Jpn J Clin Oncol 2008;38(3)214–221
doi:10.1093/jjco/hyn002

Weekly Epoetin Beta Maintains Haemoglobin Levels and Improves Quality of Life in Patients with Non-Myeloid Malignancies Receiving Chemotherapy

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Received July 23, 2007; accepted December 28, 2007; published online February 21, 2008

Objective: This study was aimed at investigating the effectiveness and safety of once-weekly epoetin beta for anaemic cancer patients receiving chemotherapy.
Methods: A total of 104 patients with a haemoglobin level of ≤11.0 g/dL were enrolled. Patients received a once-weekly subcutaneous dose of 36 000 IU epoetin beta for 12 weeks. If the increase in the haemoglobin level was <1.0 g/dL after 6 weeks, or a red blood cell transfusion was required between days 15 and 42, the dose of epoetin beta was increased to 54 000 IU from the subsequent week. The primary endpoint was the percentage of patients who achieved a haemoglobin increase of ≥2.0 g/dL; the haemoglobin response rate. Quality of life (QOL) was assessed using the Functional Assessment of Cancer Therapy-Anaemia (FACT-An) questionnaire.
Results: The haemoglobin response rate was 66.3% among the 98 patients (breast cancer: $n = 25$; malignant lymphoma: $n = 21$; ovarian cancer: $n = 20$; lung cancer: $n = 15$; other cancers: $n = 17$) assessable for a haemoglobin response. Thirty-nine patients (39.8%) required a dose escalation to 54 000 IU. At the end of the study, QOL assessable patients ($n = 96$) showed a mean improvement in the FACT-An total fatigue subscale score (FSS) of 0.3 points from baseline. Patients with a haemoglobin response had a mean change in the total FSS of +3.2, compared with −3.4 for patients without a haemoglobin response. No serious adverse event of epoetin beta was observed.
Conclusions: Epoetin beta administered at an initial dose of 36 000 IU once-weekly was well tolerated, with increased haemoglobin levels and improved QOL in anaemic cancer patients receiving myelosuppressive chemotherapy.

Key words: anaemia – erythropoietin – cancer – chemotherapy – quality of life

INTRODUCTION

Anaemia is a common complication of cancer patients undergoing chemotherapy. Symptoms of anaemia, including fatigue, palpitations, dizziness and dyspnea markedly reduce patient activity, resulting in impaired quality of life (QOL). In most cases, however, physicians hesitate to prescribe red blood cell (RBC) transfusions until the haemoglobin level is <8.0 g/dL, even if the patient has symptoms related to anaemia, such as fatigue. Although the safety of blood transfusion has improved in recent years, risks still remain, such as viral infections, graft versûs host disease and haemolytic reactions.

In Europe and the United States, erythropoietin (EPO) agents have widely been used since the 1990s for the treatment of chemotherapy-induced anaemia. Although a three-times weekly dosing schedule was initially introduced (1–3), this schedule was inconvenient for outpatients. Several studies reported that once-weekly dosing of EPO increased the

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haemoglobin level and improved QOL in a manner comparable with those obtained by three-times weekly dosing (4,5).

Since EPO agents have not been approved for the treatment of chemotherapy-induced anaemia in Japan, we previously conducted a dose-finding study of weekly epoetin beta in patients with malignant lymphoma or lung cancer, resulting in a recommended weekly dose of 36 000 IU (6). In this prospective study, we investigated the haemoglobin response, the effects on QOL and the safety of once-weekly epoetin beta in anaemic patients with non-myeloid malignancies. We also investigated the effects of dose escalation to 54 000 IU in patients showing insufficient haemoglobin increase.

PATIENTS AND METHODS

Patient Eligibility

Inclusion criteria were as follows: (a) histological or cytological confirmation of non-myeloid malignancy diagnosis, (b) treatment with cyclic chemotherapy, (c) anaemia (haemoglobin level $\leq$11.0 g/dL) considered to be primarily chemotherapy-induced, (d) life expectancy of at least 4 months, (e) aged between 20 and 79 years, (f) Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 to 2, (g) eligibility for the QOL questionnaire and (h) adequate hepatic and renal function.

Exclusion criteria included: (a) iron deficiency (mean corpuscular volume $<80\ \mu m^3$ or iron saturation [{Fe/(Fe+ unsaturated iron-binding capacity)} $\times$ 100] $<$15.0%); (b) surgery scheduled during the study period; (c) EPO therapy within 4 weeks prior to the study; (d) documented haemorrhagic lesions; (e) pregnancy, breastfeeding or non-use of adequate birth control measures; (f) history of myocardial, pulmonary, cerebral infarction, serious drug allergy, uncontrolled hypertension, hypersensitivity to any EPO agent or any serious complication; and (g) tumor in the central nervous system.

Study Design and Treatment Schedule

This multicentre, open-label study was conducted at 14 sites in Japan.

The protocol was approved by the institutional review board of the respective hospitals, and written informed consent was obtained from all patients who participated in the study.

The initial dose of epoetin beta (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan) was 36 000 IU, and a once-weekly treatment was administered subcutaneously for 12 weeks. If the patient's haemoglobin level did not increase by $\geq$1.0 g/dL from baseline after 6 weeks of treatment, or an RBC transfusion was required between days 15 and 42, the dose of epoetin beta was increased to 54 000 IU weekly from the subsequent week. If the haemoglobin level increased to $\geq$14.0 g/dL, epoetin beta was discontinued until the haemoglobin level decreased to $\leq$12.0 g/dL, and was then restarted at two-thirds (24 000 IU or 36 000 IU) of the previous dose (36 000 IU or 54 000 IU). RBC transfusion was allowed at the discretion of the investigator during the study. An oral daily dose of 100–200 mg elemental iron was recommended if the mean corpuscular volume was $<80\ \mu m^3$ or the iron saturation was $<$15.0%.

QOL was evaluated at baseline and week 12 using the Japanese Functional Assessment of Cancer Therapy-Anaemia (FACT-An) questionnaire (7,8), a well-validated instrument. In this study, the FACT-An total fatigue subscale, which consists of 13 fatigue related questions, was mainly analysed. The FACT-An total fatigue subscale scores (FSS) range from 0 to 52, with higher scores indicating less fatigue.

Evaluation of Efficacy and Safety

The American Society of Clinical Oncology/The American Society of Hematology guidelines (9) stipulate that the criteria for the haemopoietic effect should be an increase in haemoglobin level $\geq$1.0–2.0 g/dL in 6–8 weeks. Furthermore, there are reports (2,6), which showed that QOL is improved in patients with an increase in haemoglobin level of $\geq$2.0 g/dL.

The primary endpoint of the study was the percentage of patients achieving an increase in the haemoglobin level of $\geq$2.0 g/dL from the baseline between weeks 4 and 12, the haemoglobin response rate, excluding the data within 28 days after an RBC transfusion. The secondary endpoint was the change in FSS after 12 weeks of treatment. The percentage of patients receiving RBC transfusions between day 28 and the end of the study was also assessed. It was not expected that treatment with an EPO agent could influence transfusion requirements before day 28.

Adverse events (AEs) were assessed during the 12-week treatment period and during a 1-week observation period after the last dosing. Anti-erythropoietin antibodies were measured by the enzyme-linked immunosorbent assay and radio-immunoprecipitation (RIP) assay, and detection by either was judged as positive.

Statistical Analysis

We expected that 90 patients would need to be enrolled in the study to obtain a haemoglobin response rate of 70 $\pm$ 10% (95% confidence interval [CI]), as the primary endpoint.

Patients who received at least one dose of the study drug comprised the safety population. For efficacy analysis, the full analysis set (FAS) population was defined as eligible patients who received at least one dose of the study drug.

The changes in the haemoglobin level and FACT-An scores were calculated by subtracting each patient's baseline values from the last values. The rates of increase in haemoglobin before and after dose escalation were compared using a linear mixed-effects model. The potential factors influencing the change in FSS were examined by multiple

regression analysis. Pearson correlation coefficients were calculated to assess the association between changes in the haemoglobin level and FACT-An scores.

RESULTS

Demographics and Baseline Characteristics

A total of 104 patients were enrolled in the study between February and November 2004. Five patients discontinued the study before the first dosing for the following reasons: patient eligibility criteria violation, $n = 3$; patient denial, $n = 1$; and disease progression, $n = 1$. Thus, 99 patients were administered epoetin beta. One patient was excluded because of non-compliance with the eligibility criteria, leaving 98 patients as the FAS population. Eighty-seven patients (88.8%) completed all 12 weeks of the study. Eleven patients (11.2%) withdrew from the study. The primary reasons for withdrawal were progressive disease and AEs.

The demographics and baseline characteristics of the FAS population are listed in Table 1. Common types of cancer were breast ($n = 25$), malignant lymphoma ($n = 21$), ovarian ($n = 20$) and lung ($n = 15$). The mean age was 58.4 years (range: 23–78), and the mean body weight was 50.7 kg (range: 31.7–74.0). Most of the patients had an ECOG PS of 0 or 1 and a tumour stage of III or IV. The main chemotherapeutic agents used during the study were platinum for lung and other types of cancer, anthracycline for malignant lymphoma, taxane for breast cancer and platinum plus taxane for ovarian cancer. All patients met the criterion that they should not be iron-deficient at the time of enrollment.

Haemoglobin Response

The mean change in the haemoglobin level from baseline to the end of the study was 2.47 g/dL (standard deviation [SD]: 2.09; range: −2.8 to 6.0), as shown in Fig. 1. Figure 1 shows the mean changes in haemoglobin levels by tumour type. The pattern of changes in haemoglobin level was similar for the different tumour types. The mean increase in the haemoglobin level in patients with and without an initial EPO level of ≥100 mIU/mL were 1.76 g/dL (SD: 2.60) and 2.50 g/dL (SD: 1.85), respectively.

The haemoglobin response rates, defined as the percentage of patients achieving an increase in haemoglobin level of ≥2.0 g/dL from the baseline between weeks 4 and 12, are listed in Table 2. The overall haemoglobin response rate was 66.3% (65 of 98 patients). The median time to the haemoglobin response was 56 days from the first dosing, analysed by the Kaplan–Meier method. The percentage of patients with a haemoglobin level of ≥12.0 g/dL between weeks 4 and 12 was 59.2% (58 of 98 patients).

The percentage of patients who required dose escalation to 54 000 IU was 39.8% (39 of 98 patients). In these patients, the haemoglobin level increased after dose escalation, and the change in the haemoglobin level was 1.23 g/dL (SD: 2.19) at the end of the study. The haemoglobin response rate was 33.3% (13 of 39 patients) in patients who required dose escalation. The rate of haemoglobin increase before and after dose escalation was 0.023 g/dL/week (Weeks 0–6) and 0.266 g/dL/week (Weeks 7–12), respectively ($P = 0.0055$).

For three patients, the drug treatment was discontinued when the haemoglobin level exceeded 14.0 g/dL, and was restarted at a dose of 24 000 IU when the haemoglobin level decreased to ≤12.0 g/dL.

Quality of Life

Overall compliance in terms of the percentage of patients who completed the FACT-An was 100% at baseline and 97% (95 of 98 patients) at the end of the study. For three patients who dropped out due to progressive disease and were regarded as missing not at random, the scores at the end of the study were substituted with the minimum scores for all patients. Two patients were excluded from the evaluation of the change in the FSS because the responses to some items were missing.

The mean baseline FSS was 31.8 (SD: 11.4, $n = 98$) points. At the end of the study, the mean change from baseline was 0.3 (SD: 11.8, $n = 96$) points. The mean FSS change in the patients with progressive disease, as judged by each investigator, was −3.8 (SD: 16.7, $n = 15$) points (haemoglobin change: 2.4 g/dL). On the other hand, the mean change in patients without progressive disease was 1.9 (SD: 9.6, $n = 78$) points (haemoglobin change: 2.3 g/dL). These data indicated that progressive disease may be one of the independent variables affecting the change in FSS.

Relationship Between Haemoglobin Response and QOL Score

The results of a multiple regression analysis suggested that the change in the haemoglobin level ($P = 0.014$), the FSS at the initiation of dosing ($P < 0.0001$) and the PS at the end of the study ($P < 0.0001$) largely contributed to the change in the FSS. The correlation coefficient between the change in the FSS and the changes in the haemoglobin level was 0.280, indicating a significant correlation ($P = 0.006$, $n = 96$).

Patients who achieved an increase in the haemoglobin level of ≥2.0 g/dL experienced a 3.2-point mean change in FSS. On the other hand, patients who did not achieve an increase in haemoglobin level of ≥2.0 g/dL experienced a −3.4-point change (Fig. 2). There were no differences in the FSS at the initiation of dosing between patients with and without a change in haemoglobin level of ≥2.0 g/dL (32.0 versus 31.6). These data indicate that the change in FSS is dependent on the change in the haemoglobin level.

Concerning the relationship between the FSS at the initiation of dosing and the change in the FSS, patients with a baseline FSS of ≤36.0 reported greater improvement (mean ± SD: 1.6 ± 13.0) in the FSS at the end of the study (Table 3).

Table 1. Characteristics of the full analysis set population

	Characteristics	Total	Lung	Malignant Lymphoma	Breast	Ovarian	Other types
Sex	Male	27	11	10	9	0	6
	Female	71	4	11	25	20	11
Age (years)	Mean ± SD	58.4 ± 10.8	60.5 ± 10.5	56.5 ± 13.4	58.2 ± 9.0	54.4 ± 11.0	63.8 ± 8.0
	Range	23–78	41–78	23–74	39–77	30–75	40–76
ECOG performance status	0	48	1	9	14	13	11
	1	39	12	9	6	6	6
	2	11	2	3	5	1	0
Tumour stage	I	6	0	1	3	2	0
	II	17	1	4	7	4	1
	III	15	0	3	0	9	3
	IIIA	2	1	0	1	0	0
	IIIB	8	6	0	2	0	0
	IV	50	7	13	12	5	13
Treatment regimen	Platinum based	17	7	2	0	1	7
	Taxane based	28	5	0	19	3	1
	Anthracycline based	28	1	18	6	0	3
	Platinum, Anthracycline based	4	0	1	0	1	2
	Platinum,Taxane based	21	2	0	0	15	4
Weight (kg)	Mean ± SD	50.7 ± 8.2	53.8 ± 8.7	52.7 ± 9.9	47.9 ± 7.2	49.3 ± 6.6	50.9 ± 7.4
	Range	31.7–74.0	38.0–70.7	31.7–74.0	34.0–63.0	34.1–60.0	37.7–65.5
Haemoglobin (g/dL)	Mean ± SD	9.3 ± 1.4	9.6 ± 1.4	9.3 ± 1.4	9.4 ± 1.4	9.2 ± 1.6	9.1 ± 1.4
	Range	5.6–11.9	6.4–11.2	6.5–11.3	5.7–11.9	6.4–11.7	5.6–11.1
MCV (fL)	Mean ± SD	92.3 ± 6.5	89.0 ± 6.4	90.0 ± 5.4	91.9 ± 5.8	94.6 ± 7.5	95.7 ± 5.3
	Range	79.9–107.5	79.9–99.3	80–101	80.3–103.2	81.9–107.5	84–103.4
Reticulocyte (‰)	Mean ± SD	19.7 ± 16.4	20.8 ± 15.1	24.2 ± 24.1	18.0 ± 13.2	21.1 ± 15.6	14.1 ± 10.3
	Range	1–106	2–50	1–106	1–58	1–54	1.1–35.1
Transferrin saturation (%)	Mean ± SD	29.7 ± 22.3	22.4 ± 7.1	41.5 ± 30.6	21.9 ± 16.9	31.1 ± 24.3	30.5 ± 18.0
	Range	4.8–92.9	12.5–35.5	9.9–92.9	4.8–80.6	7.2–90.7	14.0–90.3
Serum endogenous erythropoietin (mIU/mL)	Mean ± SD	119.1 ± 310.5	64.3 ± 69.9	80.7 ± 108.0	68.4 ± 107.1	125.9 ± 144.8	252.0 ± 706.0
	Range	15.7–2970	15.7–234	17.3–399	16.7–472	23.2–578	20.4–2970
Baseline QOL:FACT-An							
Anemia subscale (0–80)	Mean ± SD	50.8 ± 14.5	47.0 ± 15.9	50.6 ± 11.7	47.1 ± 13.7	53.5 ± 11.1	56.7 ± 19.5
	Range	16–80	17–74	26–67	20–71	34–75	16–80
Fatigue subscale (0–52)	Mean ± SD	31.8 ± 11.4	29.6 ± 12.9	30.3 ± 10.6	29.7 ± 10.7	33.9 ± 8.7	36.5 ± 14.1
	Range	4–52	4–52	10–43	7–50	20–48	8–52

SD, standard deviation; ECOG, Eastern Cooperative Oncology Group; QOL, quality of life; FACT-An, Functional Assessment of Cancer Therapy-Anemia; MCV, mean corpuscular volume.

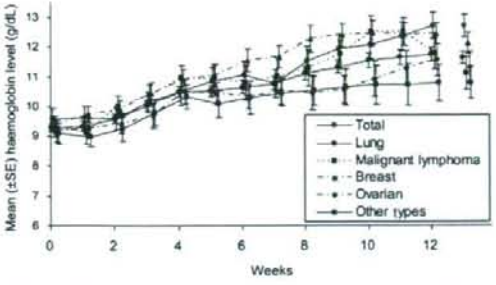

Figure 1. Change in haemoglobin level by tumor type. Mean weekly haemoglobin levels for the FAS population. Haemoglobin values within 28 days after RBC transfusion were excluded. FAS, full analysis set; RBC, red blood cell.

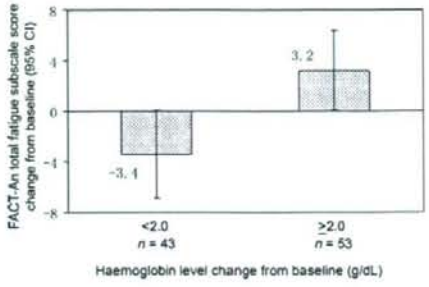

Figure 2. Changes in the FACT-An total fatigue subscale score by change in haemoglobin level. FACT-An, Functional Assessment of Cancer Therapy-Anaemia.

RBC Transfusion Requirement

The percentage of patients who received RBC transfusions between day 28 and the end of the study was only 6.1% (6 of 98 patients). The mean pretransfusion haemoglobin level at the time of the first transfusion was 6.2 g/dL (range: 5.4–7.3 g/dL). The percentage of patients whose haemoglobin level had decreased to <8.0 g/dL or who received an RBC transfusion between day 28 and the end of the study was 20.4% (20 of 98 patients).

Safety

AEs reported by at least 20% of the patients are summarised in Table 4. Death as a result of disease progression was not reported as an AE. Adverse drug reactions reported by at least 5% of patients are listed in Table 5. Among the 133 events in 48 patients (48.5%) that were considered related to the study drug, Grade III events were headache, hypertension, diarrhea, decreased serum potassium, impaired consciousness, anorexia and decreased serum phosphate. Three events (3.0%) of hypertension were reported as possibly related to epoetin beta treatment. An antihypertensive drug was administered after the onset of hypertension in one patient, who had hypertension as a comorbidity before the study. One patient (65-year-old female with malignant lymphoma) experienced a thrombovascular event, a lacunar infarction, at week 6. This event was evaluated as being unrelated to epoetin beta and was attributed to aging.

The incidence and type of AEs in patients who required dose escalation did not differ from those in patients who did not.

In two patients with ovarian and gastric cancer, anti-erythropoietin antibodies were detected only by RIP assay.

Table 2. Haemoglobin response rate by baseline haemoglobin, tumour type and dose escalation

	%	*n*
Response rate*	66.3	65/98
Response rate by baseline haemoglobin, g/dL		
<10.0	68.8	44/64
≥10.0	61.8	21/34
Response rate by tumour type		
Lung	80.0	12/15
Malignant lymphoma	66.7	14/21
Breast	76.0	19/25
Ovarian	65.0	13/20
Other types	41.2	7/17
Response rate by dose escalation		
Yes	33.3	13/39
No	88.1	52/59

*All patients, including those receiving transfusions.

Table 3. Changes in the FACT-An total fatigue subscale score by baseline FSS and final PS

Time period	Baseline		End of treatment		
	n	Mean score (SD)	*n*	Mean score (SD)	Mean change from baseline (SD)
Total	98	31.8 (11.4)	96*	31.8 (13.5)	0.3 (11.8)
Baseline FSS					
≤36.0	62	24.8 (7.9)	62	26.5 (12.0)	1.6 (13.0)
>36.0	36	43.9 (4.0)	34*	41.5 (10.3)	−2.2 (8.8)
Final PS					
0	58	35.5 (11.3)	56*	37.4 (10.3)	2.4 (10.2)
1	28	27.4 (9.1)	28	29.0 (11.5)	1.6 (12.2)
2	4	19.3 (9.4)	4	11.8 (11.4)	−7.5 (7.9)
3	3	29.7 (15.9)	3	21.0 (7.2)	−8.7 (13.8)
4	5	25.7 (7.3)	5	6.4 (7.1)	−19.3 (6.4)

*Two patients missing FSS. Collected but could not be calculated.
FSS, FACT-An total fatigue subscale score; PS, performance status.

Table 4. Frequencies of adverse events ($n = 99$)

Event	n	%	Grade*				
			I	II	III	IV	V
Neutropenia	83	83.8	3	11	24	45	0
Leukopenia	78	78.8	2	16	41	19	0
Nausea	57	57.6	38	11	8	0	0
Thrombocytopenia	55	55.6	21	9	23	2	0
Lymphopenia	52	52.5	0	18	34	0	0
Anorexia	46	46.5	22	13	10	1	0
Fatigue	39	39.4	22	14	3	0	0
Vomiting	36	36.4	18	16	2	0	0
Diarrhea	33	33.3	23	6	4	0	0
Increased lactate dehydrogenase	32	32.3	25	6	1	0	0
Peripheral neuropathy	26	26.3	21	5	0	0	0
Fever	26	26.3	17	7	2	0	0
Constipation	24	24.2	3	13	7	1	0
Increased alanine aminotransferase	24	24.2	15	6	3	0	0
Alopecia	22	22.2	7	15	0	0	0

*National cancer institutes common toxicity criteria, version 2.0.

Table 5. Frequencies of adverse drug reactions ($n = 99$)

Event	n	%	Grade*				
			I	II	III	IV	V
Increased lactate dehydrogenase	10	10.1	9	1	0	0	0
Headache	7	7.1	6	0	1	0	0
Nausea	7	7.1	5	2	0	0	0
Rash	5	5.1	3	2	0	0	0
Back pain	5	5.1	5	0	0	0	0

*National cancer institutes common toxicity criteria, version 2.0.

Neutralisation of EPO activity was detected in neither patient, and the haemoglobin level was elevated after dosing with the study drug. The investigators judged that the antibody did not cause pure red cell aplasia.

When re-examined six months after the last observation, one of these patients (ovarian cancer) was antibody negative, whereas the other (gastric cancer) could not be re-examined, having died of the underlying disease.

DISCUSSION

Several studies have been conducted to assess the effects of EPO agents in anaemic cancer patients, and increased haemoglobin levels and improvement in QOL that correlated with the increased haemoglobin level were reported (1,10).

The objectives of our study were to investigate the effects of an initial once-weekly 36 000 IU dose of epoetin beta on haemoglobin levels and QOL in patients with non-myeloid malignancy undergoing chemotherapy. The criterion for a haemoglobin response, an increase in the haemoglobin level of $\geq$2.0 g/dL, was based on a report that symptoms of anaemia assessed by the FACT-An are improved in patients with a change in the haemoglobin level of $\geq$2.0 g/dL (2,6). According to this index, the haemoglobin response rate in the present study was 66.3% (65 of 98 patients). The increases in haemoglobin levels that were observed were independent of the tumour type or the baseline haemoglobin level. None of the investigators performed a randomised comparison of a dose increase versus an unchanged dose in EPO low responders. In the present study, there was an increase in the rate of haemoglobin increase after dose escalation to 54 000 IU, and the haemoglobin response rate for patients who required a dose escalation was 33.3% (13 of 39 patients).

The secondary endpoint, the change in the FSS, showed an increase of 0.3 points; however, in patients who showed an increase in the haemoglobin level of $\geq$2.0 g/dL, the FSS was increased by 3.2 points, which was significantly higher than the $-$3.4-point change in patients whose haemoglobin level increased by $<$2.0 g/dL. A 3.2-point increase is comparable with the 3 points considered to be a clinically significant change in FSS (11). In addition, the mean change in FSS for patients with progressive diseases (PD) was $-$3.8 points (median: $-$6.5 points, range: $-$37 to 35 points) even though correction of anaemia was observed. In total, excluding PD cases, a 1.9- point improvement was observed.

Investigating the relationship between the FSS at the initiation of dosing and the change in the FSS showed that greater improvements in FSS were observed in patients with lower FSS. The FSS before treatment with epoetin beta was 31.8 $\pm$ 11.4 points, which is higher than the scores (FSS: 22.1–29.7 points, change in FSS: 1.6–5.2 points) in cancer patients with anaemia reported in several randomised trials (1,10,12–14). Nevertheless, the mean initial haemoglobin level (9.3 g/dL) in the present study was equal to the levels in the other trials (9.2–10.1 g/dL). Since it has been reported that the FSS after treatment with an EPO agent is aggravated in patients with an FSS exceeding 36.0 at the initiation of dosing (15), the scores were analysed after stratification at 36.0. This resulted in improved scores (1.6 $\pm$ 13.0 points) for those patients with a baseline score of $\leq$36.0, when compared with patients with a score $>$36.0 ($-$2.2 $\pm$ 8.8 points). The results of a multiple regression analysis of the change in the FSS demonstrated that the change in the haemoglobin level, the FSS at the initiation of dosing and the PS at the end of the study were factors that largely contributed to the change in the FSS. A positive and significant association was observed between

the degree of increase in the haemoglobin level and the degree of improvement in the FSS ($r = 0.280$, $P = 0.006$). It was comparable with the results ($r = 0.2879$, $P = 0.0002$; $r = 0.35$, $P = 0.001$ and $r = 0.2893$, $P < 0.0001$) of three other studies (1,10,16).

The RBC transfusion rate was only 6.1% (6 of 98 patients) between day 28 and the end of the study. As reported for once-weekly epoetin alfa administered to patients with various types of cancer (14), the transfusion rates between week 5 and the end of treatment were 14.5% (24 of 166 patients) for epoetin alfa and 29.3% (48 of 164 patients) for placebo. Furthermore, the mean pretransfusion haemoglobin levels for the first transfusion reported in the previous trial in the United States (7.9 and 7.8 g/dL, respectively) were higher than those (6.2 g/dL) in the present study in Japan. To evaluate the effect of EPO agents, the percentage of patients whose haemoglobin level had decreased to <8.0 g/dL or who received an RBC transfusion was considered to be a more objective index than the RBC transfusion rate in Japan, because RBC transfusion itself is prescribed at the discretion of the investigator and when the haemoglobin level is low.

Epoetin beta was well tolerated in the present study. Most of the AEs were consistent with the underlying disease or with the chemotherapy. Hypertension, which was judged to be related to epoetin beta was observed in three patients. It was alleviated either by no treatment or the administration of hypotensive agents. Lacunar infarction was also observed in one patient. A relationship to epoetin beta was ruled out, however, and this event was judged to be due to aging. Two recently published studies (17,18) targeting higher haemoglobin levels, in which survival was a primary endpoint, have raised concerns that EPO agents may have a negative impact on survival in cancer patients. A meta-analysis of 57 studies, including these two recent studies revealed an overall survival hazard ratio of 1.08 (95%CI: 0.99–1.18) and that uncertainties remain as to whether EPO agents affected survival (19). The FDA has provided new safety information on erythropoiesis-stimulating agents (ESAs), in which the target haemoglobin level is not to exceed 12 g/dL, because analyses of other studies in patients with cancer found a higher chance of serious and life-threatening adverse drug reactions or deaths with the use of ESAs (20). Although, in the present studies, there was no problem with safety when the haemoglobin level at which dosing was withheld was set at 14 g/dL, in consideration of FDA ALERTs, etc., we intend to investigate the use of lower values for target haemoglobin level and haemoglobin level at which dosing should be withheld.

In conclusion, once-weekly epoetin beta treatment increased the haemoglobin level and correspondingly improved the QOL in anaemic patients with non-myeloid malignancies receiving chemotherapy. Additionally, haemoglobin levels could be improved and controlled by once-weekly treatments at an initial dose of 36 000 IU followed by dose adjustment in the range of 24 000–54 000 IU.

Acknowledgements

The authors thank all investigators of Japan Erythropoietin Study Group: Tokai University School of Medicine, National Cancer Center Hospital, National Cancer Center Hospital East, Aichi Cancer Center Hospital, Saitama Medical University, Kinki University School of Medicine, Nihon University School of Medicine, Saitama Cancer Center, Tsukuba University Hospital, Niigata Cancer Center Hospital, National Hospital Organization Nagoya Medical Center, Niigata University Medical & Dental Hospital, Tochigi Cancer Center and Osaka City General Hospital.

Funding

This study was supported by Chugai Pharmaceutical Co., Ltd, Tokyo, Japan.

Conflict of interest statement

One of the authors, Hironobu Minami, receives honoraria from Chugai Pharmaceutical Co., Ltd. and Kirin Pharma Co., Ltd.

One of the authors, Yasuo Ohashi, consults on design and data analysis of clinical trials for Chugai Pharmaceutical Co., Ltd.

One of the authors, Nagahiro Saijo, holds stock option for Takeda Pharmaceutical Co., Ltd.

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British Journal of Cancer (2008) 98, 693–696

www.bjcancer.com

Short Communication

Randomised phase II trial of irinotecan plus cisplatin *vs* irinotecan, cisplatin plus etoposide repeated every 3 weeks in patients with extensive-disease small-cell lung cancer

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Patients with previously untreated extensive-disease small-cell lung cancer were treated with irinotecan 60 mg m^{-2} on days 1 and 8 and cisplatin 60 mg m^{-2} on day 1 with ($n = 55$) or without ($n = 54$) etoposide 50 mg m^{-2} on days 1–3 with granulocyte colony-stimulating factor support repeated every 3 weeks for four cycles. The triplet regimen was too toxic to be considered for further studies.

British Journal of Cancer (2008) **98**, 693–696. doi:10.1038/sj.bjc.6604233 www.bjcancer.com
Published online 5 February 2008

Keywords: small-cell lung cancer; chemotherapy; irinotecan; etoposide; three drug combination

Small-cell lung cancer (SCLC), which accounts for approximately 14% of all malignant pulmonary tumours, is an aggressive malignancy with a propensity for rapid growth and early widespread metastases (Jackman and Johnson, 2005). A combination of cisplatin and etoposide (PE) has been the standard treatment, with response rates ranging from 60 to 90% and median survival times (MSTs) from 8 to 11 months in patients with extensive disease (ED)-SCLC (Fukuoka *et al*, 1991; Roth *et al*, 1992). A combination of irinotecan and cisplatin (IP) showed a significant survival benefit over the PE regimen (MST: 12.8 *vs* 9.4 months, $P = 0.002$) in a Japanese phase III trial for ED-SCLC (Noda *et al*, 2002), although another phase III trial comparing these regimens failed to show such a benefit (Hanna *et al*, 2006). Thus, irinotecan, cisplatin and etoposide are the current key agents in the treatment of SCLC. A phase II trial of the three agents, IPE combination, in patients with ED-SCLC showed a promising antitumour activity with a response rate of 77%, complete response (CR) rate of 17% and MST of 12.9 months (Sekine *et al*, 2003).

We have developed these IP and IPE regimens in a 4-week schedule where irinotecan was given on days 1, 8 and 15. The dose of irinotecan on day 15, however, was frequently omitted because of toxicity in both regimens (Noda *et al*, 2002; Sekine *et al*, 2003). The objectives of this study were to evaluate the toxicities and antitumour effects of IP and IPE regimens in the 3-week schedule in patients with ED-SCLC and to select the right arm for subsequent phase III trials.

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Received 15 October 2007; revised 2 January 2008; accepted 9 January 2008; published online 5 February 2008

PATIENTS AND METHODS

Patient selection

Patients were enrolled in this study if they met the following criteria: (1) a histological or cytological diagnosis of SCLC; (2) no prior treatment; (3) measurable disease; (4) ED, defined as having distant metastasis or contralateral hilar lymph node metastasis; (5) performance status of 0–2 on the Eastern Cooperative Oncology Group (ECOG) scale; (6) predicted life expectancy of 3 months or longer; (7) age between 20 and 70 years; (8) adequate organ function as documented by a white blood cell (WBC) count $\geqslant 4.0 \times 10^3\,\mu l^{-1}$, neutrophil count $\geqslant 2.0 \times 10^3\,\mu l^{-1}$, haemoglobin $\geqslant 9.5\,g\,dl^{-1}$, platelet count $\geqslant 100 \times 10^3\,\mu l^{-1}$, total serum bilirubin $\leqslant 1.5\,mg\,dl^{-1}$, hepatic transaminases $\leqslant 100\,IU\,l^{-1}$, serum creatinine $\leqslant 1.2\,mg\,dl^{-1}$, creatinine clearance $\geqslant 60\,ml\,min^{-1}$, and $PaO_2 \geqslant 60$ torr; and (9) providing written informed consent.

Patients were not eligible for the study if they had any of the following: (1) uncontrollable pleural, pericardial effusion or ascites; (2) symptomatic brain metastasis; (3) active infection; (4) contraindications for the use of irinotecan, including diarrhoea, ileus, interstitial pneumonitis and lung fibrosis; (5) synchronous active malignancies; (6) serious concomitant medical

illness, including severe heart disease, uncontrollable diabetes mellitus or hypertension; or (7) pregnancy or breast feeding.

Treatment schedule

In the IP arm, cisplatin, 60 mg m^{-2}, was administered intravenously over 60 min on day 1 and irinotecan, 60 mg m^{-2}, was administered intravenously over 90 min on days 1 and 8. Prophylactic granulocyte colony-stimulating factor (G-CSF) was not administered in this arm. In the IPE arm, cisplatin and irinotecan were administered at the same dose and schedule as the IP arm. In addition, etoposide, 50 mg m^{-2}, was administered intravenously over 60 min on days 1–3. Filgrastim 50 μg m^{-2} or lenograstim 2 μg kg^{-1} was subcutaneously injected prophylactically from day 5 to the day when the WBC count exceeded 10.0×10^3 μl^{-1}. Hydration (2500 ml) and a $5HT_3$ antagonist were given on day 1, followed by an additional infusion if indicated in both arms. These treatments were repeated every 3 weeks for a total of four cycles.

Toxicity assessment, treatment modification and response evaluation

Toxicity was graded according to the NCI Common Toxicity Criteria version 2.0.

Doses of anticancer agents in the following cycles were modified according to toxicity in the same manner in both arms. Objective tumour response was evaluated according to the Response Evaluation Criteria in Solid Tumors (RECIST) (Therasse *et al*, 2000).

Study design, data management and statistical considerations

This study was designed as a multi-institutional, prospective randomised phase II trial. This study was registered on 6 September 2005 in the University hospital Medical Information Network (UMIN) Clinical Trials Registry in Japan (http://www.umin.ac.jp/ctr/index.htm), which is acceptable to the International Committee of Medical Journal Editors (ICMJE) (http://www.icmje.org/faq.pdf). The protocol and consent form were approved by the Institutional Review Board of each institution. Patient registration and randomisation were conducted at the Registration Center. No stratification for randomisation was performed in this study. The sample size was calculated according to the selection design for pilot studies based on survival (Liu *et al*, 1993). Assuming that (1) the survival curve was exponential for survivals; (2) the MST of the worse arm was 12 months and that of the better arm was 12 months × 1.4; (3) the correct selection probability was 90%; and (4) additional follow-up in years after the end of accrual was 1 year, the estimated required number of patients was 51 for each arm. Accordingly, 55 patients for each arm and their accrual period of 24 months were planned for this study.

The dose intensity of each drug was calculated for each patient using the following formula as previously described:

$$\text{The dose intensity (mg m}^{-2}\text{ week}^{-1}\text{)} = \frac{\text{Total milligrams of a drug in all cycles per body surface area}}{\text{Total days of therapy}/7}$$

where total days of therapy is the number of days from day 1 of cycle 1 to day 1 of the last cycle plus 21 days for both arms (Hryniuk and Goodyear, 1990).

Differences in the reason for termination of the treatment and the frequencies of grade 3–4 toxicities were assessed by χ tests. Survival was measured as the date of randomisation to the date of death from any cause or the date of the most recent follow-up for overall survival and to the date of disease progression or the date of death for progression-free survival (PFS). The survival of the arms was estimated by the Kaplan–Meier method and compared in an exploratory manner with log-rank tests (Armitage *et al*, 2002).

RESULTS

Patient characteristics

From March 2003 to May 2005, 55 patients were randomised to IP and 55 patients to IPE. One patient in the IP arm was excluded because the patient was ineligible and did not receive the study treatment. The remaining 109 patients were included in the analyses of toxicity, tumour response and patient survival. There were no differences between the two arms in any demographic characteristics listed (Table 1).

Treatment delivery

Treatment was well tolerated with respect to the number of cycles delivered in both arms (Table 2). Among reasons for termination of the treatment, disease progression was noted in nine (17%)

Table 1 Patient characteristics

	IP ($n=54$)	IPE ($n=55$)
Sex		
Female	11	8
Male	43	47
Age (years)		
Median (range)	63 (42–70)	62 (48–70)
PS		
0	11	12
1	42	41
2	1	2
Weight loss		
0–4%	38	43
5–9%	10	10
⩾10%	6	2

Table 2 Treatment delivery

	IP ($n=54$) No. (%)	IPE ($n=55$) No. (%)
Number of cycles delivered		
6[a]	—	1 (2)
4	41 (76)	36 (65)
3	6 (11)	6 (11)
2	3 (6)	6 (11)
1	4 (7)	6 (11)
Reasons for termination of the treatment[†]		
Completion	40 (74)	35 (64)
Disease progression	9 (17)	2 (4)
Toxicity	3 (6)	13 (24)
Patient refusal	2 (4)	4 (7)
Others	0 (0)	1 (2)
Total number of cycles delivered	192 (100)	186 (100)
Total number of omission on day 8	35 (18)	37 (17)
Total number of cycles with dose reduction	28 (15)	31 (17)

[†]$P=0.013$ by χ^2 test. [a]Protocol violation.

patients in the IP arm and in two (4%) patients in the IPE arm, whereas toxicity was noted in three (6%) patients in the IP arm and 13 (24%) patients in the IPE arm ($P=0.013$) (Table 2). The dose of irinotecan on day 8 was omitted in 35 (18%) cycles in the IP arm and 37 (17%) cycles in the IPE arm (Table 2). The total dose and dose intensity of cisplatin and etoposide were similar between the IP and IPE arms in the present study (Table 3).

Toxicity

The myelotoxicity was more severe in the IPE arm (Table 4). Grade 3 febrile neutropaenia was noted in 5 (9%) patients in the IP arm and 17 (31%) patients in the IPE arm ($P=0.005$). Packed red blood cells were transfused in 4 (7%) patients in the IP regimen and 14 (26%) patients in the IPE regimen ($P=0.011$). Platelet concentrates were needed in none in the IP regimen and 2 (4%) patients in the IPE regimen ($P=0.16$). Grade 3–4 diarrhoea was observed in 8 (15%) patients in the IP arm and 13 (24%) patients in the IPE arm ($P=0.262$). Grade 3–4 fatigue was more common in the IPE arm with marginal significance (2 *vs* 11%, $P=0.054$). The severity of other non-haematological toxicities did not differ significantly between the arms. No treatment-related death was observed in this study.

Table 3 Total dose and dose intensity

	3-week regimens in this study		4-week regimen*
	IP ($n=54$) Median (range)	IPE ($n=55$) Median (range)	IPE ($n=30$) Median (range)
Total dose ($mg\,m^{-2}$)			
Cisplatin	240 (60–240)	240 (60–360)	240 (60–240)
Irinotecan	420 (60–480)	390 (60–720)	563 (60–720)
Etoposide	0	600 (150–900)	600 (150–600)
Dose intensity ($mg\,m^{-2}\,week^{-1}$)			
Cisplatin	19 (14–25)	20 (16–34)	15 (12–15)
Innotecan	33 (14–40)	35 (15–55)	35 (19–45)
Etoposide	0	48 (34–68)	37 (28–38)

*From our previous study (Sekine *et al*, 2003).

Table 4 Grade 3–4 toxicities

	IP ($n=54$)			IPE ($n=55$)		
	Grade 3	4	3+4 (%)	Grade 3	4	3+4 (%)
Leukocytopaenia	9	1	10 (19)	18	11	29 (53)*
Neutropaenia	17	11	28 (52)	24	28	52 (95)*
Anaemia	18	0	18 (25)	16	9	25 (45)
Thrombocytopaenia	2	0	2 (4)	13	0	13 (13)†
Febrile neutropaenia	5	0	5 (9)	17	0	7 (13)
Diarrhoea	8	0	8 (15)	11	2	13 (24)
Vomiting	4	0	4 (7)	3	0	3 (5)
Fatigue	1	0	1 (2)	5	1	6 (11)†
Hyponatraemia	9	3	12 (22)	11	2	13 (24)
AST elevation	0	0	0 (0)	3	0	3 (5)
CRN elevation	1	0	1 (2)	0	0	0 (0)

*$P<0.001$; †$P<0.01$; and ‡$P=0.054$ by χ^2 test.

Response, treatment after recurrence and survival

Four CRs and 37 partial responses (PRs) were obtained in the IP arm, resulting in the overall response rate of 76 with 95% confidence interval (CI) of 65–87%, whereas six CRs and 42 PRs were obtained in the IPE arm, and the overall response rate was 87% with a 95% CI of 79–96% ($P=0.126$). Median PFS was 4.8 months (95% CI, 4.0–5.6) in the IP and 5.4 months (95% CI, 4.8–6.0) in the IPE arm ($P=0.049$) (Figure 1A). After recurrence, 22 (44%) patients in the IP arm and 8 (16%) patients in the IPE arm received etoposide-containing chemotherapy. The MST and 1-year survival rate were 12.4 months (95% CI, 9.7–15.1) and 54.8% (95% CI, 41.4–68.2%) in the IP and 13.7 months (95% CI, 11.9–15.5) and 61.5% (95% CI, 48.6–74.4%) in the IPE arm ($P=0.52$), respectively (Figure 1B).

DISCUSSION

This study showed that the IPE regimen in a 3-week schedule with CSF support produced a promising response rate, PFS and overall survival. Haematological toxicity in the IPE arm, however, was very severe in spite of the G-CSF support with the grade 3 febrile neutropaenia noted in 31% of patients.

In comparison between the 3-week IPE regimen in this study and the 4-week IPE regimen in the previous study, the delivery of cisplatin and etoposide was improved in the 3-week IPE regimen when compared with the 4-week IPE regimen at the cost of the irinotecan total dose. The response rate and MST were 87% and 13.7 months, respectively, in the 3-week IPE regimen and 77% and 12.9 months in the previous 4-week schedule, and toxicity profiles were comparable to each other (Sekine *et al*, 2003).

The MST of 12.4 months in the IP arm in this study was comparable to that of the previous phase III study, with an MST of 12.8 months (Noda *et al*, 2002). Thus, this study showed the reproducible excellent survival outcome of patients with ED-SCLC who were treated with the IP combination. In contrast, a recent American phase III study of the PE regimen *vs* IP regimen failed to show the superiority of the IP regimen to the PE regimen; the MST

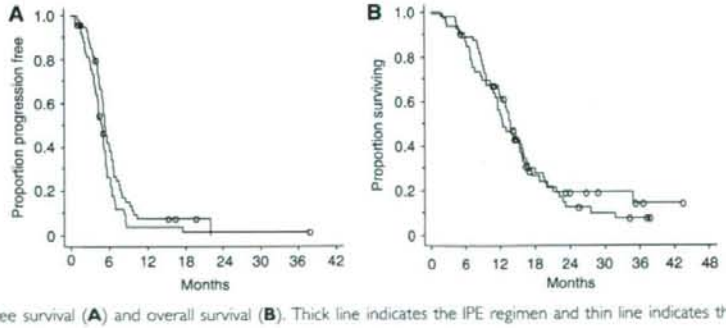

Figure 1 Progression-free survival (**A**) and overall survival (**B**). Thick line indicates the IPE regimen and thin line indicates the IP regimen.

for the PE regimen was 10.2 months and that for the IP regimen was 9.3 months (Hanna *et al*, 2006). The discrepancy between the Japanese and American trials may be explained by the different cisplatin dose schedules; cisplatin was delivered at a dose of 60 mg m^{-2} on day 1 every 3 or 4 weeks in the Japanese trials, whereas cisplatin was delivered at a dose of 30 mg m^{-2} on days 1 and 8 every 3 weeks in the American one. A platinum agent administered at divided doses was associated with poor survival in patients with ED-SCLC in our previous randomised phase II study (Sekine *et al*, 2003).

The issue of adding further agents to the standard doublet regimen has been investigated in patients with ED-SCLC. The addition of ifosfamide or cyclophosphamide and epirubicin to the cisplatin and etoposide combination produced a slight survival benefit, but at the expense of greater toxicity (Loehrer *et al*, 1995; Pujol *et al*, 2001). Phase III trials of cisplatin and etoposide with or without paclitaxel showed unacceptable toxicity with 6–13% toxic deaths in the paclitaxel-containing arm (Mavroudis *et al*, 2001; Niell *et al*, 2005). The results in these studies and the current study are consistent in the increased toxicity despite the G-CSF support and no definite survival benefit in the three or four drug combinations over the standard doublet in patients with ED-SCLC.

In conclusion, the IPE regimen was marginally more effective than the IP regimen, but was too toxic despite the administration of prophylactic G-CSF.

ACKNOWLEDGEMENTS

This study was supported, in part, by Grants-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan. We thank the following doctors for their care for patients and valuable suggestion and comments on this study: Takahiko Sugiura, Aichi Cancer Center; Yoshinobu Ohsaki, Asahikawa Medical College; Shinzo Kudoh, Osaka City University Medical School; Makoto Nishio, Cancer Institute Hospital; Hiroshi Chiba, Kumamoto Community Medical Center; Koichi Minato, Gunma Prefectural Cancer Center; Naoyuki Nogami, Shikoku Cancer Center; Hiroshi Ariyoshi, Aichi Cancer Center Aichi Hospital; Takamune Sugiura, Rinku General Medical Center; Akira Yokoyama, Niigata Cancer Center Hospital; and Koshiro Watanabe, Yokohama Municipal Citizen's Hospital. We also thank Fumiko Koh, Yuko Yabe and Mika Nagai for preparation of the paper.

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Prospective Study of the Accuracy of *EGFR* Mutational Analysis by High-Resolution Melting Analysis in Small Samples Obtained from Patients with Non–Small Cell Lung Cancer

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Abstract **Purpose:** Epidermal growth factor receptor (*EGFR*) mutations, especially in-frame deletions in exon 19 (DEL) and a point mutation in exon 21 (L858R), predict gefitinib sensitivity in patients with non–small cell lung cancer (NSCLC). In this study, we verified the accuracy of *EGFR* mutation analysis in small samples by high-resolution melting analysis (HRMA), which is a rapid method using PCR amplification with a dye to analyze the melting curves in NSCLC.

Experimental Design: We designed a prospective study to compare the sensitivity and specificity of HRMA and DNA sequencing with laser capture microdissection. Eligible patients with lung lesions were screened by bronchoscopy or percutaneous needle biopsy to histologically confirm the diagnosis, followed by surgical resection of the NSCLC. Small diagnostic specimens were analyzed for *EGFR* mutations by HRMA, and the surgically resected specimens were examined for mutations by HRMA and DNA sequencing.

Results: The analyses for *EGFR* mutations were conducted in 52 eligible cases of the 92 enrolled patients. *EGFR* mutations were detected in 18 (34.6%) patients. The results of HRMA from surgically resected specimens as well as DNA sequencing revealed 100% sensitivity and specificity. On the other hand, the sensitivity and specificity of HRMA from the small diagnostic specimens were 83.3% and 100%, respectively.

Conclusions: In this study, we showed that HRMA is a highly accurate method for detecting DEL and L858R mutations in patients with NSCLC, although it is necessary to consider the identification of patients with a false-negative result when the analysis is conducted using small samples.

Somatic mutations in the kinase domain of the epidermal growth factor receptor (*EGFR*) have been reported in patients with non–small cell lung cancer (NSCLC; refs. 1–3). Although many types of *EGFR* mutations have been identified, they seem to be concentrated in exons 18 to 21 of *EGFR*; ~85% to 90% of *EGFR*-mutant patients have mutations in two hotspots: a short in-frame deletion in exon 19 (DEL) and a point mutation at codon 858 in exon 21 (L858R; ref. 4). Several studies have revealed that *EGFR* mutations are strongly associated with the tumor response and clinical outcome in patients with NSCLC receiving treatment with EGFR tyrosine kinase inhibitors, such as gefitinib (Iressa, AstraZeneca; refs. 5–7). The mutational status of *EGFR*, especially the presence/absence of DEL and L858R, is a strong predictor of the sensitivity to EGFR tyrosine kinase inhibitor, and the detection of *EGFR* mutations is useful for decision-making by both patients and physicians (4, 8). Recently, a laboratory test for *EGFR* mutations has become clinically available for guiding treatment decisions.

Until now, screening for these mutations has most commonly been conducted using DNA sequencing methods. In our previous study, we used methanol-fixed, paraffin-embedded surgical specimens and performed direct sequencing and pyrosequencing with laser capture microdissection (LCM) to ensure high-quality genetic analysis of archived tissues (5, 9). However, these approaches are not useful in clinical practice for two reasons. First, although the sequencing methods require a high ratio of tumor-to-normal tissue DNA for optimal results, the diagnostic specimens obtained from cases of advanced NSCLC may contain only a small amount of tumor cells and

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Received 12/19/07; revised 4/19/08; accepted 4/21/08.

Grant support: Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, a Health and Labour Science Research grant from the Ministry of Health, Labour and Welfare, Japan, and a grant-in-aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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doi:10.1158/1078-0432.CCR-07-5207

are highly contaminated with normal cells. Secondly, *EGFR* mutation analysis based on DNA sequencing requires special instruments and is also time-consuming and expensive. Therefore, some simple and highly sensitive nonsequencing methods to detect *EGFR* mutations have been reported (10–22). However, the accuracy of these methods for clinical use have not been assessed in prospective studies.

High-resolution melting analysis (HRMA) using the LCGreen I (Idaho Technology) dye was introduced as an easy, quick, and inexpensive method for the screening of mutations (23), and we established and validated the HRMA method to detect DEL and L858R mutations in cases of NSCLC (9, 10). Our cell line study revealed that DEL and L858R mutations could be detected using HRMA in the presence of 10% and 0.1% of mutant cells, respectively (10). We also showed that the two major mutations could be identified by HRMA retrospectively using DNA extracted from archived Papanicolaou-stained cytologic slides with 88% sensitivity and 100% specificity (9). Furthermore, it was shown that among patients treated with gefitinib, the response rate (78% versus 8%), time-to-progression (median, 9.2 versus 1.6 months), and overall survival (median, 21.7 versus 8.7 months) were significantly better in patients with *EGFR* mutations than with wild-type *EGFR* ($P < 0.001$), as detected by HRMA (9). These results suggest that this easy, quick, and inexpensive method which was done using diagnostic small samples of advanced NSCLC tumors is one of the most useful and precise methods to detect *EGFR* mutations in clinical practice.

In this study, we designed a prospective study to detect two major *EGFR* mutations by HRMA using small diagnostic cytologic or biopsy specimens and surgically resected specimens, and the results were compared with the results of DNA sequencing methods combined with LCM, which we consider as the "gold standard" for such detection, applied to methanol-fixed, paraffin-embedded surgically resected specimens. We evaluated the diagnostic sensitivity, specificity, predictive values, and accuracy of the detection of *EGFR* mutations using HRMA and revealed that this method is feasible for clinical use to detect *EGFR* mutations in small samples obtained from patients with NSCLC.

Patients and Methods

Patients and materials. Patients with lung lesions, which were suspected clinically to be operable NSCLC, were enrolled in this prospective study. The patients were scheduled for bronchoscopy or percutaneous needle biopsy to establish the histologic diagnosis, and informed consent was obtained from each of the patients prior to these diagnostic procedures. Thereafter, the patients diagnosed with NSCLC underwent lung surgery at our hospital. In this study, mutational analysis of *EGFR* was done by HRMA or DNA sequencing methods combined with LCM in all the patients in which both the preoperatively obtained diagnostic specimens and the resected specimens were histologically confirmed by a certified pathologist to contain malignant cells.

Based on a protocol approved by the Institutional Review Board of the National Cancer Center, we did mutational analyses of *EGFR* to detect DEL and L858R in the eligible patients. The Papanicolaou-stained cytologic slides ($n = 35$), formalin-fixed, paraffin-embedded transbronchial or percutaneous needle biopsy specimens ($n = 34$), and methanol-fixed, paraffin-embedded surgically resected specimens subjected to LCM using a PixCell II LCM system (Arcturus Engineering, Inc.; $n = 52$) were collected prospectively. DNA was extracted using the QIAamp DNA Micro Kit (Qiagen), as described in our previous report (10).

HRMA. PCR was done to amplify exons 19 or 21 of *EGFR* using LCGreen I (Idaho Technology) on a LightCycler (Roche Diagnostics) and primers designed as previously described (10). If the first PCR products were not available for the mutational analyses of the melting curves, we did a second PCR using the same primers. These PCR products were denatured at 95°C for 10 min and cooled to 40°C to promote the formation of heteroduplexes. The LightCycler capillary was transferred to an HR-1 (Idaho Technology), an HRMA instrument, and heated at a transition rate of 0.3°C/s. Data were acquired and analyzed using the accompanying software (Idaho Technology). After normalization and temperature-adjustment steps, melting curve shapes from 78.5°C to 85.5°C were compared between the tumor samples and control samples. Human Genomic DNA (Roche Diagnostics) was used as the negative control sample with wild-type *EGFR*. Samples revealing skewed or left-shifted curves as compared with the control samples were judged to have mutations without positive controls (9, 10). All analyses were done in a blinded fashion by two researchers (T. Fukui and T. Takano). After independent evaluation by the two researchers, the final judgment was arrived at by consensus after joint viewing of the melting curves from both.

DNA sequencing methods with LCM. In our previous study, we did a direct sequencing or pyrosequencing of *EGFR* in patients with recurrent NSCLC after primary surgery (5). Based on the results of our previous study, we consider direct sequencing with LCM for the detection of DEL and pyrosequencing with LCM for the detection of L858R as the gold standard in relation to *EGFR* mutational analysis. DNA was extracted from methanol-fixed, paraffin-embedded surgical specimens by LCM, according to a previously described method (24). Direct sequencing of the PCR products for DEL was done using ABI PRISM3700 and 3100 DNA sequencers (Applied Biosystems). Pyrosequencing to analyze L858R was done using Pyrosequencing PSQ 96MA (Pyrosequencing; refs. 5, 25). The *EGFR* mutational analysis using DNA sequencing methods was done in a blinded fashion by a researcher (H. Sakamoto) according to a previously described method (5), and then compared with the corresponding results obtained using HRMA.

Statistical analysis. The primary end point of this study was the sensitivity and specificity of the results obtained using HRMA as compared with those of the results obtained using DNA sequencing with LCM. The sample size was calculated using a statistical power level of 0.80 and two-sided α level of 0.1 on the basis of an estimated sensitivity of at least 0.80 and an expected value of 0.95 for HRMA, a minimum of 20 patients with *EGFR*-mutated tumors were required. Because the percentage of NSCLC patients with *EGFR* mutations was expected to be 40% in this study population composed of only Japanese, approximately 50 patients with NSCLC were needed. Therefore, considering a specificity of at least 0.80 and the expected value of 0.95 for HRMA, 30 patients with wild-type tumors showed a statistical power level of 0.90 using a two-sided α level of 0.1.

The associations between mutational status and patient characteristics were assessed by a χ^2 test using the SPSS statistical package (SPSS version 11.0 for Windows; SPCC, Inc.).

Results

Patient characteristics. From December 2005 to December 2006, 92 patients with clinically suspected operable NSCLC were enrolled in this study. The following diagnostic procedures were done preoperatively in 90 patients: bronchoscopy ($n = 57$), percutaneous needle biopsy ($n = 27$), or bronchoscopy followed by percutaneous needle biopsy ($n = 6$). The patient characteristics are shown in Table 1. All the patients were Japanese. Among the patients, a definitive diagnosis was established in 85 patients by bronchoscopy in 43 of 59 patients

Table 1. Patient characteristics

(A) Characteristics of all the patients enrolled in this study (n = 92)

	All (n = 92)	BF (n = 64)	PNB (n = 34)*
Age, year, median (range)	64 (34-84)	64 (38-84)	62 (41-79)
Gender (male/female)	58/34	41/23	23/11
Smoking history (N/F/C)	29/30/33	23/19/22	7/14/13
Tumor size, mm, average (range)	27.2 (10.2-73.4)	28.3 (13.8-56.6)	24.5 (10.2-73.4)
Accuracy of the diagnostic procedure (%)	66/85 (77.6)	43/59 (72.9)	25/31 (80.6)
Accuracy of the cytologic slides (%)	54/85 (63.5)	31/59 (52.5)	23/30 (76.7)
Accuracy of the biopsy specimens (%)	42/62 (67.7)	35/54 (64.8)	7/9 (77.8)

(B) Characteristics of the patients who underwent analysis of the EGFR mutations in this study (n = 52)

	All (n = 52)	BF (n = 38)	PNB (n = 17)†
Age, year, median (range)	64.5 (34-84)	64.5 (34-84)	64 (47-78)
Gender (male/female)	36/16	25/13	14/3
Smoking history (N/F/C)	16/17/19	15/11/12	1/7/9
Tumor size, mm, average (range)	27.0 (11.0-56.6)	28.3 (20.6-56.6)	24.1 (11.0-48.8)
Postoperative diagnosis (Ad/Sq/LCNEC)	45/5/2	34/4/0	12/3/2
Pathologic stage (IA/B, IIA/B, IIIA/B)	19/13, 3/5, 9/2	15/8, 3/2, 8/2	7/5, 0/2, 3/0

NOTE: Never smokers were defined as patients who had never smoked, former smokers were defined as patients who had stopped smoking at least 1 y before the diagnosis, and current smokers were defined as patients who were still smoking at the time of the diagnosis.
Abbreviations: BF, bronchoscopy; PNB, percutaneous needle biopsy; N, never smoker; F, former smoker; C, current smoker; Ad, adenocarcinoma; Sq, squamous cell carcinoma; LCNEC, large cell neuroendocrine carcinoma.
*Including six patients in whom bronchoscopy was done followed by percutaneous needle biopsy.
†Including three in whom bronchoscopy was done followed by percutaneous needle biopsy.

(72.9%) and by percutaneous needle biopsy in 25 of 31 patients (80.6%); in 18 of the 85 (21.2%) patients, the histologic diagnosis could not be established preoperatively by bronchoscopy and/or percutaneous needle biopsy, the patients underwent lung surgery for suspicious malignant lung lesion, and examination of the resected specimens revealed the diagnosis of primary NSCLC in 17 and malignant lymphoma in 1 of the 18 patients. Among the 76 patients diagnosed to have primary NSCLC, 73 consented to undergo lung surgery. Finally, the analysis for *EGFR* mutations was done on 52 patients with a definitive histologic diagnosis of primary NSCLC, established both by examination of the preoperative diagnostic specimens and of the corresponding resected specimens (Fig. 1).

Mutational analyses. We analyzed 35 cytologic samples and 34 biopsy specimens obtained from 52 patients by HRMA, and

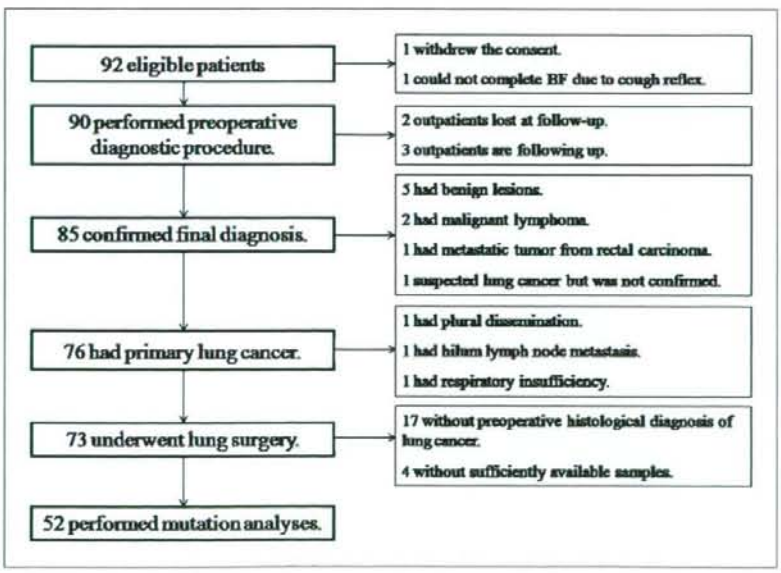

Fig. 1. Flowchart of the analyses conducted in 92 enrolled patients with lung tumors in this study.

Table 2. EGFR mutation status among the patient subgroups

	n	*EGFR* mutations*				*P*
		DEL	L858R	Total	%	
Total	52	5	13	18	34.6	—
Gender						
Women	16	2	9	11	68.8	0.001
Men	36	3	4	7	19.4	
Smoking history						
Never	16	3	8	11	68.8	0.001 †
Former	17	2	4	6	35.3	
Current	19	0	1	1	5.3	
Histology						
Ad	44	5	13	18	100	0.025 ‡
Sq	6	0	0	0	0	
LCNEC	2	0	0	0	0	

Abbreviations: DEL, deletional mutations in exon 19; L858R, a point mutation at codon 858 in exon 21; Ad, adenocarcinoma; Sq, squamous cell carcinoma; LCNEC, large cell neuroendocrine carcinoma.
*The EGFR mutations were analyzed by DNA sequencing with LCM.
†Comparison between never smokers and others.
‡Comparison between adenocarcinoma and others.

did both HRMA and DNA sequencing with LCM in the 52 resected specimens corresponding to the 52 patients. Among the 52 surgically resected specimens analyzed by DNA sequencing with LCM, there were 18 (34.6%) samples with *EGFR* mutations, 5 with DEL mutations, and 13 with L858R mutations. As shown in Table 2, the *EGFR* mutations were detected more frequently in women, never-smokers, and patients with a histologic diagnosis of adenocarcinoma. All results from HRMA done in a blinded fashion by two researchers (T. Fukui and T. Takano) were consistent.

HRMA could be conducted using small diagnostic samples from all 52 patients, although the analysis needed to be conducted using the second PCR product in 15 cases. In the analysis of exon 19, 5 samples revealed different curves from the control and 47 samples revealed almost the same curves as the control; therefore, we judged that the five former patients had DEL mutations (Fig. 2A). In the analysis of exon 21, 10 samples revealed a left-shift from the control and 42 samples revealed almost the same curves as the control; therefore, we judged that the 10 former patients had L858R mutations

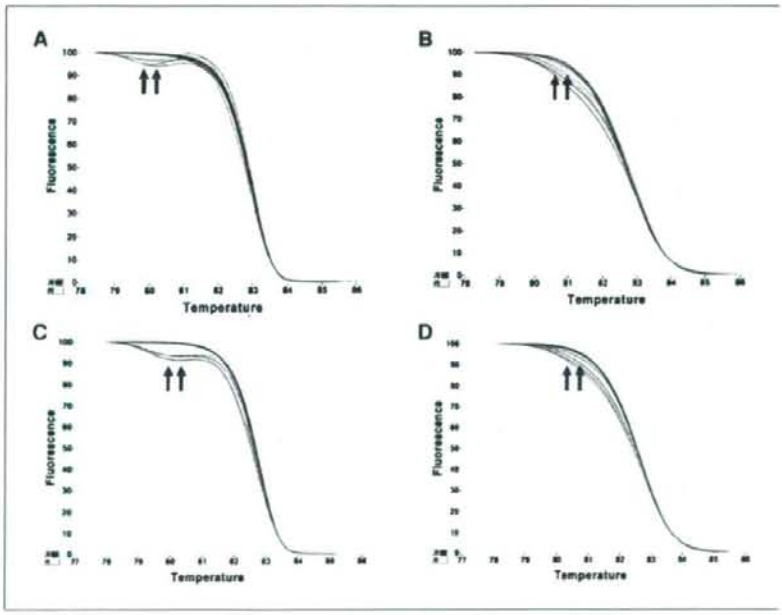

Fig. 2. Adjusted melting curves obtained by HRMA of the samples in this study to detect *EGFR* mutations (↑), in-frame deletions in exon 19 (*A*, small samples; *C*, resected specimens) and a point mutation in exon 21 (*B*, small samples; *D*, resected specimens). Each sample that revealed a skewed or left-shifted curve from those of the control sample was judged to have a mutation.

Table 3. Results of the EGFR mutation analyses in patients with *EGFR* mutation–positive tumors

No. of patients	Small samples	Surgically resected specimens	
	HRMA	HRMA	Sequence with LCM
13	DEL	DEL	DEL1*
26	DEL	DEL	DEL1*
32	DEL	DEL	DEL2†
40	DEL	DEL	DEL2†
47	DEL	DEL	DEL1*
5	L858R‡	L858R	L858R
6	Wild-type	L858R	L858R
12	L858R	L858R	L858R
18	L858R	L858R	L858R
21	L858R	L858R	L858R
23	L858R‡	L858R	L858R
25	Wild-type	L858R	L858R
27	L858R‡	L858R	L858R
28	L858R	L858R	L858R
31	Wild-type‡	L858R	L858R
41	L858R‡	L858R	L858R
53	L858R	L858R	L858R
54	L858R‡	L858R	L858R

Abbreviations: DEL, deletional mutations in exon 19; L858R, a point mutation at codon 858 in exon 21.
*DEL1: del E746-A750 (del 2235-2249).
†DEL2: del E746-A750 (del 2236-2250).
‡The analyses by HRMA were done using second PCR products.

(Fig. 2B). All the 52 surgically resected specimens analyzed by DNA sequencing with LCM could also be analyzed by HRMA, although the analysis needed to be conducted using the second PCR product in two cases. DEL mutations were detected in 5 patients (Fig. 2C) and L858R mutations in 13 patients (Fig. 2D) among the 52 patients. Of the 52 specimens, both cytologic slides and biopsy specimens were analyzed in 17 cases. Discrepant results were obtained by HRMA in one of the cases, with L858R mutation being detected in the cytologic slides but not in the biopsy specimens. We included this patient in the population with L858R mutations.

The results of HRMA were consistent with the results of DNA sequencing with LCM in all the surgically resected specimens analyzed by the two methods. On the other hand, HRMA using small diagnostic specimens revealed the wild-type curve in three cases, although analysis of the corresponding surgically resected specimens analyzed by pyrosequencing with LCM revealed the L858R mutation (Table 3). Thus, the results for these samples obtained by HRMA were considered as false-negative results. Neither method of analysis yielded any false-positive cases. The results of the *EGFR* mutational analysis by HRMA compared with DNA sequencing with LCM using surgically resected specimens were shown in Table 4. The sensitivity, specificity, and accuracy of HRMA using small diagnostic specimens were 83.3%, 100%, and 94.2%, respectively. Using surgically resected specimens, those of HRMA were all 100%.

Discussion

In this prospective study, we showed the high accuracy of the HRMA method for detecting two major *EGFR* mutations, DEL and L858R in patients with NSCLC. The accuracy of HRMA was clearly equal to that of DNA sequencing with LCM for the detection of mutations in surgically resected specimens. On the other hand, the sensitivity and specificity of HRMA were 83.3% (90% confidence interval: 68.9-97.7%) and 100%, respectively, when the small diagnostic samples were analyzed. Although the sensitivity of HRMA which was estimated to be at least 0.80 did not reach statistical significance, we consider HRMA as one of the available methods for the detection of *EGFR* mutations in clinical practice because the specificity, which is important for clinical decision-making, of HRMA was 100% and the *EGFR* mutation rate was less than the expected 40% to secure enough statistical power in this study.

Recently, many researchers reported establishing simple and highly sensitive nonsequencing methods for detecting *EGFR* mutations using small tumor samples (11–22), and the results of several mutation analyses were correlated with the clinical outcome of EGFR tyrosine kinase inhibitor treatment (17–19). Using serial dilution studies, some researchers have reported methods that are able to detect mutations in samples containing ~0.1% to 10% mutated DNA (13, 14, 16–18, 20–22), as opposed to direct DNA sequencing which requires the presence of at least 10% to 30% of mutated DNA in the samples (18, 20). Additionally, several novel methods offered higher sensitivity and specificity than DNA sequencing to identify the mutations in clinical samples. But almost none of the methods were validated for diagnostic accuracy in a prospective study, and we therefore consider these methods to still be unsuitable for routine clinical examination. Although these nonsequencing methods were not mutually compared, based on our previous results of retrospectively verifying the accuracy of HRMA (9, 10), we thought to develop in this prospective study an easy, quick (PCR for ~1 hour and HRMA for 2 to 3 minutes), and inexpensive (at a running cost per sample of approximately $7.50, which consisted of $5.50 for the DNA extract and less than $2.00 for PCR using LCGreen I dye) method that might be useful in clinical practice with a great advantage over DNA sequencing, which requires the

Table 4. Comparison of the sensitivity, specificity, predictive values, and accuracy between HRMA and DNA sequencing with LCM (n = 52)

	HRMA using small samples	HRMA using surgically resected specimens
True-positive	15	18
True-negative	34	34
False-positive	0	0
False-negative	3	0
Sensitivity	83.3 (68.9-97.8)	100
Specificity	100	100
NPV	91.9 (84.5-99.3)	100
PPV	100	100
Accuracy	94.2 (88.9-99.5)	100

NOTE: The results of these analyses were compared with those of DNA sequencing with LCM (used as the gold standard in this study). Data are presented as % or % (90% confidence interval). True-positive is defined as the correct detection of DEL in exon 19 or L858R in exon 21.
Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

Table 5. Results of HRMA using cytologic slides or biopsy specimens

	Cytologic slides (n = 35)		Biopsy specimens (n = 34)	
	First PCR	Second PCR	First PCR	Second PCR
Successfully analyzed	29 (83.0%)	35 (100%)	5 (15.0%)	34 (100%)
True-positive	7	11	1	10
True-negative	19	21	4	22
True-negative	0	0	0	0
False-positive	3	3	0	2
Sensitivity	70.0% (7/10)	78.6% (11/14)	100% (1/1)	83.3% (10/12)
Specificity	100% (19/19)	100% (21/21)	100% (4/4)	100% (22/22)
NPV	100% (7/7)	100% (11/11)	100% (1/1)	100% (10/10)
PPV	86.4% (19/22)	87.5% (21/24)	100% (4/4)	91.2% (22/24)
Accuracy	89.7% (26/29)	91.4% (32/35)	100% (5/5)	94.1% (32/34)

NOTE: The results of these analyses were compared with those of DNA sequencing with LCM (used as the gold standard in this study). True-positive is defined as the correct detection of DEL in exon 19 or L858R in exon 21.
Abbreviations: NPV, negative predictive value; PPV, positive predictive value.

extraction of high-quality DNA from an adequate amount of pure tumor cells, takes a long time, and is expensive.

In this study, the three patients with L858R detected by DNA pyrosequencing with LCM using the surgically resected specimens were labeled as having the wild-type *EGFR* in the analyses conducted using the small diagnostic samples. With regard to these false-negative results, the following three points need to be discussed: first, our previous study, conducted using human lung cancer cell lines, showed that HRMA can detect the mutations, even when samples contain only a small proportion (DEL, 10%; L858R, 0.1%) of mutant cells (10). In this study, the sensitivity of HRMA was also considered to be sufficiently high for the detection of *EGFR* mutations, especially L858R, even when the analysis was conducted using small samples after evaluation by a clinical pathologist to determine if they contained benign or malignant cells. Thus, we assume a higher accuracy of HRMA when using small samples in clinical practice. Although it still needs to be comparatively analyzed with the previously reported non-sequencing methods, HRMA can be considered as one of the sensitive methods available for the detection to *EGFR* mutations in clinical practice.

Second, high-quality DNA should be preserved in clinical samples to obtain the best results. There always remains the risk of an indeterminate or false-negative result because the DNA might have degenerated during sampling or during the preservation of clinical samples. In a comparison between the cytologic slides and biopsy specimens, better results were obtained from analyses of the first PCR products using the cytologic slides rather than the results obtained using the biopsy specimens, regardless of the amount of tumor cells examined (Table 5). This could probably be explained by the differences in the method of sample fixation between the two types of specimens. It has been suggested by a previous report that DNA is preserved better in the methanol-fixed samples than in the formalin-fixed specimens (26). Therefore, if we used methanol for specimen fixation of biopsy specimens, the results of HRMA using the first PCR products from small biopsy samples might improve. Hereafter, we propose to perform mutation analyses using methanol-fixed specimens, if possible.

Finally, we need to consider the possibility of intratumoral heterogeneity, and small diagnostic samples and surgically resected specimens may each represent overlapping but different populations of these tumor cells. A lack of association in the immunohistochemical expression profile between lung biopsy specimens and the corresponding resected tumor specimens has been reported (27). Furthermore, intratumoral heterogeneity was shown not only in terms of microheterogeneity of the tumor cell phenotype (28), but in terms of genetic heterogeneity in cancer (29, 30). In particular, the intratumoral genetic heterogeneity of *EGFR* mutations may explain the variable clinical response of NSCLC to gefitinib. It is also possible that the small diagnostic samples contain only wild-type cells, even if the tumor, overall, shows mutations, because the small samples yield only small part of the tumor. It is always necessary to consider the possibility of a false-negative result of mutational analyses conducted using the small samples.

In the current prospective study, we showed the feasibility and high accuracy of using HRMA for detecting two major *EGFR* mutations, DEL and L858R, in patients with NSCLC. Although HRMA showed high accuracy, the possibility of indeterminate or false-negative results, and because of the sensitivity of this method, the quality of DNA preservation in the clinical samples or intratumoral genetic heterogeneity, must be borne in mind to a certain extent when this analysis is conducted using small diagnostic samples. Therefore, HRMA should not be used to exclude patients from EGFR tyrosine kinase inhibitor treatment on the basis of the negative results only. Based on the results of this prospective study, we suggest that this method is very useful for clinical decision-making, especially in patients with a positive result.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Kiyoaki Nomoto, Karin Yokozawa, Chizu Kina, Sachiko Miura, Misuzu Okuyama, Sachiyo Mimaki, and Chie Hirama for their technical support.

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thus influenced the results including those assessed (overall survival) and not assessed (disease free survival and time to progression). Future studies on the efficacy of docetaxel as a second line agent should serve to address issues like the optimal dose regimen and intensity as well as adjust for potential confounders.

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Reply: Higher Intensity Does Not Necessary Yield Better Survival in Second-Line Chemotherapy for NSCLC

To the Editor:

We would like to thank Singh et al. for suggesting that the dose of docetaxel and previous treatment modality may have an impact on second-line therapy in non-small cell lung cancer (NSCLC). Herein, we discuss the dose of docetaxel and the influence of previous chemotherapy in relation to second-line treatment of NSCLC.

In second-line chemotherapy for NSCLC, whether a higher dose of an anticancer agent would inevitably yield a longer survival is open to question. In a study comparing docetaxel 100 mg/m^2, docetaxel 75 mg/m^2 and best supportive care, the overall survivals were 5.9, 7.5, and 7.0 months, respectively.[1] Docetaxel 100 mg/m^2 was also found to be inferior to docetaxel 75 mg/m^2 in terms of the 1-year survival rate in another phase III study.[2] A similar tendency was also observed for another agent in the second-line setting; pemetrexed 500 mg/m^2 and 900 mg/m^2 were compared, and the overall median survivals were 6.7 and 6.9 months, respectively, and the hazard ratio was 1.013 (95% confidence interval, 0.837–1.226).[3] Even the response rate in the 900 mg/m^2 arm did not exceed that in the 500 mg/m^2. Thus, finding the optimal dose of docetaxel or other agents for second-line chemotherapy may be an intriguing issue.[4]

Meanwhile, docetaxel 60 mg/m^2 is the standard therapeutic dose in Japan, since a Japanese phase I trial determined the maximum tolerated dose to be 70 mg/m^2.[5] Even though this dose of docetaxel is lesser than that used in other countries, this may be the optimal dose for Japanese. In a phase II study of docetaxel for previously untreated NSCLC conducted in Japan, the response rate to docetaxel 60 mg/m^2 was 19%, no less than that to the higher doses used in other countries.[6] A retrospective study evaluating docetaxel 60 mg/m^2 for previously treated NSCLC also showed a response rate of 18.5%, comparable with that reported for higher doses.[7] This difference in the dose requirement in Japanese may be attributed to ethnic differences between the Japanese and other populations, but the issue remains under debate.

The previously employed treatment modality differed between those who had received a combination of carboplatin and paclitaxel (group P) and those who had received a combination of a platinum and an agent other than paclitaxel [group nonpaclitaxel (NP)] in our study. We consider, however, that this difference had only a small impact on our study results, for three reasons. Firstly, all the patients in our study had metastatic disease at the time of recurrence and start of docetaxel therapy. Secondly, although 29% of patients in group NP had received radiotherapy, the response rate to the previous treatment in group NP was the same as that in group P (45.0 versus 44.9%, respectively). In general, the response rate to chemoradiotherapy is higher than that to chemotherapy alone. This difference may have disappeared in our study, probably because we only recruited patients who developed recurrence after chemoradiotherapy. Finally, no previous studies of second-line chemotherapy for NSCLC have dealt with these issues. Even though multiple modalities may have been used in previous treatment, we can only evaluate the integrated result of the treatment. It is impossible to distinguish between the efficacy of chemotherapy and radiotherapy if both are undertaken simultaneously.

In conclusion, further investigation of the optimal dose of chemotherapeutic agents for second-line chemotherapy of NSCLC is warranted. The efficacy of previous chemotherapy, whether or not administered in combination with radiotherapy, is a useful reference for subsequent docetaxel therapy.

Disclosure: The authors declare no conflicts of interest.

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ISSN: 1556-0864/08/0309-1079