the Cockcroft and Gault formula), lung function (functional oxygen saturation [SpO_2] $\geq$92%) and normal electrocardiogram.

Patients were excluded from this study for active infection, symptomatic brain metastasis, a wide-spread diffuse shadow in the lung caused by interstitial pneumonitis diagnosed by chest X-ray, pregnancy, serious concomitant systemic disorders incompatible with the study, clinically significant effusions, Common Terminology Criteria for Adverse Events (CTCAEs) v3 grade $\geq$2 peripheral neuropathy, the inability to discontinue aspirin and other non-steroidal anti-inflammatory agents or the inability or unwillingness to take folate and vitamin B_{12} during the study.

This study was conducted in compliance with the guidelines of good clinical practice and the Declaration of Helsinki, and it was approved by the local institutional review boards. All patients gave written informed consent before study entry. The Efficacy and Safety Evaluation Committee (ESEC), an independent body, was consulted if any efficacy and safety issues arose in the study.

Study Design

This was a Phase I/II, multicenter, single-arm, open-label study, performed in two steps. The RD level established in Step 1 was carried forward in Step 2. Patients enrolled in Step 1 at the RD level could continue in Step 2 unless otherwise indicated. The planned number of patients in total of Steps 1 and 2 treated with Pem/Cis was 18–38 for examination of efficacy and safety profile. In Step 1, six patients were to be enrolled in each dose level. The lower number of the planned number of patients, 18, was set as the minimum number of patients needed to confirm that the response rate of the study drugs was significantly larger than the threshold rate of 10% at one-sided significant level 0.05 with $\geq$80% power.

Study Treatment

Pemetrexed was intravenously administered as a 10-min infusion on Day 1 of a 21-day cycle, followed by cisplatin administration intravenously as a 2-h infusion 30 min after pemetrexed administration. Patients were instructed to take a daily 1 g multivitamin containing 500 μg of folate beginning 1 week prior to Day 1 of Cycle 1 until study discontinuation. Vitamin B_{12} (1000 μg) was intramuscularly injected, starting 1 week prior to Day 1 of Cycle 1 and repeated every 9 weeks until study discontinuation. Patients remained on study unless they were discontinued, for instance, due to disease progression and unacceptable adverse events.

Determination of RD for Step 2

In Step 1 (Phase I), four escalating dose levels were planned: pemetrexed at 500 (Level 1), 700 (Level 2), 900 (Level 3) and 1000 mg/m^2 (Level 4) with cisplatin held at 75 mg/m^2. In addition, a lower dose level (Level −1) was planned at pemetrexed 500 mg/m^2 and a lower dose of cisplatin 60 mg/m^2 for a failure case of dose-escalation in Level 1. In the dose-escalation procedure, the starting dose of pemetrexed was set to be 500 mg/m^2 which is ca. 40% of the maximum tolerated dose (MTD) of pemetrexed monotherapy with folic acid and vitamin B_{12} supplementation determined in a Japanese Phase I study; the MTD and RD of pemetrexed were determined to be 1200 and 1000 mg/m^2, respectively (7). The percentage of the starting dose to the MTD was based on a guideline for Phase I/II study on anticancer drugs (10). For escalation of pemetrexed dose, a modified Fibonacci dose-escalation method was used (2). Dose level reduction or escalation depended on the incidence of dose-limiting toxicity (DLT) at a given dose level (Fig. 1). If two of six patients at Levels 1, 2 or 3 developed DLT, that dose level was considered the RD for Step 2 (Phase II) of the study, and then Step 2 was initiated. This was also the case for Level −1 or 4 if 0–2 patients developed DLT. If three or more patients developed DLT at a given dose level (except dose Level −1), the next lower dose level was considered the RD level for Step 2. If three or more patients had DLT at Level −1, a decision was made as to whether the study should be continued.

A DLT was defined as a toxicity occurring in Cycle 1 meeting one of the following criteria: any grade ≥3 non-hematologic toxicity (except nausea, vomiting, anorexia and fatigue), grade ≥2 peripheral neuropathy or hearing loss/impairment, grade ≥3 febrile neutropenia (<1000/mm^3 with ≥38.5°C), grade 4 leukopenia (<1000/mm^3) or neutropenia (<500/mm^3) lasting ≥3 days, thrombocytopenia (<25000/mm^3), or thrombocytopenia requiring platelet transfusion. A failure to start the second cycle by Day 29 due to toxicity was also considered a DLT. All toxicities were assessed according to CTCAE.

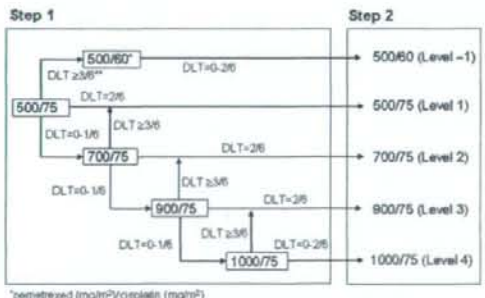

Figure 1. Scheme of dose-escalation Steps 1 and 2. DLT, dose-limiting toxicity.

Treatment Assessments

Anti-tumor Activity

Disease staging was assessed according to International Mesothelioma Interesting Group Tumor Node Metastasis (IMIG TNM) staging criteria (13). Within 28 days before the first treatment and approximately every 4 weeks after the first treatment, computer tomography or X-ray imaging of each lesion was performed. Tumor response was assessed using the modified Southwest Oncology Group (SWOG) criteria. Unidimensionally measurable lesions were defined as *Measurable disease*, and assessed objectively by the sum of the greatest diameters of them. Bidimensionally measurable lesions defined in the standard SWOG criteria (5) were assessed in the similar way. Best overall response selected from total overall response assessments was determined according to assessment of the Extramural Case Judgment Committee (E-CJC). Duration of response was measured as from the date of the first objective assessment of complete response (CR) or partial response (PR) until the date of the first assessment of progression of disease (PD). PFS was measured as from the registration date of Cycle 1 treatment until the first date of PD or death from any cause. Overall survival time was measured as from the registration date of Cycle 1 treatment until the date of death from any cause or until the last follow-up date in survival surveillance period.

QOL Assessments and Pulmonary Function Tests

QOL surveillance was employed using the following questionnaires: QOL questionnaire for cancer patients treated with anticancer drugs (QOL-ACD), and functional assessment of cancer therapy for lung cancer (FACT-L). These questionnaires were used on Day 1 of Cycles 1 and 2, and on 3 months after Day 1 of Cycle 1. QOL-ACD consists of four subscales (activity, physical condition, psychological condition and social relationships) and a total QOL scale (face scale) (4). The lung cancer subscale (LCS) score of FACT-L was used (3). As pulmonary function tests, forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1) and vital capacity (VC) were measured using a spirometer in the sitting position. All tests followed the Japanese Respiratory Function Test guidelines (14).

Safety

Adverse events were recorded throughout the study and after the last drug administration until signs of recovery were evident. Adverse events were evaluated according to treatment-emergent adverse events (TEAEs) definitions, and coded using the Medical Dictionary for Regulatory Activities (MedDRA v9.0). The severity (grade) of an adverse event was assessed according to CTCAE v3.

Statistical Analysis

The evaluation period of efficacy and safety in this study was defined as from the beginning of the study treatment to 5 months after the last patient began study treatment. For the

evaluations of overall survival time and 1-year survival rate, survival surveillance period was defined as from the beginning of the study treatment to 1 year after the last patient began study treatment. Patients who received the study drugs and complied with all inclusion/exclusion criteria were included in full analysis set (FAS). Patients who were treated with the RD level in Step 1 or 2 among FAS were included in efficacy analysis set for efficacy evaluation. Patients who received the study drugs at least once were included in safety analysis set for safety evaluation.

Assessment results of the best overall response by the E-CJC were used for efficacy analysis. Statistical tests based on binominal distribution were done to confirm that the response rate of the study drugs was significantly larger than the threshold rate of 10% at one-sided significant level 0.05. The threshold rate 10% was set on the basis of historical data on the response rate of cisplatin alone arm reported in other studies (15,16).

RESULTS

Patient Characteristics

From 2005 to 2006, a total of 25 Japanese patients with MPM were enrolled in Steps 1 and 2 at seven centers in Japan. All patients met the eligibility criteria and received study treatment; all were included in FAS. One patient was still receiving the study drug at the time of the efficacy and safety evaluations in this report.

Patient characteristics are summarized in Table 1. The majority of patients were male (22 patients, 88.0%). The median age was 61 years (range: 50–74 years). Most patients had a PS of 1 (18 patients, 72.0%) and clinical stage IV (21 patients, 84.0%). The predominant histologic subtype was epithelial in 64% of patients. Two demographic characteristics showed differences among dose levels. There were more patients with PS 0 in Level −1 (50.0%) than in Level 1 (21.1%). All six (100%) patients in Level −1 had the epithelial subtype versus 10 (52.6%) patients in Level 1.

Table 1. Patient characteristics

	Step 1 Level −1 ($n = 6$)	Level 1 ($n = 19$)	All treated ($n = 25$)
Gender			
Male	5	17	22
Female	1	2	3
Age			
Mean	61	61	61
SD	3.9	6.3	5.8
Med	61	59	61
Weight(kg)			
Mean	62.8	58.1	59.2
SD	8.51	11.19	10.65
Performance status prior to Cycle 1			
0	3	4	7
1	3	15	18
Histological subtype			
Epithelioid mesothelioma	6	10	16
Sarcomatoid mesothelioma	0	5	5
Biphasic mesothelioma	0	4	4
Other	0	0	0
Asbestos exposure			
Had no exposure	2	3	5
Had exposure	4	16	20
Stage of disease			
Ia	0	0	0
Ib	0	1	1
II	0	1	1
III	1	1	2
IV	5	16	21

Level 1: pemetrexed 500 mg/m^2 + cisplatin 75 mg/m^2
Level −1: pemetrexed 500 mg/m^2 + cisplatin 60 mg/m^2
SD, standard deviation.

Dose-Escalation, Dose-Limiting Toxicity and RD

One patient in Level 1 of Step 1 died on Day 14 of Cycle 1 due to exacerbation of pneumonia, respiratory failure (hypoxia) and disseminated intravascular coagulation (DIC). The ESEC evaluated the case of the early death. Since the patient had had the shadow of the lung detected by radiographic image prior to receiving study treatment, it was unlikely that the administration of pemetrexed was the primary cause of the pneumonia. The autopsy of this patient showed that interstitial changes in the lung were mild and the pathological diagnosis was an organizing pneumonia. The result of the autopsy was compatible with the clinical course and suggested that the direct cause of the death was not the drug-induced interstitial pneumonia but the exacerbation of infectious pneumonia, worsened by the study treatment. The case, therefore, was considered not appropriate for the DLT evaluation.

One patient was added in this dose level to assess the safety profile additionally. Among the six patients in Level 1 excluding the case inappropriate for the DLT evaluation, two patients showed DLTs: drug-induced pneumonitis in one patient and dose delay of Cycle 2 initiation due to decreased neutrophil count in the other. According to the protocol definition, Level 1 was determined to be an RD for the next phase (Fig. 1).

The ESEC, however, recommended examining the treatment at Level −1 (pemetrexed 500 mg/m^2 and cisplatin 60 mg/m^2) exploratively to accumulate more safety information. Accordingly, six patients were enrolled and treated at Level −1, and no DLTs were observed in this dose level.

Evaluating the data of these two levels together, the ESEC agreed to continue Step 2 carefully with the dose of Level 1. The sponsor decided to carry forward into Step 2 with

an RD of Level 1 (pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2). In Step 2, 12 patients were treated at Level 1.

Efficacy

Nineteen patients (7 in Step 1 and 12 in Step 2) in Level 1 were included in the efficacy analysis set and of 19 patients, seven patients had PR, five patients had stable disease (SD), six patients had PD and one patient was classified as not evaluated. An overall response rate (ORR) was 36.8% [95% confidence interval (CI): 16.3%–61.6%]. The 95% one-sided confidence lower limit was 18.8%, exceeding the threshold level of 10%. The six patients in Level −1 had PR; thus, the ORR for all 25 patients treated with the study drug reached 52.0% (13 total PR, 95% CI: 31.3%–72.2%).

The secondary efficacy variables were time-to-event outcomes (the duration of response, PFS and overall survival time), 1-year survival rate, QOL and pulmonary function test. The median duration of response was 5.2 months (95% CI: 4.3–7.3 months) for the seven responders in the efficacy analysis set (Table 2). The median duration of response for the six responders at Level −1 was again 5.2 months. For the efficacy analysis set, median PFS was 4.7 months (95% CI: 1.3–6.5 months) and MST was 7.3 months (95% CI: 4.6–14.2 months, Fig. 2) with 1-year survival rate of 36.8% (95% CI: 15.2%–58.5%). Median PFS for the six patients at Level −1 was 10.1 months. MST at Level −1 could not be calculated by Kaplan–Meier method. The 1-year survival rate of Level −1 (66.7%) was beyond 50%.

The QOL-ACD and FACT-L measures were used for QOL evaluation. There were no major changes from prior to Cycle 1 to 3 months after Cycle 1 treatment in the mean scores for the activity and physical condition subscales of QOL-ACD (Table 3); however, mean scores from prior to Cycle 1 to 3 months after Cycle 1 treatment for the psychological condition and social relationships subscales numerically increased. The mean LCS score of FACT-L did not change substantially from prior to Cycle 1 to 3 months after Cycle 1 treatment (data not shown). These score changes indicate that QOL of the patients was maintained without worsening from baseline. Pulmonary function was also maintained with no worsening from baseline observed in the pulmonary function tests (FEV_1, FVC and VC) in the efficacy analysis set (data not shown).

Safety

Of 25 patients of the safety analysis set, three died during the study period: one (Level 1, Step 1) from exacerbation of pneumonia as a pre-existing complication, respiratory failure, and DIC, as described earlier, and the other two (Step 2) due to study disease. Two patients experienced non-fatal serious adverse events (fever and aspiration pneumonia, respectively). A causal relationship between fever and the study drugs could not be ruled out, but the aspiration pneumonia was not considered related to study drugs. Adverse events leading to discontinuation from study treatment were observed in six patients: one patient at Level 1 and three patients at Level −1 in Step 1 and in two patients in Step 2. Adverse event leading to discontinuation in two or more patients was increased blood creatinine (two patients).

Table 2. Summary of time-to-event outcomes and 1-year survival rates

	Step 1 Level −1 (n = 6)	Level 1 (n = 19)	All treated (n = 25)
Duration of response (months)			
Responders	6	7	13
Med	5.2	5.2	5.2
(95% CI)	3.1 − *	4.3–7.3	4.3–7.3
Range	2.7–9.6	2.0–7.3	2.0–9.6
Censored (%)	50	14.3	30.8
Progression free survival (months)			
Med	10.1	4.7	4.8
(95% CI)	4.3 − *	1.3–6.5	2.5–7.1
Range	3.3–12.1	0.5–9.6	0.5–12.1
Censored (%)	50	10.5	20
Overall survival (months)			
Med	NA	7.3	9.2
(95% CI)	11.1 − *	4.6–14.2	5.8–14.4
Range	8.6–19.3	0.5–21.5	0.5–21.5
Censored (%)	66.7	21.1	32
1-year survival rate (%)			
	66.7	36.8	44.0
(95% CI)	28.9–100.0	15.2–58.5	24.5–63.5

*Not calculated. NA, not assessed.
Level 1: pemetrexed 500 mg/m^2 + cisplatin 75 mg/m^2.
Level −1: pemetrexed 500 mg/m^2 + cisplatin 60 mg/m^2.
CI, confidence interval.

Grade 3 or more laboratory TEAEs were observed in 16 patients: four patients at Level 1 and five patients at Level −1 in Step 1 and in seven patients in Step 2. Laboratory TEAEs observed in at least half of the 25 patients were decreased-hemoglobin, decreased red blood cell count, decreased neutrophil count, decreased white blood cell count, decreased lymphocyte count, increased blood urea and decreased body weight (Table 4). Grade 3 or more non-laboratory TEAEs were observed in eight patients: three patients at Level 1 and one patient at Level −1 in Step 1 and in four patients in Step 2. Non-laboratory TEAEs observed in at least half of the 25 patients were nausea, anorexia, vomiting and malaise. No major differences between Levels 1 and −1 (Step 1) in the incidence of TEAEs were noted.

For the 19 patients at Level 1, laboratory TEAEs of grade 3 or higher, possibly related to drug, and observed in at least two patients were decreased neutrophil count (seven patients, 36.8%), decreased hemoglobin (six patients, 31.6%), decreased white blood cell count (five patients, 26.3%), decreased lymphocyte count (five patients, 26.3%),

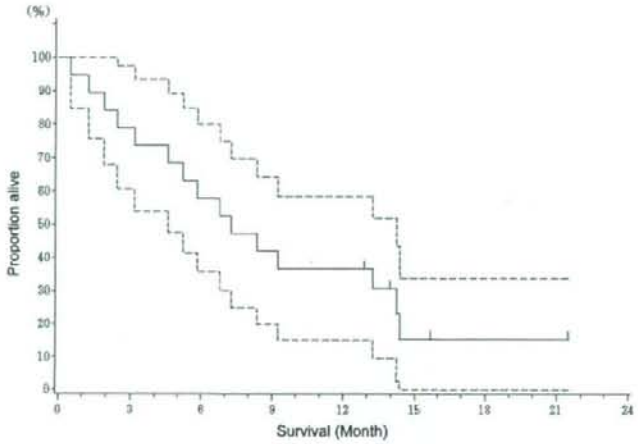

Figure 2. Kaplan–Meier plot of overall survival in the efficacy analysis set. Solid lines, overall survival; dotted lines, high and low limits of 95% confidence interval.

decreased platelet count (two patients, 10.5%) and decreased blood potassium (two patients, 10.5%). Non-laboratory adverse drug reactions of grade 3 or higher observed in at least two patients were vomiting (three patients, 15.8%), anorexia (three patients, 15.8%), nausea (two patients, 10.5%) and malaise (two patients, 10.5%). Adverse drug reactions of grade 3 or higher for the six patients in Level −1 were decreased neutrophil count (three patients), decreased-hemoglobin (two patients), decreased lymphocyte count (two patients) and decreased red blood cell count (one patient).

DISCUSSION

This Phase I/II study reports the first experience of the combination of pemetrexed and cisplatin therapy in Japanese patients. The RD of Pem/Cis combination therapy was established at pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2, with pemetrexed administration on Day 1 of each 21-day cycle followed by cisplatin, which is the same regimen used in worldwide for patients with MPM (16).

Of the 19 patients evaluable for efficacy at the RD level, there were PRs in seven patients, for an ORR of 36.8% (95% CI: 16.3%–61.6%). A pivotal Phase III study of the same regimen as that applied of the present study, yielded a response rate of 41.3% (95% CI: 34.8%–48.1%) in 225 patients (16). The response rates from both studies are comparable despite of the large difference in sample size.

The response rate of all the 25 treated patients was higher than the response rate for the 19 patients treated at the RD (52.0% versus 36.8%). This is due to the fact that all the six patients in Level −1 had PR. The excellent outcome observed in Level −1 may be attributed to differences between those patients who received the RD and those patients in Level −1 in the histological subtype of mesothelioma. All six patients in Level −1 had an epithelial subtype, which is known as a favorable prognostic factor, while only about half of the 19 patients at the RD had this subtype. In addition, the PS of the patients in Level −1 was better than the patients at RD.

A secondary efficacy endpoint MST showed 7.3 months in this study, shorter than that of the Pem/Cis arm in the Phase III study (12.1 months) (16). Although it would be difficult to compare MST of this study derived from a small sample size with the large Phase III study ($n = 226$), the discrepancy of survival between the two studies could be ascribed for the demographic characteristics of patients in both. There are less patients who had good prognostic factors in this study than in the Pem/Cis arm of the Phase III study: epithelial subtype: 52.6% versus 68.1%, a good PS: 21.1% (PS = 0) versus 51.8% (Karnofsky PS = 90/100) and clinical stage I/II: 8.0% versus 22.6% (16).

In this study, the most common adverse events (>50% of patients) were decreased-hemoglobin, erythropenia, neutropenia, leukopenia and lymphopenia for laboratory parameters, and nausea, anorexia, and vomiting for non-laboratory parameters. These hematologic and gastrointestinal events were similarly observed in the Pem/Cis arm of the pivotal Phase III study (16). No grade 3/4 febrile neutropenia toxicity which is a potentially life-threatening event was reported in our study. One death by pneumonitis was observed in this study; however, the patient was considered to have a pre-existing condition before initial treatment with study therapy. Adverse events observed in this study were predictable from safety profile observed in overseas trials and market experiences of pemetrexed and cisplatin combination therapy.

Table 3. Summary of QOL questionnaire for cancer patients treated with anticancer drugs (Level 1, $n = 19$)

Subscale	Measurement Point	n	Mean	SD	Min	Med	Max
Activity							
	Prior to Cycle1	19	62.9	25.35	20.0	60.0	100.0
	Prior to Cycle2	15	61.8	32.27	5.0	70.0	100.0
	Prior to Cycle3	14	69.6	21.79	20.0	75.0	95.0
	Cycle1 + 3M	11	60.5	32.13	5.0	70.0	100.0
Physical							
	Prior to Cycle1	19	64.7	22.33	15.0	70.0	100.0
	Prior to Cycle2	15	64.3	18.11	20.0	65.0	95.0
	Prior to Cycle3	14	66.2	18.33	30.0	70.0	85.0
	Cycle1 + 3M	11	61.4	21.46	35.0	60.0	95.0
Psychological							
	Prior to Cycle1	19	53.2	20.62	12.5	56.3	81.3
	Prior to Cycle2	15	59.6	24.87	12.5	62.5	100.0
	Prior to Cycle3	14	58.0	17.41	31.3	56.3	87.5
	Cycle1 + 3M	11	61.4	18.07	37.5	68.8	87.5
Social							
	Prior to Cycle1	19	32.9	21.56	5.0	25.0	75.0
	Prior to Cycle2	15	33.7	19.13	0.0	25.0	70.0
	Prior to Cycle3	14	43.6	19.94	10.0	42.5	85.0
	Cycle1 + 3M	11	36.4	22.59	10.0	30.0	85.0
Face scale							
	Prior to Cycle1	19	50.0	23.57	0.0	50.0	100.0
	Prior to Cycle2	14	55.4	24.37	0.0	50.0	100.0
	Prior to Cycle3	14	64.3	23.44	25.0	50.0	100.0
	Cycle1 + 3M	11	63.6	20.50	25.0	75.0	100.0

Level 1: pemetrexed 500 mg/m^2 + cisplatin 75 mg/m^2. M, months.
QOL, quality of life.

CONCLUSION

The RDs for the Pem/Cis combination are pemetrexed 500 mg/m^2 and cisplatin 75 mg/m^2, which is the same regimen used in worldwide for patients with MPM. The combination shows promising efficacy with an acceptable safety profile in Japanese patients with MPM.

On January 2007, Pem/Cis combination therapy was approved and launched for the treatment of patients with MPM in Japan. Intensive post-marketing surveillance in patients with MPM is ongoing.

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Conflict of interest statement

S.A. and Y.N. are employed by the sponsor, Eli Lilly Japan K.K.; N.S. and M.F. are paid consultants to the sponsor.

Table 4. Summary of treatment-emergent adverse events (TEAEs) reported >25% patients

System organ class preferred term	Step 1 Level −1 ($n = 6$)	Level 1 ($n = 19$)	All treated ($n = 25$)
Patients with ≥1 TEAEs	6	19	25
Laboratory			
Hemoglobin decreased	6	18	24
Red blood cell count decreased	6	16	22
Neutrophil count decreased	5	16	21
White blood cell count decreased	5	15	20
Lymphocyte count decreased	5	12	17
Blood urea increased	5	11	16
Weight decreased	3	12	15
Blood albumin decreased	2	10	12
Platelet count decreased	4	8	12
Protein total decreased	3	9	12
Blood creatinine increased	4	7	11
Neutrophil count increased	2	8	10
White blood cell count increased	2	8	10
Blood sodium decreased	2	7	9
Alanine aminotransferase increased	1	7	8
Protein urine present	1	7	8
Aspartate aminotransferase increased	1	6	7
Blood magnesium decreased	2	5	7
Blood potassium decreased	0	7	7
Non-laboratory			
Nausea	6	18	24
Anorexia	6	16	22
Vomiting	3	15	18
Malaise	5	10	15
Constipation	3	9	12
Hiccups	3	5	8
Rash	2	6	8
Diarrhoea	1	6	7
Oedema	2	5	7
Pyrexia	2	5	7
Dysgeusia	3	4	7
Headache	1	6	7

Level 1: pemetrexed 500 mg/m^2 + cisplatin 75 mg/m^2
Level −1: pemetrexed 500 mg/m^2 + cisplatin 60 mg/m^2
MedDRA Ver 9.0.

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ORIGINAL ARTICLE

mRNA expression of RRM1, ERCC1 and ERCC2 is not associated with chemosensitivity to cisplatin, carboplatin and gemcitabine in human lung cancer cell lines

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mRNA expression of RRM1, ERCC1 and ERCC2 is not associated with chemosensitivity to cisplatin, carboplatin and gemcitabine in human lung cancer cell lines
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Background and objective: Expression of genes involved in DNA repair and/or DNA synthesis, including ribonucleotide reductase M1 (RRM1) and excision repair cross-complementation 1 (ERCC1) has been reported to be associated with chemosensitivity to platinum agents and gemcitabine. The aim of this study was to test whether similar associations would be seen between mRNA expression for the RRM1, ERCC1 and ERCC2 genes and *in vitro* chemosensitivity in lung cancer.

Methods: Using a panel of 20 lung cancer cell lines, including 15 NSCLC and 5 small cell lung cancers (SCLC), the mRNA expression levels for the RRM1, ERCC1 and ERCC2 genes were examined by quantitative real-time reverse transcription PCR. The *in vitro* cytotoxicity of cisplatin, carboplatin and gemcitabine was assessed using a tetrazolium-based colorimetric assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) assay).

Results: Significantly, higher RRM1 mRNA expression was found in SCLC compared with NSCLC. However, there were no correlations between mRNA expression of the ERCC1, ERCC2 and RRM1 genes and chemosensitivity to cisplatin, carboplatin or gemcitabine.

Conclusions: These *in vitro* results suggest that further studies are needed to evaluate the expression of the RRM1, ERCC1 and ERCC2 genes as predictive biomarkers for sensitivity to platinum agents and gemcitabine.

Key words: chemosensitivity, DNA repair, DNA synthesis, lung cancer, predictive biomarker.

INTRODUCTION

Lung cancer is a leading cause of cancer deaths both in Japan and the USA.[1,2] Despite advances in the molecular biology, diagnosis and treatment of non-small cell lung cancer (NSCLC), which accounts for about 85% of all lung cancers, the improvement in long-term survival has only been marginal.[3] The best prospects of a cure are offered by surgical removal of early stage lung cancer, followed by concurrent chemoradiotherapy for locally advanced lung cancer. Chemotherapy for advanced lung cancer offers mild benefits in improvement of quality of life and increased survival time.

The common first-line chemotherapeutic regimens for advanced NSCLC are platinum-based combinations. The combinations of cisplatin or carboplatin with another cytotoxic agent such as paclitaxel, docetaxel, gemcitabine, vinorelbine or irinotecan produce similar response rates of about 30–40% and a median survival time of about 1 year.[4,5] To improve clinical outcomes in advanced NSCLC, clinical integration of molecular biomarkers that predict

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responses to chemotherapeutic or molecularly targeted agents, leading ultimately to individualized chemotherapy, may be important. Despite intensive studies, however, only mutations of the epidermal growth factor receptor (EGFR) gene have been validated as correlating with the clinical efficacy of EGFR tyrosine kinase inhibitors.[6]

Recently, expression of genes involved in DNA repair and/or DNA synthesis have been reported to be associated with chemosensitivity to platinum agents and gemcitabine, as well as clinical outcomes in patients with surgically resected early stage NSCLC.[7–9] Excision repair cross-complementation 1 (ERCC1) is one of the key enzymes in the nucleotide excision repair (NER) pathway.[10] Platinum agents such as cisplatin and carboplatin induce monoadducts and intrastrand or interstrand cross-linking of DNA.[10] The removal of adducts from genomic DNA is mediated by the NER pathway, in which ERCC1 forms a heterodimer with the xeroderma pigmentosum group F (XPF) protein and excises the nucleotide segment containing the adducts in coordination with XPG. ERCC2/XPD is also a component of the NER mechanism.[11] Enhanced gene expression in the NER pathway has been thought to be a major cause of resistance to cisplatin and other DNA-damaging chemotherapeutic agents. Ribonucleotide reductase M1 (RRM1) is involved in DNA synthesis, catalysing the biosynthesis of deoxyribonucleotides from the corresponding ribonucleotides, which is the molecular target of gemcitabine.[12] Earlier work had suggested that patients with low levels of tumour RRM1 mRNA expression had improved survival compared with those with high RRM1 mRNA expression levels, when treated with gemcitabine.[11] Therefore, analysis of the expression of these genes could be useful in the development of predictive biomarkers for NSCLC.

The identification of molecular biomarkers with the potential to predict treatment outcomes is essential for triaging patients to the most beneficial therapy. As one of the multiple approaches to establishing robust predictive biomarkers, we evaluated whether there would be associations between mRNA expression of the ERCC1, ERCC2 and RRM1 genes and *in vitro* chemosensitivity to cisplatin, carboplatin and gemcitabine.

METHODS

Cell lines

Fifteen NSCLC and five small cell lung cancer (SCLC) cell lines were used. Two NSCLC and 4 SCLC sell lines, with the prefix ACC-LC-, were established in our laboratories at Aichi Cancer Center. These cell lines were derived from lymph node metastases (–80, –94), pleural effusions (–49, –319) or pericardial effusions (–48, –172). NCI-H460 and A549 were purchased from the American Type Culture Collection (Manassas, VA, USA). PC-1 and PC-10 were generously provided by Dr Y. Hayata (Tokyo Medical University, Tokyo, Japan). The remaining 10 cell lines (VMRC-LCD, RERF-LC-MT, -AI, Calu1, Calu6, SK-MES-1, SK-Lu-1 and SK-LC-2, –3 and –6) were generous gifts from Dr Old and Dr M. Akiyama. All cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum.

Drugs

Gemcitabine (Gemzar) was provided by Eli Lilly, Kobe, Japan. Cisplatin and carboplatin were provided by Bristol-Myers Squibb, Tokyo, Japan.

Cytotoxicity assay

Exponentially growing cells were harvested and resuspended at a final concentration of $1–20 \times 10^4$ cells/mL in fresh medium. Cell suspensions (100 μL) were dispensed into 96-well tissue culture plates and after 24 h at 37°C, various concentrations of the anticancer agents were added and incubated for 3 days. Cytotoxicity was evaluated by complete dose–response curves in the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT assay) as described previously.[13] The per cent cytotoxicity was calculated as: % cytotoxicity = {1-[Optical Density (OD) treated]/(OD control)} × 100. Each experiment was repeated at least three times. The cytotoxic effect of each treatment was assessed as the IC50 (drug concentration inducing a 50% reduction in cell survival in comparison with the control untreated cells), which was calculated from the dose–response curves.

RNA preparation

Cells were lysed with 1 mL of Isogen (Nippongene, Toyama, Japan) and total RNA was extracted according to the manufacturer's protocol, with the addition of glycogen to facilitate RNA precipitation. The RNA was further purified and treated with DNase (RNeasy kit, Qiagen, Valencia, CA, USA) according to the manufacturer's protocol, and stored at –80°C until use.

Reverse transcriptase-PCR amplification

Total RNA (50 ng) extracted from each cell line was subjected to one-step real-time reverse transcriptase (RT)-PCR for absolute quantitation of the mRNA levels of the ERCC1, ERCC2, RRM1 and β-actin genes, using the Applied Biosystems 7500F PCR system (Applied Biosystems, Foster City, CA, USA). The assays were performed in 20 μL reaction mixtures, using a One-step SYBR PrimeScript RT-PCR kit (TAKARA, Ohtsu, Japan) according to the manufacturer's protocol. The sequences of the primers are shown in Table 1. The RT-PCR condition was an initial incubation at 42°C for 5 min followed by 10-s incubation at 95°C, then 40 cycles at 95°C (5 s), 60°C (34 s). Linear regression analysis of standard curves demonstrated a strong correlation for all genes ($r^2 > 0.98$). The

Table 1 The primer sequences and PCR reaction conditions

	Forward primer sequence	Reverse primer sequence
ERCC1	CTCAAGGAGCTGGCTAAGATGT	CATAGGCCTTGTAGGTCTCCAG
ERCC2	CTGGAGGTGACCAAACTCATCTA	CCTGCTTCTCATAGAAGTTGAGC
RRM1	CGCTAGAGCGGTCTTATTTGTT	TTGCTGCATCAATGTCTTCTTT
β-actin	TTCTACAATGAGCTGCGTGTG	CAGCCTGGATAGCAACGTACA

ERCC1, excision repair cross-complementation 1; ERCC2, excision repair cross-complementation 2; RRM1, ribonucleotide reductase M1.

Table 2 IC50 values for cisplatin, carboplatin and gemcitabine in lung cancer cell lines

Cell line	Histology	Cisplatin (μmol/L)	Carboplatin (μmol/L)	Gemcitabine (μmol/L)
ACC-LC-94	Ad	1.14	18.4	0.0119
ACC-LC-319	Ad	16.5	284	>128
SK-LC-3	Ad	39.7	512	>128
A549	Ad	4.22	47	0.00821
SK-Lu-1	Ad	40.2	512	1
VMRC-LCD	Ad	14.3	147	7.17
RERF-LC-MT	Ad	5.21	92.9	>128
Calu1	Sq	9.96	89.9	0.398
SK-MES-1	Sq	1.81	28.1	0.00411
PC-1	Sq	0.127	1.84	0.00303
RERF-LC-AI	Sq	2.69	33	0.00394
PC-10	Sq	8.23	430	>128
NCI-H460	La	3.83	49.4	0.0135
Calu6	La	0.939	15.5	0.00778
SK-LC-6	La	2.35	37.3	0.00244
ACC-LC-48	SCLC	3.2	35.8	0.0191
ACC-LC-49	SCLC	3.71	52.8	1
ACC-LC-80	SCLC	3.18	43.7	0.0344
ACC-LC-172	SCLC	2.78	35.2	0.0125
SK-LC-2	SCLC	7.91	50.9	>128

Ad, adenocarcinoma; La, large cell lung cancer; SCLC, small cell lung cancer; Sq, squamous cell lung cancer.

relative gene expression levels were normalized to those of the house keeping gene, β-actin.

Statistical analysis

The strength of the association between the expression of ERCC1, ERCC2 and RRM1 and chemosensitivity of the cell lines was calculated using either Pearson's correlation coefficient or linear regression analysis. Correlations were considered significant at $P < 0.05$. One-way analysis of variance (ANOVA) followed by the Bonferroni post-test was used for comparison of RRM1 expression levels among the different cell lines. All analyses was performed using Stat View version 5.0 software.

RESULTS

Chemosensitivities to cisplatin, carboplatin and gemcitabine were examined in 20 human lung cancer cell lines, including 15 NSCLC and 5 SCLC cell lines. Cytotoxicity was measured by the MTT assay following 72 h of continuous exposure to the drugs. The IC50 values for these agents on each cell line are shown in Table 2. The IC50 values of gemcitabine for ACC-LC-319, SK-LC-3, RERF-LC-MT and PC-10 and SK-LC-2 were greater than 128 μmol/L, which was above the clinically achievable plasma concentration. There were statistically significant positive correlations between the cytotoxicities of cisplatin and carboplatin among the 15 NSCLC cell lines ($r = 0.966$; $P < 0.0001$), as well as for all 20 lung cancer cell lines, including the 5 SCLC cell lines ($r = 0.956$; $P < 0.0001$), suggesting that these agents induced similar cytotoxic effects in lung cancer cells (Fig. 1). There was a relatively weak but statistically significant correlation between the cytotoxicity of gemcitabine and that of cisplatin or carboplatin among the 15 NSCLC cell lines ($r = 0.715$; $P < 0.001$ for cisplatin, $r = 0.792$; $P < 0.001$ for carboplatin), as well as for all 20 lung cancer cell lines ($r = 0.701$; $P < 0.001$ for cisplatin, $r = 0.733$; $P < 0.001$ for carboplatin, data not shown).

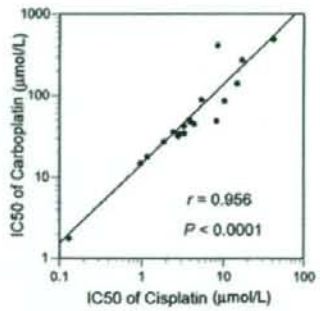

Figure 1 Correlation between chemosensitivities to cisplatin and carboplatin.

Table 3 Relative mRNA expression for ERCC1, ERCC2 and RRM1 in lung cancer cell lines

Cell line	RRM1	ERCC1	ERCC2
ACC-LC-94	1.046	1.090	1.045
ACC-LC-319	1.438	0.480	0.307
SK-LC-3	1.416	0.899	0.588
A549	1.628	0.767	0.203
SK-Lu-1	1.956	0.751	0.553
VMRC-LCD	3.291	0.744	0.671
RERF-LC-MT	1.593	0.225	0.167
Calu1	2.268	0.438	0.531
SK-MES-1	1.459	0.735	0.236
PC-1	2.889	0.749	0.713
RERF-LC-AI	3.739	0.327	0.303
PC-10	1.993	0.864	0.269
NCI-H460	2.002	0.671	0.431
Calu6	0.745	0.725	0.348
SK-LC-6	2.47	0.782	0.508
ACC-LC-48	2.388	0.414	0.257
ACC-LC-49	4.602	0.670	0.455
ACC-LC-80	3.826	1.080	0.435
ACC-LC-172	3.896	0.472	0.841
SK-LC-2	4.688	3.402	1.906

ERCC1, excision repair cross-complementation 1; ERCC2, excision repair cross-complementation 2; RRM1, ribonucleotide reductase M1.

Expression of mRNA for the ERCC1, ERCC2 and RRM1 genes was quantified by real-time PCR and normalized to β-actin mRNA expression (Table 3). mRNA expression for RRM1 was higher in SCLC cell lines compared with NSCLC cell lines. There were statistically significant differences in RRM1 expression between SCLC and adenocarcinoma, and between SCLC and large cell carcinoma (Fig. 2). There was also a statistically significant correlation between ERCC1 mRNA expression and ERCC2 mRNA expression among the 15 NSCLC cell lines ($r = 0.547$; $P < 0.05$, Fig. 3a), as well as for all 20 lung cancer cell lines ($r = 0.666$; $P < 0.005$, data not shown). However, there were no associations between RRM1 mRNA

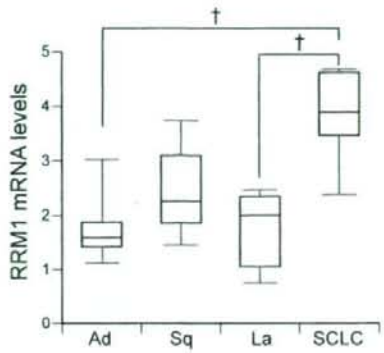

Figure 2 Predominant mRNA expression of the RRM1 gene in SCLC cell lines compared with NSCLC cell lines. Box plots show relationships between RRM1 mRNA expression and the four histological types of lung cancer. The line within each box indicates the median value. †$P < 0.005$ by ANOVA with Bonferroni correction.

expression and either ERCC1 mRNA (Fig. 3b) or ERCC2 mRNA (Fig. 3c) expression in these cell lines.

The chemosensitivity data were analysed in relation to mRNA expression of the ERCC1, ERCC2 and RRM1 genes using linear regression analysis. No significant associations were observed between the IC50 values of cisplatin, carboplatin and gemcitabine and mRNA expression for ERCC1 (Fig. 4a), ERCC2 (Fig. 4b) or RRM1 (Fig. 4c) among the 15 NSCLC cell lines. Similar results were obtained for all 20 lung cancer cell lines, including the five SCLC cell lines (data not shown).

DISCUSSION

Better and more accurate definition of the biological characteristics of the tumour is considered important for improving clinical outcome in advanced NSCLC especially in predicting response to combination chemotherapy.[14] Several reports have been published on the molecular and/or immunohistochemical analysis of molecules involved in DNA repair and/or DNA synthesis, using transbronchial and percutaneous biopsy samples from locally advanced or metastatic NSCLC.[7,11,15–17] However, there are several problems associated with mRNA and/or protein expression analyses using small tissue samples obtained by lung biopsy,[18,19] including the considerable intratumour heterogeneity, mRNA fragmentation, inevitable contamination with normal fibroblasts, the fixation procedure and storage conditions.[20] As mRNA extracted from formalin-fixed paraffin-embedded tissues is considerably fragmented, quantitative RT-PCR often yields unsatisfactory results.[21] In addition, problems with the specificity of the antibodies used for immunohistochemical analyses have been reported.[22] These limitations may result in misleading molecular

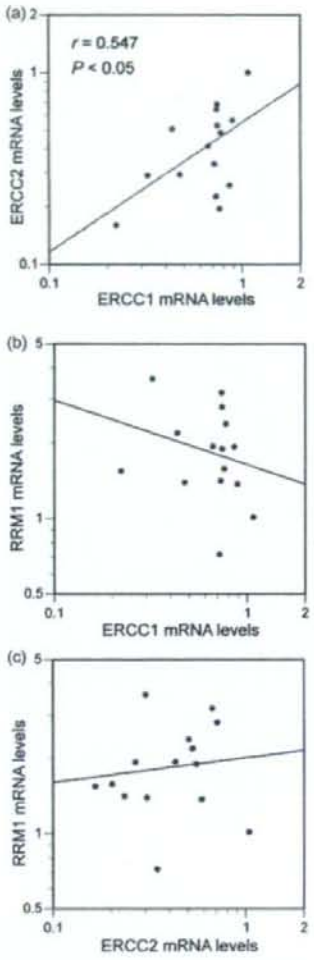

Figure 3 Correlations between (a) ERCC1 and ERCC2 mRNA expression, (b) ERCC1 and RRM1 mRNA expression and (c) ERCC2 and RRM1 mRNA expression.

analyses from clinical trials, in which the expression of biomarkers in transbronchial and percutaneous lung biopsy samples is evaluated. Thus, as one of many approaches to integrating molecular analysis with individualized chemotherapy, the *in vitro* associations between mRNA expression of the ERCC1, ERCC2 and RRM1 genes and chemosensitivity to platinum agents and gemcitabine was assessed. However, the behaviour of cell lines adapted to grow *in vitro* may differ from the *in vivo* situation, and laboratory findings may not always accurately model the clinical situation.

RRM1 expression is reported to be associated with the response to gemcitabine *in vitro*.[23] Increased RRM1 expression has been reported in two gemcitabine-resistant NSCLC cell lines. In addition, upregulation of RRM1 has been reported in different gemcitabine-resistant cell lines,[24–26] and in a murine colon cancer model.[27] Reduced RRM1 expression has also been reported to be associated with increased sensitivity to gemcitabine in the human NSCLC H23 cell line using transfection and knockdown techniques.[7] Low levels of RRM1 expression are associated with poor survival among patients with resected NSCLC.[28] Association of increased RRM1 expression with resistance to gemcitabine was also reported in the setting of preoperative NSCLC, as well as in advanced NSCLC. In a prospective induction phase II clinical trial of chemotherapy with platinum and gemcitabine RRM1 mRNA expression was correlated with tumour response.[29] However, in the present study there was no correlation between RRM1 mRNA expression and chemosensitivity to gemcitabine, cisplatin or carboplatin. Possible explanations for the differences between this study and other *in vitro* studies are the use of tissues from different sources and the use of different assay systems, such as overexpression and/or knockdown techniques for molecular biomarkers in a limited number of cell lines. The discrepancy between this study and *in vivo* studies might be explained by possible technical limitations such as the quality of mRNA extracted from the small samples obtained by lung biopsy and the specificity of the antibody used.

The association between ERCC1 and chemosensitivity to cisplatin has been evaluated in many *in vitro* and *in vivo* studies. Increased expression of ERCC1 was associated with cisplatin resistance in ovarian cancer cells.[30] Transfection of the ERCC1 gene into an ERCC1-deficient Chinese hamster ovary (CHO) cell line conferred DNA adduct repair capability and cisplatin resistance.[31] In a human colon cancer cell line with mismatch repair deficiency, ERCC1 antisense RNA abrogated the synergistic cytotoxicity of gemcitabine and cisplatin *in vitro*.[32] The association between ERCC1 mRNA expression and chemoresponsiveness to cisplatin has been observed in primary gastric cancer and in ovarian cancer.[33–35] In the present study, there was no association between ERCC1 mRNA expression and chemoresponsiveness to either cisplatin or gemcitabine. The lack of association between ERCC1 mRNA expression and chemoresponsiveness to cisplatin is consistent with a previous *in vivo* study, of mRNA from formalin-fixed paraffin-embedded primary tumour specimens from patients with advanced NSCLC before treatment with cisplatin and gemcitabine. However, low ERCC1 mRNA expression was associated with longer survival and a trend towards a higher response rate.[16] A recent study also reported no association between ERCC1 mRNA expression and chemoresponsiveness or survival in patients with advanced NSCLC treated with platinum-based chemotherapy.[36]

ERCC1 mRNA expression in formalin-fixed paraffin-embedded tumour specimens obtained by bronchoscopic fine needle aspiration biopsy[15] is a prognostic factor in patients with resected NSCLC,[37] and patients with advanced NSCLC treated with

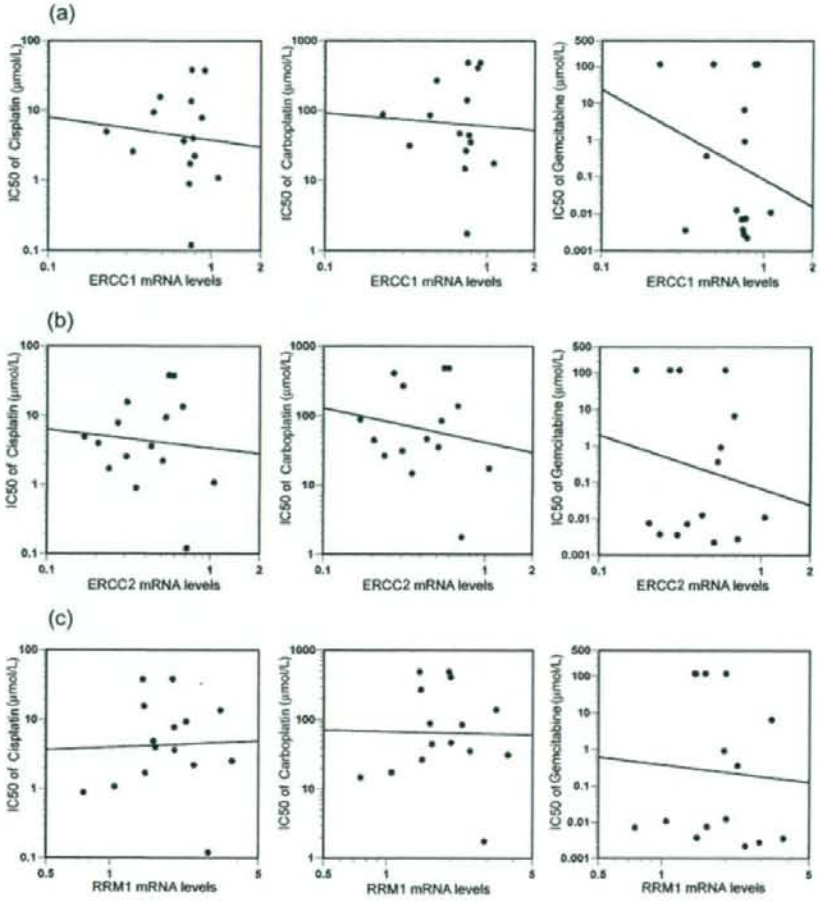

Figure 4 Associations between mRNA expression for (a) ERCC1, (b) ERCC2 and (c) RRM1 and chemosensitivities to cisplatin, carboplatin and gemcitabine.

cisplatin and gemcitabine. Furthermore, ERCC1 protein, as measured by immunohistochemical scoring, is a determinant of survival after surgical treatment of early stage NSCLC. ERCC1 protein is a prognostic factor for clinical outcome and a predictive biomarker for cisplatin-based adjuvant chemotherapy in patients with completely resected ERCC1-negative NSCLC,[8] although a problem with the specificity of the anti-ERCC1 mAb 8F1 has been reported.[22] Thus, further studies are needed to establish the role of ERCC1 in NSCLC.

The ERCC2 gene codes for a DNA helicase, which is a member of the multi-step NER pathway. The Asp312Asn polymorphism, resulting from a G/A substitution in exon 10 of the ERCC2 gene has been highly conserved through evolution, and has been reported to be a prognostic factor in patients with advanced NSCLC treated with cisplatin.[38] In addition, an *in vitro* study showed that ERCC2 overexpression leads to cisplatin resistance in a glioma cell line,[39] suggesting that expression of the ERCC2 gene may be associated with chemosensitivity to cisplatin in lung cancer cells. However, the present study failed to show associations with sensitivity to platinum agents and gemcitabine. Therefore, ERCC2 also needs further evaluation in lung cancer.

Five SCLC cell lines were included to determine whether the associations between ERCC1, ERCC2 and RRM1 mRNA expression and chemosensitivity to platinum agents and gemcitabine reported for NSCLC could be extended to SCLC. Platinum agents are key drugs and gemcitabine has modest activity in the treatment of SCLC with response rates of 11.9–13%.[40,41] However, the present study failed to show any associations. These findings are supported by a previous study, in which gene expression and the growth

inhibitory activities of various anticancer agents were similar for 19 NSCLC and 10 SCLC cell lines.[42]

There have been no *in vitro* studies examining the association between RRM1, ERCC1 or ERCC2 and chemosensitivity to platinum agents and gemcitabine, except for studies using overexpression and/or knockdown techniques. Although this *in vitro* study did not show associations in a panel of lung cancer cell lines, definitive conclusions cannot be drawn from the data, because only a limited number of cell lines were used. Exploration of the relationship between drug response phenotype and tumour genome mRNA expression profile, using cell line panels and/or tumour tissues together with cDNA and oligonucleotide arrays, would be a promising approach in the search for predictive biomarkers.[43,44] Finally, in order to validate pharmacogenetic or pharmacoproteomic candidates for lung cancer in clinical settings, further careful and more comprehensive studies using multiple approaches are warranted.

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