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A Randomized, Double-Blind, Phase IIa Dose-Finding Study of Vandetanib (ZD6474) in Japanese Patients With Non-Small Cell Lung Cancer

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Introduction: Vandetanib (ZACTIMA™) is a once-daily, oral anticancer drug that selectively inhibits vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) signaling. Vandetanib was evaluated as a monotherapy in a randomized, double-blind, dose-finding study in Japan.

Patients and Methods: Eligible patients with locally advanced or metastatic (stage IIIB/IV) or recurrent non-small cell lung cancer, previously treated with chemotherapy, were randomized to receive once-daily oral vandetanib 100, 200, or 300 mg (1:1:1). The primary objective was to determine the objective response rate for each vandetanib dose.

Results: Fifty-three patients received vandetanib (100 mg, $n = 17$; 200 mg, $n = 18$; 300 mg, $n = 18$). The objective response rate in each dose arm was 17.6% (3 of 17; 100 mg), 5.6% (1 of 18; 200 mg), and 16.7% (3 of 18; 300 mg). Common adverse events included rash, diarrhea, hypertension, and asymptomatic QTc prolongation. The adverse event profile was generally consistent with that reported previously for agents that inhibit the VEGFR or EGFR signaling pathways. Among the three responders evaluated for *EGFR* mutation, two had no mutation, and in one case, the *EGFR* mutation status could not be determined by direct DNA sequencing and amplification refractory mutation system assay of *EGFR* exons 19–21. Baseline plasma VEGF levels appeared to be lower in patients who experienced clinical benefit after vandetanib treatment.

Conclusion: In Japanese patients with advanced non-small cell lung cancer, vandetanib monotherapy (100–300 mg/d) demonstrated antitumor activity with an acceptable safety and tolerability profile.

Key Words: Non-small cell lung cancer, Vandetanib, EGFR, VEGFR.

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Non-small cell lung cancer (NSCLC) accounts for approximately 75% of lung cancers and is the leading cause of cancer-related death worldwide.[1] Despite the introduction of more effective chemotherapeutic agents, new approaches are required to further improve patient outcome and survival. A major focus of new anticancer research is the targeting of cell-signaling pathways that contribute to tumor growth and progression.

Vascular endothelial growth factor receptor (VEGFR) and epidermal growth factor receptor (EGFR) are key drivers of tumor angiogenesis and cell proliferation, respectively, and both pathways have been validated as clinically relevant targets in NSCLC. The addition of bevacizumab, a humanized anti-VEGF-A monoclonal antibody, to paclitaxel and carboplatin has demonstrated clinical benefit in patients with NSCLC,[2] and the EGFR inhibitors gefitinib and erlotinib have demonstrated clinical activity as single agents in NSCLC.[3,4] Furthermore, EGFR is known to regulate the production of VEGF and other proangiogenic factors[5] and resistance to EGFR inhibition has been associated with increased expression of VEGF in a human tumor xenograft model of NSCLC.[6] Therefore, targeting the VEGFR and EGFR pathways may be more effective than inhibiting either pathway alone. This hypothesis is supported by the promising results from early clinical evaluation of erlotinib and bevacizumab in combination in patients with recurrent NSCLC.[7]

Vandetanib (ZACTIMA™) is a once-daily, orally available anticancer drug that inhibits VEGFR- and EGFR-dependent signaling,[8] as well as the RET (REarranged during

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Transfection) receptor tyrosine kinase, which is an important growth driver in certain types of thyroid cancer.[9] Early clinical evaluation of vandetanib has demonstrated a promising efficacy and safety profile in a broad population of patients with advanced cancer. Phase I studies in advanced solid tumors conducted in the USA/Australia[10] and Japan[11] showed that once-daily doses of vandetanib (up to and including 300 mg) were generally well tolerated. In the Japanese study, objective tumor responses were observed in 4 of 9 patients with refractory NSCLC. Subsequent phase II studies in advanced NSCLC demonstrated antitumor activity both as a monotherapy and in combination with certain chemotherapy.[12–14] The positive outcome of these phase II trials led to the ongoing phase III evaluation of vandetanib in previously treated advanced NSCLC.

The primary objective of this randomized phase IIa study was to assess the objective response rate (ORR) to vandetanib (100, 200, or 300 mg/d) in Japanese patients with refractory NSCLC. The three doses investigated were selected based on the outcome of the Japanese phase I trial.[11]

PATIENTS AND METHODS

Patients

Patients with histologic or cytologic confirmation of locally advanced/metastatic (stage IIIB/IV) or recurrent NSCLC after failure of 1 or 2 platinum-based chemotherapy regimens were recruited from eight centers in Japan. The main eligibility criteria were age ≥20 years, a WHO performance status of 0 to 2, an estimated life expectancy ≥12 weeks, and completion of prior chemotherapy and/or radiotherapy at least 4 weeks before study entry (8 weeks for chest radiation and 6 weeks for mitomycin C). Patients with squamous cell histology were also eligible, and brain metastases were permitted if patients were asymptomatic and did not require corticosteroid treatment. Key exclusion criteria were a mixed small-cell and non-small cell histology, evidence of severe or uncontrolled systemic diseases, poorly controlled hypertension, a QTc interval ≥460 milliseconds by electrocardiogram during the screening period, and prior treatment with EGFR or VEGFR signaling inhibitors. All patients provided written informed consent. The study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki, applicable guidelines on good clinical practice, local Institutional Review Board approval, and the AstraZeneca policy on Bioethics.

Study Design and Treatments

This was a randomized, double-blind, parallel-group, phase IIa dose-finding multicenter study to assess the efficacy and safety of vandetanib. A total of 53 patients were randomized (1:1:1) to receive once-daily oral vandetanib (100, 200, or 300 mg/d; Figure 1). Patients were stratified by histology (adenocarcinoma versus others), gender (male versus female), and smoking history (smoker versus nonsmoker). Treatment continued until a withdrawal or dose-interruption criterion was met. These criteria included progressive disease (PD), unacceptable toxicity, protocol noncompliance, or voluntary discontinuation by the patient.

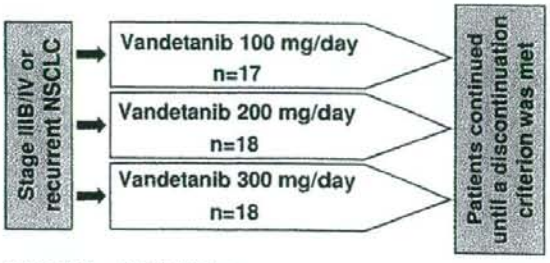

FIGURE 1. Study design.

Efficacy

The primary objective of the study was to determine ORR with vandetanib monotherapy, using the Response Evaluation Criteria in Solid Tumors (RECIST); assessments were performed at baseline and every 4 weeks for the first 24 weeks of treatment, and then every 8 weeks until withdrawal. A confirmed complete response or partial response (PR) was considered to be an objective tumor response. Investigator assessment of best overall tumor response was used for the primary analysis and these assessments were subsequently submitted to AstraZeneca for review by the response evaluation committee. Secondary efficacy endpoints included time to progression (TTP), duration of response (the time interval between the date of first documented objective tumor response until the date of PD or death), and disease control rate (DCR) for each dose of vandetanib. Time to progression was calculated from the date of randomization until the date of PD or death (in the absence of progression) and estimated using the Kaplan–Meier method. DCR was defined as confirmed complete response, PR, or stable disease (SD) ≥8 weeks.

Safety and Tolerability

Safety was assessed by monitoring for adverse events (AEs) and collecting laboratory data. All AEs were collected for up to 30 days after the last dose of vandetanib and were graded according to Common Terminology Criteria for Adverse Events (CTCAE, version 3). Unless otherwise clinically indicated, 12-lead electrocardiograms were performed twice at screening, weekly for the first 8 weeks of treatment, and then once every 4 weeks thereafter. Vandetanib treatment was interrupted following: a single QTc measurement ≥550 milliseconds; 2 consecutive QTc measurements ≥500 milliseconds but <550 milliseconds; an increase of ≥100 milliseconds from baseline; or an increase of ≥60 milliseconds from baseline QTc to a QTc value ≥460 milliseconds. Upon resolution of QTc prolongation, vandetanib treatment was recommenced at a reduced dose.

Pharmacokinetics

To investigate the pharmacokinetic (PK) profile of vandetanib, blood samples were collected on the same days as scheduled electrocardiogram measurements. Plasma concentrations of vandetanib were determined using reversed-phase liquid chromatography–mass spectrometry. The col-

lected data were related to a nonlinear mixed effects model to estimate population PK using NONMEM V (v 1.1).

Tumor Biomarkers

An exploratory objective of this study was to investigate how variations in copy number or mutational status of the *EGFR* gene affect tumor response in advanced NSCLC patients receiving vandetanib treatment. Tumor biopsy samples were obtained from consenting patients, formalin-fixed, and embedded in paraffin. Gene copy number was investigated by fluorescence in situ hybridization using the LSI EGFR SpectrumOrange/CEP 7 SpectrumGreen probe (Vysis, Abbott Laboratories, IL) according to a previously published method.[15] Tumor samples had a high *EGFR* gene copy number if there was high gene polysomy (≥4 *EGFR* gene copies in ≥40% of tumor cells) or gene amplification (presence of tight *EGFR* gene clusters, an *EGFR* gene to chromosome 7 ratio of ≥2, or ≥15 copies of the *EGFR* gene per tumor cell in ≥10% of analyzed cells).

EGFR mutations were analyzed by DNA sequencing of exons 19–21, and additionally by using the amplification refractory mutation system (ARMS) assay to detect the exon 21 L858R point mutation and the most common exon 19 deletion (del G2235–A2249).[16]

Plasma Biomarkers

Plasma samples were collected from patients at baseline, day 29, and day 57, and stored at −70°C. The concentrations of the following angiogenic markers were determined by colorimetric Sandwich ELISA (R&D Systems, Minneapolis, USA): VEGF (Cat. #DVE00), the soluble angiopoietin receptor Tie-2 (Cat. #DTE200), and VEGFR-2 (Cat. #DVR200).

RESULTS

Patient Characteristics

Fifty-three patients were recruited from eight centers in Japan between December 27, 2004, and September 30, 2005. All were randomized on this study and received study drug. Patient characteristics and baseline demographics were generally similar in the three arms, and the patient populations were considered to be appropriate for the dose-finding objectives of this study (Table 1). At the time of data cut-off (23 January 2006), 11 patients were ongoing; PD was the most common reason for discontinuation (n = 35). Other reasons for discontinuation were AEs (n = 6) and withdrawal of consent (n = 1).

Efficacy

The overall ORR was 13.2% (95% CI: 5.5–25.3%) (7 of 53 patients), and all 7 responders were PRs (Table 2). According to vandetanib dose received, the ORRs were 17.6% (95% CI: 3.8–43.4%) (3 of 17 patients; 100 mg), 5.6% (95% CI: 0.1–27.3%) (1 of 18 patients; 200 mg), and 16.7% (95% CI:3.6–41.4%) (3 of 18 patients; 300 mg). In all cases, the response evaluation committee assessment of tumor responses was similar to the investigator assessments. The characteristics of those patients who achieved a PR are described in Table 3. Secondary efficacy assessments are presented in Table 2 and Figure 2.

Safety

Overall, the most common AEs were rash, diarrhea, hypertension, and QTc prolongation (Table 4). In general, no major differences were observed in the incidences of

TABLE 1. Patient Demographic and Baseline Characteristics (Full Analysis Set)

	Vandetanib 100 mg/d (n = 17)	Vandetanib 200 mg/d (n = 18)	Vandetanib 300 mg/d (n = 18)	Total (n = 53)
Median age, yr (range)	58 (30–78)	61 (43–77)	61 (44–77)	60 (30–78)
Male (%)	11 (64.7)	12 (66.7)	11 (61.1)	34 (64.2)
Female (%)	6 (35.3)	6 (33.3)	7 (38.9)	19 (35.8)
Smoking history[a]				
No (%)	5 (29.4)	8 (44.4)	7 (38.9)	20 (37.7)
Yes (%)	12 (70.6)	10 (55.6)	11 (61.1)	33 (62.3)
WHO performance status 0/1/2	5/12/0	7/11/0	6/12/0	18/35/0
Previous chemotherapy				
One regimen (%)	13 (76.5)	9 (50.0)	14 (77.8)	36 (67.9)
Two regimens (%)	4 (23.5)	9 (50.0)	4 (22.2)	17 (32.1)
Staging (%)				
IIIB	2 (11.8)	3 (16.7)	1 (5.6)	6 (11.3)
IV	14 (82.4)	12 (66.7)	15 (83.3)	41 (77.4)
Recurrent	1 (5.9)	3 (16.7)	2 (11.1)	6 (11.3)
Histology (%)				
Squamous	5 (29.4)	6 (33.3)	4 (22.2)	15 (28.3)
Adenocarcinoma	11 (64.7)	12 (66.7)	12 (66.7)	35 (66.0)
Other	1 (5.9)	0	2 (11.1)	3 (5.7)
Brain metastasis at study entry (%)	4 (23.5)	3 (16.7)	5 (27.8)	12 (23.6)

[a] No, patients who have smoked <100 cigarettes in their lifetime; Yes, patients who have smoked >100 cigarettes in their lifetime.

TABLE 2. Efficacy Summary

	Vandetanib 100 mg/d (n = 17)	Vandetanib 200 mg/d (n = 18)	Vandetanib 300 mg/d (n = 18)
Primary efficacy assessment			
Best response (RECIST)			
Partial response, n (%)	3 (17.6)	1 (5.6)	3 (16.7)
Stable disease ≥8 wk, n (%)	5 (29.4)	6 (33.3)	8 (44.4)
Disease progression, n (%)	9 (52.9)	10 (55.6)	7 (38.9)
Not evaluable, n (%)	0	1 (5.6)	0
Secondary efficacy assessments			
Disease control ≥8 wk, n (%)	8 (47.1)	7 (38.9)	11 (61.1)
Duration of response (wk)			
Median (range)[a,b]	na	na	15.9 (7.3–20.1)
Time to progression (wk)			
Median (range)[a]	8.3 (4.0–40.7)	12.3 (0–40.3)	12.3 (1.4–32.7)
No. of events	12	13	13

na, not applicable; RECIST, Response Evaluation Criteria in Solid Tumors.
[a] Median estimated using the Kaplan–Meier method.
[b] This parameter could not be estimated in the 100 and 200 mg/d arms owing to the lack of progressions by the date of data cut-off.

TABLE 3. Characteristics of Patients Who Were Partial Responders

Treatment (initial dose)	Gender	Age (yr)	Smoking History[a]	Histology	Previous Chemotherapy Regimens	Time to PR (d)	Duration of Response (d)
100 mg	Male	65	Yes	Adenocarcinoma	1	28	204[b]
100 mg	Female	72	No	Adenocarcinoma	1	78	141[b]
100 mg	Male	52	No	Adenocarcinoma	1	143	141[b]
200 mg	Female	69	No	Adenocarcinoma	1	26	140[b]
300 mg[c]	Male	69	Yes	Adenocarcinoma	2	31	51
300 mg	Female	68	No	Adenocarcinoma	1	28	81[b]
300 mg	Female	55	No	Adenocarcinoma	1	82	141

[a] No, patients who have smoked <100 cigarettes in their lifetime; Yes, patients who have smoked >100 cigarettes in their lifetime.
[b] Censored on the day of last tumor evaluation due to absence of disease progression (response ongoing at data cut-off).
[c] Patient started study treatment with 300 mg and the treatment was stopped 29 d after the start due to QTc prolongation. The patient re-started at a reduced dose level (200 mg) 35 d after the start.

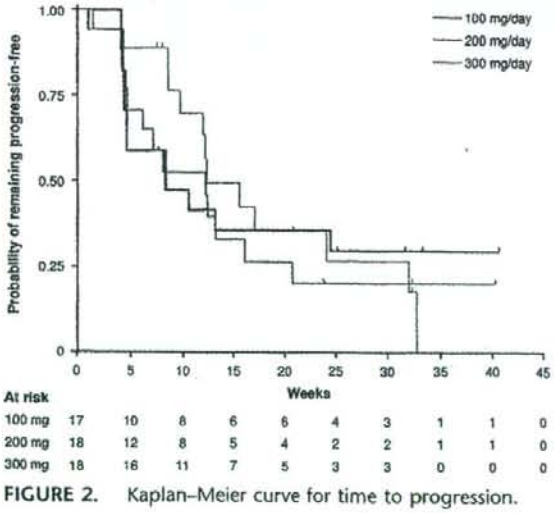

FIGURE 2. Kaplan–Meier curve for time to progression.

the common AEs across the three vandetanib arms, although the incidences of diarrhea, constipation, and abnormal hepatic function were numerically higher in the vandetanib 300 mg arm compared with the 100 or 200 mg arms. A dose-dependent increase in the incidence of CTC grade 3 and 4 events was observed; the incidence of these events in the 100, 200, and 300 mg dose arms were 29.4% (5 of 17 patients), 38.9% (7 of 18 patients), and 66.7% (12 of 18 patients), respectively. Of the 24 CTC grade 3 or 4 AEs considered by the investigator to be vandetanib-related, hypertension (100 mg, n = 4; 200 mg, n = 3; 300 mg, n = 3), and asymptomatic QTc prolongation (200 mg, n = 1; 300 mg, n = 1) were reported in more than one patient. Across the three dose levels, the AEs in this study were generally manageable with symptomatic treatment, dose interruption, or reduction.

Six patients discontinued vandetanib because of an AE considered by the investigator to be vandetanib-related: cryptogenic organizing pneumonia (COP), hepatic steatosis, and photosensitivity reaction (each n = 1, 200 mg arm); QTc prolon-

TABLE 4. Number of Patients With Most Commonly Reported Adverse Events (Occurring in ≥10% Across all Treatment Groups), Regardless of Causality

MedDRA Preferred Term[a]	Vandetanib 100 mg/d (n = 17)	Vandetanib 200 mg/d (n = 18)	Vandetanib 300 mg/d (n = 18)	Total (n = 53)
Rash (%)	10 (59)	9 (50)	9 (50)	28 (53)
CTC grade 3/4	0/0	1/0	0/0	1/0
Diarrhea (%)	8 (47.1)	8 (44)	11 (61)	27 (51)
CTC grade 3/4	0/0	1/0	1/0	2/0
Hypertension (%)	8 (47)	10 (56)	7 (39)	25 (47)
CTC grade 3/4	4/0	3/0	3/0	10/0
ECG QTc prolonged (%)	4 (24)	9 (50)	8 (44)	21 (40)
CTC grade 3/4	0/0	1/0	1/0	2/0
Photosensitivity reaction (%)	2 (12)	5 (28)	5 (28)	12 (23)
CTC grade 3/4	0/0	0/0	0/0	0/0
Nasopharyngitis (%)	3 (18)	4 (22)	4 (22)	11 (21)
CTC grade 3/4	0/0	0/0	0/0	0/0
Dry skin (%)	2 (12)	4 (22)	5 (28)	11 (21)
CTC grade 3/4	0/0	0/0	0/0	0/0
Nausea (%)	3 (18)	3 (17)	4 (22)	10 (19)
CTC grade 3/4	0/0	0/0	0/0	0/0
Constipation (%)	2 (12)	1 (6)	6 (33)	9 (17)
CTC grade 3/4	0/0	0/0	0/0	0/0
Fatigue (%)	4 (24)	1 (6)	2 (11)	7 (13)
CTC grade 3/4	0/0	0/0	0/0	0/0
ECG QT prolonged (%)	1 (6)	2 (11)	4 (22)	7 (13)
CTC grade 3/4	0/0	0/0	0/0	0/0
Hepatic function abnormal (%)	1 (6)	1 (6)	4 (22)	6 (11)
CTC grade 3/4	0/0	0/0	1/0	1/0
Hematuria (%)	2 (12)	2 (12)	2 (12)	6 (11)
CTC grade 3/4	0/0	0/0	0/0	0/0

[a] MedDRA version 8.1.

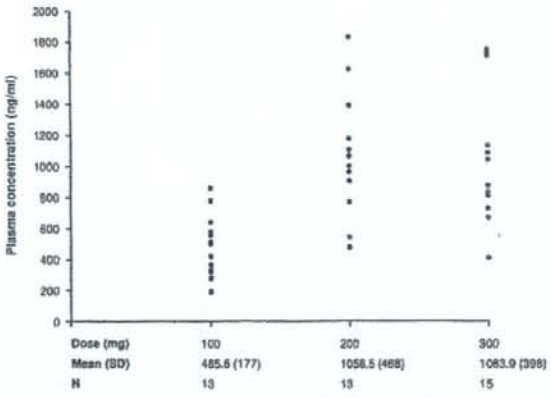

FIGURE 3. Observed maximum vandetanib plasma concentration at day 28. Patients who received dose reduction within the first 28 days were excluded.

gation, alanine aminotransferase increased, and erythema multiforme (each n = 1, 300 mg arm). Only COP was classed as a serious AE. Six patients had vandetanib dose reductions due to AEs (100 mg, n = 1; 200 mg, n = 1; 300 mg, n = 4).

Seven patients experienced eight respiratory-related events (COP, dyspnoea, interstitial lung disease [ILD], hypoxia, pneumonitis [all n = 1], and pneumonia [n = 3]). The incidence of these events in the three dose levels was 5.9% (1 of 17 patients; 100 mg), 11.1% (2 of 18 patients; 200 mg) and 22.2% (4 of 18 patients; 300 mg), respectively. Four of these events were considered to be related to vandetanib (COP, ILD, pneumonia [n = 2]). The ILD event was reported in a 64-year-old male patient in the 300 mg arm and resulted in patient death. This event was reported 8 days after vandetanib 300 mg was discontinued because of disease progression. No postmortem examination was performed and the investigator and a third-party physician considered the cause of death to be ILD.

All QTc prolongation was asymptomatic and manageable with dose interruption and/or reduction. The incidence of QTc prolongation was lower in the vandetanib 100 mg (24%) arm compared with the 200 mg (50%) and 300 mg (44%) arms. The mean change in QTc interval from baseline to week 3 (when maximum prolongation was observed) in the 100, 200, and 300 mg arms was +14 milliseconds (range, −25 to 29 milliseconds), +16.5 milliseconds (range, −36 to 49 milliseconds), and +27.6 milliseconds (range, 4 to 51 milliseconds), respectively. Protocol-defined QTc prolongation determined at the treatment site resulted in dose reduc-

come. In contrast, plasma levels of VEGFR-2 showed a trend to decrease over the same period, whereas plasma Tie-2 levels did not seem to change (Table 6). Baseline plasma VEGF levels appeared to be lower in patients who experienced clinical benefit following vandetanib treatment: PR (median 22.3 pg/ml, $n = 6$) and SD (median 37.0 pg/ml, $n = 16$) versus PD (median 63.7 pg/ml, $n = 21$). Patients with a low (below median) baseline plasma VEGF level had a longer TTP (median, 24.1 week) than those with a high (above median) baseline VEGF level (median, 8.3 weeks) (Figure 4). No clear relationship was apparent between baseline levels of plasma Tie-2 and VEGFR-2 and tumor response.

DISCUSSION

The primary objective of this phase IIa study was to assess the ORR to three doses of vandetanib (100, 200, and 300 mg/d) in Japanese patients with advanced or recurrent NSCLC. These doses of vandetanib were selected based on the outcomes of a Japanese phase I study where it was observed that vandetanib was well tolerated up to a dose of 300 mg and objective tumor responses were observed in 4 of 9 patients with NSCLC at doses of either 200 or 300 mg.[11]

In this study, objective tumor responses were observed at all three doses of vandetanib. The ORR in the 100, 200, and 300 mg arms was 17.6% (3 of 17 patients), 5.6% (1 of 18 patients), and 16.7% (3 of 18 patients), respectively. The DCR and TTP were similar across the three dose arms. It was noted that 50% (9 of 18) of the patients in the 200 mg arm had failed two previous chemotherapy regimens, compared with 23.5% (4 of 17 patients) and 22.2% (4 of 18 patients) in the 100 and 300 mg arms, respectively. It is possible that these differences contributed to the lower ORR observed in the 200 mg arm, although the number of patients in each dose arm was too small to allow any definitive conclusions to be made.

Vandetanib was well tolerated at 100, 200, and 300 mg dose levels in this study. Overall, AEs were generally mild and manageable with symptomatic treatment, dose interruption or reduction. In addition, the AE profile was consistent with that determined during phase I evaluation in patients with advanced solid tumors[10,11] and phase II monotherapy data in NSCLC.[12] Furthermore, the AE profile was also consistent with that reported previously for agents that inhibit the VEGFR[17,18] or EGFR[4,19] signaling pathways. In general, no apparent dose dependence was noted in the incidence of the common AEs in this study except for asymptomatic QTc prolongation (24%, 56%, and 44% for the 100, 200, and 300 mg dose arms, respectively), an event that was manageable by dose interruption/reduction.

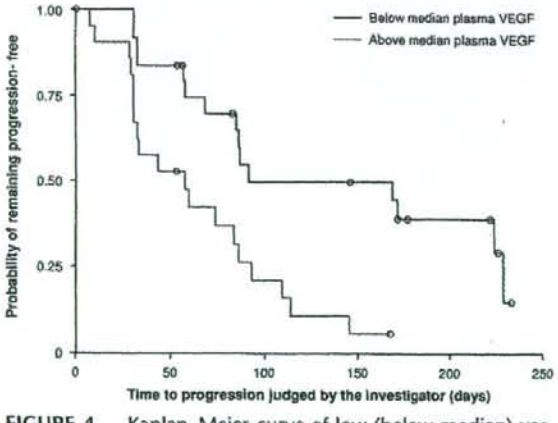

FIGURE 4. Kaplan–Meier curve of low (below median) versus high (above median) baseline plasma VEGF and time to progression.

A notable feature of this study, and the phase II program for vandetanib in NSCLC, is that patients with squamous cell histology or stable brain metastases were permitted to enter the trials. Both of these factors have been associated with an increased risk of bleeding, including severe life-threatening hemoptysis in NSCLC patients with squamous histology in a randomized phase II study of bevacizumab with carboplatin and paclitaxel.[20] These events have also been reported with other inhibitors of VEGF/VEGFR signaling, such as sunitinib and sorafenib.[17,18] Importantly, no CNS hemorrhage AEs or hemoptysis attributable to vandetanib were reported in this study.

The PK profile in this NSCLC patient population was consistent with that seen previously during Phase I evaluation in Japanese and USA/Australian patients with a range of solid tumors.[10,11]

In patients with NSCLC, specific *EGFR* mutations are associated with increased sensitivity to EGFR tyrosine kinase inhibitors,[21,22] and a better survival outcome with gefitinib has been shown to correlate with high *EGFR* gene copy number.[23] In this study, an exploratory analysis of tumor samples for amplification of *EGFR* gene copy number and somatic mutations of the *EGFR* gene revealed no clear relationship between *EGFR* mutation or gene amplification status and clinical outcome in patients receiving vandetanib. The *EGFR* mutation frequency of 4% (1 of 27 patients) is lower than that previously reported,[24,25] and further studies are needed to evaluate *EGFR* mutation status as a possible predictive marker for vandetanib therapy in advanced NSCLC.

In addition to *EGFR* mutation/amplification status, plasma profiling of cytokines and angiogenic factors may be a feasible approach for identifying blood-based prognostic and activity markers for therapies in NSCLC. Preliminary analysis of plasma concentrations of the angiogenesis markers VEGF and VEGFR-2 in the present study revealed that patients with PR or SD were more likely to have low baseline levels of VEGF than those with PD. It has been shown previously that low pretreatment levels of circulating VEGF correlated with a good response to gefitinib treatment in patients with NSCLC.[26] The significance of the relationship between these biomarkers and clinical outcome requires further investigation.

In conclusion, vandetanib monotherapy (100–300 mg/d) demonstrated antitumor activity with an acceptable safety and tolerability profile in Japanese patients with advanced NSCLC. Based only on this study, there is no com-

pelling evidence to identify the optimal dose of vandetanib monotherapy in this population of patients; further investigation of vandetanib doses in the range 100 to 300 mg is warranted in Japanese patients with advanced NSCLC. Other randomized phase II studies of vandetanib in advanced NSCLC have demonstrated improvements in progression-free survival with vandetanib 300 mg as a monotherapy versus gefitinib[12] and with the combination of vandetanib 100 mg and docetaxel.[14] Phase III evaluation of vandetanib in a broad population of patients, both as monotherapy at 300 mg (versus placebo in patients previously treated with anti-EGFR therapy [ZEPHYR]; versus erlotinib [ZEST]) and at 100 mg in combination with docetaxel (ZODIAC) or pemetrexed (ZEAL), has been initiated in global trials.

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Mutations in the LKB1 tumour suppressor are frequently detected in tumours from Caucasian but not Asian lung cancer patients

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Somatic mutations of *LKB1* tumour suppressor gene have been detected in human cancers including non-small cell lung cancer (NSCLC). The relationship between *LKB1* mutations and clinicopathological characteristics and other common oncogene mutations in NSCLC is inadequately described. In this study we evaluated tumour specimens from 310 patients with NSCLC including those with adenocarcinoma, adenosquamous carcinoma, and squamous cell carcinoma histologies. Tumours were obtained from patients of US ($n=143$) and Korean ($n=167$) origin and screened for *LKB1*, *KRAS*, *BRAF*, and *EGFR* mutations using RT—PCR-based SURVEYOR-WAVE method followed by Sanger sequencing. We detected mutations in the *LKB1* gene in 34 tumours (11%). *LKB1* mutation frequency was higher in NSCLC tumours of US origin (17%) compared with 5% in NSCLCs of Korean origin ($P=0.001$). They tended to occur more commonly in adenocarcinomas (13%) than in squamous cell carcinomas (5%) ($P=0.066$). *LKB1* mutations associated with smoking history ($P=0.007$) and *KRAS* mutations ($P=0.042$) were almost mutually exclusive with *EGFR* mutations ($P=0.002$). The outcome of stages I and II NSCLC patients treated with surgery alone did not significantly differ based on *LKB1* mutation status. Our study provides clinical and molecular characteristics of NSCLC, which harbour *LKB1* mutations.
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Clinical Studies

Peutz–Jeghers syndrome (PJS) is caused by mutations in the *LKB1* tumour suppressor gene (Hemminki *et al*, 1998). LKB1 is serine–threonine kinase, which has been shown to regulate cell cycle progression, apoptosis, and cell polarity (Tiainen *et al*, 1999). The major target of LKB1 kinase activity is thought to be AMP-activated protein kinase (AMPK). AMPK is activated under low cellular energy conditions by raising AMP levels and it phosphorylates multiple downstream targets including tuberosclerosis complex 2 gene, which represses mTOR signalling. Phosphorylation of AMPK by LKB1 is needed for full activity of AMPK and suppression of mTOR activity under low energy conditions (Shaw *et al*, 2004). The hallmarks of PJS include mucocutaneous pigmentation and hamartomatous polyps of the gastrointestinal tract. Patients with PJS have an increased risk of developing gastrointestinal, pancreatic, breast, gynecological, and non-small cell lung cancers (NSCLC). The overall risk for cancers is increased 5- to 12-fold in different age groups compared with the general population (Hearle *et al*, 2006). Somatic mutations of the *LKB1* tumour suppressor have rarely been found in cancers from patients who do not have PJS except for NSCLC (Avizienyte *et al*, 1999). Previous reports have suggested the *LKB1* mutation rate to be as high as 30% in NSCLC tumours and cell lines derived from patients of Caucasian origin (Carretero *et al*, 2004; Matsumoto *et al*, 2007) and to be infrequent in NSCLC patients of Asian origin (3%) (Onozato *et al*, 2007). Furthermore, *LKB1* mutations have been shown to be associated with adenocarcinoma histology, male gender, and smoking history (Matsumoto *et al*, 2007). A recent report of using a mouse model for *lkb1* inactivation in NSCLC has provided insights into the role of the gene in this cancer. This study showed that *lkb1* inactivation in combination with activating mutations of *kras* using inducible promoters in the lung was associated with decreased survival compared with *kras* mutation alone (Ji *et al*, 2007).

Current screening techniques for *LKB1* tumour suppressor mutations rely on conventional exonic sequencing of the DNA, which can identify single base pair changes and small deletions/insertions (Ballhausen and Gunther, 2003). The addition of multiple ligation-dependent probe amplification (MLPA), which enables detection of exonic and whole gene deletions, with exonic sequencing has increased the mutation detection rates to 80% in patients with PJS phenotype (Volikos *et al*, 2006). Conventional

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sequencing has also been used to detect mutations of *LKB1* at mRNA level and some mutations missed by sequencing at the DNA level have been discovered by mRNA-based approaches (Abed *et al*, 2001). However, mutant forms of *LKB1* mRNAs can have a shortened half-life because of nonsense-mediated decay, which can potentially interfere with mutation detection (Abed *et al*, 2001).

We have recently described a rapid and sensitive enzymatic method to detect mutations in epidermal growth factor (*EGFR*) of DNA from fresh tissue and paraffin-embedded tissues (Janne *et al*, 2006). This method includes amplification of region of interest with PCR, SURVEYOR endonuclease digestion of the products, which cleaves mismatched heteroduplex DNAs, and detection of DNA fragments by sensitive high-performance liquid chromatography (HPLC) WAVE HS system. Subsequently, SURVEYOR-positive specimens are fractionated in partially denaturing conditions and are Sanger-sequenced. The major advantages of SURVEYOR-WAVE method are the fast exclusion of wild-type specimens without laborious conventional sequencing and high sensitivity. The SURVEYOR-WAVE method is more sensitive than conventional sequencing as it can detect mutant DNA sequences when they are present in 1% or more of total DNA (Janne *et al*, 2006).

The current study was designed to analyse the incidence of *LKB1* mutations in NSCLC. Furthermore, we wanted to investigate the *LKB1* mutational frequency in different histologies and ethnic backgrounds, and assess their correlation to smoking history, gender, stage, survival, and other oncogenic mutations in NSCLC.

MATERIALS AND METHODS

Cell lines and tumour specimens

The NSCLC cell lines A549, NCI-H1395, NCI-H1650, NCI-H1666, NCI-H1781, NCI-1975, NCI-H23, NCI-H2126, NCI-H441, NCI-H820, HCC2935, HCC4006, and HCC827 were purchased from ATCC (Manassas, VA, USA). H3255, H3255GR, HCC2279, and PC-9 have been previously described (Ono *et al*, 2004; Tracy *et al*, 2004; Engelman *et al*, 2006). Ma1, and Ma70 are NSCLC cell lines harbouring *EGFR* mutations that were established at the Kinki University, Osaka, Japan. A549, NCI-H1395, NCI-H1666, NCI-H23, NCI-H2122, NCI-H2126, and NCI-H460 have previously been reported to contain *LKB1* mutations (Sanchez-Cespedes *et al*, 2002; Bamford *et al*, 2004; Carretero *et al*, 2004).

NSCLC tumours ($n=310$) were collected from surgical resections from patients with stages I–IV NSCLC when sufficient material for RNA extraction was available. The majority of the specimens ($n=167$) was collected at the Samsung Medical Center, Seoul, Korea. Frozen tumour tissues were collected from 809 out of 2442 patients who underwent curative resection for NSCLC from November 1995 to February 2007 at Samsung Medical Center. One or two pieces from the periphery of the tumour masses – avoiding necrotic regions – were immediately frozen at −80°C until retrieved. Medical records and haematoxylin and eosin-stained slides of the specimen were reviewed by a single pathologist. Only frozen tumour tissues from adenocarcinoma or squamous cell carcinoma (according to the 2004 World Health Organization histopathological criteria) were included. Only frozen tumour tissues with a tumour cell content of more than 70% were used for further analysis. In addition, frozen tumour tissues of the following patients were excluded from the study: patients who had received preoperative neoadjuvant treatments, patients with double primary lung cancer, and patients who had undergone incomplete resections or who had not been subjected to mediastinal lymph node dissections. Selected frozen tumour tissues were used for the microdissection. Briefly, frozen tissues were lightly stained with haematoxylin–eosin to improve visualisation, and necrotic tumour tissues and intervening normal tissues were removed. Each of the microdissected tumour tissues with a tumour cell content of more than 90% was placed in 1 ml Easy Blue reagent of a commercially available RNA isolation kit (easy-spin™ Total RNA Extraction Kit, iNtRON Biotechnology, Gyeonggi-do, Korea), immediately homogenised by vortexing, and the total RNA was extracted. The quantity and quality of RNA were analysed using a spectrometer (Nanodrop Technologies, Rockland, DE, USA) and Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit, Agilent Technologies Inc., Böblingen, Germany), respectively. Finally, 167 frozen tissues with acceptable quality of RNA (RNA Integrity Number (RIN) value over 7.0) were used for the current studies. All patients provided written informed consent.

The tumours from Caucasian patients ($n=143$) were collected at the Brigham and Women's Hospital, Boston, MA, USA between 1991 and 1997 and have been previously published for patient characteristics and histology, and for expression profile-based clustering of the tumours (Bhattacharjee *et al*, 2001; Hayes *et al*, 2006). Frozen samples of resected lung tumours were obtained within 30 min of resection and subdivided into 100 mg samples and snap frozen at −80°C. Each specimen was associated with an immediately adjacent sample embedded for histology in an optimal cutting temperature medium and stored at −80°C. Six micrometres of frozen sections of embedded samples stained with haematoxylin and eosin were used to confirm the postoperative pathological diagnosis and to estimate the cellular composition of adjacent samples. All specimens underwent pathological review by two pathologists. In all 109 tumours obtained during the same time period were excluded because they did not meet one or more of the eligibility criteria. Tissue samples were homogenised in Trizol (Life Technologies, Gaithersburg, MD, USA) and RNA was extracted and purified by using the RNeasy column purification kit (Qiagen, Chatsworth, CA, USA). Denaturing formaldehyde gel electrophoresis followed by northern blotting using a β-actin probe assessed RNA integrity. Samples were excluded if β-actin was not full length. All patients provided written informed consent. The US cohort included specimens that have previously undergone analyses and the results have been published for *EGFR*, *KRAS*, and *BRAF* mutations (Bhattacharjee *et al*, 2001; Naoki *et al*, 2002; Hayes *et al*, 2006). We reconfirmed the mutations in 30 of these specimens using the SURVEYOR-based analysis (see section SURVEYOR digestion and HPLC analysis) and found 100% concordance between the two methods (data not shown).

Cell line specimens were snap frozen and stored at −80°C. RNA was extracted from tumours and cell lines using Trizol (Invitrogen, Carlsbad, CA, USA), purified with Rneasy Mini Kit (Qiagen, Valencia, CA, USA) and was used for cDNA synthesis using the QuantiTect reverse transcription kit (Qiagen, Valencia, CA, USA).

PCR primers and cycling conditions

For *LKB1* gene analysis, PCR primers were designed to amplify the cDNA in two amplicons. PCR primers of the first amplicon were designed to hybridise to the noncoding area of the mRNA upstream of exon 1 (5′-agggaagtcggaacacaagg-3′) and to exon 5 (5′-ccagatgtccaccttgaagc-3′) generating a PCR product of 797 bp. The primers for the second amplicon located at exon 5 (5′-aacggcc tggacaccttct-3′) and to noncoding exon 10 (5′-gaaccggcaggaagact gag-3′) generating a product of 702 bp, which has an overlapping part with first amplicon. For SURVEYOR-WAVE analysis of *KRAS*, PCR primers (5′-ggcctgctgaaaatgactga-3′, 5′-tcctgagcctgttttgtgtct-3′) were designed to generate an amplicon of 407 bp covering codons 12, 13 and 61, which are the codons commonly mutated in lung cancers. For SURVEYOR-WAVE mutation analysis of *BRAF*, cDNA was amplified in two overlapping amplicons (5′-aggattt cgtggtgatggag-3′, 5′-gatgacttctggtgccatcc-3′, and 5′-gacgggactcgagt gatgat-3′, 5′-ggtatcctcgtcccaccata-3′) covering codons 387–673. For SURVEYOR-WAVE analysis of *EGFR*, PCR amplification was done in a single amplicon (5′-ggagcctcttacacccagtg-3′,

5′-aggtcatcaactcccaaacg-3′), which covered exons 18–21 of the gene. PCR amplification was done using JumpStart Taq (Sigma, St Louis, MO, USA) under the manufacturer's guidelines. A part of the specimens ($n=103$) was previously characterised for *KRAS*, *BRAF*, *EGFR* mutations using reverse transcritase (RT)–PCR and direct sequencing of the PCR products (Naoki *et al*, 2002; Hayes *et al*, 2006).

SURVEYOR digestion and HPLC analysis

SURVEYOR digestion and HPLC analysis were carried out as described previously (Janne *et al*, 2006). In brief, PCR products were digested in reaction mixture containing equal volumes of SURVEYOR enzyme (Transgenomics, Omaha, NE, USA) and Enhancer (Transgenomics, Omaha, NE, USA) at 42°C for 20 min followed by termination of the reaction by Stop Solution (Transgenomics, Omaha, NE, USA). Specimens were then loaded to the WAVE HS HPLC (Transgenomics, Omaha, NE, USA) at 50°C, eluted with an increasing acetonitrile gradient, and detected by UV detector using DNA intercalating fluorescence dye (Transgenomics, Omaha, NE, USA). When cell lines known to be homozygous for specific mutation were analysed, PCR products were mixed 1:1 with PCR products of a wild-type cell line, denatured by heating, and slowly renatured to generate heteroduplexes.

Sequencing and fractionation

Specimens that showed an altered pattern on the SURVEYOR tracings were purified using QIAquick kit (Qiagen, Valencia, CA, USA) and sequenced bi-directionally by molecular biology core facility of Dana–Farber Cancer Institute. If a specimen showed an altered pattern on the SURVEYOR tracing but had a wild-type sequence by direct DNA sequencing, it underwent fractionation by WAVE HS HPLC in partially denaturing conditions. Running temperatures for specific amplicons were calculated by the Navigator Software (Transgenomics, Omaha, NE, USA). Collected fragments were amplified with PCR using the same primers as in the original amplification, purified and sequenced as previously described above.

Statistical analysis

Fisher's exact test was used to assess the association of *LKB1* mutation status with other clinical, pathological, and genetic characteristics. To adjust for any difference between ethnic groups, the association between *LKB1* mutation rate and each characteristic was also evaluated as stratified contingency tables. If we did not reject that the odds ratios were the same across ethnic groups, we then tested whether the common odds ratios were unity based on the stratified Mantel–Haenszel estimate (Breslow and Day, 1980). Overall survival was estimated using the Kaplan–Meier method, with differences between the groups compared using the log-rank test. All *P*-values were based on a two-sided hypothesis, with $P<0.05$ considered to be statistically significant and $0.05<P<0.10$ considered to be borderline significant.

RESULTS

SURVEYOR-WAVE mutation detection of *LKB1* tumour suppressor in NSCLC cell lines

The impact of the stability of *LKB1* mRNA on detecting *LKB1* mutations was tested using RT–PCR with mRNA extracted from NSCLC cell lines that had previously been characterised for *LKB1* mutations. These included NCI-H441 (wild type) and A549, NCI-H1395, NCI-H23, and NCI-H2126 (all containing *LKB1* mutations). Reverse transcriptase–PCR amplification of the whole coding region of the *LKB1* mRNA showed that cell lines with nonsense (A549, NCI-H23) mutations or 1 bp deletion (H1395) expressed mRNA with comparable size to the wild-type H441 cell line (1460 bp). H2126 cell line, which is known to have homozygous deletion of exons 4–6, expressed mRNA with substantially smaller size (~1000 bp) corresponding to deletion of 398 bp. RT–PCR revealed no major difference in *LKB1* mRNA expression levels between *LKB1* mutant or wild-type cell lines (Figure 1A).

As *LKB1* mutant and wild-type cell lines expressed comparable amounts of *LKB1* mRNA with RT–PCR, we studied the cDNA for mutations using the SURVEYOR-WAVE method. The WAVE HPLC provides a system to analyse DNA fragments smaller than 900 bp and therefore we designed two overlapping amplicons covering exons 1–5 (797 bp) and 5–9 (702 bp) to amplify the whole coding region of *LKB1* mRNA. PCR products of *LKB1* mutant cell lines were mixed 1:1 with the products from wild-type cell lines (H441) to generate heteroduplexes as *LKB1* mutant cell lines were previously reported to be homozygous for the inactivation of the gene. SURVEYOR-WAVE analysis of the amplicon covering exons 1–5 revealed novel peaks with the cDNA for A549, and NCI-H1395 cell lines compared with the wild type from NCI-H441 (Figure 1B). SURVEYOR-WAVE analysis of exons 5–9 showed novel peaks for the NCI-H23 cell line as well. The mutations detected with SURVEOR-WAVE were confirmed by conventional DNA sequencing and they corresponded to previous reports (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004). We could not detect the *LKB1* mutation of H2126 cell line with SURVEYOR-WAVE method using a two-amplicon approach because this cell line has a homozygous deletion of exons 4–6 and the reverse primer of the first amplicon and the forward primer of the second amplicon, which lie on the deleted part of the gene (data not shown).

LKB1 tumour suppressor gene mutations in NSCLC tumours

We next used the SURVEYOR-WAVE method to screen NSCLC tumour specimens ($n=310$) for *LKB1* mutations. We detected 34 *LKB1* mutations (11%) in the NSCLC tumour specimens (Table 1). The majority of the *LKB1* mutations detected was deletions or insertions ($n=25$, 74%). The remainder was missense ($n=7$, 21%) and nonsense ($n=2$, 6%) mutations (Table 2, Figure 1C). About one-half of the deletions and insertions were small, covering <15 bp ($n=14$, 56%), whereas larger deletions ($n=11$, 44%) covering hundreds of base pairs were detected in the remaining specimens. Some mutational hotspots were discovered. The areas that had the same mutation in more than one tumour specimen included deletion of exon 4 ($n=4$), deletion of exons 2 and 3 ($n=3$), D194Y ($n=2$), and P281L ($n=2$). Interestingly, a significant portion of the mutations was located in exon 1 ($n=11$, 32%) but there was no area of recurrent mutations in this exon (Table 2). Of the missense mutations detected in the current study, all except R426W are in the kinase domain of the protein. Missense mutations in codons 176 and 194 have been previously characterised in PJS (Launonen, 2005). We also found four F354L alterations (data not shown) but we did not consider these as missense mutations as this alteration has previously been reported to be a rare polymorphism of the gene (Launonen *et al*, 2000). We did not have access to the corresponding normal tissues and therefore, we could not verify if some of the missense mutations were somatic or germline.

Association of *LKB1* tumour suppressor mutations in NSCLC with clinicopathological characteristics

The mutation frequency of *LKB1* gene was significantly higher in NSCLCs in the Caucasian cohort (Table 1). Twenty-five (17% of specimens) of the *LKB1* mutations were detected in NSCLCs

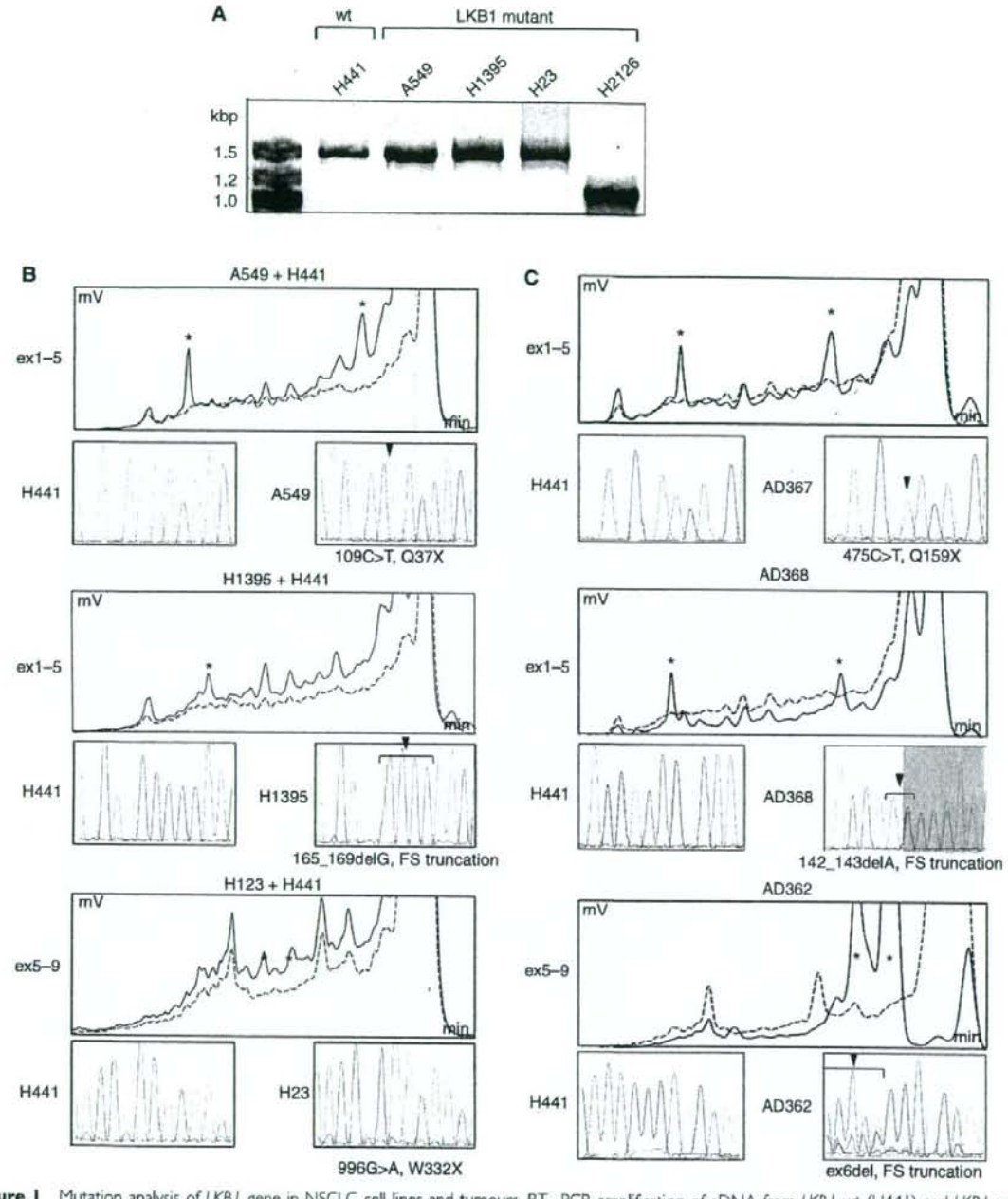

Figure 1 Mutation analysis of *LKB1* gene in NSCLC cell lines and tumours. RT–PCR amplification of cDNA from *LKB1* wt (H441) and *LKB1* mutant (A549, H1395, and H23) cell lines display the full length LKB1 mRNA (1.4 kbp) while the *LKB1* mutant cell line, H2126 with a deletion of exons 4–6 expresses a shorter mRNA (1.0 kbp) (**A**). HPLC tracings of SURVEYOR-WAVE mutation analysis of NSCLC cell lines A549, H1395, or H23 (continuous line), and H441 (dashed line). Time in minutes is shown on the *X*-axis, voltage in mV on the *Y*-axis (**B**). A549 and H1395 show novel peaks (*) in the amplicon covering exons 1–5 (ex1–5) corresponding to 109C>T, Q37X and 165_169delG, frameshift and truncation (FS truncation) mutations. The analysis from H23 demonstrates novel peaks in the amplicon covering exons 5–9 (ex5–9) corresponding to 996G>T, W332X mutation. *LKB1* wild-type cDNA (H441) was added to PCR products 1 : 1, denatured by heating and slowly renaturated to generate heteroduplexes since A549, H1395, and H23 have previously reported to be homozygous for the *LKB1* mutations. SURVEYOR-WAVE mutation analyses of NSCLC tumours (**C**). AD367 and AD368 tumours showed novel peaks in the ex1–5 amplicon corresponding to 475C>T, Q159X, and 142_143delA, FS, truncation mutations. AD362 tumour had novel peaks in ex5–9 amplicon corresponding to deletion of exon 6. Mutant sequences for AD367 and AD368 are displayed from sequences using the forward primer while mutation of the AD362 is showed with reverse primer.

Table 1 Frequency of *LKB1* mutations in NSCLC tumours and their association with clinicopathological characteristics

	LKB1 mutation		
	+	–	P-value*
All tumours	34 (11%)	276 (89%)	
Age, median	61.2	62.2	
Ethnicity			
Caucasian cohort	25 (17%)	118 (83%)	0.001
Asian cohort	9 (5%)	158 (95%)	
Gender			
Male	20 (11%)	167 (89%)	NS
Female	14 (12%)	107 (88%)	
Smoking			
Never (<10 py)	2 (3%)	70 (97%)	0.007
Smoker (>10 py)	26 (14%)	161 (86%)	
Tumour stage			
I	19 (10%)	169 (90%)	NS
II	8 (14%)	51 (86%)	
III	5 (11%)	42 (89%)	
IV	1 (12%)	7 (88%)	
Histology			
Adenocarcinoma	27 (13%)	180 (87%)	0.047
Squamous carcinoma	5 (5%)	87 (95%)	
Adenosquamous	2 (22%)	7 (78%)	

*Fisher's exact test, NS = not statistically significant ($P > 0.05$).

collected from patients in the United States, whereas only nine mutations (5% of specimens) were detected in the Korean cohort ($P = 0.001$) (Table 1). The *LKB1* mutation rate tended to be higher in adenocarcinomas (13%) compared with squamous cell carcinomas (5%) ($P = 0.067$). Differences in histological subgroups were relatively modest in the US cohort with mutations in 18 out of 94 (19%) adenocarcinomas *vs* 5 out of 38 (13%) in squamous cell cancers ($P = 0.461$). This is in contrast to the findings in the Asian patients where all of the *LKB1* mutations were detected in adenocarcinomas (9 out of 113 (8%)) and none were detected in squamous cell cancers (0 out of 54 (0%); $P = 0.032$). Nevertheless, the higher rate of *LKB1* mutation in adenocarcinomas compared with squamous cell carcinomas retains the same level of statistical significance (stratified $P = 0.064$) after adjusting for fluctuation between ethnic groups. The US cohort also included nine specimens from adenosquamous carcinomas and two out of nine (22%) had *LKB1* mutations, which is similar to the frequency in adenocarcinomas in this population (Table 1). There was no association between *LKB1* mutations and the clinical stage of the NSCLC patients. Kaplan–Meier survival curves of stages I and II NSCLC patients showed a tendency for shorter survival in patients with *LKB1* mutant tumours but this, however, did not reach statistical significance ($P = 0.17$) (Figure 2). No differences in survival were observed in patients who harboured both *LKB1* and *KRAS* mutations compared with those with *KRAS* or *LKB1* alone but the total number of patients with both mutations who had stages I or II NSCLC was small ($n = 9$; data not shown). We detected an association of *LKB1* mutations with a smoking history ($P = 0.007$) and only two mutations were detected in tumours from 72 NSCLC patients who were either never or light (≤10 pack years) former smokers (Table 1). After adjusting for ethnic group, the higher rate of *LKB1* mutation among patients with a smoking history is borderline significant (stratified $P = 0.067$). The reduction in statistical significance is likely owing to the loss of power associated with the overall rarity of *LKB1* mutations among never or light former smokers. For these analyses we combined both never smokers and light (≤10 pack years) smokers as the frequency of mutations in other oncogenes such as *EGFR* is similar in these two patient groups (Pham *et al*, 2006). There were no correlations between *LKB1* mutations and gender or age of a patient.

Table 2 The specific *LKB1* mutations in NSCLC tumours

Mutation type	No. (%)	Mutation	Amino acid change	Exon	Histology
Missense	7 (21)	*526G>T	D176Y	4	Ad
		*580G>T	D194Y	4	Ad
		580G>T	D194Y	4	Sq
		829G>T	D277Y	6	AdSq
		*842C>T	P281L	6	Ad
		*842C>T	P281L	6	Ad
		1276C>T	R426W	9	Ad
Nonsense	2 (6)	206C>A	S69X	1	Ad
		475C>T	Q159X	4	Ad
Deletion/ insertion	25 (74)	*75_76del2&insT	FS, truncates	1	Ad
		120_130del11	FS, truncates	1	Ad
		125_127insGG	FS, truncates	1	Ad
		128_129delC	FS, truncates	1	Ad
		142_143delA	FS, truncates	1	Ad
		180delC	FS, truncates	1	Ad
		209delA	FS, truncates	1	Ad
		227_228delC	FS, truncates	1	Ad
		47_651del604	FS, truncates	1-5	Sq
		153_536del384	FS, truncates	1-4	AdSq
		*exon 2-3del	Truncates	2-3	Sq
		*exon 2-3del	Truncates	2-3	Ad
		exon 2-3del	Truncates	2-3	Ad
		exon 2-4del	FS, truncates	2-4	Sq
		464_465del2insTTTGCT	FS, truncates	3-4	Sq
		562_563delG	FS, truncates	4	Ad
		*exon 4del	FS, truncates	4	Ad
		exon 4del	FS, truncates	4	Ad
		exon 4del	FS, truncates	4	Ad
		exon 4del	FS, truncates	4	Ad
		610_623del14	FS, truncates	5	Ad
		*837_844delC	FS, truncates	6	Ad
		837_844insC	FS, truncates	6	Ad
		exon 6del	FS, truncates	6	Ad
		1038_1040insG	FS, truncates	8	Ad

Ad = Adenocarcinoma; AdSq = Adenosquamous carcinoma; Sq = Squamous cell carcinoma; . *These mutations were detected in Korean NSCLC patients.

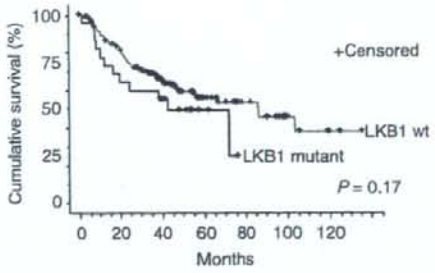

Figure 2 Kaplan–Meyer survival curves of stage I and II NSCLC patients with LKB1 wildtype (red line, $n = 198$) *vs* LKB1 mutant (blue line, $n = 23$) tumours.

Association of *LKB1* mutations with *K-Ras*, *B-Raf*, and *EGFR* mutations in NSCLC

Previous reports have suggested that in NSCLC cell lines, *LKB1* mutations often occur concurrently with *KRAS* or *BRAF* mutations

(Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004). Furthermore, *EGFR* mutations are often mutually exclusive with *KRAS* mutations in NSCLC (Kosaka *et al*, 2004; Marchetti *et al*, 2005). We used combined data from previous papers (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004) and from Sanger institute's databases (Bamford *et al*, 2004) to analyse association of *LKB1* mutations with mutations of *KRAS*, *BRAF*, and *EGFR*. Analysis of *LKB1* mutation harbouring NSCLC cell lines (A-427, A549, NCI-H1395, NCI-H1666, NCI-H2122, NCI-H2126, NCI-H23, and NCI-H460) showed that five of the cell lines (63%) had concurrent *LKB1* and *KRAS* mutations, two (25%) had concurrent *LKB1* and *BRAF* mutations, and only one (13%) had neither *KRAS* nor *BRAF* mutations. None of these cell lines had EGFR mutations.

As our findings in NSCLC cell lines suggested concurrency of *KRAS* or *BRAF* and mutual exclusiveness of *EGFR* mutations with LKB1 mutations, we analysed the mutational status of these genes in our primary NSCLC tumour specimens. *KRAS* mutations were detected in 49 (16% in the whole tumour set, 25% in Caucasian and 8% in Asian specimens) tumour specimens with 10 (20% of *KRAS* mutants) of these occurring concurrently with an *LKB1* mutation ($P=0.042$) (Table 3). Four *BRAF* mutations (1%) were found in the tumour set (G465V, N581S, L596R, and T599I) and one of these (N581S) occurred concurrently with *LKB1* mutation ($P=0.373$). Seventy tumours (23% in the whole tumour set, 9% in Caucasian, 34% in Asian specimens) had *EGFR* kinase domain mutations with only one of them occurring concurrently with an *LKB1* mutation ($P=0.002$). The tumour with a concurrent *EGFR* and *LKB1* mutation had a missense mutation of *LKB1* outside the kinase domain (R426W). No germ line DNA was available from this patient. However, a recent report has suggested that R426W is in fact a rare polymorphism of the gene (Onozato *et al*, 2007). Taken together our findings suggest that unlike *KRAS*, mutations in *EGFR* and *LKB1* are mutually exclusive in NSCLC.

Previous reports (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004) and Cancer Genome Project by Sanger Institute (Bamford *et al*, 2004) have extensively characterised *LKB1* mutations in NSCLC cell lines with *KRAS* and *BRAF* mutations, but *LKB1* status of *EGFR* mutant NSCLC cell lines has not been extensively analysed. Therefore, we analysed the *LKB1* genotype of NSCLC cell lines with known *EGFR* or *ERBB2* mutations. Twelve *EGFR* mutant and one *ERBB2* mutant cell lines were analysed for *LKB1* genotype. No *LKB1* mutations were detected in these cell lines (Table 4).

Table 3 Association of *LKB1* mutations with *KRAS*, *BRAF*, and *EGFR* mutations in NSCLC tumours

		LKB1 mutation		
		+	−	P-value*
EGFR mutation	+	1	69	0.002
	−	33	207	
K-Ras mutation	+	10	39	0.042
	−	24	237	
B-Raf *mutation*	+	1	3	0.373
	−	33	273	

*Fisher's exact test.

Table 4 LKB1 genotypes of NSCLC cell lines with EGFR or ERBB2 mutations

Cell line	*EGFR* genotype	*HER2* genotype	*LKB1* genotype
H1650	E746_A750del	Wt	Wt
H1781	Wt	G776V, Cins	Wt
H1975	L858R, T790M	Wt	Wt
H3255	L858R	Wt	Wt
H3255GR	L858R, T790M	Wt	Wt
H820	L747_L751del, T790M	Wt	Wt
HCC2279	E746_A750del	Wt	Wt
HCC2935	E746_T751del, S752I	Wt	Wt
HCC4006	L747_E749del, A750P	Wt	Wt
HCC827	E746_A750del	Wt	Wt
Ma-1	E746_A750del	Wt	Wt
Ma-70	L858R	Wt	Wt
PC-9	E746_A750del	Wt	Wt

Wt = wild type.

DISCUSSION

The present study characterised *LKB1* mutation frequency in NSCLC using one of the largest tumour sets to date ($n=310$). Our study analysed tumours from different histologies and of both a US and Korean origin to determine potential histological and ethnic variation in *LKB1* mutational frequency. The large size of our study enabled us to study associations of *LKB1* mutations with clinicopathological factors, which have been incompletely characterised in previous studies (Sanchez-Cespedes *et al*, 2002; Carretero *et al*, 2004; Fernandez *et al*, 2004; Matsumoto *et al*, 2007; Onozato *et al*, 2007). In addition, we used a modification of a sensitive mutation screening technique that we have previously developed to facilitate the rapid detection of *LKB1* mutations (Janne *et al*, 2006).

Findings from our study confirm the high frequency of *LKB1* mutations in NSCLC (11%), which in contrast, are rare (0–4%) in other common solid malignancies (Avizienyte *et al*, 1998, 1999). The reason behind these observations is presently unknown but might reflect the differences in carcinogen exposure in the lungs compared with other tissues. In support of this hypothesis, we find that *LKB1* mutations are significantly ($P=0.007$) more common in smokers than in never or light ($\leqslant$10 pack years) cigarette smokers (Table 1). Male PJS patients (age $\geqslant$50 years) have an increased risk of developing lung cancer compared with the general population but the relationship of smoking and the increased risk of lung cancer in PJS is unknown (Hearle *et al*, 2006). Interestingly, the *LKB1* mutation spectrum found in the current study is very similar to those previously published for PJS (deletions 34%, insertions 15%, splice site mutations 14%, missense mutations 21%, and nonsense mutations 12%) (Launonen, 2005) and, as in PJS, no clear mutational hotspots were detected.

Our study also demonstrated that the *LKB1* mutation frequency was significantly higher in cancers derived from a US population compared with those found in Korean patients (17 *vs* 5%; $P=0.001$). These differences also track with cigarette smoking, as the number of never/light former smokers was much higher in the Korean cohort compared with the US cohort of patients (38 *vs* 13%). Similarly, a recent study of 100 Japanese NSCLCs found that only 3% contained an *LKB1* mutation (Onozato *et al*, 2007). These findings are in contrast to *EGFR* mutations, which are more frequently detected in tumours from never/light cigarette smokers and from Asian patients (Janne and Johnson, 2006). Our studies further highlight ethnic and environmental differences in the origins of NSCLC.

Given the differences in *LKB1* mutation frequencies in smokers *vs* never/light smokers and in the US compared with Korean patients, we further determined whether these were also associated with other oncogene mutations known to vary in these subgroups of patients. Consistent with prior studies we found a significant association with concurrent *KRAS* mutations, which are common in smokers (Ahrendt *et al*, 2001), in one out of three of NSCLC with *LKB1* mutations (Table 3). In contrast, there was a significant inverse relationship of *LKB1* mutations with *EGFR* mutations in both NSCLC tumours and cell lines, which has not previously been

described (Tables 3 and 4). These differences may relate to the biological role of LKB1 in lung cancer. It is possible that in *EGFR* mutant lung cancers there is already maximal activation of the PI3K/Akt/mTOR signalling pathway and thus an *LKB1* mutation may not be required to further potentiate this signalling pathway. In contrast in *KRAS* mutant cancers, a concurrent *LKB1* mutation may be required to enhance mTOR activation. Mice with concurrent *KRAS* mutations and *LKB1* inactivation have more aggressive tumours and a shorter survival than those with only *KRAS* mutant cancers (Ji *et al*, 2007). In our study, we were not able to detect a significant survival difference for patients whose tumours contained *LKB1* mutations alone or concurrently with *KRAS* mutations (data not shown) likely because of the limited number of tumour specimens. Additional studies are needed to clarify the prognostic impact of *LKB1* mutations in humans with NSCLC. In the present study ~2 out of 3 of *LKB1* mutant tumours were *KRAS* wild type (Table 3). One possibility is that such tumours contain a concurrent mutation in another oncogene that activates the same signalling pathway as *KRAS*. For this reason, we examined our tumours for *BRAF* mutations, which are found in 1–2% of NSCLC (Naoki *et al*, 2002). We detected a concurrent *LKB1* mutation in one of the four *BRAF* mutant tumours (Table 3). This tumour was wild type for *KRAS* (data not shown). In addition, some of the *BRAF* mutant NSCLC cell lines (NCI-H1395, G469A; NCI-H1666, G466V) also contain a concurrent *LKB1* mutation (Sanchez-Cespedes *et al*, 2002; Bamford *et al*, 2004; Carretero *et al*, 2004). Future studies will help further clarify whether *LKB1* mutations occur concurrently with other genomic alterations in NSCLC and the impact of this on patient outcome.

Our study employed a mutation scanning technology to screen for *LKB1* mutations at the cDNA level (Janne *et al*, 2006). This was advantageous as the entire coding region of *LKB1* could be rapidly screened for a mutation using just two overlapping cDNA fragments. *LKB1* is a challenging gene to analyse at the genomic DNA level because of its high guanine–cytosine content. In addition, as many of the *LKB1* mutations are small deletions (Table 2) or involve deletions of entire exons, these would be missed using exon-specific genome sequencing methods. Our method, however, does have limitations as it would miss deletions at the site of PCR primers, whole gene deletions, or deletions within the promoter region all of which have been infrequently detected in PJS (Volikos *et al*, 2006). Thus our studies may underestimate the true *LKB1* mutation frequency in NSCLC. In addition, our method is limited to the analysis of fresh tumour specimens, which are available only from the minority of NSCLC patients. Furthermore, as techniques isolating mRNA from formalin-fixed paraffin-embedded tumour specimens continue to improve, this rapid mutation scanning technique can be used to analyse broader populations of tumours from NSCLC patients. Future studies may need to employ a combination of *LKB1* mutation detection methodologies including the current method, MLPA and direct sequencing.

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Efficacy and Safety of Two Doses of Pemetrexed Supplemented with Folic Acid and Vitamin B_{12} in Previously Treated Patients with Non-Small Cell Lung Cancer

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Abstract **Purpose:** The objective of this study was to evaluate the efficacy and safety of two doses of pemetrexed supplemented with folic acid and vitamin B_{12} in pretreated Japanese patients with advanced non-small cell lung cancer (NSCLC).

Experimental Design: Patients with an Eastern Cooperative Oncology Group performance status 0 to 2, stage III or IV, and who received previously one or two chemotherapy regimens were randomized to receive 500 mg/m^2 pemetrexed (P500) or 1,000 mg/m^2 pemetrexed (P1000) on day 1 every 3 weeks. The primary endpoint was response rate.

Results: Of the 216 patients evaluable for efficacy (108 in each arm), response rates were 18.5% (90% confidence interval, 12.6-25.8%) and 14.8% (90% confidence interval, 9.5-21.6%), median survival times were 16.0 and 12.6 months, 1-year survival rates were 59.2% and 53.7%, and median progression-free survival were 3.0 and 2.5 months for the P500 and P1000, respectively. Cox multiple regression analysis indicated that pemetrexed dose was not a significant prognostic factor. Drug-related toxicity was generally tolerable for both doses; however, the safety profile of P500 showed generally milder toxicity. Main adverse drug reactions of severity grade 3 or 4 were neutrophil count decreased (20.2%) and alanine aminotransferase (glutamine pyruvic transaminase) increased (15.8%) in P500 and neutrophil count decreased (24.3%), WBC count decreased (20.7%), and lymphocyte count decreased (18.0%) in P1000. One drug-related death from interstitial lung disease occurred in the P500.

Conclusion: P500 and P1000 are similarly active with promising efficacy and acceptable safety outcomes in pretreated patients with NSCLC. These results support the use of P500 as a second- and third-line treatment of NSCLC.

Pemetrexed (LY231514; Alimta), a multitargeted antifolate, has shown antitumor activity as a single agent or in combination with other anticancer agents (1, 2). Pemetrexed at doses of 500 or 600 mg/m^2 has been evaluated in various clinical settings in a broad range of tumors including lung (non-small cell and mesothelioma), colorectal, gastric, pancreatic, head and neck, bladder, cervical, and breast cancers (3-13). In a randomized phase III trial that compared 3-week regimens of single-agent 500 mg/m^2 pemetrexed versus 75 mg/m^2 docetaxel in pretreated patients with non-small cell lung cancer (NSCLC), respective response rates (9.1% versus 8.8%) and median survival times (MST; 8.3 versus 7.9 months) did not differ between pemetrexed and docetaxel. However, fewer hematologic adverse effects, such as grade 3 or 4 neutropenia, febrile neutropenia, and neutropenic fever, were observed in patients treated with pemetrexed (3).

Myelosuppression is the predominant dose-limiting toxicity of pemetrexed as reported in phase I studies (14-16). A multivariate analysis identified the correlation between poor folate status (as indicated by elevated plasma homocysteine levels) and increased toxicity to pemetrexed, which led to the requirement that patients in all pemetrexed studies receive folic acid and vitamin B_{12} supplementation (2, 17). This has been shown to decrease toxicity to pemetrexed without compromising efficacy (18). Without supplementation, the maximum tolerated dose of pemetrexed, given every 3 weeks, has been shown to be 600 mg/m^2 in heavily pretreated patients (14); however, with supplementation, higher pemetrexed doses have been given without limiting side effects. In a Japanese phase I

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Note: The results of this study have been reported at American Society of Clinical Oncology, World Conference on Lung Cancer, and European Cancer Conference in 2007.

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study of pemetrexed that included folic acid and vitamin B_{12} supplementation, the maximum tolerated dose of pemetrexed was 1,200 mg/m^2 and recommended dose was 1,000 mg/m^2 given every 3 weeks (19). Pemetrexed pharmacokinetics in Japanese patients was not overtly different from those observed in Caucasian patients.

In view of these data, we conducted a randomized, phase II study that confirmed the efficacy and safety of a standard dose of pemetrexed (500 mg/m^2; P500) with that of a higher dose (1,000 mg/m^2; P1000), including folic acid and vitamin B_{12} supplementation, in previously treated NSCLC patients. The primary endpoint was evaluation of response rate. Secondary endpoints were assessments of response duration, progression-free survival (PFS), 1-year survival rate, MST, quality of life (QoL), and adverse events.

Materials and Methods

Patient selection. Men and women, between 20 and 75 years old, with a life expectancy of at least 12 weeks and histologically and/or cytologically confirmed advanced NSCLC were eligible for the study. In addition, all patients met the following inclusion criteria: stage III or IV disease, at least one target lesion, one or two prior chemotherapeutic regimens, an Eastern Cooperative Oncology Group performance status (PS) of 0 to 2, adequate bone marrow function (neutrophils ≥2,000/mm^3, platelets ≥100,000/mm^3, and hemoglobin ≥9.0 g/dL), hepatic function [total bilirubin within 1.5 times the upper normal limit, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) within 2.5 times the upper normal limit, and serum albumin ≥2.5 g/dL], renal function (serum creatinine ≤1.2 mg/dL and creatinine clearance ≥45 mL/min), and pulmonary function (functional oxygen saturation ≥92%).

Patients were excluded from the study for radiographic signs of interstitial pneumonitis or pulmonary fibrosis, serious or uncontrolled concomitant systemic disorders, active infections, the need for chronic administration of systemic corticosteroids, active double cancer and/or brain metastases, treatment with third-space fluid collections within 2 weeks of signing the informed consent or the need of such treatment, grade 3 or 4 toxicity, peripheral sensory neuropathy, previous pemetrexed therapy, unable or unwilling to take folic acid or vitamin B_{12} supplementation, or pregnant or breast-feeding.

This study was conducted in compliance with the guidelines of good clinical practice and the principles of the Declaration of Helsinki, and it was approved by the local institutional review boards. All patients gave written informed consent before study entry.

Study design and sample size. This open-label multicenter study had response rate as the primary objective, and 244 patients were enrolled and 226 were allocated to either 500 mg/m^2 (P500) or 1,000 mg/m^2 (P1000) randomly.

The sample size was calculated to ensure that the response rate in each group exceeded 5%. Based on the results from previous study, assuming a 13% true response rate, 5% one-sided significance level for the test with exact probability based on binomial distribution, and 90% power, at least 107 patients in each treatment arm (total of 214) were necessary. Assuming a 10% dropout rate, 240 patients were planned for the study (actual: 244 patients).

The randomization was done by an independent registration center and was dynamically balanced for PS, previous platinum chemotherapy, disease stage, gender, time from prior chemotherapy to the enrollment, and hospital. Patients were balanced with respect to the study drug in each stratum for each prognostic factor using the minimization method.

Treatment plan. Pemetrexed was administered as an i.v., 10-min infusion on day 1 of a 21-day cycle. Patients were instructed to take orally 1 g/d of a multivitamin containing 500 μg folic acid from 1 week before day 1 of course 1 until 22 days after the last administration of pemetrexed. Vitamin B_{12} (1000 μg) was injected i.m. 1 week before day 1 of course 1 and repeated every 9 weeks until 22 days after the last administration of pemetrexed. Patients were discontinued from the study for disease progression, unacceptable adverse events, inadvertent enrollment, use of excluded concomitant therapy, a cycle delay of >42 days, or if the patient requested to discontinue the study.

Administration of pemetrexed was delayed if patients met any of the following criteria: neutrophils <2,000/mm^3, hemoglobin <9.0 g/dL, platelets <100,000/mm^3, AST/ALT >2.5 times the upper normal limit, total bilirubin >1.5 times the upper normal limit, serum creatinine >1.2 mg/dL, PS 3 or 4, or grade ≥3 nonhematologic toxicity (except for anorexia, nausea, vomiting, and fatigue). The dose of pemetrexed was decreased to 400 mg/m^2 in the P500 arm and to 800 mg/m^2 in the P1000 arm, if any of the following events occurred in the previous course: grade 4 leukopenia or neutropenia, grade ≥3 febrile neutropenia, thrombocytopenia, or platelet transfusion, grade ≥3 nonhematologic toxicity (except for grade 3 anorexia, nausea, vomiting, and fatigue), or AST/ALT increased. The pemetrexed dose was similarly reduced if initiation of the next course was postponed after day 29 due to drug-related adverse events. Patients who continued to show evidence of toxicity after reducing the pemetrexed dose were discontinued from the study.

Baseline and treatment assessments. Pretreatment assessments included chest X-ray, electrocardiogram, blood chemistry, urinalysis, pregnancy test, creatinine clearance, functional oxygen saturation, vital signs, PS, body weight, and use of prior therapies. Tumor size was examined using X-ray, computer tomography, or magnetic resonance imaging done within 28 days before the planned day of the first treatment. This was repeated about every 4 weeks after the first examination.

Tumor response rate was assessed as the percentage of patients in whom complete response (CR) and partial response (PR) were confirmed based on the best overall response of the tumor response evaluation. Response was evaluated according to the Response Evaluation Criteria in Solid Tumors (20). Objective tumor responses in all responding patients were evaluated by an external review committee given no information on the treatment groups.

Duration of overall response (CR + PR) was measured from the date of the first objective assessment of CR or PR until the date of progressive disease. PFS was measured from the date of registration (for the initiation of course 1) until the date of progressive disease or death. One-year survival rate was defined as the percentage of patients who survived for 1 year from the registration date. Survival was measured from the registration date to the date of death (regardless of cause).

QoL was assessed by the QoL Questionnaire for Cancer Patients Treated with Anticancer Drugs and the Functional Assessment of Cancer Therapy for Lung Cancer (Japanese version; refs. 21–23).

Assessments of QoL were done before treatment, before the second and third courses of chemotherapy, and 3 months after the start of treatment.

Adverse events were recorded throughout the study and after the last drug administration until signs of recovery were evident. All such events were evaluated according to the Common Terminology Criteria for Adverse Events version 3.0.

Statistical analysis. Efficacy measurements were done according to the guidelines for clinical evaluation methods of anticancer drugs. Efficacy analysis was done on patients who met all selection criteria and received at least one dose of pemetrexed. Safety analysis was done on patients who received at least one dose of pemetrexed.

Statistical tests were done to establish a pemetrexed response rate of >5%; 90% confidence intervals (CI) for the objective response rate were constructed for each arm. All survival curves for time-to-event variables were created using the Kaplan-Meier method; 95% CIs were calculated for each arm. Response rate, response duration, and PFS were compared between the two arms using the χ^2 test. Cox multiple regression analysis was done on all evaluable patients from two combined arms to

identify significant prognostic factors for survival. Covariates evaluated were pemetrexed dose, gender, age, PS, disease stage, histology, interval from prior chemotherapy to registration for the first treatment course, the number of prior chemotherapeutic regimens, and use of prior platinum chemotherapy. For the QoL analysis, distributions of subscales were summarized for each arm using descriptive statistics (mean, SD, minimum, median, and maximum). As a retrospective analysis for safety, major grade 3 to 4 drug-related adverse events were compared between the two arms using the χ^2 test.

Results

Patient disposition and characteristics. From October 2004 to October 2005, a total of 244 Japanese patients with advanced NSCLC were enrolled at 28 centers. Of the 244 patients enrolled, 226 were randomly assigned (114 to the P500 arm and 112 to the P1000 arm) at least 1 week before treatment after receiving folic acid and vitamin B_{12} supplementation. A total of 225 patients (114 in the P500 arm and 111 in P1000 arm) were evaluable for safety. Of these patients, 216 (108 in each arm) were evaluable for efficacy. Gender, age, PS, histology, stage, and prior platinum chemotherapy were well balanced across the two arms (Table 1).

Efficacy evaluation. Objective tumor response rates and durations of overall response are shown in Table 2. Of the 108 patients evaluable for efficacy in the P500 arm, 20 achieved PR for an objective response rate of 18.5% (90% CI, 12.6-25.8%); the median duration of response was 4.9 months (95% CI, 3.8-8.7 months). Of the 108 patients evaluable for efficacy in the P1000 arm, 16 achieved PR for an objective response rate of 14.8% (90% CI, 9.5-21.6%); the median duration of response was 3.0 months (95% CI, 2.8-6.1 months). As seen above, the lower limits of the 90% CI in both arms were >5%, showing a statistically significant objective response rate >5% in each of the arms. The differences between arms in response rate and response duration were not statistically significant (P = 0.5839 and 0.1740).

By October 2006, 125 of the 216 evaluable patients had died. The MST and 1-year survival rate were 16.0 months and 59.2% in the P500 arm and 12.6 months and 53.7% in the P1000 arm (P = 0.1463, log-rank test for survival; Fig. 1). Median PFS was 3.0 months (95% CI, 2.0-3.5 months) in the P500 arm and 2.5 months (95% CI, 1.8-3.2 months) in the P1000 arm (P = 0.7139, log-rank test).

Cox multiple regression analysis indicated that pemetrexed dose was not a significant prognostic factor; however, gender (female), PS (0), disease stage (III), histologic type (non-squamous cell carcinoma), and longer intervals from prior chemotherapy were shown to be good prognostic factors (Fig. 2). Of note, patients with non-squamous cell carcinoma had a longer MST compared with those with other histologic types (16.0 versus 9.3 months; P = 0.00264, Cox regression analysis). Pretreatment QoL assessments in both arms were relatively high and showed neither worsening nor improvement following pemetrexed treatment (Table 3).

Safety evaluation. A total of 225 patients (114 for P500 and 111 for P1000) were evaluable for safety. Leukopenia, neutropenia, lymphopenia, anemia, elevation of AST/ALT, lactate dehydrogenase, and rash were commonly reported; however, no grade 4 leukopenia or febrile neutropenia was observed (Table 4). Other grade 4 toxicities were uncommon. Gastrointestinal toxicities such as nausea, vomiting, and anorexia were mostly mild and more frequently reported in the P1000 arm. As a retrospective analysis for safety, major grade 3 to 4 drug-related adverse events were compared

Table 1. Patient characteristics

Variable	P500	P1000
Patients who were given at least one dose of pemetrexed	114	111
Gender		
Male	72	71
Female	42	40
Age, median (range)	61.0 (37-74)	62.0 (26-74)
Eastern Cooperative Oncology Group PS		
0	45	37
1	63	68
2	6	6
Histology		
Adenocarcinoma	79	82
Squamous cell carcinoma	25	26
Others	10	3
Disease stage		
III	22	22
IV	92	88
No. prior chemotherapies		
1	44	53
2	67	57
3	3	1
Prior platinum chemotherapy		
Yes	108	104
No	6	7
Interval from prior chemotherapy to registration for the first course starts (mo)		
<3	72	66
3	42	45

Table 2. Objective tumor response and median response duration

Variable	P500 (n = 108)	P1000 (n = 108)
Objective tumor response		
CR	0	0
PR	20	16
Stable disease	40	34
Progressive disease	48	58
Response rate (90% CI), %	18.50 (12.6-25.8)	14.80 (9.5-21.6)
Median response duration (95% CI), mo	4.9 (3.8-8.7)	3.0 (2.8-6.1)

between the two arms using the χ^2 test. Grade 3 or 4 anorexia was reported more frequently in the P1000 arm (10.8% versus 2.6%; P = 0.0284). Drug-related rash was observed in 67.5% and 80.2% of the patients treated with P500 and P1000, respectively. However, all severities were grade 1 or 2. Five of the P500 patients and 3 of the P1000 patients developed interstitial lung disease related to pemetrexed treatment that resulted in the death of one patient (P500 arm). The other 7 patients recovered from their illness after discontinuing the study drug. A total 16 (14.0%) patients in the P500 arm and 26 (23.4%) patients in the P1000 arm discontinued the treatment because of drug-related adverse events.

Dose administration. The median number of treatment courses completed in both arms was 3 (range, 1-24+). Eleven percent of patients in the P500 arm and 8% in the P1000 arm completed at least 10 courses. Dose reduction occurred in 20 (17.5%) patients in the P500 arm and 27 (24.3%) patients in the P1000 arm. The most frequent cause of dose reduction was ALT elevation. Relative dose intensities were 89.6% in the P500 group and 89.8% in the P1000 group.

Discussion

This phase II, randomized study is the first report on the efficacy and safety of a higher dose of pemetrexed (1,000 mg/m^2) in pretreated Japanese patients with NSCLC. Most patients (>50%) received two courses of prior chemotherapy, and the vast majority or patients (>90%) received prior platinum-based chemotherapy. The response data indicate promising tumor reduction activity and are noteworthy in pretreated patients. The survival data are also promising and better than those reported in second- and third-line settings and comparable with those reported in first-line settings (3, 24, 25). In the phase III study (3) comparing pemetrexed with docetaxel, the response

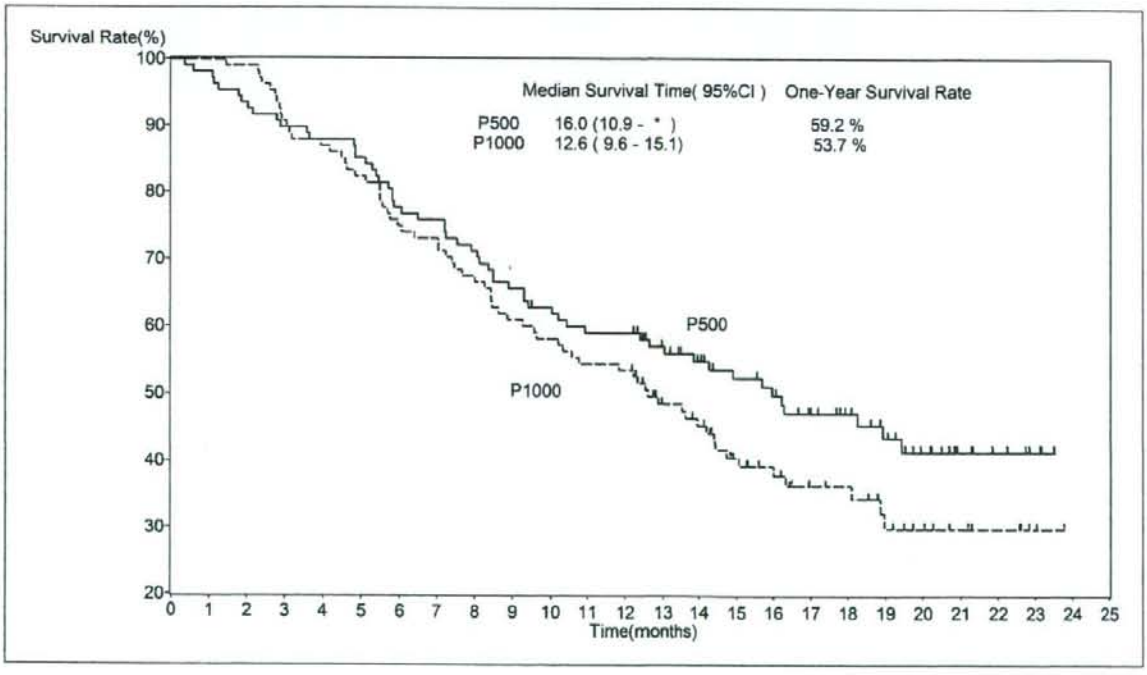

Fig. 1. Kaplan-Meier curve showing the overall survival for each arm. *Asterisk*, upper limit could not be calculated because of the censoring at the end of study period.