1. Introduction

Lung cancer remains the leading cause of cancer related deaths worldwide.[1] Non-small-cell lung cancer (NSCLC) accounts for 80% of all lung cancer cases and approximately 30% of patients have locally advanced lung cancer.[2] The standard treatment for locally advanced NSCLC patients involves concurrent thoracic radiotherapy (TRT) and chemotherapy.[3]

A treatment regimen has been developed in Japan using cisplatin and vinorelbine concurrently administered with thoracic radiotherapy at a total dose of 60 Gy to patients with locally advanced NSCLC.[4,5] To improve survival, docetaxel consolidation therapy is conducted following the same regimens administered to NSCLC patients.[6] This is based on the concept of clinical trial SWOG 9504[7] that suggested that consolidation chemotherapy was a promising strategy for the treatment to NSCLC patients. However, a drawback is the fact that a majority of the patients in the Japanese study were not able to continue with the consolidation of docetaxel due to treatment related pneumonitis.[6]

S-1 is an oral fluoropyrimidine agent designed to enhance anticancer activity and reduce toxicity through the combined use of an oral fluoropyrimidine agent (tegafur), a dihydropyrimidine dehydrogenase inhibitor (5-chloro-2,4-dihydroxypyridine) and an orotate phosphoribosyl transferase inhibitor.[8] S-1 was shown to produce active response as a single agent for metastatic NSCLC with minimal toxicity.[9] S-1 has been launched for use as an adjuvant therapy for early stage lung cancer,[10] chemoradiotherapy for stage III,[11] frontline chemotherapy[12] and 2nd or 3rd[13] line chemotherapy in advanced stages of the disease.

Based on a promising efficacy with S-1, we hypothesised that chemoradiotherapy followed by S-1 consolidation would be feasible and clinically active. Hence, the Japan National Hospital Organization Study Group for Lung Cancer (JNHOSGLC) conducted a multicentre, phase II study for patients with unresectable stage III NSCLC, where chemoradiotherapy was administered to patients followed by S-1 consolidation therapy (UMIN000002381). The primary objective was to determine the response rate, while secondary objectives were to determine the safety of this new regimen and to estimate progression-free and overall survival.

2. Patients and methods

2.1. Eligibility criteria

Patients with histologically or cytologically confirmed NSCLC at unresectable stage III disease were eligible for this study. Stage III was decided based on the 6th AJCC Cancer Staging Manual.[14] Eligible stage IIIA disease was defined by the presence of multiple and/or bulky N2 mediastinal lymph nodes on computed tomography (CT). Eligible stage IIIB disease was assigned either by N3 (contralateral mediastinal) or by T4 from invasion of mediastinal structures, heart, great vessels, trachea, carina, oesophagus or vertebral body. Confirmation of T4 or N3 status was established according to T4 involvement found at the time of thoracotomy or thoracoscopy; involvement of the trachea or carina by bronchoscopy; unequivocal invasion of the heart, oesophagus, aorta or vertebral body by CT scan, or magnetic resonance imaging; or biopsy of contralateral mediastinal N3 nodes. Eligible patients also needed to meet the following criteria: measurable disease of 20 mm or more in size; no prior history of chemotherapy or TRT; Eastern Cooperative Oncology Group performance status of 0 or 1; aged between 20 and 74 years; have leucocytes $\geqslant$ 4000/mL, platelets $\geqslant$ 100,000/mL, and haemoglobin $\geqslant$ 9.5 g/dL, serum creatinine < institutional upper limit of normal, and partial pressure of arterial oxygen $\geqslant$ 70 mmHg. Patients were excluded if they had infections; apparent interstitial pneumonitis or fibrosis on chest CT; irradiation field larger than half of an ipsilateral lung; severe complications; another active cancer. The ethics committee of each participating institution approved the protocol, and all patients provided written informed consent before the start of the study. For staging, all patients underwent CT of the thorax and abdomen, and either a brain CT scan or magnetic resonance imaging (MRI). A radio isotopic bone scan was also performed for all patients. Positron emission tomography was not necessary for enrolment.

2.2. Therapy

Treatment consisted of a chemoradiotherapy phase with two cycles of cisplatin and vinorelbine followed by a consolidation phase with two cycles of S-1. Chemoradiotherapy consisted of cisplatin at 80 mg/m^2 on days 1 and 29; vinorelbine at 20 mg/m^2 on days 1, 8, 29 and 36; and concurrent TRT at a total dose of 60 Gy. Sequential S-1 consolidation therapy at doses of 80-120 mg/body twice per day was started on day 57 with two cycles of 4 weeks administration and 2 weeks withdrawal. The dose of S-1 was determined based on body surface area (BSA): 80 mg was delivered when BSA was less than 1.25/m^2, 100 mg when 1.25/m^2 < BSA < 1.50/m^2 and 120 mg when BSA $\geqslant$ 1.50 m^2.

Concurrent TRT began on day 2 of chemotherapy by using a linear accelerator (6–10 megavolt), in 2-Gy, single and daily fractions for five consecutive days per week to provide a total dose of 60 Gy. A curative radiation field was constructed by using a plain chest radiograph and a contrast-enhanced computed tomography (CT) scan. The initial dose (approximately 40 Gy) was administered to the primary tumour, the ipsilateral hilum with a 2-cm margin, and involved mediastinal lymph nodes with a 1-cm margin. Prophylactic radiation fields were not planned except for subcarinal lymph nodes. Subsequently, a 20-Gy dose was given as a booster in accordance with tumour shrinkage. An initial TRT dose of 40 Gy was administered to the antero-posterior parallel-opposed pair of portals. Oblique anterior and posterior fields were required to avoid over dosage of the spinal cord.

The criteria for starting consolidation chemotherapy included completion of two cycles of cisplatin and vinorelbine, a full dose of thoracic radiotherapy, and the absence of a progressive disease, as well as being in good general condition.

2.3. Evaluation

All eligible patients who received treatment were considered assessable for response and toxicity measures. Chest X-rays,

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blood counts and blood chemistry studies were repeated once a week during the treatment period. Follow-up studies including CT scan were performed once a month during the treatment period and every 3 months after treatment. The response was evaluated in accordance with Response Evaluation Criteria in Solid Tumours (RECIST). For evaluation of the antitumour effects, an extramural review was conducted. Acute toxicity was graded according to the NCI Common Toxicity ver. 3.0.

2.4. *Statistical methods*

We calculated the sample size based on Fleming's single-stage design for phase II study. We set a response rate of 60% as a baseline survival rate and 75% as the high level of interest with a power of 0.8 at a one-sided significance level of .05, requiring an accrual of at least 62 eligible patients. Assuming the loss of follow-up cases, a minimum of 65 patients was required for this study. Progression-free and overall survival was estimated using the Kaplan–Meier method, with corresponding two-sided 95% confidence interval (CI) for median times. For progression-free survival, follow-up measures were conducted during the study enrolment to document evidence of disease progression or death, or last documented progression-free status. Overall survival was measured from the study enrolment to the date of death or last contact. Statistical analyses were performed with SAS version 9.2 software (SAS Institute, Cary, NC).

3. Results

3.1. *Patient characteristics*

Sixty-six patients were enrolled between January 2006 and July 2009. One patient that did not receive any protocol treatment was not assessable and therefore not included in the analysis. Baseline patient characteristics and demographics are listed in Table 1. The median age was 63 years (range, 45–73 years), and 55 patients were male and 10 patients were female. Thirty patients (46%) were at stage IIIA and 35 patients (54%) were at stage IIIB. Histological studies showed squamous cell carcinoma in 33 patients, adenocarcinoma in 23 patients and other cancers in nine patients.

3.2. *Treatment delivery*

Of the 65 patients, 57 patients (87.7%) completed the concurrent portion of the regimen. Failure to complete the concurrent therapy was due to toxicities such as grade 3 pneumonitis ($n = 1$) and ileus ($n = 1$), delay in chemotherapy for more than 2 weeks ($n = 3$), pneumonia ($n = 1$), deteriorating condition ($n = 1$) and surgery ($n = 1$). Forty-five patients (69.2%) proceeded to consolidation therapy. Reasons for failing to proceed to consolidation therapy included chemoradiotherapy toxicities such as persistent neutropenia ($n = 2$) and renal failure ($n = 2$), pneumonia ($n = 1$) declining performance status ($n = 1$), cardiac ischaemia unrelated to the treatment ($n = 1$), progressive disease documented on restaging after completion of the concurrent therapy ($n = 1$), vertigo ($n = 1$), refusal to undergo consolidation therapy ($n = 1$) and surgery ($n = 1$). A total of 31 patients (47.6%) completed the two cycles of consolidation therapy. Early discontinuation of the consolidation therapy included toxicity of more than grade 2 pneumonitis ($n = 9$), declining performance status ($n = 1$), disease progression ($n = 2$), cerebral infarction unrelated to the treatment ($n = 1$) and refusal of the therapy ($n = 1$).

3.3. *Response and survival*

The overall response rate during the study was 61.5% (95% CI, 48.6–73.3%) with one complete response and 39 partial responses. Stable disease and progressive disease occurred in 19 patients (29.2%) and six patients (9.2%), respectively. One patient had an inadequate reassessment. The estimated median progression-free survival was 10.2 months (95% CI, 8.6–13.7 Fig. 1). Kaplan–Meier estimates of progression-free survival were 44.6% (95% CI, 32.1–57.1%) at one year and 17.9% (95% CI, 6.3–29.5%) at three years. The estimated median duration of survival in all patients was 21.8 months (95% CI, 15.6–27.6; Fig. 2). Twenty-three patients remained alive after a median follow-up of 37.7 months (range, 12.5–54.3 months). Kaplan–Meier estimates of overall survival were 73.9% (95% CI, 63.2–84.5%) at one year and 34.0% (95% CI, 21.2–46.9%) at three years.

3.4. *Toxicity*

Grade 3 or 4 toxicities for the concurrent treatment phase are summarised in Table 2. Among the 65 assessable patients, one patient had a grade 3 pneumonitis (1.5%) while there were no grade 3 or 4 treatment-associated oesophagitis. The most common grade 3 or 4 haematological toxicities were leukopaenia (56.8%) and neutropenia (53.7%).

Table 3 summarises the grade 3 or 4 toxicities for the 44 patients who received consolidation therapy. It is apparent that minimal toxicity was observed in patients who received consolidation therapy. The most common grade 3 or 4 toxicity was anaemia (8.9%). Leukopaenia or neutropenia was observed in just three patients (6.7%) and severe oesophagitis was not observed. Seven patients developed grade 2 pneumonitis and two grade 3 pneumonitis during consolidation therapy. One patient died three months after chemoradiotherapy as the result of pneumonitis.

4. Discussion

This is the first phase II study to investigate the use of the oral fluoropyrimidine agent S-1 as a consolidation drug after chemoradiotherapy in stage III NSCLC. Our data indicated a reasonable survival with a median survival time (MST) of 21.8 months and a three-year survival rate of 34.0%. In addition, tumour response was demonstrated to be 61.5% and clinically active. However, less than half of the patients completed this regimen (47.6%) and it is unlikely that this treatment is feasible.

This study was originally designed to extend and enhance the concept of consolidation as reported in SWOG 9504,[7] a phase II study, where docetaxel was administered after cisplatin, etoposide (PE) and TRT to patients with stage III

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NSCLC. Although a significant MST of 26 months was observed in that study, this finding could not be replicated in a phase III study. Dr. Hanna and colleagues reported that consolidation with docetaxel after PE and TRT could not improve survival compared with chemoradiotherapy alone with the same MST range of 23 months in each arm.[15]

Previous studies in Japan showed that chemoradiotherapy using cisplatin and vinorelbine elicits high response and survival rates in patients with stage III NSCLC. A phase I study showed an MST of 30.4 months with a three-year survival rate of 50% in 18 patients.[4] A retrospective study using the recommended dose demonstrated an MST of 21 months and a three year survival rate of 33% in 73 patients, where the chemotherapy cycle was originally planned with a maximum of three cycles but with a median of two (mean 2.4, ranges 1–3).[5] Our treatment regimen was also designed based on the aforementioned phase I trial and is almost identical to that of the retrospective study with the exception of using consolidation S-1, and the results indicated an MST of 21.8 months and a three-year survival rate of 35%. Considering the retrospective study as a historical control, the comparable survival data between the two studies suggest that the effect of S-1 consolidation is marginal and unclear. Although a phase III trial is needed to conclude the benefit of consolidation of S-1, different administrative methods for the drug may be more appropriate to patients with stage III NSCLC, as chemoradiotherapy including cisplatin and S-1 was reported to be active and promising.[21]

Table 1 – Patient Characteristics (N = 65).

	No. of patients	%
Gender		
Male	55	84.6
Female	10	15.4
Age, years		
Median	63	
Range	45–73	
Performance status		
0	27	41.5
1	38	58.5
Stage		
IIIA	30	46.2
IIIB	35	53.8
Histology		
Squamous cell	33	50.7
Adenocarcinoma	23	35.4
Large cell	2	3.1
Other	7	10.8

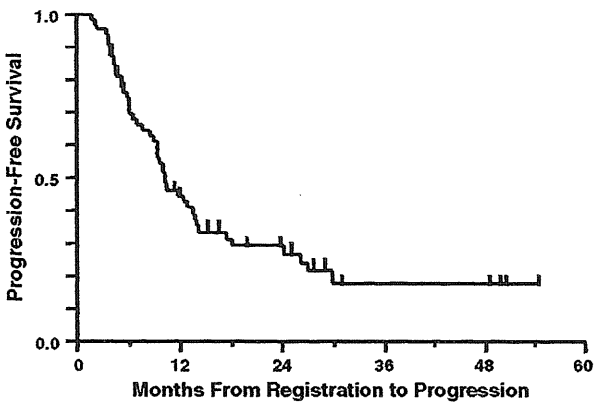

Fig. 1 – Progression-free survival of patients treated with cisplatin + vinorelbine + concurrent thoracic radiotherapy followed by S-1.

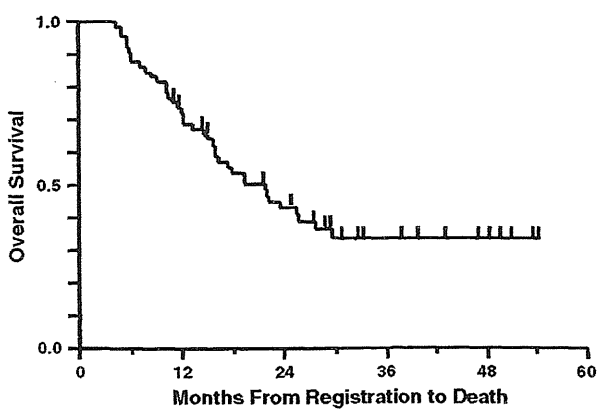

Fig. 2 – Overall survival of patients treated with cisplatin + vinorelbine + concurrent thoracic radiotherapy followed by S-1.

Cisplatin, vindesine, and mytomicin (MVP) were used for chemoradiotherapy in patients with stage III NSCLC in other studies, which is a preceding regimen of cisplatin and vinorelbine. In a phase III study of WJTOG 0105,[16] the standard treatment arm of MVP and concurrent TRT yielded an MST of 20.5 months with four cycles of chemotherapy. In another phase III trial in Japan,[17] the same regimens produced an MST of 23.7 months with two cycles of chemotherapy. Although the difference in MST may come from a split form of radiotherapy delivery in the WJTOG study, no survival benefits were observed from the addition of two cycles of chemotherapy. Again, these results are consistent with our finding that the effect of consolidation is marginal.

Feasibility is another problem in this study. Although 57 patients (87.7%) completed the concurrent portion of the regimen, only 31 patients (47.6%) finished the consolidation phase. Nine developed grade 2 or 3 pneumonitis in the 45 patients during the S-1 consolidation. In previous study, docetaxel consolidation following cisplatin, vinorelbine and TRT was reported as not feasible in Japanese patients. Almost the same 86% completed chemoradiotherapy, however, 34 patients (37%) finished consolidation therapy, whereas 14 of the 25 patients that participated in the consolidation phase developed pneumonitis.[6] On the other hand, in the aforementioned SWOG 9504, 74 patients (88%) completed chemoradiotherapy and 49 patients (59%) finished consolidation. An ethnic difference has been suggested in toxicity in NSCLC patients[18] and it is possible that pneumonitis is more common in Japanese compared to Caucasian, and further research will be required. In haematological toxicities in our study, the incidence of grade 3 or 4 neutropenia and leukopaenia were 53.7% and 56.8%, respectively, which is similar to previous reports.[5] Considering other side effects, a lower incidence of oesophagitis was

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Table 2 – Major toxicities, chemoradiotherapy ($N = 65$).

	Grade 3		Grade 4	
	No.	%	No.	%
Haematologic				
Leukopaenia	27	41.5	10	15.3
Neutropenia	25	38.4	10	15.3
Anaemia	3	4.6	2	3.0
Thrombocytopenia	0	0.0	0	0.0
Neutropenic fever	4	6.1	0	0.0
Nonhaematologic				
Nausea	2	3.0	0	0.0
Vomiting	0	0.0	0	0.0
Anorexia	2	3.0	0	0.0
Oesophagitis	0	0.0	0	0.0
Pneumonitis	1	1.5	0	0.0

Table 3 – Major toxicities, consolidationl S-1 ($N = 45$).

	Grade 3		Grade 4	
	No.	%	No.	%
Haematologic				
Leukopaenia	2	4.4	0	0.0
Neutropenia	1	2.2	0	0.0
Anaemia	3	6.7	1	2.2
Thrombocytopenia	0	0.0	0	0.0
Neutropenic fever	0	0.0	1	2.2
Nonhaematologic				
Nausea	0	0.0	0	0.0
Vomiting	0	0.0	0	0.0
Anorexia	0	0.0	0	0.0
Oesophagitis	0	0.0	0	0.0
Pneumonitis	2	4.4	0	0.0

observed in our study. Severe radiation-related oesophagitis usually occurred in concurrent chemoradiotherapy and the incidences were reported to be in the range of 17–28%.[15,19] However, there have been several reports that minimal side-effects of oesophagitis were seen in the regimes using vinca alkaloids[5,16,17] and including ours, and further study is needed to confirm this association.

In conclusion, chemoradiotherapy with cisplatin and vinorelbine followed by S-1 consolidation demonstrated a reasonable overall survival in patients with stage III NSCLC. However, considering the questionable feasibility and marginal additional effect of S-1, it is recommended that chemoradiotherapy alone is still the standard patient treatment.

Conflict of interest statement

None declared.

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A Phase I Study of Amrubicin and Fixed Dose of Irinotecan (CPT-11) in Relapsed Small Cell Lung Cancer

Japan Multinational Trial Organization LC0303

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Purpose: To determine the maximum tolerated dose of amrubicin (AMR) with a fixed dose of irinotecan (CPT-11).

Methods: Patients having pathologically proven small cell lung cancer (SCLC) relapsed after one or two chemotherapies, and Eastern Cooperative Oncology Group performance status of 0 to 2 were eligible for the study. CPT-11 was delivered as 50 mg/m^2 on days 1 and 8, every 21 days. AMR was delivered on day 1. Doses of AMR were level 1: 80 mg/m^2, level 2: 90 mg/m^2, and level 3: 100 mg/m^2. Dose elevation was determined using the modified continuous reassessment method. Tolerability was assessed after the first cycle. Another two cycles were conducted when disease progression or unacceptable toxicities were not observed.

Results: Eighteen patients (mean age: 66.3 years) were enrolled. A total of 40 courses were conducted. Grade 3/4 toxicities of the first cycle were leukocytopenia: 11 (61%, grade 3/4: 8/3); neutropenia: 15 (83%, grade 3/4: 6/9); and thrombocytopenia: three (17%, grade 3/4: 2/1). Other grade 3 toxicities observed were febrile neutropenia, one; infection, three; diarrhea, one; and dyspnea, one. Dose-limiting toxicity was observed in two of six patients at level 2 (neutropenia and febrile neutropenia) and in one of six at level 3 (thrombocytopenia and infection). The maximum tolerated dose was level 3, and so, the recommended dose for phase II trials was judged to be 90 mg/m^2. Objective response was obtained in four of eight patients who were able to evaluate responses. Median survival time was 13 months, with 68% at 1-year survival rate.

Conclusions: This combination was well tolerated and showed encouraging activities in SCLC. Randomized phase II trials are being planned in chemonaive SCLC.

Key Words: Small cell lung cancer, Amrubicin, Irinotecan, Modified continuous reassessment method, Relapse.

(*J Thorac Oncol.* 2011;XX: 395–399)

Lung cancer is the most common and deadliest cause of cancer death worldwide.[1] Small cell lung cancer (SCLC) represents 13% of all lung cancer and is the most aggressive form of lung cancer, with an overall 5-year survival rate less than 5%.[2] SCLC is one of the most chemo-sensitive solid tumors, and the outcome for patients with SCLC is slowly improving.[3,4] Nevertheless, despite a good initial response to therapy, most patients with SCLC develop chemotherapy resistance and relapse. Second-line chemotherapy should then be applied. Although the introduction of new agents has improved the outcome of patients with SCLC,[5] the development of more active chemotherapy, and especially the introduction of more effective new drugs, is warranted to continue to improve the survival of patients with SCLC.

Amrubicin (AMR), a totally synthetic 9-aminoanthracycline, inhibits DNA topoisomerase II activity. AMR is converted to an active metabolite, amrubicinol, by reduction of its C-13 ketone group to a hydroxy group. AMR has either an equivalent or a stronger antitumor effect in comparison with doxorubicin in nude mice transplanted with human tumor cells.[6–8] In a phase I trial of AMR for 3 consecutive days at 3-week intervals in patients with advanced non-SCLC (NSCLC) without prior chemotherapy, the maximum tolerated dose (MTD) and the recommended dose were estimated to be 50 mg/m^2 and 45 mg/m^2, respectively, The major dose-limiting toxicity (DLT) was myelosuppression.[9] In a phase II study of AMR using a schedule of 45 mg/m^2 on days 1 to 3 every 3 weeks, in 33 previously untreated patients with extensive disease (ED) SCLC, an overall response rate of

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76% and a complete response rate of 9% were reported.[10] Median survival time was 11.7 months. When 3-day AMR is combined with another chemotherapeutic agent such as cisplatin, carboplatin, or irinotecan (CPT-11), the incidence of myelosuppression increases, which makes it impossible to use clinically.[11]

On the other hand, in a phase I study of the administration of AMR on day 1, 29 evaluable courses of treatment were conducted in groups at doses increasing from 10 to 130 mg/m^2 of AMR on day 1.[12] Myelosuppression was the DLT, and a MTD was 130 mg/m^2. Nonhematologic side effects were mild grastrointestinal symptoms and hair loss. The recommended dose was 100 mg/m^2.

Although there is no study to compare the 3-day AMR with single-day AMR, the decision of 3-day AMR regimen is mainly derived from the results of NSCLC.

Irinotecan is a water-soluble semisynthetic camptothecin derivative that inhibits topoisomerase I. Irinotecan is one the most active drugs used in the treatment of SCLC and NSCLC.[13,14] The Japan Clinical Oncology Group reported that the combination of cisplatin and irinotecan allows for significantly better survival than the combination of cisplatin and etoposide for previously untreated ED SCLC.[15]

Combinations of topoisomerase I and topoisomerase II inhibitors have been reported to be active against SCLC based on in vitro and in vivo animal model.[16]

Based on this background, a phase I study was designed to determine the MTD of 1-day AMR and irinotecan by applying the modified continual reassessment method (CRM) and to obtain preliminary evidence of the therapeutic activity of this combination in patients with relapsed SCLC.

PATIENTS AND METHODS

Patient Selection

Patients were required to fulfill the following eligibility criteria: pathologically or cytologically diagnosed SCLC; relapsed after one or two regimens of chemotherapy; adequate reserves of hematological function (neutrophil count ≥ 1500/μl and ≤7000/μl, platelet count ≥ 100,000/μl, and hemoglobin > 8.5 mg/dl); adequate hepatic function (bilirubin ≤ 1.5 mg/dl, aspartate aminotransferase [≤2x the upper limit of normal], and alanine aminotransferase [≤2x the upper limit of normal]); adequate renal function (creatinine [≤2x the upper limit of normal] and pulmonary function [Pao_2 ≥ 70 torr]); Eastern Cooperative Oncology Group performance status (PS) of 0, 1, or 2; expected survival more than 3 months; and acquisition of written informed consent. Baseline pretreatment evaluations included a complete history, physical examination, laboratory tests, chest radiograph, electrocardiogram, computed tomography scans of the chest and abdomen, magnetic resonance imaging of the brain, and a radionuclide bone scan. The protocol was approved by the institutional review board of each participating institute.

Exclusion criteria included the following: massive pleural effusion, pericardial effusion, or ascites; symptomatic brain metastasis; uncontrollable hypertension, unstable angina, heart failure, and myocardial infarction within 1 year; uncontrollable diabetes mellitus; watery diarrhea and ileus; pulmonary fibrosis in chest x-ray; history of anthracyclin use; and severe infection.

Drug Administration

The protocol treatment consisted of three courses, each requiring 21 days to complete. In each course, irinotecan was diluted in 500 ml of normal saline for administration and then administered to the patient at a fixed dose of 50 mg/m^2 as an intravenous infusion on days 1 and 8. After the completion of irinotecan infusion, AMR dissolved in 20-ml saline was delivered intravenously as a 5-minute infusion on day 1 alone, every 21 days. The dose levels of AMR consisted of level 1: 80 mg/m^2, level 2: 90 mg/m^2, and level 3: 100 mg/m^2. Which dose level of AMR should be allocated to each of the patients except the first three patients was determined by applying the CRM.[17] The CRM, which is comparable with the traditional phase I design in terms of study duration and proportion of patients treated at a dose greater than the MTD, can take account of the cumulative DLT data of all the treated patients and determine the dose allocation to the next cohort.

Granulocyte colony stimulating factor (G-CSF) was allowed to use at more than grade 3 leukocytopenia or neutropenia, and prophylactic use were not allowed.

Trial Design

Toxicity was graded according to the National Cancer Institute-Common Toxicity Criteria version 2.0. The scheme of this trial is shown in Figure 1. Tolerability was assessed after the first course. Another two courses were conducted when disease progression or unacceptable toxicities were not observed. DLT was defined using the National Cancer Institute Common Toxicity Criteria (version 2.0) as development of at least one of the following adverse events occurring during the first course (21 days) of protocol treatment: grade 4 neutropenia lasting more than 7 days; febrile neutropenia (>38.5°C, grade 3 or 4 neutrophils) for more than 1 day; grade 4 thrombocytopenia (<10,000/μl); more than 2 weeks delay of next course due to neutropenia (<1500/μl) or thrombycytopenia (<75,000/μl); >grade 2 liver or renal dysfunction; and other >grade 2 toxicities except for alopecia, nausea, vomiting, and appetite loss. The MTD was defined a priori as the highest dose at which a maximum of 33% of patients were expected to experience a DLT during

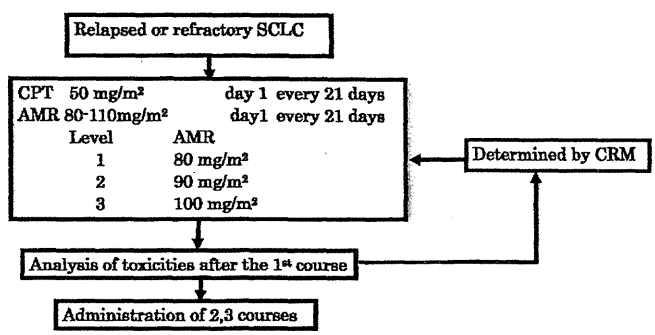

FIGURE 1. Schema of phase I study using CRM. SCLC, small cell lung cancer; CPT, irinotecan; AMR, amrubicin;CRM, continuous reassessment method.

the first course. The first three patients were treated at level 1 for reasons of safety. Nevertheless, if one of three or more patients had the DLT at level 1, then AMR would be decreased to the dose level of 70 mg/m^2. The dose level that was the closest to the current estimate of the MTD was identified by the CRM and allocated to each of the succeeding patients. Both dose escalation and deescalation were permitted.

Statistical Analysis

The MTD was estimated by the CRM at the end of the trial. For accrued patients into whom a part or all the protocol treatment are administered, the incident frequency and severity of adverse events, including DLTs, were reported. Objective tumor assessments were conducted using Response Evaluation Criteria Solid Tumors.[18] Overall survival was measured from the date of study treatment to the date of death from any cause. One-year survival rate were estimated by Kaplan-Meier method.

RESULTS

Between June 2004 and October 2006, 18 patients participated in this trial at five institutions. Patient characteristics are listed in Table 1. Fifteen patients were male, and three were female, and the median age was 66 years. Four patients had PS 0, and 14 patients had PS 1. Two patients had limited disease, and 16 patients had ED at relapse. Numbers of prior chemotherapy regimens were 1 in 11 patients and 2 in seven patients. Previous chemotherapy consisted of cisplatin and etoposide in 10 patients; cisplatin and irinotecan in six patients; carboplatin and etoposide in four patients; carboplatin and irinotecan in three patients; and etoposide in one patient. The responses to the prior chemotherapy were complete response in two patients and partial response in 15 patients. All the responders were sensitive relapse, which is defined as disease that responded to first-line chemotherapy and relapsed later than 90 days after the last dose of first-line chemotherapy. Only one had progression of disease. Other treatments included thoracic radiotherapy in 12 patients and surgery in one patient.

TABLE 1. Patient Characteristics

Characteristics	
Total no. of patients	18
Sex	
Male	15
Female	3
Age (yr)	
Mean	66.3
Range	57–79
Performance status (ECOG)	
0	4
1	14
Disease extent at relapse	
Limited disease	2
Extensive disease	16
Prior therapy	
No. of prior chemotherapy regimens	
1	11
2	7
Chemotherapy	18
Cisplatin + etoposide	10
Cisplatin + irinotecan	6
Carboplatin + etoposide	4
Carboplatin + irinotecan	3
Etoposide	1
Response to prior chemotherapy	
CR	2
PR	15
SD	
PD	1
Chemotherapy-free interval (d)	
<60	1
≥60	17
Thoracic radiotherapy	12
Surgery	1

ECOG, Eastern Cooperative Oncology Group; CR, complete response; PR, partial response; SD, stable disease; PD, progression disease.

Toxicities during the first course are listed in Table 2. Six patients each were enrolled at dose levels 1 (AMR, 80 mg/m^2), 2 (AMR, 90 mg/m^2), and 3 (AMR, 100 mg/m^2). No DLT were observed during the first course of level 1. At level 2, DLT was observed in two patients. One patient had neutropenia lasting > 7 days, and the other patient had febrile neutropenia. At level 3, DLT was observed in two patients: one had neutropenia and the other had thrombocytopenia and infection.

On the basis of these cumulative DLT data on 18 patients, the DLT probabilities at levels 1, 2, and 3 were calculated by the CRM as 0.000, 0.205, and 0.348, respectively. Because the target DLT probability was specified as 33% as mentioned (see Trial Design section), the MTD was estimated to be level 3.

A total of 40 courses were conducted. Grade 3/4 hematological toxicities of first cycle were leukocytopenia: 61% (grade 3/4: 8/3); neutropenia: 83% (grade 3/4: 6/9); and thrombocytopenia: 17% (grade 3/4: 2/1). Grade 3 febrile neutropenia occurred in one patient (6%). Grade 3 infection occurred in three patients (17%). Grade 3 diarrhea and dyspnea occurred in one patient (6%) each. In the first course, DLT was observed in two of six patients at dose level 2 (prolonged grade 4 neutropenia and febrile neutropenia) and in one of six at dose level 3 (thrombocytopenia and infection).

For supportive care during the entire courses, G-CSF was administered in 16 patients (88%). Antibiotics were used for three patients (17%). Blood transfusion was required in one patient (6%).

Response and Survival

Tumor responses were observed in eight patients. Four patients of eight patients had partial response, three had stable disease, and one had disease progression. The overall response rate was 50.0% (4/8). The median survival time was 13 months, with 68% at 1-year survival rate.

TABLE 2. Toxicities During the First Course

	Grade (National Cancer Institute-Common Toxicity Criteria)															
	Level 1 (n = 6) 80 mg/m^2, Day 1 50 mg/m^2, Days 1 and 8					Level 2 (n = 6) 90 mg/m^2, Day 1 50 mg/m^2, Days 1 and 8					Level 3 (n = 6) 100 mg/m^2, Day 1 50 mg/m^2, Days 1 and 8					
Amrubicin Irinotecan	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4	Grades 3 and 4 in All Levels
Leukopenia		1	2	3			1	1	3	1			2	2	2	11/18 (61%)
Neutropenia		1	2	2	1				1	5				3	3	15/18 (83%)
Thrombocytopenia	4	2				4	1		1		2	2		1	1	3/18 (17%)
Hemoglobin febrile		5	1			1	4		1		1	2	3			1/18 (6%)
Neutropenia	1															1/18 (6%)
Infection	5		1			6					3			3		3/18 (17%)
Nausea	4	2				3	3				3	1	2			0/18 (0%)
Diarrhea	2	4				5			1		3	2	1			1/18 (6%)
Dyspnea	6					5			1		6					1/18 (6%)

DISCUSSION

This demonstrated that the MTD of AMR determined by the CRM was level 3, 100 mg/m^2, and thus, the recommended dose for phase II trials was judged to be 90 mg/m^2, one dose level below the MTD, with the fixed dose of irinotecan delivered as 50 mg/m^2 on days 1 and 8, every 21 days.

This study was for previously treated SCLC. Objective response was obtained in four patients (29%). Median survival time was 13 months with 68% at 1-year survival rate. It should be noted that AMR was administered on day 1 alone without the use of G-CSF. So far, there has been no study using AMR only on day 1 with irinotecan.

Combinations of irinotecan and 3-day AMR in lung cancer have been reported recently. Yanaihara et al.[19] conducted a phase I trial, in which 11 patients with NSCLC were treated at 3-week intervals with AMR on days 1 to 3 plus 60 mg/m^2 of irinotecan on days 1 and 8. The 30 mg/m^2 of AMR dose was one dose level above the MTD. Diarrhea and leukopenia were the DLT. The recommended dose for phase II studies is 60 mg/m^2 of irinotecan on days 1 and 8 and 25 mg/m^2 of AMR on days 1 to 3 every 3 weeks. They also reported that AMR did not affect the pharmacokinetics of irinotecan, SN-38 or SN-38 glucuronide.

Kaneda et al.[11] started the dose of AMR from 35 mg/m^2 on days 1 to 3 and irinotecan 50 to 60 mg/m^2 after the completion of AMR on days 1 and 8, every 3 weeks in phase I for patients with advanced lung cancer. The most frequent toxicities were bone marrow suppression followed by infection, diarrhea, and pneumonitis. As a consequence of these toxicities, the MTD and the recommended dose could not be determined. They concluded that this combination is not tolerated and is inactive against both NSCLC and SCLC. This indicates that 35 mg/m^2 of AMR on days 1 to 3 may be too toxic.

Oshita et al.[20] conducted dose escalation study of AMR with fixed-dose irinotecan in patients with ED SCLC. Thirteen previously untreated patients were treated with irinotecan at 60 mg/m^2 day 1 and dose-escalated AMR on days 1 to 3 with prophylactic G-CSF on days 5 to 9, every 2 to 3 weeks. A total of 31 courses were administered at dose level 2 (35 mg/m^2/d) in six patients, and grade 4 neutropenia was observed during five courses (16.1%). Irinotecan at 60 mg/m^2 on day 1 and AMR at 35 mg/m^2/d on days 1 to 3 with G-CSF support every 3 weeks are recommended. The above data indicate that 35 mg/m^2/d on 1 to 3 could not be administered without G-CSF.[20]There are three phase II trials published on AMR monotherapy for patients with relapsed SCLC. The Thoracic Oncology Research Group conducted a single-arm phase II study AMR on 16 chemotherapy refractory and 44 sensitive patients.[21] When given at a dose of 40 mg/m^2/d (days 1–3) every 3 weeks, their results demonstrated a 50% response rate in the refractory group and 52% in the sensitive group. The progression-free survival, overall survival, and 1-year survival in the refractory group and the sensitive group were 2.6 and 4.2 months, 10.3 and 11.6 months, and 40% and 46%, respectively. In previously treated patients with SCLC, AMR 40 mg/m^2/d (days 1–3) every 3 weeks was administered in a phase II trial.[22] Twenty-six patients (nine sensitive and 17 refractory patients) received a median number of three cycles of therapy. The response rate was 46.2% (55.6% in sensitive patients and 41.2% in refractory patients). The median survival time was 9.4 months (11.0 months for sensitive patients and 5.7 months for refractory patients). Grade 4 neutropenia occurred in 73.1% of patients. Grade 3 or 4 thrombocytopenia occurred in 50% of patients. A comparison of AMR (40 mg/m^2/d, d1–3) with topotecan (1.0 mg/m^2/d, days 1–5) for previously treated SCLC (n = 59, 36 sensitive relapsed and 23 refractory relapsed) in a randomized phase II trial was reported by Inoue et al.[23] Overall response rates were 38% (53% in sensitive relapse and 17% in refractory) for the AMR arm and 13% (21% and 0% in sensitive and refractory) for the topotecan arm. AMR may be superior to topotecan. Nevertheless, neutropenia was severe, and one treatment-related death due to infection was observed in the AMR arm.

Although this study is a phase I study, the response rate of 50% and median survival time of 13 months for the previously treated patients with SCLC indicate that the combination of AMR and irinotecan is active and encouraging.

As a result of our study, AMR 90 mg/m^2 on day 1 with irinotecan 50 mg/m^2 on days 1 and 8 were recommended for

further study because of the increased incidence of grade 4 myelosuppression at level 3 (AMR, 100 mg/m^2). Our data indicate that AMR on day 1 alone with fixed-dose irinotecan without G-CSF was feasible and demonstrated activity in relapsed SCLC.

A randomized phase II trial comparing AMR (90 mg/m^2, day 1) and irinotecan (50 mg/m^2) with cisplatin (60 mg/m^2 on day 1) and irinotecan (60 mg/m^2 on days 1 and 8) is ongoing for patients with chemonaive ED SCLC.

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Enzastaurin has anti-tumour effects in lung cancers with overexpressed JAK pathway molecules

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BACKGROUND: Enzastaurin, an oral serine–threonine kinase inhibitor, was initially developed as an ATP-competitive selective inhibitor against protein kinase Cβ. However, the mechanism by which enzastaurin contributes to tumourigenesis remains unclear.

METHODS: We analysed the anti-tumour effects of enzastaurin in 22 lung cancer cell lines to ascertain the potential for enzastaurin-based treatment of lung cancer. To identify molecules or signalling pathways associated with this sensitivity, we conducted a gene, receptor tyrosine kinases phosphorylation and microRNA expression profiling study on the same set of cell lines.

RESULTS: We identified eight genes by pathway analysis of molecules having gene-drug sensitivity correlation, and used them to build a support vector machine algorithm model by which sensitive cell lines were distinguished from resistant cell lines. Pathway analysis revealed that the JAK/STAT signalling pathway was one of the main ones involved in sensitivity to enzastaurin. Overexpression of JAK1 was observed in the sensitive cells by western blotting. Simultaneous administration of enzastaurin and JAK inhibitor inhibited enzastaurin-induced cell growth-inhibitory effect. Furthermore, lentiviral-mediated JAK1-overexpressing cells were more sensitive to enzastaurin than control cells.

CONCLUSION: Our results suggested that the JAK1 pathway may be used as a single predictive biomarker for enzastaurin treatment. The anti-tumour effect of enzastaurin should be evaluated in lung cancer with overexpressed JAK pathway molecules.

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Keywords: lung cancer; enzautaurin; PKC inhibitor; gene expression; drug sensitivity

Translational Therapeutics

Non-small-cell lung cancer (NSCLC) patients are usually diagnosed with advanced disease, and their prognosis remains poor despite improvements in chemotherapies (Mountain, 1997; Schiller *et al*, 2002; Ohe *et al*, 2007; Jemal *et al*, 2009). Recently, molecular-targeted therapies have been developed for NSCLC treatment. For example, NSCLC patients with epidermal growth factor receptor (EGFR) mutations have shown a dramatic response to EGFR inhibitors such as gefitinib and erlotinib (Mok *et al*, 2009; Maemondo *et al*, 2010). However, there remain many other molecular abnormalities in lung cancer that are as yet unexplored (Salgia and Skarin, 1998).

The protein kinase C (PKC) family of serine–threonine protein kinases has been implicated in several important cellular functions including proliferation, motility, invasion and apoptosis (Livneh and Fishman, 1997). Among the PKC isoforms, PKCβ is known to be an important mediator of vascular endothelial growth factor (VEGF) (Xia *et al*, 1996; Yoshiji *et al*, 1999), the most potent angiogenic factor found in various tumours. Increased invasion and proliferation in tumours have also been associated with PKCβ (Zhang *et al*, 2004). Overexpression and increased activity of PKCβ have been implicated in transformation and tumourigenesis in lung cancer (Barr *et al*, 1997; Lahn *et al*, 2006). In several human cancers, PKCβ expression is linked to poor prognosis, most notably in B-cell lymphoma (Shipp *et al*, 2002; Li *et al*, 2007). Biochemical analysis demonstrated that PKCβ could target the phoshatidylinositol 3-kinase (PI3K)/AKT pathway and other signal transduction pathways (Graff *et al*, 2005; Rascoe *et al*, 2005). However, the mechanism by which PKCβ contributes to tumourigenesis remains unclear.

The PKCβ inhibitor enzastaurin, an oral serine–threonine kinase inhibitor, was initially developed as an ATP-competitive selective inhibitor against PKCβ (Faul *et al*, 2003). Enzastaurin is now being evaluated in several phase II studies across a variety of more common tumour types including: breast, ovarian colon and prostate cancers (Mina *et al*, 2009; Vergote *et al*, 2009; Dreicer *et al*, 2010; Glimelius *et al*, 2010). It has also been evaluated as second- or third-line therapy for NSCLC in a phase II study (Oh *et al*, 2008; Chiappori *et al*, 2010). *In vitro*, sequence-dependent, synergistic anti-proliferative and proapoptotic effects of the combination of cytotoxic drugs and enzastaurin have been found in NSCLC cells (Rademaker-Lakhai *et al*, 2007; Morgillo *et al*, 2008; Tekle *et al*, 2008). These studies suggest that enzastaurin may have an activity against lung cancer.

In this study, we analysed the anti-tumour effects of enzastaurin in a panel of 22 lung cancer cell lines to ascertain the potential for enzastaurin-based treatment of lung cancer. We also conducted gene, receptor tyrosine kinases (RTKs) phosphorylation and microRNA (miRNA) profiling on the same set of cell lines to identify the molecules associated with sensitivity of lung cancer to enzastaurin treatment. The correlation between the cytotoxic activity of enzastaurin and the corresponding gene, RTKs phosphorylation and miRNA expression patterns has been examined to clarify the

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responsible mechanisms of the signalling pathway involved in the response of lung cancers to enzastaurin treatment.

MATERIALS AND METHODS

Cell lines

We used 22 lung cancer cell lines: A549, PC3, PC7, PC9, PC14, LC2/ad, ABC-1, RERF-LC-KJ, RERF-LC-MS, RERF-LC-AI adenocarcinoma (AC) cell lines and PC1, PC10, LK2, SQ5, QG56, EBC-1, LC1/sq squamous-cell carcinoma (SCC) cell lines and NCI-H69, NCI-N231, Lu135, SBC3, MS-1 small-cell lung carcinoma (SCLC) cell lines for this study. In addition, five cell lines comprising H1650, H1975, LC-1F, RERF-LC-OK and VMRC-LCD, were used as the test set for a validation study. A549, NCI-H69, NCI-N231, H1650 and H1975 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA); RERF-LC-KJ, RERF-LC-AI, RERF-LC-OK, LC2-ad, SQ5, LC2/Ad, LC1/Sq, LC-1F and MS-1 were obtained from the RIKEN Cell Bank (Ibaraki, Japan) and PC1, PC3, PC7, PC9, PC10 and PC14 were obtained from Immuno-Biological Laboratories (Gunma, Japan); RERF-LC-MS, ABC-1, EBC-1, LK2, QG56 and VMRC-LCD were purchased from Health Science Research Resources Bank (Osaka, Japan). Lung cancer cell lines were maintained in RPMI 1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum.

Drugs and growth-inhibition assay

Enzastaurin was kindly provided by Ely Lilly. Growth inhibition was assessed by MTS assay to examine the effect of enzastaurin on lung cancer cell lines. Cell suspensions (5000 cells per well) were seeded into 96-well plates and increasing concentrations of enzastaurin (0, 0.01, 0.1, 1.0, 10 and 100 μM) were added. After incubation for 72 h at 37 °C, MTS was added to each well and incubated for 2 h at 37 °C, after which absorbance was measured using a microplate reader with a test wavelength of 450 nm. The IC_{50} value was defined as the concentration needed for 50% reduction of the growth by treatment with enzastaurin.

JAK inhibitor (JAK inhibitor I, Cat. No 420099) was purchased from Calbiochem (San Diego, CA, USA). A549 and RERF-LC-KJ cells (5000 cells per well) were seeded into 96-well plates. After 24 h, the cells were incubated for 72 h in the various concentrations of enzastaurin (0, 0.01. 0.1, 1.0, 10 and 100 μM), with or without low-dose (1 μM) JAK inhibitor.

RNA isolation, cDNA array, RTKs phosphorylation antibody array and miRNA array

Total RNA was isolated from lung cancer cell lines with the use of TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. High-density oligonucleotide array analysis was carried out using Affymetrix HG-U133A (22 282 probe sets) expression array, as previously described (Gemma *et al*, 2006). Scanning was performed with the GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA), and GeneChip analysis was based on the Affymetrix GeneChip Manual with GeneChip Operating Software version 1.0 (Affymetrix), and Microarray Database software. We also performed human RTKs phosphorylation antibody array, including 71 antibodies (RayBiotech, Inc., Norcross, GA, USA). MicroRNA expression profiles were analysed by TaqMan MicroRNA Array set version 2.0 containing 667 miRNAs and validated by TaqMan MicroRNA assay (Applied Biosystems, Foster City, CA, USA).

Western blot analysis

Cells were lysed in buffer containing 50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 0.1% sodium dodecyl sulphate, 1% Nonidet P-40 and 0.5% sodium deoxycholate. The lysates were kept on ice for 30 min, and then centrifuged at 13 000 g for 30 min. The supernatant was collected and 10 μg of protein were separated by gel electrophoresis on 10% gels, transferred to nitrocellulose membranes and detected by immunoblotting using a chemiluminescence system (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). The antibodies detecting JAK1, STAT3, phospho-STAT3 (p-STAT) and β-actin were purchased from Cell Signaling Technology (Beverley, MA, USA).

Lentiviral-mediated JAK1-overexpressing cells

Expression plasmid vector pEZ-Lv151 was used for lentiviral vector production (GeneCopoeia, Rockville, MD, USA). The coding sequence of human JAK1 or enhanced green fluorescent protein (EGFP) was inserted under the transcriptional control of the CMV promoter in pEZ-Lv151. The human JAK1 lentiviral expression plasmid (Ex-T8644-Lv151) or EGFP plasmid (Ex-EGFP-Lv151) was cotransfected into 293Ta cells with the Lenti-Pac HIV Packaging Mix (GeneCopoeia). Lentivirus-containing supernatants were harvested 48 h after transfection. The lentivirus particles were purified and stored at −80 °C in aliquots until use.

To establish stable JAK1-overexpressing cell lines, A549 cells were transduced with serial dilutions of lentiviral supernatant in the presence of 5 $\mu g\,ml^{-1}$ polybrene and selected by 0.8 $ng\,ml^{-1}$ geniticine. After antibiotic selection for 3 weeks, stable overexpressing JAK1 cells (LV-JAK1 A549 cells) were obtained.

Statistical analyses

Data analysis for the correlation coefficients that revealed the correlation between the drug activity patterns and the gene expression patterns was principally done by a modified National Cancer Institute programme (Miyanaga *et al*, 2008). We used pathway analysis to provide a viewpoint of the biological function of genes within the proposed classifier. Pathway analysis was done using the Pathway Architect software (Stratagene, La Jolla, CA, USA). The pathways showing the relationships among the genes on the list were drawn by selecting all molecules on the pathway edit window. All relationships among the molecules were retrieved from the database, with this information being derived from PubMed abstracts by natural language processing technology. The function was done by selecting the data of maximum reliability (MAX) by choosing all modes of interactions including 'Promoter Binding', 'Regulation', 'Protein Modification' and 'Expression' and by taking the relationships supported by three or more consistent data sources. Next, we picked out the incorporated genes from the imported gene list used at the onset of the pathway analysis, except the subunits of the target gene. Thus, a list of the genes associated with drug response was established with respect to not only gene expression profile data but also the biological functions of altered/associated genes. Data from the listed genes were used to build a support vector machine (SVM) model with ArrayAssist software (Stratagene) to predict the drug response (IC_{50}). The SVM algorithm model with Gaussian kernels was used to distinguish sensitive cells from resistant cells, using biomarkers identified by the gene expression-enzastaurin drug sensitivity correlation and pathway analysis. The classification ability of the genes was evaluated using leave-one-out cross-validation.

RESULTS

Effect of enzastaurin on the growth of lung cancer cells

Growth-inhibitory effects of enzastaurin on lung cancer cell lines were assessed by MTS assay.

Figure 1 shows the sensitivity to enzastaurin among the 22 lung cancer cells. Based on the IC_{50}, the 22 cell lines were classified into two groups, namely: enzastaurin sensitive and enzastaurin

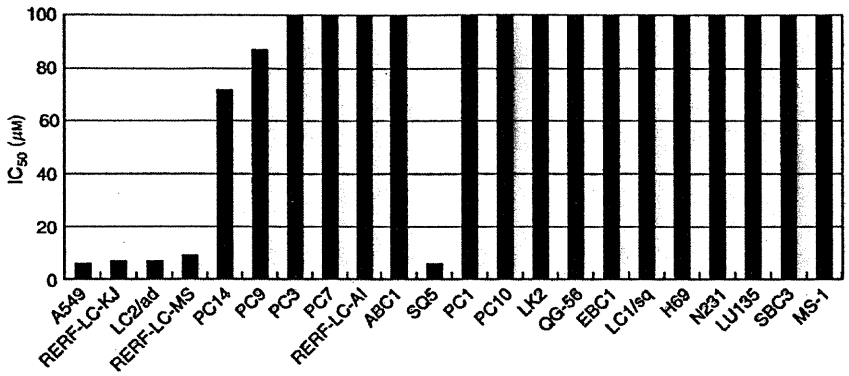

Figure 1 IC_{50} values for 22 lung cancer cell lines responding to enzastaurin treatment by MTS assay. According to sensitivity to enzastaurin, these 22 cell lines were classified as sensitive (IC_{50} of $\leqslant 10\,\mu M$) or resistant (IC_{50} of $>50\,\mu M$).

Table 1 Unique genes correlated with sensitivity to enzastaurin

Gene symbol	Gene title	F-statistic	*P*-value	Correlation coefficients	Eight-gene predictor
DUSP1	Dual specificity phosphatase 1	49.2	8.39E−07	−0.69	*
ILF3	Interleukin enhancer binding factor 3, 90 kDa	48.5	1.10E−06	0.67	*
LITAF	Lipopolysaccharide-induced TNF factor	36.0	9.75E−06	−0.70	*
JAK1	Janus kinase 1 (a protein tyrosine kinase)	27.1	6.36E−05	−0.65	*
COPS7B	COP9 constitutive photomorphogenic homologue subunit 7B (Arabidopsis)	19.3	5.48E−04	0.66	*
RAD23A	RAD23 homologue A (*S. cerevisiae*)	23.0	9.10E−04	0.74	*
TNFAIP1	Tumour necrosis factor, α-induced protein 1 (endothelial)	19.5	0.002	−0.65	*
MIRN21///TMEM49	Transmembrane protein 49///microRNA 21	14.1	0.003	−0.66	*
PSEN1	Presenilin 1 (Alzheimer disease 3)	9.5	0.012	−0.65	
PPAP2A	Phosphatidic acid phosphatase type 2A	11.3	0.014	−0.75	
IGF1R	Insulin-like growth factor 1 receptor	10.6	0.019	−0.66	
SART3	Squamous cell carcinoma antigen recognised by T cells 3	9.4	0.019	0.65	
NDFIP1	Nedd4 family interacting protein 1	6.0	0.029	−0.66	
MLPH	Melanophilin	8.2	0.034	−0.65	
SEMA3C	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted (semaphorin) 3C	5.9	0.056	−0.67	
UGDH	UDP-glucose dehydrogenase	5.9	0.062	−0.68	

Abbreviations: ANOVA = analysis of variance; TNF = tumour necrosis factor. Note: F-statistic and *P*-values were calculated by ANOVA. *Genes used as eight-gene predictor are shown.

resistant. Five cell lines (A549, RERF-LC-KJ, LC2/ad, RERF-LC-MS and SQ5) were sensitive (IC_{50} of $\leqslant 10\,\mu M$), and the remaining 17 cell lines were resistant to enzastaurin (IC_{50} of $>50\,\mu M$). The five cell lines sensitive to enzastaurin consisted of four AC (4/10, 40%) and one SCC (1/7, 14%) cell line; no SCLC (0/5) cell lines were enzastaurin sensitive. These results suggest that enzastaurin has anti-tumour activity against NSCLC.

Gene expression-drug sensitivity correlation

We have previously performed gene expression profile analysis of the same set of 22 lung cell lines by Affymetrix GeneChip (Gemma *et al*, 2006). First, we used the MTS results for enzastaurin for the development of a molecular model of sensitivity to enzastaurin. Twenty-three genes were significantly correlated with sensitivity to enzastaurin (correlation coefficients of >0.65). Next, pathway analysis was performed using the 23 genes to provide a viewpoint of the biological function of the genes, as previously described (Miyanaga *et al*, 2008). Pathway analysis removed the incorporated genes out of the imported 23 genes. Sixteen genes, associated with sensitivity to enzastaurin, were identified based on the biological functions of altered/associated genes (Table 1; Figure 2A). Pathway analysis revealed that JAK1 was the final target gene for the sensitivity to enzastaurin in lung cancer cells (Figure 2A). We next identified the optimal number of genes whose expression could accurately distinguish the sensitive cells from the resistant ones. Analysis of variance (ANOVA) was done to remove the genes with variance. The top eight genes (DUSP1, ILF3, LITAF, JAK1, COPS7B, RAD23A, TNFAIP1 and MIRN21/TMEM49) according to the ANOVA were subsequently found to be the minimum number necessary for prediction of drug response (Figure 2B; Table 1). We used the eight most strongly correlated genes to build an SVM algorithm model by which the five sensitive cells were distinguished from the 17 resistant cells. Overall, the SVM classification based on the above-mentioned eight genes, correctly classified the sensitivity to enzastaurin of all of the 22 cells (data not shown). Next, we examined the robustness of the eight-gene predictor, for classifying cells into the enzastaurin-sensitive group, in an independent set of NSCLC cells, and found that the eight-gene predictor correctly classified all five resistant cells (Table 2). Thus, we had ultimately identified an eight-gene signature that was validated for its ability to predict the sensitivity to enzastaurin in an independent set of lung cancer cells.

RTKs phosphorylation and miRNA expression-drug sensitivity correlation

Pathway analysis revealed that JAK1 was an important gene for the sensitivity to enzastaurin in lung cancer cells. JAK1 and its downstream STAT3 gene expression levels of sensitive cells were

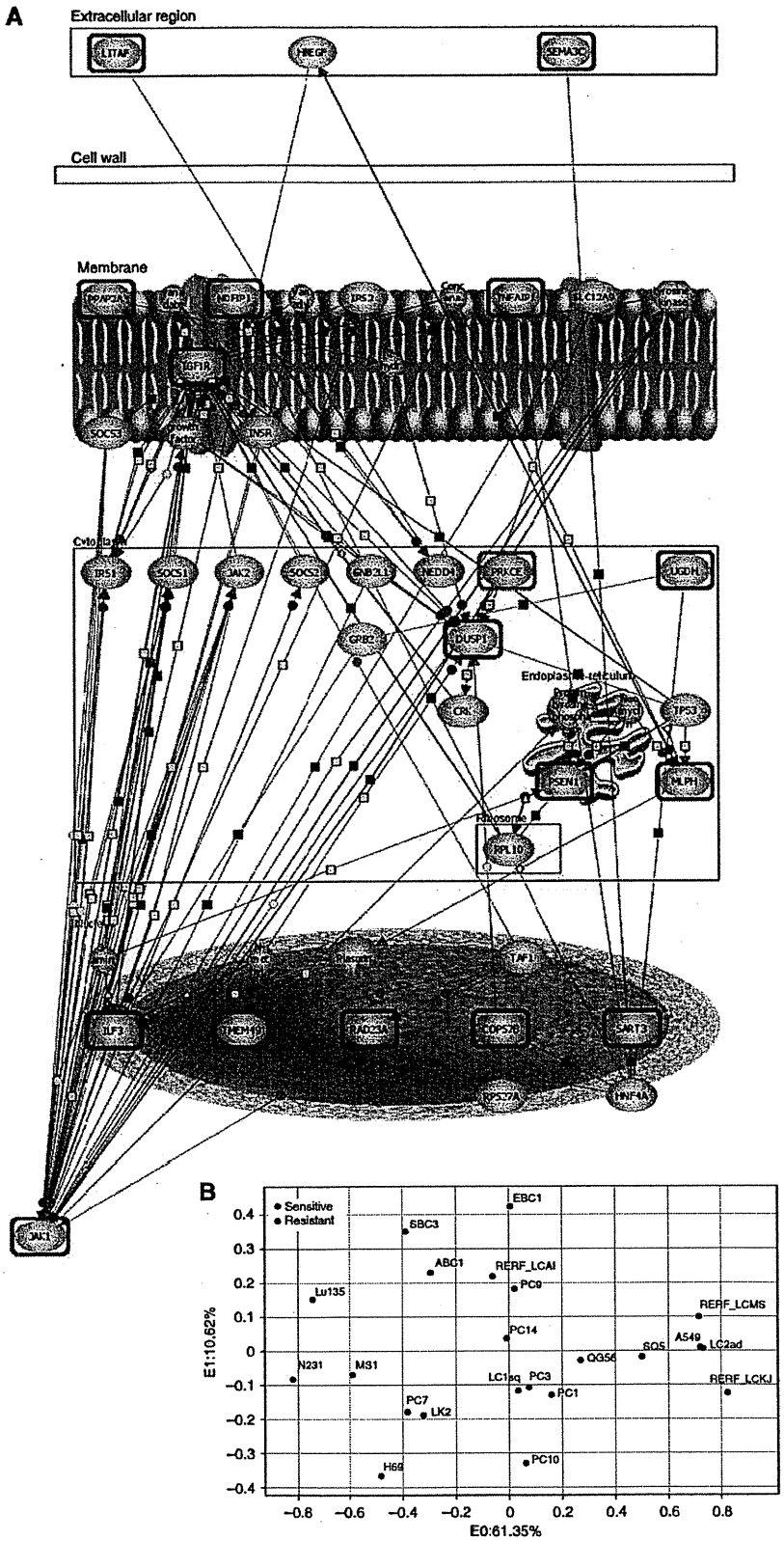

Figure 2 Sixteen genes associated with enzastaurin response were established by pathway analyses and prediction of drug response using an eight-gene signature. (**A**) Sixteen genes (blue circle) associated with enzastaurin response and PKC (red circle) belonged to the same signal pathway. (**B**) Principal component analysis based on the eight-gene profile correctly distinguished the sensitive cells from the resistant ones. The colour reproduction of this figure is available at the *British Journal of Cancer* online.

significantly higher than those of resistant cells (Figures 3A and B). To further clarify the signalling mechanism correlated with the sensitivity to enzastaurin, we also examined RTKs phosphorylation expression profiles of the same set of 22 lung cancer cells. The top 10 RTKs phosphorylation associated with enzastaurin sensitivity are shown in Table 3 (correlation coefficients of >0.50). Pathway analysis using the 23 genes and 10 RTKs phosphorylation associated with sensitivity to enzastaurin also revealed that JAK/STAT signal pathway was mainly involved in the drug response (data not shown). Among the 10 RTKs phosphorylation, the expression of two RTKs mainly associated with angiogenesis and lymphangiogenesis (VEGFR2 and VEGFR3) was significantly elevated in sensitive cells compared with in resistant cells (Figures 3C and D).

Table 2 Validation of the eight-gene predictor by examining the SVM value in an independent set of five NSCLC cell lines

		Histology	IC_{50} (μM)	Predicted class*
1	H1650	AC	>100	Resistant
2	H1975	AC	>100	Resistant
3	RERF-LC-OK	AC	>100	Resistant
4	VMRC-LCD	AC	>100	Resistant
5	LC-1F	SCC	>100	Resistant

Abbreviations: AC = adenocarcinoma; SVM = support vector machine; NSCLC = non-small-cell lung cancer. Note: *Cell lines were classified as sensitive (IC_{50} of $\leqslant 10\,\mu$M) and resistant (IC_{50} of $>50\,\mu$M) to enzastaurin.

Table 3 Kinase and miRNA correlated with the sensitivity to enzastaurin

	Kinase	F-statistic	*P*-value	Correlation coefficients
(a)				
1	M-CSFR	11.51	0.02	−0.82
2	VEGFR2	9.17	0.03	−0.68
3	FER	9.00	0.02	−0.60
4	EphA1	7.58	0.02	−0.61
5	VEGFR3	6.76	0.05	−0.58
6	TNK1	4.45	0.09	−0.71
7	NGFR	3.73	0.11	−0.68
8	MATK	2.95	0.15	−0.52
9	Hck	2.26	0.20	−0.53
10	SYK	1.82	0.23	−0.58
	miRNA	**F-statistic**	***P*-value**	**Correlation coefficients**
(b)				
1	hsa-miR-15a*	18.56	0.0004	0.51
2	hsa-miR-454*	16.65	0.0006	0.53
3	hsa-miR-92a	15.96	0.0007	0.52
4	hsa-miR-301b	12.49	0.0021	0.54
5	hsa-miR-130b	11.85	0.0026	0.54
6	hsa-miR-106b*	11.42	0.0032	0.52
7	hsa-miR-345	9.25	0.01	0.54
8	hsa-miR-31	7.25	0.05	−0.76
9	hsa-let-7a	4.04	0.09	0.54
10	hsa-miR-193b	2.76	0.14	−0.64
11	hsa-miR-193b*	2.76	0.15	−0.61
12	hsa-miR-21	2.24	0.18	−0.53
13	hsa-miR-30c-2*	1.93	0.24	−0.52

Abbreviations: ANOVA = analysis of variance; miRNA = microRNA. Note: F-statistic and *P*-values were calculated by ANOVA. *The miRNA name used in TaqMan microRNA array analysis.

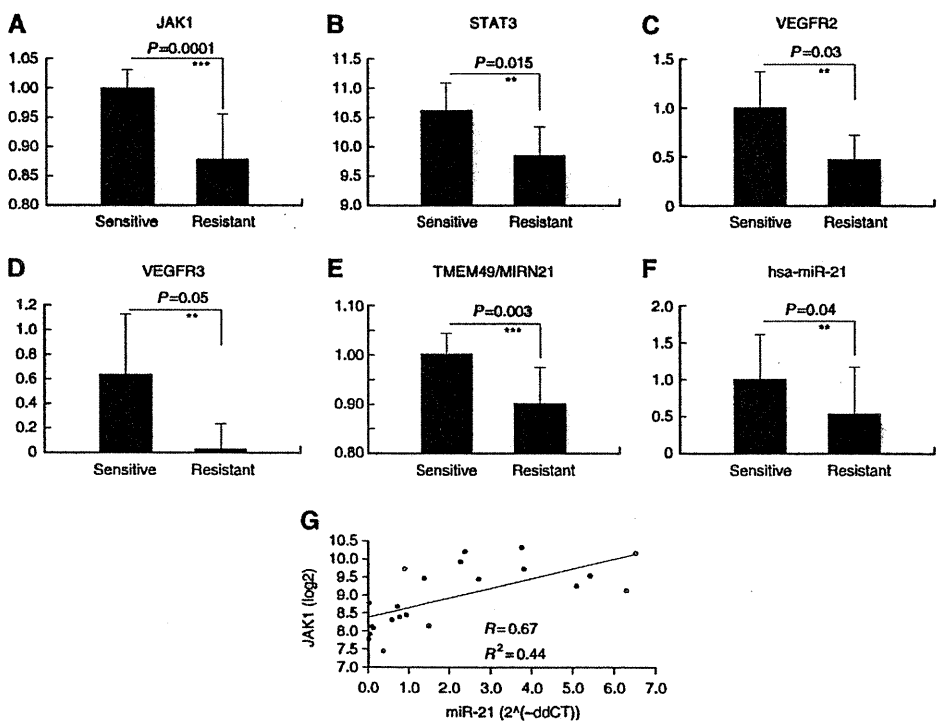

Figure 3 JAK1, VEGFR2, VEGFR3 and miR-21 were correlated with drug response. (**A** and **B**) JAK1 and STAT3 gene expression levels were significantly higher in the sensitive cell group than in the resistant cell group. (**C** and **D**) Elevated levels of VEGFR2 and VEGFR3 expression were observed in sensitive cells. (**E**) Expression of MIRN21/TMEM49 was significantly higher in sensitive cells than in resistant cells, by gene-chip analysis. (**F**) Mature miR-21 expression was significantly higher in sensitive cells than in resistant cells by quantitative RT–PCR analysis. (**G**) Quantitative comparison of miR-21 and JAK1 showed a significant positive correlation between these two molecules. $^{**}P<0.05$ when compared with the resistant cells. $^{***}P<0.01$ when compared with the resistant cells.

In order to investigate post-transcriptional regulation, miRNA microarray analysis of the 22 cells was also performed. We identified 13 miRNAs correlated with enzastaurin sensitivity (correlation coefficients of >0.50) (Table 3). Interestingly, MIRN21/TMEM49, a host gene of miR-21, was included among the eight genes associated with enzastaurin sensitivity, and was expressed at significantly higher levels in sensitive cells compared with in resistant cells (Figure 3E). In addition, a correlation between miR-21 and enzastaurin sensitivity was found in miRNA array analysis (correlation coefficients -0.53) (Table 3). Recent reports demonstrated that miR-21 is a major miRNA that may play an oncogenic role in lung carcinogenesis (Volinia *et al*, 2006; Yanaihara *et al*, 2006; Seike *et al*, 2009). The expression levels of miR-21 were examined by real-time quantitative RT–PCR. miR-21 expression was significantly higher in sensitive cells than in resistant cells ($P<0.05$, paired *t*-test) (Figure 3F). The quantitative comparison of miR-21 and JAK1 showed a significant positive correlation between these two (Pearson's correlation, $r=0.67$, $P<0.05$) (Figure 3G). We ultimately recognised JAK1, VEGFR2, VEGFR3 and miR-21 as factors concerned with sensitivity to enzastaurin. In particular, JAK1 is the most significant molecule involved in drug response.

JAK1 expression effect on drug sensitivity in A549 cells

To investigate further the effect of JAK1 on sensitivity to enzastaurin, JAK1 protein expression of 11 NSCLC cells was evaluated by western blot analysis. Elevated JAK1 protein was observed in enzastaurin-sensitive NSCLC cells (Figure 4A). Next, we inhibited JAK1 protein using JAK1 inhibitor in enzastaurin-sensitive A549 and RERF-LC-KJ cells. After the treatment of JAK inhibitor (1 μM), JAK1 and its downstream p-STAT3 expression was completely diminished until 72 h in A549 cells (Figure 4B). We examined the effect of enzastaurin and JAK inhibitor combination therapy on cell growth. Concurrent JAK inhibitor and enzastaurin therapy significantly decreased the growth-inhibitory effect of enzastaurin, compared with enzastaurin monotherapy in enzastaurin-sensitive A549 cells (Figure 4C). Enzastaurin therapy after JAK inhibitor 1 μM treatment also diminished the growth-inhibitory effect of enzastaurin, compared with enzastaurin monotherapy in A549 cells (Figure 4D). The IC_{50} values of concurrent enzastaurin with JAK inhibitor and enzastaurin therapy after JAK inhibitor were 76 and 83, respectively, whereas that of enzastaurin monotherapy was 5.8 (Figures 4C and D). In addition, RERF-LC-KJ cells, which are also sensitive to

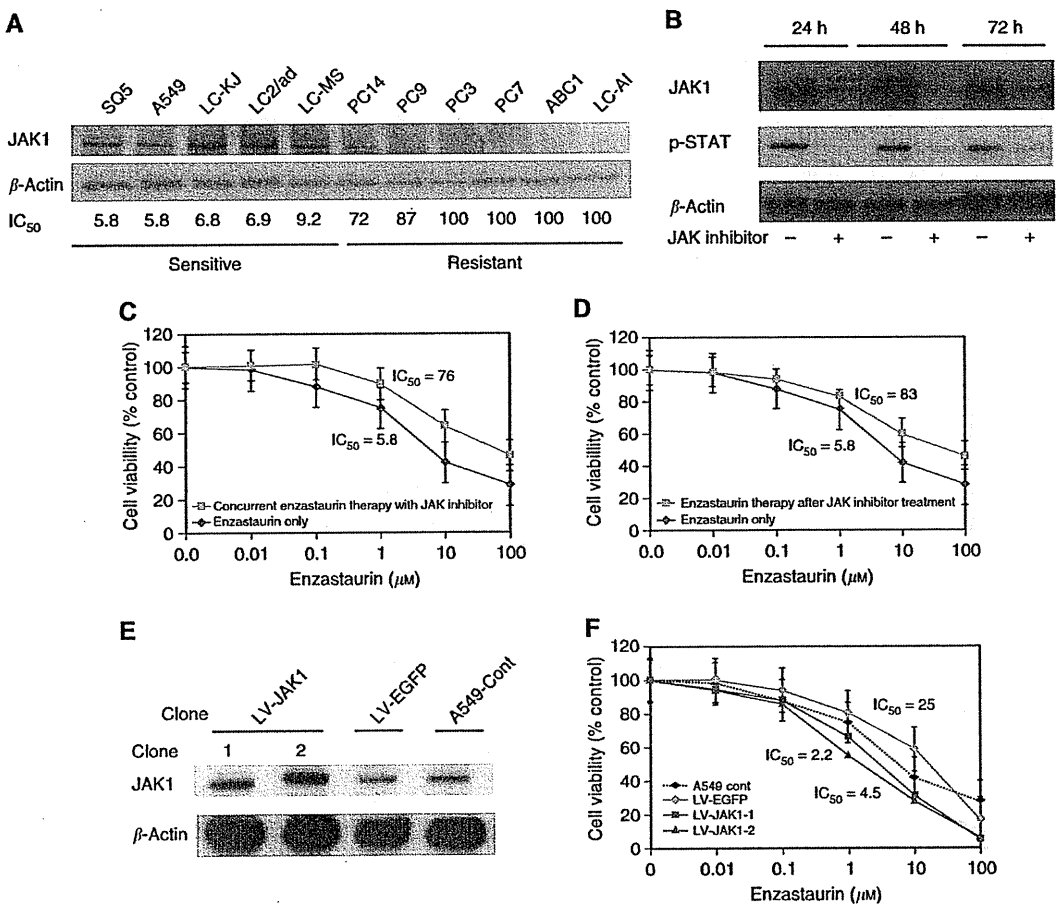

Figure 4 Effect of combination therapy with enzastaurin and JAK1 expression on cell growth in lung cancer cells. (**A**) JAK1 expression levels were significantly higher in the sensitive cell group than in the resistant cell group, by western blotting. (**B**) Completed inhibition of JAK1/STAT signalling by JAK1 inhibitor in A549 cells. P-STAT3 was completely inhibited until 72 h after the treatment of 1 μM JAK inhibitor. (**C**) Enzastaurin treatment with JAK inhibitor for 72 h was examined in A549 cells. Each result is expressed as cell viability in treated samples compared with the untreated sample (100%) for enzastaurin alone and concurrent therapy with the 1 μM JAK inhibitor treatment. (**D**) The effect of JAK inhibitor treatment (1 μM) for 24 h followed by enzastaurin treatment for 72 h was examined in A549 cells. (**E**) Lentiviral-mediated production of JAK1 in A549 cells. Western blotting showed that JAK1 expression levels were significantly higher in two LV-JAK1 clones than in the control clones. (**F**) Enzastaurin treatment for 72 h was examined in LV-JAK1-A549 cells. Each result is expressed as cell viability in the treated samples compared with the untreated sample (100%) for enzastaurin therapy.

enzastaurin, showed resistance after JAK inhibitor therapy in combination with enzastaurin (data not shown). In RERF-LC-KJ cells, both IC_{50} values of concurrent enzastaurin with JAK inhibitor and enzastaurin therapy after JAK inhibitor were over 100, whereas that of enzastaurin monotherapy was 6.8. To confirm further the ability of JAK1 to indicate drug sensitivity to enzastaurin, we developed a lentiviral vector for the expression of JAK1 and established stable JAK1-overexpressing A549 cells (LV-JAK1-A549 cells). Western blot analysis showed the overexpression of JAK1 in LV-JAK1-A549 cells (Figure 4E). The growth-inhibitory effect of enzastaurin on LV-JAK1-A549 cells was assessed by MTS assay. The drug sensitivities of two LV-JAK1-A549 cells were greater than those in the control cells (Figure 4F). The IC_{50} values of two LV-EGFP A549 cells were 2.2 and 4.5, respectively, whereas that of LV-EGFP A549 cells was 25 (Figure 4F). These results indicate that JAK1 expression contributed to the drug sensitivity and could be used as a drug-sensitive marker to enzastaurin in lung cancer cells.

JAK/STAT3 pathway directly activates miR-21

A significant correlation between JAK1 and miR-21 was found in our set of NSCLC cells (Figure 3G). STAT3 is a transcription factor activated by JAK1, and its binding to the target sites in miR-21 promoter upon IL-6 induction has been reported previously (Löffler *et al*, 2007; Iliopoulos *et al*, 2010). To verify the association between JAK1 and miR-21, miR-21 expression was quantified after the stimulation of IL-6 by qRT–PCR analysis. Upon IL-6 exposure, p-STAT3 expression was significantly upregulated, resulting in the overexpression of miR-21 at 24 h in A549 cells (Figures 5A and B). We also evaluated the miR-21 expression in LV-LAK1 A549 cells. In the JAK1-overexpressing cells, miR-21 expression was significantly higher than in parent cells (Figure 5C). These results supported the concept that miR-21 is directly induced by JAK/STAT signalling in NSCLC cells.

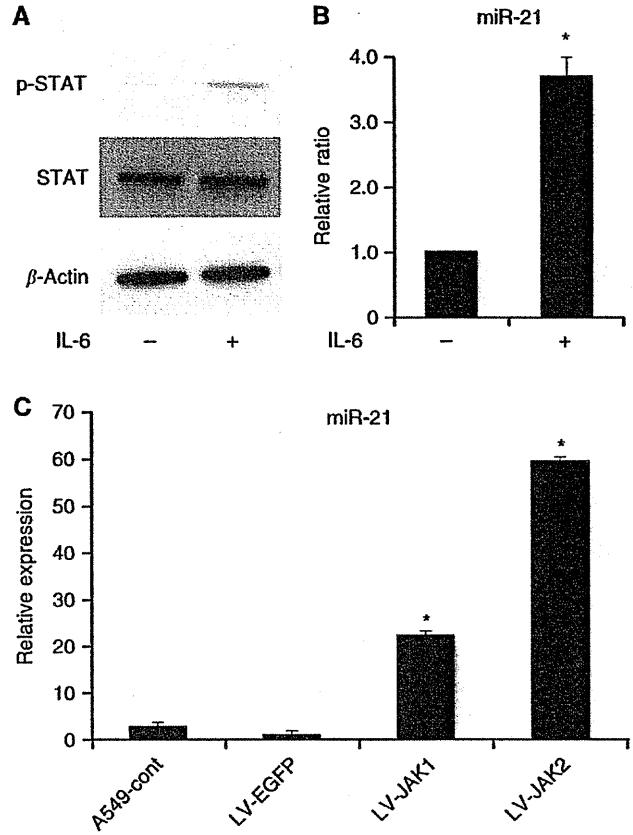

Figure 5 Association between JAK1 and miR-21 expression. (**A**) p-STAT3 was overexpressed after IL-6 stimulation of A549 cells for 24 h. (**B**) After IL-6 stimulation, miR-21 expression was significantly increased, as measured by qRT–PCR analysis. (**C**) MiR-21 expression of two LV-JAK1 cells was significantly higher than in the control cells, as measured by qRT–PCR analysis. Data were mean ± s.d. from three independent experiments. *$P<0.05$ when compared with the respective parent cells.

DISCUSSION

Enzastaurin has recently been evaluated as second- or third-line therapy of NSCLC in a phase II study (Oh *et al*, 2008; Chiappori *et al*, 2010). Synergistic effects of the combination of enzastaurin and cytotoxic drugs including cisplatin, gemcitabine and pemetrexed have been found in NSCLC cells in an *in vitro* study (Rademaker-Lakhai *et al*, 2007; Morgillo *et al*, 2008; Tekle *et al*, 2008). A recent study showed that enzastaurin inhibited *in vivo* metastasis of NSCLC cells (Körner *et al*, 2010). It is known that PKCs mediate the regulation of the cell cycle; enzastaurin is also able to inhibit several proteins involved in cell-cycle regulation, for example, E2F-1 associated with G1/S checkpoint and Cdc25C resulting in G2/M checkpoint (Tekle *et al*, 2008). These checkpoint arrests provide the tumour cells with the opportunity to repair their DNA, which has been damaged by cytotoxic drugs. Reduction of E2F-1 expression and phosphorylated Cdc25C by enzastaurin might explain the abrogation of the checkpoint arrest and could facilitate cytotoxic drug-damaged cells to undergo apoptosis. Furthermore, a recent study demonstrated that enzastaurin had a cooperative effect with gefitinib and was able to revert gefitinib resistance in cancer cells through the inhibition of Akt and VEGF pathways (Gelardi *et al*, 2008). These studies suggest that enzastaurin might be a promising novel agent in NSCLC patients.

Enzastaurin inhibited the downstream PKCβ signalling, PI3K/AKT pathway and the phosphorylation of glycogen synthase kinase-3β (Keyes *et al*, 2002; Graff *et al*, 2005). Anti-tumour and anti-angiogenic activity of enzastaurin was also demonstrated in tumour xenograft models, including NSCLC, and was confirmed using a standardised clonogenic assay in patient-derived tumour explants (Keyes *et al*, 2004). Significant reduction of VEGF protein levels following enzastaurin treatment, together with a significant decrease in intratumoural vessel density, has been demonstrated *in vivo* (Keyes *et al*, 2004). In the current study using a RTKs phosphorylation antibody array, we found elevated levels of VEGFR2 and VEGFR3 in the enzastaurin-sensitive cells. Our results are in agreement with previous data concerning enzastaurin and anti-angiogenic activity. These findings demonstrated that lung cancer cases with activated angiogenic activity should respond to enzastaurin treatment.

In this study, using gene-chip and pathway analysis, we identified 16 genes that correlated with sensitivity to enzastaurin. Pathway analysis also revealed that JAK1 was the most important molecule affected by enzastaurin treatment of NSCLC. The JAK is a non-RTK and can activate STAT3 transcriptional factor. The STAT3 is also persistently activated in about half of NSCLC tumours and is involved in tumour invasion, metastasis and angiogenesis through differential gene regulation (Haura *et al*, 2005; Song *et al*, 2011). Increased levels of JAK1 and STAT3 were observed in the sensitive cells in this study. Knockdown of JAK resulting in p-STAT3 also diminished the growth-inhibitory effect of enzastaurin in the sensitive cells. In contrast, overexpression of JAK1 by lentiviral-mediated production enhanced the drug sensitivity to enzastaurin in the sensitive cells. These results suggest that JAK expression levels can be used as predictive markers of enzastaurin sensitivity. Non-small-cell lung cancer patients with an activated JAK/STAT3 pathway are suitable cases for enzastaurin treatment.

MicroRNAs are small non-coding RNA molecules of about 20 nucleotides that are frequently located at chromosomal regions deleted or amplified in cancers, suggesting that miRNAs are a new class of genes involved in human tumourigenesis (Lu *et al*, 2005; Volinia *et al*, 2006; Yanaihara *et al*, 2006; Seike *et al*, 2009). Recently, miRNAs have been demonstrated as diagnostic and prognostic markers in lung cancer (Yanaihara *et al*, 2006; Seike *et al*, 2009). We previously reported that the inhibition of miR-21, whose upregulation is associated with EGFR mutations, can be a therapeutic strategy, either as a monotherapy or in combination with EGFR-TKI treatment (Seike *et al*, 2009). In this study, expression of miR-21 and its host gene, TMEM49, were significantly higher in enzastaurin-sensitive cells than in enzastaurin-resistant cells. In addition, a significant positive correlation was observed between miR-21 and JAK1. The STAT3 reportedly signals IL-6-induced upregulation of miR-21 in multiple myeloma cells (Löffler *et al*, 2007). We confirmed that JAK1 and its downstream target STAT3, containing three binding sites of miR-21 promoter, directly activated miR-21 in NSCLC cells. These results suggest that, in lung cancer, miR-21 affects the response to enzastaurin through the JAK/STAT signalling pathway.

In conclusion, we have identified unique molecules; genes, RTKs and miRNAs that are correlated with sensitivity to enzastaurin and have constructed an eight-gene signature to distinguish the sensitive cells from the resistant cells. Furthermore, we demonstrate that JAK1 is the most significant factor concerned in response to enzastaurin. Patient selection based on the JAK expression might be useful for future clinical development of enzastaurin therapy in NSCLC.

ACKNOWLEDGEMENTS

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Conflict of interest

The authors declare no conflict of interest.

Translational Therapeutics

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Clarification of clinical features of interstitial lung disease induced by irinotecan based on postmarketing surveillance data and spontaneous reports

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Irinotecan-induced interstitial lung disease (ILD) requires accurate diagnosis, followed by prompt and appropriate treatment. This study was conducted to compile information and imaging data to define the characteristics of irinotecan-induced ILD. Searches were performed on information collected for a drug reexamination application and on data from spontaneous safety reports submitted to Daiichi Sankyo Company, Limited. These database searches revealed 153 cases of serious ILD that occurred in association with irinotecan therapy, and which were reported as adverse drug reactions. Computed tomographic findings obtained after the onset of ILD were categorized based on four typical patterns. A total of 66 patients (including 15 for whom a relationship between death and serious ILD could not be excluded; incidence of serious ILD: 0.74%; death rate of ILD: 0.17%) were detected during the postmarketing surveillance along with 87 patients (22 deaths) that were identified from spontaneous reports. Within 16 weeks of starting treatment, 80.7% of the patients developed ILD. A total of 61.3% of the cases treated using steroids responded to the steroid therapy. These results indicate that there is no specific clinical or imaging feature associated with ILD related to irinotecan and that the prognosis of ILD related to irinotecan was poor in patients with preexisting ILD. The relative risk calculated for the association between preexisting ILD and death was 2.25 ($P=0.29$). During irinotecan treatments, patients need to be carefully observed for symptoms, especially at 16 weeks after starting treatment. In addition, when patients are receiving this type of therapy, they also need to undergo chest imaging studies. *Anti-Cancer Drugs* **22:563–568 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

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Introduction

Interstitial lung disease (ILD) is one of the typical adverse drug reactions (ADRs) seen with the use of anticancer agents and is a serious event that must be taken into consideration by physicians at all times. In recent years, problems associated with pulmonary disorders induced by anticancer agents, especially ILD, have led to the publication of many studies that have examined the mechanisms involved, ethnic differences associated with the incidence, and the risk/prognostic factors. This information has helped to improve our understanding of ILD caused by anticancer therapy.

During treatment with Topotecin (irinotecan), ILD has been shown to have a lower incidence (approximately 1%, regardless of the seriousness according to postmarketing surveillance) than either diarrhea or myelosuppression, although it can progress to respiratory failure and have a fatal outcome in some cases. Thus, ILD is considered to be a severe irinotecan-caused ADR [1–3]. Irinotecan-induced ILD, similar to that induced by other anticancer agents such as gefitinib and gemcitabine, requires accurate diagnosis, followed by prompt and appropriate treatment.

Anticancer drugs can cause pulmonary damage that manifests as a diffuse change of the lung fields. This can be detected by imaging studies, provided the patient is suspected of having ILD. Accordingly, a high index of suspicion, based on the risk factors for each drug or patient, is important for early detection and treatment of this ADR.

Marketing of irinotecan in Japan was approved in 1994 and this drug has been used to treat an estimated 200 000 patients as of May 2008. With the help of attending physicians, this study was designed to collect and compile clinical information and imaging data for use in helping to define the characteristics of irinotecan-induced ILD.

This study was conducted under the Pharmaceutical Affairs Act, Good Post-Marketing Surveillance Practice, and Good Vigilance Practice in Japan.

Authors' contributions: Akihiko Gemma and Noritoshi Yoshii adjudicated imaging findings. Noritoshi Yoshii, Koji Kakihata, and Tadamichi Suzuki conceived the analysis. Masaki Nagashima performed the analysis. All authors discussed the results and commented on the manuscript.

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