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Influence of Previous Chemotherapy on the Efficacy of Subsequent Docetaxel Therapy in Advanced Non-small Cell Lung Cancer Patients

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Purpose: To identify factors, particularly the previous use of paclitaxel, that might influence the efficacy of subsequent docetaxel therapy.

Patients and Methods: The patient characteristics, responses, and survivals were compared between the two groups that had received a combination of carboplatin and paclitaxel (group P), and a combination of a platinum and an agent other than paclitaxel (group NP).

Results: A total of 227 patients (127 in group P, and 100 in group NP) were recruited from a hospital-based registry. Two hundred twenty patients were evaluated for the survival, and 210 patients were evaluated for the response of docetaxel therapy. The response rate to docetaxel therapy (14.2% versus 16.0%, $p = 0.702$) or the median survival time (10.9 months versus 11.1 month, $p = 0.567$) did not differ between groups P and NP. The results of multivariate analysis, adjusted for sex, age, and performance status at the start of docetaxel therapy, showed that not the regimen per se, but the response to previous chemotherapy significantly influenced the response rate of docetaxel therapy (odds ratio [OR]: 1.38, 95% confidential interval [CI]: 0.63–3.01; and OR: 2.93, 95% CI: 1.28–6.72, respectively). As for the overall survival, neither the response to nor the previous chemotherapy regimen had any impact (hazard ration [HR]: 0.90, 95% CI 0.66–1.22; HR 0.88, 95% CI 0.65–1.20, respectively).

Conclusion: The previous use of paclitaxel had no impact on the response or survival to subsequent docetaxel therapy. In contrast, the response to previous chemotherapy had a predictive value in relation to responses to subsequent docetaxel therapy in patients with advanced non-small cell lung cancer.

Key Words: Non-small cell lung cancer, Second-line chemotherapy, Docetaxel.

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Lung cancer is a leading cause of cancer-related deaths worldwide.[1] Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all cases of lung cancer. For chemotherapy-naive, patients with advanced NSCLC, with a good performance status (PS), platinum -based chemotherapy has been shown to offer a modest survival benefit over best supportive care alone.[2,3] A high proportion of patients, however, shows disease relapse after initial clinical responses, or progress during the chemotherapy. Thus, a large percentage of patients is moved on to second-line chemotherapy, even though it should only be considered in selected patients with a good PS.[4]

In the landmark study by Shepherd et al., second-line docetaxel thearpy was demonstrated to improve the outcome over best supportive care alone in patients with a history of previous chemotherapy.[5] Since then, a number of agents have been introduced as effective agents for the second-line setting[6–8]; however, the impact of previous chemotherapy on the efficacy of subsequent chemotherapy has not been established.

In relation to small-cell lung cancer, the response of tumors to first-line therapy and recurrence more than 3 months after completion of the initial therapy is often referred to as "sensitive relapse," and absence of tumor response, tumor progression through treatment, or tumor recurrence within 3 months of discontinuation of initial therapy is termed "refractory" disease. Although both are grouped together in most second-line clinical trials, their prognosis and response to salvage therapy have been shown to be different.[9,10] Therefore, in patients with small-cell lung cancer, the efficacy of previous chemotherapy has a significant impact on selection of the subsequent chemotherapy. Whether this relationship between first-and second-line chemotherapy would also apply to cases of NSCLC has not yet been clarified.

In this study, we attempted to identify factors, particularly the previous use of paclitaxel, that might influence the response to subsequent docetaxel therapy in patients with NSCLC. Towards this objective, we divided our patients into two groups according to the previous regimen received.

PATIENTS AND METHODS

We evaluated the patients with histologically or cytologically proven unresectable locally advanced or metastatic

NSCLC, who had received a platinum-containing chemotherapy, and subsequently received docetaxel therapy. The following baseline pretreatment demographic and prognostic information was extracted: age, sex, PS (Eastern Cooperative Oncology Group scale), clinical stage at diagnosis, histology, interval between the final administration of the previous chemotherapy and the start of docetaxel, and response to previous chemotherapy. The platinum-containing therapy was continued for as long as clinical benefit could be observed. Docetaxel was administered at the dose of 60 mg/m^2 and repeated every 3 weeks or longer. We divided these patients into two groups by the initial regimen that they received, namely, combined carboplatin and paclitaxel (group P), or combination of a platinum and an agent other than paclitaxel (group NP).

Objective responses were evaluated using standard bidimensional measurements.[11] Overall survival was measured from the first day of docetaxel treatment until death or the final day of the follow-up period, analyzed using the Kaplan-Meier method, and compared using the log-rank test. Other comparisons were made by χ^2 test, Fisher exact test, and Wilcoxon's test. Factors potentially associated with the efficacy of docetaxel therapy were assessed by univariate and multivariate analysis using the logistic regression model and Cox proportional hazards model. All variables were entered in a single step. Variables tested were sex (male versus female), age (continuous variable), PS at the start of docetaxel therapy (0 versus 1 and 2), regimen of previous chemotherapy (group P versus NP), interval between previous therapy and the start docetaxel chemotherapy (continuous variable), and response to previous chemotherapy (SD/PD versus CR/PR). Differences were considered to be significant at $p < 0.05$. All analyses were performed with Dr. SPSS II (SPSS Japan Inc.).

RESULTS

Patient Characteristics and Docetaxel Delivery

A total of 227 consecutive patients were recruited from a hospital-based registry who were treated with docetaxel after previous platinum-containing chemotherapy between January 2001 and April 2006 at the National Cancer Center Hospital. Of these 127 patients were classified into group P, and 100 into group NP. Seven patients were excluded for the analysis of survival because there was no measurable lesion for the evaluation of response in the previous chemotherapy. Of these 220 patients, another 10 patients were excluded for the analysis of response to docetaxel therapy, because there was no measurable lesion for the evaluation of response in the subsequent docetaxel therapy. By the time of the analysis, 187 out of the 227 patients had died. The median follow-up duration was 10.2 months (range, 0.3–66.9 months) for all patients, and 18.9 months (range, 0.8–66.9 months) for patients who had lost for follow up or alive at the time of analysis.

The patient characteristics are listed in Table 1. The sex and age distributions were similar in the two groups. Stage III disease and a history of previous radiation therapy were slightly predominant in group NP, because concurrent chemoradiotherapy was only administered with the cisplatin (CDDP) and vinorelbine regimen. The response to initial therapy did not differ between the two groups.

TABLE 1. Patient and Disease Characteristics in the Two Groups

	Group P (N = 127)		Group NP (N = 100)		
Characteristics	**No.**	**(%)**	**No.**	**(%)**	p
Sex					
Male	90	(70.9)	79	(79.0)	0.161
Female	37	(29.1)	21	(21.0)	
Age, yr					
Median	58	60		0.072	
Range	30–77		34–75		
Performance status at the start of docetaxel therapy					
0	22	(17.3)	26	(26.0)	0.262
1	101	(79.5)	72	(72.0)	
2	4	(3.2)	2	(2.0)	
Stage at diagnosis					
III	34	(26.8)	51	(51.0)	0.002
IV	72	(56.7)	39	(39.0)	
Recurrence	21	(16.5)	10	(10.0)	
Histology					
Adenocarcinoma	90	(70.9)	68	(68.0)	0.262
Squamous cell carcinoma	23	(18.1)	15	(15.0)	
Large cell carcinoma	2	(1.6)	0	(0)	
Other	12	(9.4)	17	(17.0)	
Interval between the final administration of the previous chemotherapy and the start of docetaxel (wk)					
Median	17		17		0.285
Range	3–134		2–141		
Response to previous chemotherapy					
CR	0	(0)	2	(2.0)	0.031
PR	57	(44.9)	43	(43.0)	
SD	49	(38.6)	46	(46.0)	
PD	17	(13.4)	6	(6.0)	
NE	4	(3.1)	3	(3.0)	
Other treatment					
Radiation	0	(0)	29	(29.0)	<0.001
Surgery	21	(16.5)	10	(10.0)	0.149

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable.

In group NP, the regimens used for the prior chemotherapy and the number of patients treated were as follows; CDDP and vinorelbine (n = 35), combined carboplatin and gemcitabine (n = 24), CDDP and gemcitabine (n = 19), CDDP and irinotecan (n = 18), and others (n = 4).

The median (range) number of cycles of docetaxel chemotherapy administered was 3 (1–17) in group P and 3 (1–13) in group NP.

Efficacy

The response data to docetaxel therapy are summarized in Table 2. There were no significant differences between group P and group NP in terms of the overall response rate (15.1% versus 17.6%), "clinical benefit rate" (79.8% versus 75.6%), or median survival time (6.1 month versus 6.0

TABLE 2. Summary of Docetaxel Therapy in the Two Groups

	Group P (N = 127)		Group NP (N = 100)		
Characteristics	**No.**	**(%)**	**No.**	**(%)**	***p***
Treatment administration					
Median (range)	3	1–17	3	1–13	0.596
Response to docetaxel therapy					
CR	0	(0)	1	(1.0)	0.256
PR	18	(14.2)	15	(15.0)	
SD	81	(63.8)	54	(54.0)	
PD	24	(18.9)	22	(22.0)	
NE	4	(3.1)	8	(8.0)	
CR/PR	18	(14.2)	16	(16.0)	0.702
CR/PR/SD	99	(78.0)	70	(70.0)	0.173
Median survival time, mo (95% CI)	10.9 (7.6–14.1)		11.1 (8.6–13.5)		0.567

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable.

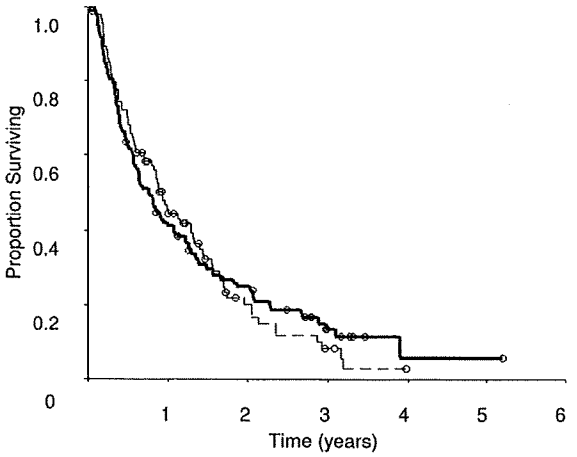

FIGURE 2. Overall survival classified by the responses to previous chemotherapy. Continuous line: SD/PD (n = 118); and dotted line: CR/PR (n = 102). Hazard ratio (95% confidence interval): 0.91 (0.68–1.23).

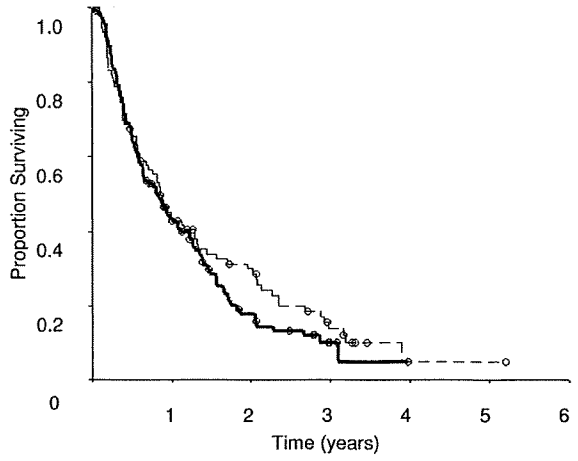

FIGURE 1. Overall survival classified by the previous chemotherapy regimens. Continuous line: carboplatin and paclitaxel (group P, n = 123); and dotted line: platinum and an agent other than paclitaxel (group NP, n = 97). Hazard ratio (95% confidence interval): 1.09 (0.81–1.47).

months) (Figure 1). The response rates to docetaxel in good and poor responders to previous chemotherapy were 21.8% and 9.4%, respectively, in group P (p = 0.074), and 25.0% and 12.0%, respectively, in group NP (p = 0.164). The overall survival did not differ between the good and poor responders (Figure 2).

The result of univariate and multivariate analysis of the response to the docetaxel are shown in Table 3. In the multivariate analysis adjusted for sex, age, PS at the start of docetaxel therapy, the response to previous chemotherapy significantly influenced the response to subsequent docetaxel therapy (odds ratio [OR]: 2.93; 95% CI: 1.28–6.72). The previous chemotherapy regimen (OR: 1.38; 95% CI: 0.63–3.01), and interval between the final administration of the previous chemotherapy and the start of docetaxel therapy (OR: 0.4; 95% CI: 0.86–1.02) were not found to be significant factors influencing the response to docetaxel therapy. The impact of the responses to the previous chemotherapy was denoted the same tendency in the analysis of each group (OR: 3.82; 95% CI: 1.09–13.5 for group P, and OR: 2.13; 95% CI: 0.67–6.70 for group NP). The result of univariate and multivariate analysis of the overall survival is shown in Table 4. Neither the response to nor the regimen used in the previous chemotherapy had significant impact. Interval between the final administration of the previous chemotherapy and the start of docetaxel therapy were statistically significant in the overall survival.

DISCUSSION

The purpose of this study was to evaluate the influence of previous chemotherapy on the efficacy of subsequent docetaxel chemotherapy. Above all, our major question was whether the regimen of previous chemotherapy, especially the use of paclitaxel, would have any influence on the subsequent docetaxel therapy. In previous studies, response to docetaxel therapy had no association with prior exposure to or the efficacy of paclitaxel therapy, but details about the paclitaxel treatment are not described in these reports.[6,7] In our study, by dividing patients according to the previous regimen received, we showed that the previous use of paclitaxel had no impact on the response to subsequent docetaxel therapy, and that the response to previous chemotherapy was associated with the response to, but not to the survival, after subsequent docetaxel therapy.

Although both paclitaxel and docetaxel are widely used, the influence of prior use of paclitaxel on the response to subsequent docetaxel therapy has not yet been thoroughly reviewed in cases of NSCLC. In the TAX320 study conducted by the Non-Small Cell Lung Cancer Study Group, 31% (114 of 373) of patients had a history of prior use of paclitaxel.[6] In that study, previous exposure to paclitaxel had

TABLE 3. Univariate and Multivariate Analyses of the Response to Docetaxel (N = 210)

	Univariate			Multivariate		
	OR	95% CI	p	OR	95% CI	p
Entire						
Response to previous chemotherapy (SD/PD vs CR/PR)	1.12	0.57–2.50	0.63	2.93	1.28–6.72	0.01
Regimen of previous chemotherapy (group P vs group NP)	0.84	0.40–1.75	0.84	1.38	0.63–3.01	0.421
Interval (with a 30-d increase)	0.97	0.91–1.05	0.48	0.94	0.86–1.02	0.14
Group P						
Response to previous chemotherapy (SD/PD vs CR/PR)	2.70	0.94–7.76	0.07	2.13	0.67–6.70	0.20
Interval (with a 30-d increase)	1.04	0.96–1.12	−0.39	1.01	0.92–1.11	0.06
Group NP						
Response to previous chemotherapy (SD/PD vs CR/PR)	2.37	0.78–7.19	0.13	3.82	1.09–13.5	0.04
Interval (with a 30-d increase)	0.88	0.75–1.02	0.10	0.84	0.69–1.01	0.80

Multivariate analysis was adjusted for sex, age, and performance status at the start of docetaxel.

OR, odds ration; HR, hazard ration; P, carboplatin and paclitaxel; NP, platinum and an agent other than paclitaxel; Interval, days between previous therapy and the start docetaxel chemotherapy; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

TABLE 4. Univariate and Multivariate Analyses of Overall Survival (N = 220)

	Univariate			Multivariate		
	HR	95% CI	p	HR	95% CI	p
Entire						
Response to previous chemotherapy (SD/PD vs CR/PR)	0.91	0.68–1.23	0.56	0.90	0.66–1.22	0.484
Regimen of previous chemotherapy (group P vs group NP)	1.09	0.81–1.47	0.57	0.88	0.65–1.20	0.43
Interval (with a 30-d increase)	0.97	0.94–0.99	0.01	0.96	0.94–0.99	0.01
Group P						
Response to previous chemotherapy (SD/PD vs CR/PR)	0.95	0.64–1.41	0.80	0.92	0.60–1.41	0.71
Interval (with a 30-d increase)	0.98	0.94–1.02	0.32	1.01	0.92–1.11	0.13
Group NP						
Response to previous chemotherapy (SD/PD vs CR/PR)	0.86	0.55–1.34	0.86	0.89	0.57–1.40	0.63
Interval (with a 30-d increase)	0.96	0.92–0.99	0.02	0.84	0.69–1.01	0.03

Multivariate analysis was adjusted for sex, age, and performance status at the start of docetaxel.

OR, odds ration; HR, hazard ration; P, carboplatin and paclitaxel; NP, platinum and an agent other than paclitaxel; Interval, days between previous therapy and the start docetaxel chemotherapy; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease.

no impact on the survival of patients who received docetaxel as second-line treatment; however, neither the data of survival nor the details of paclitaxel therapy have been described in the report. In a study comparing pemetrexed and docetaxel in 571 patients, 153 patients (25%) had received paclitaxel.[7] Although the results of the study showed that paclitaxel sensitivity/resistance in the first-line treatment did not predict any difference in the response between pemetrexed and docetaxel used for second-line treatment (details not shown), there were no data comparing the patients according to a history of previous use of paclitaxel.[7] In a study reassessing these data, 20% (113 of 571) of patients had previously received both paclitaxel and platinum, and the previous chemotherapy regimen had no influence on the overall survival.[12] However, the method used for the analysis, namely, assessment of the overall population treated with docetaxel or pemetrexed together, is inappropriate to evaluate the association of previous paclitaxel use with the efficacy of subsequent docetaxel therapy. Patients who had no history of prior taxane treatment were even excluded in some previous phase III studies comparing docetaxel with best supportive care or other agents as second-line treatment.[5,8] In this study, by comparing the patients according to the history of previous use of paclitaxel, we could show specifically that exposure to paclitaxel had no effect on efficacy of subsequent docetaxel therapy.

Although docetaxel and paclitaxel exert their activity via a similar mechanism of action, that is, by interfering with microtubular function and promoting tubulin polymerization and inhibiting the depolymerization of microtubules, the preclinical and clinical activity profiles of the two agents have been shown to exhibit some differences, with partial cross-resistance.[13] Preclinical studies have demonstrated docetaxel to be a 100-fold more potent than paclitaxel in inducing bcl-2 phosphorylation and apoptotic cell death, and the cellular uptake of docetaxel is known to be greater than that of paclitaxel, both of which lead to greater cytotoxic activity of docetaxel.[14] There has been a phase II study of docetaxel in breast cancer patients showing resistance to paclitaxel; objective responses were seen in 18% (8 of 44) of the patients, and the dose or efficacy of previous paclitaxel administration had no impact on the frequency of objective responses. This

indicates that there was perhaps a partial cross-resistance between the two agents in patients of breast cancer.[15] Our study results indicate that this might also be the case in patients of NSCLC.

One of the tentative factors for better survival following second-line chemotherapy is the interval elapsed after the previous chemotherapy. This factor is a possible sign of efficacy of previous chemotherapy, but in the analysis of survival, it is difficult to distinguish whether this factor influences the response to chemotherapy or represents the characteristics of the disease in an individual. Therefore, the interval between two chemotherapy sessions has not been well established as a factor potentially influencing the response in previous studies on NSCLC patients.[5–8,16,17] Some of the studies showed that a longer interval from the last chemotherapy was significantly associated with increased survival.[7,12] In our study, interval between two chemotherapies was associated with the overall survival but not with response, which suggests that this factor have little influence on the antitumor activity of docetaxel therapy, but is representing the characteristics of the tumor.

Difference in the proportions of patients receiving surgery or radiation therapy between the two groups may be a big concern. These local therapies, however, should have only a small influence, if any, because all patients in this study had a metastatic disease at the time of recurrence and start of docetaxel therapy. Although responses to previous chemotherapy in patients treated with chemoradiotherapy could not be evaluated in the same way as the patients treated with chemotherapy alone, the response rates to previous chemotherapy did not differ between the groups P and NP (44.9% in group P, and 45.0% in group NP). Thus, we believe that these populations were appropriately included in our study.

In conclusion, the results of our study showed that docetaxel therapy was similarly active in patients with NSCLC, who had previously been treated with paclitaxel, and the response to previous chemotherapy was predictive of the response to subsequent docetaxel therapy. In the future, many promising agents, whether cytotoxic or molecule-targeted agents, may be developed for the second-line treatment of NSCLC. In the era of abundantly available agents, it will be meaningful to know which patients are likely to derive the most benefit from a particular agent. The results of this study are expected to be helpful for the selection of patients with advanced NSCLC who would benefit from docetaxel therapy.

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Epidermal Growth Factor Receptor Mutation Detection Using High-Resolution Melting Analysis Predicts Outcomes in Patients with Advanced Non–Small Cell Lung Cancer Treated with Gefitinib

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Abstract **Purpose:** Epidermal growth factor receptor (*EGFR*) mutations, especially deletional mutations in exon 19 (DEL) and L858R, predict gefitinib sensitivity in patients with non–small cell lung cancer (NSCLC). In this study, we validated *EGFR* mutation detection using high-resolution melting analysis (HRMA) and evaluated the associations between *EGFR* mutations and clinical outcomes in advanced NSCLC patients treated with gefitinib on a larger scale.

Experimental Design: The presence of DEL or L858R was evaluated using HRMA and paraffin-embedded tissues and/or cytologic slides from 212 patients. In 66 patients, the results were compared with direct sequencing data.

Results: HRMA using formalin-fixed tissues had a 92% sensitivity and a 100% specificity. The analysis was successfully completed in 207 patients, and DEL or L858R mutations were detected in 85 (41%) patients. 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 ($P < 0.001$). Even among the 34 patients with stable diseases, the time-to-progression was significantly longer in patients with *EGFR* mutations. Patients with DEL ($n = 49$) tended to have better outcomes than those with L858R ($n = 36$); the response rates were 86% and 67%, respectively ($P = 0.037$), and the median time-to-progression was 10.5 and 7.4 months, respectively ($P = 0.11$).

Conclusions: HRMA is a precise method for detecting DEL and L858R mutations and is useful for predicting clinical outcomes in patients with advanced NSCLC treated with gefitinib.

Gefitinib (Iressa; AstraZeneca) is an orally active, selective epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor. Phase II studies have shown gefitinib antitumor activity in patients with advanced non–small cell lung cancer (NSCLC; refs. 1, 2). Several studies have shown that the response rate to gefitinib is higher in women, patients with adenocarcinoma, never smokers, and Japanese or East Asians (1–3); subsequently, somatic mutations in the kinase domain of *EGFR* were suggested to be a determinant of gefitinib sensitivity (4, 5). Since then, many retrospective studies have consistently revealed that *EGFR* mutations, mainly in-frame deletions including amino acids at codons 747 to 749 in exon 19 (DEL) and a missense mutation at codon 858 (L858R) in exon 21, are associated with tumor response, time-to-progression, and overall survival in NSCLC patients treated with gefitinib (6–8).

In our previous study, which clearly showed a correlation between *EGFR* mutations and gefitinib sensitivity in patients with recurrent NSCLC after surgical resection of the primary tumor (6), we used methanol-fixed, paraffin-embedded surgical specimens and did laser capture microdissection and direct sequencing, which we considered to be the most precise methods available for identifying mutations at that time. However, these methods are not useful in clinical practice for the treatment of advanced NSCLC for two reasons. First, the diagnostic samples of advanced NSCLC tumors, unlike surgical specimens, contain a small amount of tumor cells and are highly contaminated with normal cells. Second, laser capture microdissection and direct sequencing require special

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Table 1. Patient characteristics (N = 212)

	n (%)
Age (y)	
Median (range)	62 (29-84)
Sex	
Women	92 (43)
Men	120 (57)
Smoking history*	
Never smokers	96 (45)
Former smokers	38 (18)
Current smokers	78 (37)
Histology	
Adenocarcinoma	193 (91)
Others	19 (9)
Performance status†	
0	59 (28)
1	123 (58)
2	22 (10)
3	8 (4)
Stage	
III	42 (20)
IV	75 (35)
Recurrence after surgery	95 (45)
Gefitinib therapy	
First line	89 (42)
Second line	66 (31)
Third or more line	57 (27)

*Never smokers were defined as patients who have never had a smoking habit and former smokers were defined as patients who had stopped smoking at least 1 y before diagnosis.
† At the beginning of gefitinib therapy.

instruments and cost time and money. Recently, high-resolution melting analysis (HRMA) using the dye LCGreen I (Idaho Technology) was introduced as an easy, quick, and precise method for mutation screening (9), and we established a method for detecting DEL and L858R mutations using HRMA. Our cell line study revealed that DEL and L858R mutations could be detected using HRMA in the presence of 10% and 0.1% mutant cells, respectively (10). We also showed that the two major mutations could be identified by HRMA using DNA extracted from archived Papanicolaou-stained cytologic slides with 88% sensitivity and 100% specificity (10).

In this study, we validated *EGFR* mutation detection by HRMA using DNA extracted from archived paraffin-embedded tissues. We also did the HRMA in advanced NSCLC patients treated with gefitinib on a larger scale using archived tissues and/or cytologic slides.

Patients and Methods

Patients. Among 364 consecutive patients with NSCLC who began receiving gefitinib monotherapy (250 mg/d) at the National Cancer Center Hospital between July 2002 and December 2004, 212 patients were retrospectively analyzed using HRMA. One hundred fifty-two patients were excluded from the analysis because tumor samples were not available (n = 126) or their informed consent to the genetic analysis was not obtained (n = 26).

High-resolution melting analysis. On a protocol approved by the Institutional Review Board of the National Cancer Center Hospital, we did the following genetic analyses. Formalin-fixed, paraffin-embedded tissues and/or Papanicolaou-stained cytologic slides containing sufficient tumor cells (at least 1% of nucleated cells) were selected after microscopic examination by a pathologist (K.T.). The detailed analysis method has been described previously (10). Briefly, DNA was extracted from the tissues and/or cytologic slides using a QIAamp DNA Micro kit (Qiagen). PCR was done using dye LCGreen I and primers designed to amplify a region containing E746-I759 of *EGFR* [DEL-specific primer, AAAATTCCCGTCGCTATC (forward) and AAGCAGAAACTCACATCG (reverse)] or L858 of *EGFR* [L858R-specific primer, AGATCACA-GATTTTGGGC (forward) and ATTCTTTCTCTTCCGCAC (reverse)] on a LightCycler (Roche Diagnostics). The PCR products were denatured at 95°C for 5 min and cooled to 40°C to form heteroduplexes. The LightCycler capillary was then transferred to an HR-1 (Idaho Technology), a HRMA instrument, and heated at a transition rate of 0.3°C per second. 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 samples and control samples. Human Genomic DNA (Roche Diagnostics) was used as a control sample with wild-type (WT) *EGFR*. Samples revealing skewed or left-shifted curves from those of control samples were judged to have mutations. All analyses were done in a blinded fashion.

Table 2. Clinical validation of HRMA and direct sequencing without laser capture microdissection

	HRMA without LCM			Direct sequencing without LCM (6)
	Formalin-fixed tissues	Methanol-fixed tissues	Cytologic slides (10)	
n	66	66	29	66
Successfully analyzed, *n* (%)	63 (95)	66 (100)	28 (97)	66 (100)
True positive	34	36	14	28
True negative	26	29	12	29
False positive	0	0	0	0
False negative	3	1	2	9
Sensitivity (%)	92	97	88	76
Specificity (%)	100	100	100	100
Positive predictive value (%)	100	100	100	100
Negative predictive value (%)	90	97	86	76

NOTE: The results of these analyses were compared with those of direct sequencing with LCM (used as the "gold standard" method). True positive is defined as the correct detection of deletional mutations in exon 19 or L858R.
Abbreviation: LCM, laser capture microdissection.

Table 3. *EGFR* mutations among patient subgroups

	n	*EGFR* mutations				*P*
		DEL	L858R	Total	%	
Total	207	49	36	85	41	
Sex						
Women	89	31	17	48	54	0.001
Men	118	18	19	37	31	
Smoking history						
Never smokers	93	30	19	49	53	0.002*
Former smokers	38	12	10	22	58	
Current smokers	76	7	7	14	18	
Histology						
Adenocarcinoma	189	48	35	83	44	0.007
Others	18	1†	1‡	2	11	

*Comparison between never smokers and others.
† Pleomorphic carcinoma.
‡ Adenosquamous carcinoma.

Clinical validation of HRMA. Direct sequencing with and without laser capture microdissection had been done in 66 patients with recurrent NSCLC after surgery in the previous study (6). In these patients, HRMA was done using both formalin-fixed and methanol-fixed surgical specimens without laser capture microdissection, and the results were compared with the results of direct sequencing with laser capture microdissection, which we considered to be the gold standard method.

Radiologic evaluation. One board-certified radiologist (U.T.) who was unaware of the patients' mutational statuses reviewed the baseline, the first follow-up, and confirmatory imaging studies and classified the tumor responses into complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) using standard bidimensional measurements (11). In patients without measurable lesions, significant clinical benefit and disease progression were defined as clinical PR and clinical PD, respectively. Patients who died before the follow-up imaging studies were classified as PD. SD was subdivided into minor response (MR), long SD, and short SD. MR was defined as a ≥25% decrease in the sum of the products of the perpendicular diameters of all measurable lesions, and long SD meant that SD lasted for >6 months. Responders were defined as patients with CR, PR, or clinical PR.

Statistical analysis. The associations among *EGFR* mutations, patient characteristics, and tumor responses to gefitinib were assessed using a χ^2 test. The differences in time-to-progression and overall survival according to the patient subgroups were compared using Kaplan-Meier curves and log-rank tests. The starting point of the time-to-progression and overall survival was the first administration of gefitinib. Multivariate analyses using logistic regression models and Cox proportional hazard models were done to assess the association between the clinical outcomes and the following factors: age (<70 versus ≥70 years), sex, smoking history (never smokers versus others), histology (adenocarcinoma versus others), performance status (0/1 versus 2/3), stage (recurrence after surgery versus III/IV), prior chemotherapy (yes versus no), and the mutational status of *EGFR* (mutant versus WT). All analyses were done using the SPSS statistical package (SPSS version 11.0 for Windows; SPSS, Inc.).

Results

Patient characteristics. The patient characteristics are listed in Table 1. All the patients were East Asians: 210 Japanese, 1 Korean, and 1 Chinese. The median follow-up time for the survivors was 29.7 months (range, 10.7-49.8 months).

Clinical validation of HRMA. The clinical validation of the HRMA results using various samples is shown in Table 2. The sensitivity of HRMA using DNA extracted from formalin-fixed tissues was 92%, significantly higher than that of direct sequencing without laser capture microdissection but lower than that of HRMA using methanol-fixed tissues. The specificity and positive predictive values were 100% in all the analyses.

Mutational analysis. HRMA was completed in 207 patients. Five patients could not be successfully analyzed because of incomplete PCR. Of the 207 patients, 130 were analyzed using tissue samples (96 samples were obtained by thoracotomy, 17 by mediastinoscopic lymph node biopsy, 9 by thoracoscopic lung or pleural biopsy, 5 by resection or biopsy of distant metastases, and 3 by transbronchial lung biopsy), and 117 were analyzed using cytology samples (43 samples were obtained by bronchial brushing or washing, 40 from pleural effusion, 9 by transbronchial needle aspiration, 8 from pericardial effusion, 7 by needle aspiration of superficial lymph nodes, 6 by percutaneous needle aspiration of lung tumors, and 4 from sputum). In 40 patients who were analyzed using both tissue and cytology samples, 4 had inconsistent results; mutations were detected only in tissue samples and not in cytology samples (3 patients) or vice versa (1 patient). These four patients were judged to have mutations because false-negative results were more common than false-positive results in the validation of HRMA. Consequently, DEL and L858R mutations were detected in 49 (24%) and 36 (17%) patients, respectively, and these mutations were mutually exclusive. The other 122 (59%) patients were classified as having WT *EGFR* in this study, although some of them may have had minor mutations. As

Table 4. *EGFR* mutations and response to gefitinib

	Responders		SD			PD	Response rate (%)	*P*
	CR	PR	MR	Long SD	Short SD			
WT	0	10	2	4	17	89	10/122 (8)	$<10^{-23}$
Mutant	2	64*	6	4	1	8†	66/85 (78)	
DEL	0	42	2	2	1	2	42/49 (86)	0.037
L858R	2	22	4	2	0	6	24/36 (67)	
Total	2	74	8	8	18	97	76/207 (37)	

*Including four clinical responders without measurable lesions.
† Including a patient who had no measurable lesions at baseline.

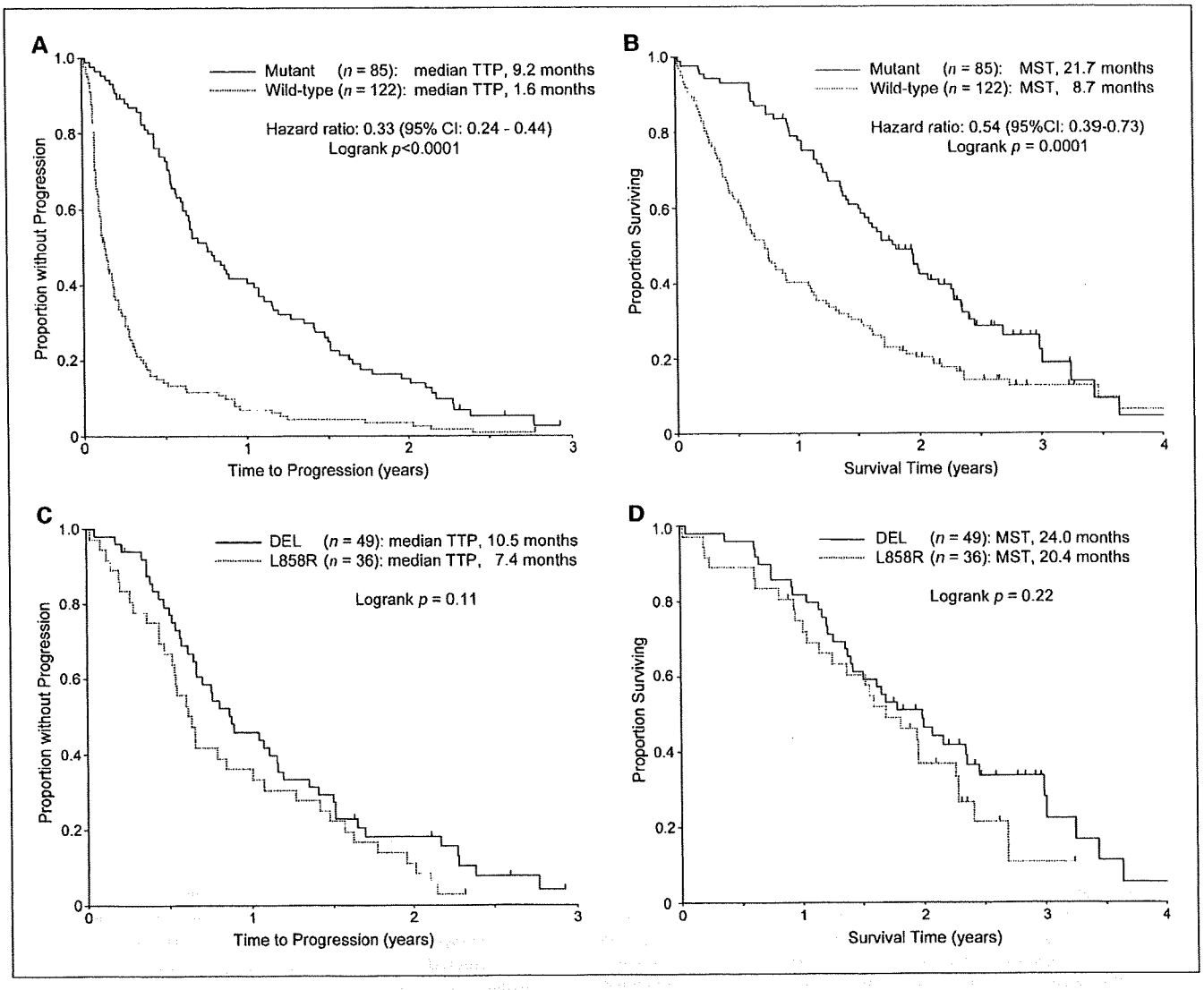

Fig. 1. Kaplan-Meier plot of time-to-progression (*A*) and overall survival (*B*) for patients with or without *EGFR* mutations. Kaplan-Meier plot of time-to-progression (*C*) and overall survival (*D*) for patients with DEL or L858R mutations. TTP, time-to-progression; MST, median survival time.

shown in Table 3, *EGFR* mutations were detected more frequently in women, never smokers, and patients with adenocarcinoma. Patient characteristics were not significantly different between patients with DEL mutations and those with an L858R mutation.

EGFR mutations and clinical outcomes. The association of the mutational status of *EGFR* and the response to gefitinib is shown in Table 4. The response rate was significantly higher in patients with *EGFR* mutations than in those with WT *EGFR* (78% versus 8%; $P < 10^{-23}$). Among patients with *EGFR* mutations, those with DEL mutations had a higher response rate than those with an L858R mutation (86% versus 67%; $P = 0.037$). Tumor responses were classified as SD in 11 patients with *EGFR* mutations and in 23 patients with WT *EGFR*. Among the patients with SD, a MR and/or a long SD (>6 months) were observed more frequently (91% versus 26%; $P = 0.0004$) and the time-to-progression was significantly longer (median, 6.9 versus 4.4 months; $P = 0.019$) in the patients with *EGFR* mutations than in the patients with WT *EGFR*.

As shown in Fig. 1, the time-to-progression (median, 9.2 versus 1.6 months; $P < 0.0001$) and overall survival (median, 21.7 versus 8.7 months; $P = 0.0001$) were significantly longer in patients with *EGFR* mutations than in those with WT *EGFR*. Patients with DEL mutations tended to have a longer time-to-progression (median, 10.5 versus 7.4 months; $P = 0.11$) and overall survival (median, 24.0 versus 20.4 months; $P = 0.22$) than those with an L858R mutation, although the difference did not reach statistical significance.

Clinical outcomes among subgroups of patients are shown in Table 5. In the univariate analysis, sex, smoking history, and histology were significant predictive factors for gefitinib sensitivity.

In the multivariate analyses, the mutational status of *EGFR* was an independent predictive factor of response [odds ratio, 38.9; 95% confidence interval (95% CI), 15.7-96.5; $P < 0.001$], time-to-progression (hazard ratio, 0.33; 95% CI, 0.24-0.45; $P < 0.001$), and overall survival (hazard ratio, 0.48; 95% CI, 0.34-0.67; $P < 0.001$). A poor performance status (2/3) was an

independent predictor of a shorter time-to-progression (hazard ratio, 1.80; 95% CI, 1.19-2.72; $P = 0.006$) and overall survival (hazard ratio, 3.97; 95% CI, 2.56-6.16; $P < 0.001$), and a history of prior chemotherapy was another independent predictor of a shorter overall survival (hazard ratio, 1.59; 95% CI, 1.14-2.23; $P = 0.006$). However, other clinical characteristics, including sex, smoking history, and histology, were not independent predictive factors for any clinical outcomes.

Discussion

In the current study, we showed the practicality of our new HRMA method for detecting two major *EGFR* mutations, DEL and L858R. The sensitivity and specificity of the analysis were 92% and 100%, respectively, when archived formalin-fixed, paraffin-embedded tissues were used without laser capture microdissection. Given the similar results that were obtained when Papanicolaou-stained cytologic slides were used (10), DEL and L858R mutations can likely be detected from such archived samples with about a 90% sensitivity and 100% specificity. Because the mutations were detected by HRMA even when only a small proportion (0.1% or 10%) of mutant cells existed (10), laser capture microdissection or other enrichment procedures are not needed in most cases. This is a major advantage of HRMA over direct sequencing because direct sequencing requires laser capture microdissection for accurate evaluation (6). However, there remained some risk of indeterminate or false-negative results because the DNA might have degenerated during sampling or the preservation of the archived samples. In fact, an analysis using methanol-fixed tissues, which are known to preserve DNA better than formalin-fixed tissues (12), was stable with no indeterminate and fewer false-negative results. Thus, an even higher sensitivity can be expected when fresh tumor samples are used. In any event, HRMA was successfully used to identify *EGFR* mutations and, more importantly, predict the clinical outcomes of gefitinib-treated patients with a high sensitivity and specificity.

Although the detection of *EGFR* mutations can provide patients with NSCLC and their physicians with critical information for optimal decision making, such tests are not common in clinical settings mainly because of the difficulty and impracticality of direct sequencing. Recently, highly sensitive nonsequencing methods to detect *EGFR* mutations in small tumor samples contaminated with normal cells have been reported (10, 13-21). Among them, HRMA has the advantages of being able to identify the mutations with less labor, time, and expense; PCR and the melting analysis can be done in the same capillary tube within a few hours, and the running cost is only about 1 U.S. dollar per sample. HRMA is expected to be one of the most practical methods for detecting *EGFR* mutations in clinical settings.

We analyzed consecutive gefitinib-treated patients in a single institution on a larger scale than any other previous report. The mutational analysis by HRMA was successful in 207 patients and confirmed strong and independent associations between the two major *EGFR* mutations and clinical outcomes. Clinical predictors, such as sex, smoking history, and histology, added little predictive information to that provided by the mutational analysis. We believe that the mutational status of *EGFR* is the most important predictor of clinical outcomes in gefitinib-treated patients.

Among the patients without the two major mutations, 8% were responders. This result may be due to false-negative HRMA results, other *EGFR* mutations, or other determinants of gefitinib sensitivity. As for other *EGFR* mutations, the direct sequencing of exons 18 to 24 was done in four responders without DEL or L858R mutations, and one of them had G719C and S768I mutations. Although missense mutations at codon 719 of *EGFR* (G719C, G719S, or G719A) may be associated with gefitinib sensitivity, the predictive significance of these mutations is unclear because the number of reported patients is small (6). At present, we consider the accurate detection of the two major *EGFR* mutations to be sufficient for optimal decision making.

Recently, the *EGFR* copy number was reported to be another predictor of gefitinib sensitivity (6, 22, 23), and Cappuzzo et al. (22) suggested that this factor was a stronger predictor of overall survival than *EGFR* mutations. Our previous study also showed that the *EGFR* copy number evaluated by quantitative

Table 5. Clinical outcomes among subgroups of patients

	n	Response rate (%)	*P*	Median TTP (mo)	*P*	MST (mo)	*P*
Total	207	37		3.7		14.5	
Sex							
Women	89	51	<0.001	5.6	0.17	18.3	0.15
Men	118	26		2.3		9.6	
Smoking history							
Never smokers	93	51	<0.001*	6.2	0.073*	16.9	0.22*
Former smokers	38	47		5.2		14.5	
Current smokers	76	14		2.2		9.1	
Histology							
Adenocarcinoma	189	40	0.004	4.3	0.060	15.1	0.10
Others	18	6		1.6		4.9	
EGFR mutations							
DEL/L858R	85	78	<0.001	9.2	<0.001	21.7	<0.001
WT	122	8		1.6		8.7	

Abbreviations: TTP, time-to-progression; MST, median survival time.
*Comparison between never smokers and others.

PCR was associated with response; however, an increased *EGFR* copy number was concentrated in patients with *EGFR* mutations and was not an independent predictor of response and overall survival (6). In the current study, we showed that *EGFR* mutations were associated with better outcomes even among patients with SD. The interpretation of this result is difficult because a long SD might be caused by intrinsic characteristics independent of treatment; however, this result suggested that *EGFR* mutations predicted not only "super responders" but also "non-super responders" who gained a clinical benefit. Contrary to these findings, Cappuzzo et al. (22) showed that *EGFR* mutations predicted only responders and were not associated with overall survival, whereas *EGFR* copy number was associated with both response and SD and was an independent predictor of overall survival. Although the reason of these discrepancies is unclear, we consider that if *EGFR* mutations are accurately identified, *EGFR* copy number adds little information for patient selection, at least in Japanese patients.

About the outcomes of patients with DEL or L858R mutations, our larger scale study produced results similar to those of some previous studies, which indicated that DEL mutations were associated with better outcomes after EGFR tyrosine kinase inhibitor treatment than an L858R mutation (24–27). Further investigations are needed to clarify the difference in the biological characteristics of the two mutations. However, in the current study, the difference was small and even patients with an L858R mutation had favorable outcomes: the response rate was 67%, the median time-to-progression was 7.4 months, and the median survival time was 20.4 months. We now think that both DEL and L858R mutations should be treated equally in clinical decision-making.

In conclusion, the detection of DEL and L858R mutations using HRMA is accurate and practical. Using HRMA, we confirmed a strong association between the two major *EGFR* mutations and clinical outcomes in patients with advanced NSCLC treated with gefitinib.

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Serum Total Bilirubin as a Predictive Factor for Severe Neutropenia in Lung Cancer Patients Treated with Cisplatin and Irinotecan

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Objective: To clarify the association between pre-treatment total bilirubin (PTB) level and severe toxicity in patients receiving cisplatin and irinotecan.
Methods: We analyzed retrospectively the relationships of grade 4 neutropenia or grade 3–4 diarrhea and clinical variables including PTB and pre-treatment neutrophil counts (PNC) using a logistic regression model.
Results: One hundred and twenty-seven patients (93 men, 34 women; median age: 61 years; range: 24–74 years) received cisplatin (60 or 80 mg/m^2) on day 1 and irinotecan (60 mg/m^2) on days 1 and 8 every 3 weeks or on days 1, 8 and 15 every 4 weeks. Grade 4 neutropenia occurred in 29 patients (23%) and grade 3–4 diarrhea occurred in 13 patients (10%). Grade 4 neutropenia was associated with a higher PTB level (odds ratio: 4.9; 95% confidence interval: 1.4–17.7), a higher cisplatin dose (2.8, 1.0–7.8) and a lower PNC (1.5, 1.0–2.3). Grade 3–4 diarrhea was associated with liver metastasis (11.2, 2.2–57.4), a higher cisplatin dose (5.0, 1.2–21.3) and a lower PNC (2.0, 1.1–3.6).
Conclusions: PTB level was associated with the severity of neutropenia caused by cisplatin and irinotecan.

Key words: irinotecan – toxicity – lung cancer

INTRODUCTION

Although irinotecan is an active agent against several solid tumors, it sometimes exhibits serious adverse effects, the most common being bone marrow toxicity, in particular leucopenia and neutropenia, and ileocolitis, which leads to diarrhea (1–4). The severity of these toxicities varies greatly between individuals, and thus identifying pre-treatment factors that predict an increased risk for severe toxicities is a critical issue in the treatment of cancer patients undergoing chemotherapy.

Irinotecan needs to be activated by systemic carboxylesterases to SN-38 to exert its anti-tumor activity, which is mediated by the inhibition of topoisomerase I (5). Glucuronidation of SN-38 (SN-38G) by UDP-glucuronosyltransferase (UGT) 1A1 during biliary excretion is the primary route of detoxification and elimination. A higher ratio of plasma SN-38 to SN38-G has been correlated with severe diarrhea, suggesting that the efficiency of SN-38 glucuronidation is an important determinant of toxicity (6–8).

Genetic polymorphisms of the UGT 1A1 gene, such as the number of TA repeats in the TATA box that are associated with reduced transcriptional efficiency and functional activity, have been reported previously (7). Some studies have demonstrated an association between UGT1A1 polymorphisms and the risk for severe toxicity from irinotecan (6, 8–11).

The UGT1A1 enzyme is also responsible for hepatic bilirubin glucuronidation. Serum bilirubin levels, therefore, may reflect UGT1A1 activity and may also be associated with irinotecan activity and toxicity. The pre-treatment serum total bilirubin (PTB) level has been shown to be related to

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severe neutropenia in patients receiving 350 mg/m^2 of irinotecan (8). We extended this observation in patients receiving cisplatin and irinotecan to clarify the association between PTB and severe toxicity, including neutropenia and diarrhea, in these patients.

PATIENTS AND METHODS

Treatment Schedule

The subjects consisted of consecutive lung cancer patients who had received cisplatin and irinotecan therapy at the National Cancer Centre Hospital between February 1999 and May 2004. Irinotecan, diluted in 500 ml of normal saline, was given intravenously over 90 min at a dose of 60 mg/m^2 on days 1 and 8 or on days 1, 8 and 15. Cisplatin was given intravenously over 60 min after the irinotecan infusion at a dose of 60 or 80 mg/m^2 on day 1 with at least 2500 ml of hydration. The first phase I trial of irinotecan and cisplatin showed that 80 mg/m^2 of cisplatin on day 1 and 60 mg/m^2 of irinotecan on days 1, 8, and 15 were the recommended dose for phase II trials (12), and this dose schedule was used for subsequent phase II and phase III trials of non-small cell lung cancer (NSCLC) (13,4,14). The second phase I trial of this combination showed that 60 mg/m^2 of cisplatin on day 1 and 80 mg/m^2 of irinotecan on days 1, 8, and 15 were the recommended dose (15). A phase II trial for small cell lung cancer, however, showed that this dose schedule was too toxic, and thereafter the dose of irinotecan was reduced from 80 to 60 mg/m^2 (16). From the above, we used 80 mg/m^2 of cisplatin and 60 mg/m^2 of irinotecan for patients with NSCLC, and 60 mg/m^2 of cisplatin and 60 mg/m^2 of irinotecan for the other patients. Administration of irinotecan was omitted if any of the following toxicities were noted on days 8 and 15: a white blood cell count $<2.0 \times 10^9$/l, a platelet count $<75 \times 10^9$/l, or grade 1–3 diarrhea. Each course was repeated every 3 or 4 weeks until the occurrence of unacceptable toxicity, disease progression, patient's refusal to continue treatment, or the investigator's medical decision to stop treatment. To control for cisplatin-induced emesis, a 5-HT3 receptor antagonist and dexamethasone were given prior to cisplatin administration.

Study Design

We retrospectively reviewed the patients' clinical records, including patient characteristics (age, sex, Eastern Cooperative Oncology Group performance status, histology of primary disease, clinical stage, prior treatment, evidence of liver metastasis), the dose and schedule of chemotherapy, and pre-treatment complete blood counts and serum chemistry profiles. We defined 'severe toxicity' as grade 4 neutropenia or grade 3–4 diarrhea during the first cycle of chemotherapy, in accordance with the NCI-CTC Version 2.0 criteria. All patients were treated as in-patients, and complete blood counts and serum chemistry profiles were assessed at least once a week. PTB was defined as the serum total bilirubin level at fasting just prior to the administration of cisplatin and irinotecan.

Table 1. Patient characteristics

		No. of patients
Sex	Male/female	93/34
Age	Median (range)	61 (24–74)
Performance status	0/1/2	34/91/2
Histology	Non-small cell lung cancer	57
	Small cell lung cancer	63
	Others	7
Liver metastasis	Yes/no	18/109
Prior chemotherapy	Yes/no	17/110
PTB (mg/m^2)	Median (range)	0.6 (0.2–2.4)
PNC ($\times 10^9$/l)	Median (range)	4.1 (1.8–8.5)
Chemotherapy	CDDP (60) day 1 + CPT-11 (60) days 1.8 q3w	32
Regimens (mg/dl)	CDDP (60) day 1 + CPT-11 (60) days 1.8.15 q4w	39
	CDDP (80) day 1 + CPT-11 (60) days 1.8 q3w	24
	CDDP(80) day1 + CPT-11 (60) days 1.8.15 q4w	32

PTB, pre-treatment total bilirubin; PNC, pre-treatment neutrophil count.

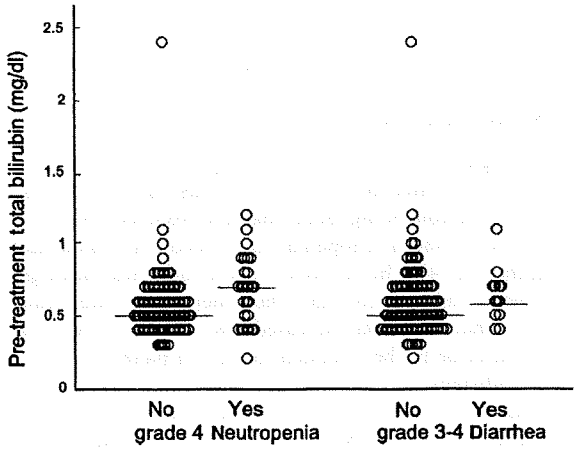

Figure 1. Association of PTB in patients who developed severe toxicity and in those who did not. The median PTB in patients who developed grade 4 neutropenia and those who did not was 0.7 (range, 0.2–1.2) mg/dl and 0.5 (range, 0.3–2.4) mg/dl, respectively ($P = 0.03$, Mann–Whitney U test). The median PTB in patients who developed grade 3–4 diarrhea and those who did not was 0.6 and 0.5 mg/dl, respectively ($P = 0.22$). The bars represent the median values.

Table 2. Univariate analysis of association between grade 4 neutropenia and pre-treatment clinical variables

	Neutropenia grade		Odds ratio (95% CI)
	Grade <4 ($n = 98$)	Grade 4 ($n = 29$)	
Sex			
Male	70	23	1
Female	28	6	0.65 (0.24–1.77)
Age			
Median (range)	61 (24–74)	65 (38–73)	1.04 (0.99–1.09)
Performance status			
0	29	5	1
1, 2	69	24	2.02 (0.70–5.80)
Liver metastasis			
No	82	27	1
Yes	16	2	0.38 (0.08–1.76)
Prior chemotherapy			
No	84	26	1
Yes	14	3	0.69 (0.19–2.60)
Treatment schedule			
Every 3 weeks	41	15	1
Every 4 weeks	57	14	0.67 (0.29–1.54)
Cisplatin dose (mg/m^2)			
60	56	15	1
80	42	14	1.24 (0.54–2.86)
AST (IU/l)			
Median (range)	22 (11–161)	22 (11–56)	0.98 (0.95–1.01)
ALT (IU/l)			
Median (range)	18 (6–266)	20 (5–67)	0.99 (0.97–1.02)
PNC ($\times 10^9$/l)			
Median (range)	4.4(2.0–8.5)	3.9 (1.8–8.3)	0.84 (0.61–1.14)
PTB (mg/dl)			
Median (range)	0.5 (0.3–2.4)	0.7 (0.2–1.2)	3.74 (0.70–19.9)
≤0.7	87	20	1
>0.7	11	9	3.56 (1.30–9.73)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PNC, pre-treatment neutrophil count; PTB, pre-treatment total bilirubin.

Statistical Methods

The Mann–Whitney U test was used to compare the PTB levels of patients who developed severe toxicity and those who did not. Possible explanatory factors were compared using a logistic regression model. A PTB threshold of ≤0.7 mg/dl was selected to categorize this variable because a total bilirubin level higher than 0.7 mg/dl has been correlated with a mutated UGT1A1 genotype and the occurrence of grade 4 neutropenia (8). Furthermore, sex, performance status, liver metastasis, prior chemotherapy, treatment schedule and cisplatin dose were defined as categorized variables, and age, AST, ALT and pre-treatment neutrophil count (PNC) were examined as continuous variables. Variables that seemed to be associated with severe toxicity ($P < 0.1$) were considered for inclusion in a multivariate analysis using a backward stepwise regression model. We performed these analyses using the SPSS statistical package (SPSS version 11.0 for Windows; SPSS Inc., Chicago, IL, USA).

RESULTS

A total of 127 consecutive patients with thoracic malignancy received cisplatin and irinotecan therapy. The patient characteristics are listed in Table 1. In all, two patients (1.5%) had

Table 3. Backward stepwise regression analysis of association between severe toxicity and pre-treatment clinical variables

Variable	Co-efficient	*P*	Odds ratio (95% CI)
Grade 4 neutropenia			
Cisplatin dose	1.04	0.04	2.84 (1.03–7.81)
PNC	0.42	0.04	1.53 (1.02–2.27)
PTB	1.59	0.02	4.93 (1.37–17.7)
Grade 3–4 diarrhea			
Liver metastasis	2.41	0.004	11.2 (2.18–57.4)
Cisplatin dose	1.61	0.03	5.00 (1.18–21.3)
PNC	0.67	0.03	1.96 (1.07–3.60)

Adjusted for age and PS.
PNC, pre-treatment neutrophil count; PTB, pre-treatment total bilirubin.

stage IIA disease, seven patients (5.5%) had stage IIIA disease, 26 patients (20%) had stage IIIB disease and 85 patients (67%) had stage IV disease. The median PTB level was 0.6 (range, 0.2–2.4) mg/dl and the median PNC was 4.1 (range 1.8–8.5) $\times 10^9$/l. A total of 93 patients (73%) received the planned doses without skipping the irinotecan administrations on day 8 or 15. Among the remaining 34 patients, the irinotecan on day 8 or 15 was omitted in 27 of 164 (16.5%) planned doses in patients with PTB level $\leq$0.7 mg/dl, while in 11 of 34 (32.4%) planned doses in patients with PTB level >0.7 mg/dl ($P = 0.053$). Thus, the actual irinotecan dose delivered was lower with marginal significance in patients with PTB level >0.7 mg/dl. Grade 4 neutropenia occurred in 29 (23%) patients and grade 3–4 diarrhea occurred in 13 (10%) patients.

The median PTB level was higher in patients who developed grade 4 neutropenia than in those who did not (0.7 and 0.5 mg/dl, respectively; $P = 0.03$) (Fig. 1), but PTB was not correlated with the presence or absence of grade 3–4 diarrhea ($P = 0.22$).

In a univariate analysis, grade 4 neutropenia was associated with only the PTB level ($\leq$0.7 versus >0.7 mg/dl; $P = 0.01$, Table 2). When PTB level was analyzed as a continuous variable, the association was not significant (OR: 3.74; 95% CI: 0.70–19.9; $P = 0.12$). In a multivariate analysis, grade 4 neutropenia was associated with the PTB level ($\leq$0.7 versus >0.7 mg/dl; $P = 0.02$), the cisplatin dose ($P = 0.04$), and PNC ($P = 0.04$, Table 3). In a univariate analysis, grade 3–4 diarrhea was associated with only liver metastasis ($P = 0.01$, Table 4). We analyzed serum levels of PTB and pre-treatment AST and ALT between patients with ($n = 18$) or without ($n = 109$) liver metastasis. The median (range) PTB was 0.6 (0.4–2.4) mg/dl in patients with liver metastasis and 0.6 (0.2–1.2) mg/dl in patients without liver metastasis ($p = 0.19$). In contrast, the median (range) levels of pre-treatment AST and ALT were 30 (16–114) IU/l and 30 (11–84) IU/l, respectively, in patients with liver metastasis and 21 (11–161) IU/l and 17 (5–266) IU/l, respectively, in patients without liver metastasis ($P = 0.0054$). In a multivariate analysis, grade 3–4 diarrhea was associated with liver metastasis ($P = 0.004$), the cisplatin dose ($P = 0.03$) and PNC ($P = 0.03$, Table 4).

DISCUSSION

This study showed that the PTB level was significantly associated with severity of neutropenia in patients treated with cisplatin and weekly irinotecan. Although irinotecan-induced toxicity can be reduced by skipping irinotecan on day 8, 15, or both, this dose modification is not enough to eliminate severe toxicity completely. In this study irinotecan was more frequently omitted on days 8 and 15 in patients with PTB level >0.7 mg/dl, and therefore, the association between PTB and irinotecan-induced toxicity may be underestimated. Thus, the PTB level, a simple routine measure in clinical practice, can be a useful predictive marker for irinotecan-induced toxicity.

The most compelling evidence for a genetic marker of toxicity caused by irinotecan therapy is seen with the *UGT* gene. In some retrospective pharmacogenetic studies, patients with at least one *UGT*1A1*28 allele encountered severe irinotecan-induced toxicity, compared with those with the wild-type genotype who were homozygous for the 6 TA repeat allele (6,9,10). In a prospective study, the *UGT*1A1 genotype was strongly associated with severe neutropenia in patients treated with irinotecan (8). More than 30 polymorphic variations have been reported to date for the *UGT*1A1 gene (17). Novel polymorphisms (*1, *6, *28,*60 and so on) in *UGT*1A1 and the functional characterization of known variants are helpful in elucidating the role of *UGT*1A1 genetic variation in irinotecan toxicity (18). The FDA has approved a *UGT*1A1 molecular assay test to detect polymorphisms in the *UGT*1A1 gene in clinical practice, so that patients with particular *UGT*1A1 gene variations that raise the risk of certain adverse effects can receive safer doses of irinotecan. This assay is intended to aid physicians to make decisions for individualized patient. Nevertheless, other important factors that affect dosing should also be considered, because severe toxicity sometimes occurs even in patients without particular *UGT*1A1 gene variations that place them at risk.

The *UGT*1A1 enzyme is responsible for hepatic bilirubin glucuronidation. A polymorphism in the *UGT*1A1 promoter has been linked with reduced *UGT*1A1 expression and is consequently associated with familiar hyperbilirubinemia. Accordingly, bilirubin levels may be associated with *UGT*1A1 function. The PTB level may reflect the total function of some polymorphisms in the *UGT*1A1 region and may be used as a simple and available surrogate marker for *UGT*1A1 function.

Recent studies have revealed that two major hepatic UGT, *UGT*1A1 and *UGT*1A9, and extra-hepatic *UGT*1A7 are involved in SN-38 glucuronidation (SN-38G) (7,19). The

Table 4. Univariate analysis of association between grade 3–4 diarrhea and pre-treatment clinical variables

	Diarrhea grade		Odds ratio (95% CI)
	Grade 0–2 ($n = 114$)	Grade 3–4 ($n = 13$)	
Sex			
Male	84	9	1
Female	30	4	1.24 (0.36–4.34)
Age			
Median (range)	65 (24–74)	65 (53–73)	1.07 (0.99–1.16)
Performance status			
0	29	5	1
1, 2	85	8	0.55 (0.17–1.80)
Liver metastasis			
No	101	8	1
Yes	13	5	4.86 (1.38–17.1)
Prior chemotherapy			
No	99	11	1
Yes	15	2	1.20 (0.20–7.04)
Treatment schedule			
Every 3 weeks	50	6	1
Every 4 weeks	64	7	0.91 (0.29–2.88)
Cisplatin dose (mg/m^2)			
60	66	5	1
80	48	8	2.20 (0.68–7.14)
AST (IU/l)			
Median (range)	21 (11–161)	23 (15–65)	1.00 (0.98–1.03)
ALT (IU/l)			
Median (range)	17 (5–266)	21 (14–84)	1.01 (0.99–1.02)
PNC ($\times 10^9$/l)			
Median (range)	4.2 (1.8–8.5)	3.5 (2.2–5.2)	0.77 (0.49–1.20)
PTB (mg/dl)			
Median (range)	0.55 (0.2–2.4)	0.6 (0.4–1.1)	1.95 (0.29–13.2)
≤ 0.7	96	11	1
>0.7	18	2	0.97 (0.20–4.75)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; PNC, pre-treatment neutrophil count; PTB, pre-treatment total bilirubin.

efficacy of irinotecan is possibly affected by the activity of these genes. Thus, the product of some genetic polymorphisms in several genes may be a better pharmacogenetic marker for selecting patients who may not respond favorably to irinotecan-containing chemotherapy.

Cisplatin and irinotecan therapy is a standard regimen for both advanced non-small cell and small cell lung cancer (4). A randomized trial of irinotecan with or without cisplatin in patients with non-small cell lung cancer showed that grade 4 neutropenia was observed more frequently in the cisplatin–irinotecan arm (37%) than in the irinotecan-alone arm (8%), whereas grade 3 and 4 diarrhea was observed at the same frequency in both arms. In the present study, a higher cisplatin dose was associated with both grade 4 neutropenia and grade 3 and 4 diarrhea. The addition of cisplatin to another anti-cancer agent aggravated diarrhea in phase III studies (20), although diarrhea was moderate in cisplatin monotherapy observed in clinical trials (21). Thus, a higher dose of cisplatin seems to be associated with diarrhea, but the mechanism for this association remains unclear.

In this study PTB level was associated with the severity of neutropenia, but not with severity of diarrhea. When SN-38G is excreted in the bile and intestines, the bacteria-derived enzyme beta-glucuronidase converts SN-38G back

into SN-38 (22,23). Presence of SN-38 in the stool is associated with the occurrence of severe diarrhea as a result of the direct enteric injury caused by SN-38 (24). This phenomenon probably occurs because UGT1A1 is not involved in this step.

Liver metastasis was associated with the development of grade 3–4 diarrhea in both univariate and multivariate analyses in this study. This may be explained by small, but statistically significant differences in the pre-treatment transaminase levels between patients with or without liver metastasis. However, in contradiction to this explanation are that: (1) neither the pre-treatment AST nor ALT level was associated with grade 3–4 diarrhea in this study, and (2) in dose-finding studies of irinotecan monotherapy in patients with liver dysfunction, patients were categorized into subgroups by the PTB and serum AST and ALT levels, criteria of which were three times or five times the upper limit of normal (25,26). Thus, the small difference in the AST and ALT levels in this study is unlikely to be significant from the medical point of view.

The PNC in patients who developed grade 3–4 diarrhea was slightly lower than that in the other patients and the PNC was associated with grade 3–4 diarrhea in the multivariate analysis. Neutrophils play an important role in maintaining the mucosal barrier of the intestine and inflammatory responses against mucosal damage (27). Thus, reduced number, dysfunction, or both, of neutrophils may lead to impairment of the mucosal integrity, rendering these patients prone to develop diarrhea. In addition, the decreased number of neutrophils in the blood is closely related to malnutrition associated with cancer (28), which may in turn be associated with enhanced toxicity during chemotherapy with irinotecan and cisplatin.

In conclusion, the PTB level was significantly associated with severity of neutropenia in patients treated with cisplatin and weekly irinotecan. This will provide a simple and useful marker required for individualized therapy to reduce the risk of harmful chemotherapy.

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Conflict of interest statement

None declared.

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CYP2C9 and CYP2C19 Polymorphic Forms Are Related to Increased Indisulam Exposure and Higher Risk of Severe Hematologic Toxicity

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Abstract

Purpose: The anticancer agent indisulam is metabolized by the cytochrome P450 of enzymes CYP2C9 and CYP2C19. Polymorphisms of these enzymes may affect the elimination rate of indisulam. Consequently, variant genotypes may be clinically relevant predictors for the risk of developing severe hematologic toxicity. The purposes of this study were to evaluate the effect of genetic variants of CYP2C9 and CYP2C19 on the pharmacokinetics of indisulam and on clinical outcome and to assess the need for pharmacogenetically guided dose adaptation.

Experimental Design: Pharmacogenetic screening of CYP2C polymorphisms was done in 67 patients treated with indisulam. Pharmacokinetic data were analyzed with a population pharmacokinetic model, in which drug elimination was described by a linear and a Michaelis-Menten pathway. The relationships between allelic variants and the elimination pharmacokinetic parameters (CL, V_{max}, K_m) were tested using nonlinear mixed-effects modeling. Polymorphisms causing a high risk of dose-limiting neutropenia were identified in a simulation study.

Results: The Michaelis-Menten elimination rate (V_{max}) was decreased by 27% ($P < 0.0001$) for heterozygous CYP2C9*3 mutants. Heterozygous CYP2C19*2 and CYP2C19*3 mutations reduced the linear elimination rate (CL) by 38% ($P < 0.0001$). The risk of severe neutropenia was significantly increased by these mutations and dose reductions of 50 to 100 mg/m^2 per mutated allele may be required to normalize this risk.

Conclusions: CYP2C9*3, CYP2C19*2, and CYP2C19*3 polymorphisms resulted in a reduced elimination rate of indisulam. Screening for these CYP2C polymorphisms and subsequent pharmacogenetically guided dose adaptation may assist in the selection of an optimized initial indisulam dosage.

Indisulam is a sulfonamide anticancer agent that disrupts cell cycle progression in the G_1-S transition (1–3). Indisulam was well tolerated, but had only moderate single-agent activity in several phase II studies (3–8). The compound is currently being evaluated as an adjuvant to standard chemotherapy in multiple phase II clinical studies for the treatment of solid tumors.

Phase I studies showed that reversible neutropenia and thrombocytopenia were the dose-limiting toxicities of indisulam (9–14). The pharmacokinetic properties of the compound have been extensively studied (9–16). Drug clearance decreased with increasing dose, which was indicative for the saturable elimination of indisulam. A semiphysiologic population pharmacokinetic-pharmacodynamic model was developed, which included two parallel pathways for drug elimination: a saturable pathway with Michaelis-Menten kinetics and a linear pathway (16, 17). The interindividual variability of the maximal rate of Michaelis-Menten elimination (V_{max}) was 45%. Differences between patients in hepatic metabolic capacity account for this variability. The pharmacokinetic-pharmacodynamic model showed a clear relationship between pharmacokinetics and hematologic toxicity (17). Patients with impaired metabolic capacity may have a relatively high risk of severe myelosuppression due to higher drug exposure.

Results of a clinical mass balance study showed that more than 98% of indisulam is metabolized before it is excreted into the urine or feces (18). No data regarding the activity or toxicity of the metabolites are available. The major metabolite, *O*-glucuronide indisulam, is formed by glucuronidation of a hydroxyl metabolite of indisulam (18, 19). The hydroxyl metabolite is highly reactive and is immediately conjugated to form *O*-glucuronide or *O*-sulfate indisulam. Therefore, the formation of this hydroxyl metabolite may be a rate-limiting process in the metabolism of indisulam.

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