Table 4. Treatment Delivered

Treatment Data	Trial: FACS (n = 145) No.	FACS (n = 145) %	LC00-03 (n = 197) No.	LC00-03 (n = 197) %	S0003 (n = 184) No.	S0003 (n = 184) %	P
Median cycles delivered	3.5		4		4		.07
Received > three cycles	35	24	118	60	100	54	< .0001
Received six cycles	16	11	58	29	68	36.5	< .0001
Dose was reduced	No data	No data	100	51	98	26	.63*

Abbreviation: FACS, four-arm cooperative study.
*Wilcoxon rank sum test to compare LC00-03 and S0003. Patient-level data not available for FACS.

dosing and drug delivery of paclitaxel plus carboplatin in the FACS Japanese study highlights the contrast.

The rationale for conducting this common-arm project specifically in collaboration with Japanese investigators was based on several factors, including the established SWOG interaction described earlier, the high quality of lung cancer investigation by Japanese cooperative groups, and prior literature that suggested that overall, Japanese patients achieve better results than their US counterparts. However, the most compelling rationale was prior pharmacogenomic literature, which suggested that relevant drug disposition differences might exist between US and Japanese populations treated with cancer chemotherapeutic agents. Well recognized here are alterations in irinotecan metabolism as a result of variability in the allelic distribution of UDP-glucuronosyltransferases, particularly *UGT1A1*28* in different ethnic groups, as Asians have a much lower frequency of variant alleles. Recently, a comparative analysis of patient-level data from phase III trials in small-cell lung cancer in Japan (J9511) and the United States (S0124) demonstrated significant differences in toxicity profiles between the two groups. In addition, a pharmacogenomic analysis of S0124 showed significant associations between genotypic variants and toxicity levels.[16,17]

Table 5. Genotype Profiles in Japanese and US Patients on LC00-03 and S0003

Polymorphism by Trial Location	No. of Patients: Com	Het	Var	P
*CYP3A4*1B*				
Japan	73	0	0	.01
United States	64	4	3	
*CYP3A5*C*				
Japan	7	16	50	.03
United States	3	7	66	
CYP2C8 (R139K)				
Japan	69	2	0	.01
United States	57	7	5	
ABCB1 (3435C→T)				
Japan	33	21	17	.11
United States	24	23	29	
NR1I2 (206 deletion)				
Japan	51	19	5	.25
United States	40	25	8	
ERCC1 (118)				
Japan	8	27	43	< .0001
United States	23	33	19	
ERCC2 (K751Q)				
Japan	73	1	0	< .001
United States	37	27	8	

NOTE. LC00-03 is the trial in Japan; S0003 is the trial in the United States. Fisher's exact test was used to determine whether allele distributions were different between the populations.
Abbreviations: Com, common allele; Het, heterozygous allele; Var, variant allele.

The genes evaluated in this study were selected on the basis of their potential to influence paclitaxel disposition or DNA damage repair. Paclitaxel is principally eliminated through multiple hydroxylation reactions mediated by cytochrome isoforms *CYP2C8, CYP3A4,* and *CYP3A5.*[18,19] The *CYP2C8*3* variant (R139K), which is associated with decreased metabolism of paclitaxel, occurs at a frequency of 9% to 15% in white patients but is rare in African and Asian populations.[20-23] In this study, the allele frequency in the US population was 12%, which was significantly different from the less-than-1% frequency in the Japanese cohort (P = .01). *CYP2C8* genotypic variability at R139K was not significantly associated with patient outcome. CYP3A isozymes account for 45% to 60% of paclitaxel metabolism.[24] In white patients, the *CYP3A5* allele is commonly nonfunctional as a result of a transition in intron 3 that produces a truncated splice variant.[25] Our findings are consistent with that of Hustert et al,[25] who reported frequencies of functional *CYP3A5* as 5% in white patients, 29% in Japanese patients, and 73% in African American patients. Of patients enrolled onto the S0003 trial conducted in the US, three of three with the functional allele (indicated as common in Table 5) were African Americans, as were three of the seven heterozygous patients. Although trends were observed, *CYP3A5*3C* genotypic variability was not significantly associated with patient outcome (overall survival *P* = .07; PFS *P* = .09), perhaps related to the small sample size. Similarly, the *CYP3A4*1B* allele was observed in seven of seven African American patients but was absent in white and Japanese patients. In vitro studies suggest that the *CYP3A4*1B* variant has enhanced activity over common allele.[26] An association was observed between occurrence of the *CYP3A4*1B* and PFS (*P* = .04); however, this association should be interpreted in the context that only African American patients harbored this allele. Thus, it remains unclear whether this potential relationship with outcome is associative or causative. The PXR (*NR1I2*-206 deletion) is a master regulator of genes involved in xenobiotic detoxification and influences transcription of *CYP3A4, CYP3A5, CYP2C8,* and *MDR-1 (ABCB1).*[27-29] Paclitaxel can activate PXR, which enhances drug clearance through increased activity of MDR1.[30] No significant differences by genotype were observed for PXR or ABCB1, although there was a trend toward

Table 6. Cox Model to Compare Outcomes by Polymorphism

Outcome by Polymorphism	Analyses			
	Comparison	HR	95% CI	*P*
ABCB1 3425				
Overall survival	Com *v* Het/Var (CC *v* CT/TT)	1.09	0.71 to 1.67	.69
PFS		1.04	0.70 to 1.56	.82
Response		0.97	0.39 to 2.38	1.00
Neutropenia		0.54	0.22 to 1.30	.19
CYP2C8 R139K				
Overall survival	Com *v* Het/Var (GG *v* GA/AA)	1.09	0.61 to 1.96	.76
PFS		1.12	0.63 to 2.00	.69
Response		1.92	0.46 to 11.11	.51
Neutropenia		1.30	0.35 to 5.00	.87
*CYP3A4***1B*				
Overall survival	Com *v* Het/Var (AA *v* AG/GG)	0.74	0.32 to 1.72	.48
PFS		0.36	0.14 to 0.94	.04
Response		0.63	0.10 to 4.76	.84
Neutropenia		0.44	0.04 to 2.94	.58
*CYP3A5***3C*				
Overall survival	Com/Het *v* Var (AA/AG *v* GG)	1.64	0.95 to 2.86	.07
PFS		1.56	0.93 to 2.63	.09
Response		1.61	0.53 to 4.76	.47
Neutropenia		1.30	0.44 to 3.85	.78
ERCC1 (118)				
Overall survival	TT *v* TC/CC	1.20	0.74 to 1.96	.45
PFS		1.11	0.69 to 1.82	.65
Response		1.45	0.48 to 4.17	.61
Neutropenia		0.57	0.20 to 1.61	.35
ERCC2 K751Q				
Overall survival	Com *v* Het/Var (AA *v* AC/CC)	0.97	0.63 to 1.49	.89
PFS		0.85	0.55 to 1.30	.45
Response		0.33	0.13 to 0.83	.02
Neutropenia		0.75	0.30 to 1.85	.63
nr1I2-206 del				
Overall survival	Com *v* Het/Var 206 deletion	0.82	0.53 to 1.25	.35
PFS		0.93	0.63 to 1.39	.75
Response		0.82	0.34 to 2.00	.77
Neutropenia		0.88	0.37 to 2.08	.90

Abbreviations: HR, hazard ratio; PFS, progression-free survival; Com, common allele; Het, heterozygous allele; Var, variant allele.

neutropenia ($P = .19$) for patients who harbored the ABCB1 3435 common allele.

The *ERCC2* gene, also known as xeroderma pigmentosum complementation group D, encodes a DNA helicase which complexes with TFIIH, a transcription factor essential for replication and nucleotide excision repair.[31] Several nonsynonymous SNPs have been described in this gene, including an Asp→Asn (G→A) at codon 312 in exon 10 and a Lys→Gln (A→C) at codon 751 in exon 23 and are likely in linkage disequilibrium with each other.[32,33] The functional consequences of these SNPs are still in contention, and the majority of studies indicate that variants in these alleles result in reduced DNA repair capacity.[34-41] Additionally, most studies indicate that *ERCC2* variants confer an increased risk of lung cancer.[32,34,35,42-48] In this study, 51% of patients (ie, 37 of 72 patients) from the US were homozygous wild type for the common (A) allele. These patients were significantly less likely to respond to treatment compared with US patients who had one or more variant alleles (A/C or C/C). However, no differences in overall survival were observed on the basis of *ERCC2* K751Q allele frequencies. In addition, this allele cannot account for the improved survival experienced by Japanese patients, as they uniformly harbored the common A/A genotype (and only one patient harbored A/C). The *ERCC1* 118 C→T SNP does not result in an amino acid substitution, although studies have nevertheless identified associations with patient outcome in various tumor types.[49] It has been suggested that this variant may modulate *ERCC1* mRNA and protein expression and/or may be in linkage disequilibrium with other functional SNPs.[14,50,51] However, three reports in NSCLC found no associations between the *ERCC1* 118 and patient outcome.[52-54] Here, we found a highly significant divergence in allele frequency between Japanese and US patients ($P < .0001$); however, no impact on patient outcome was observed.

In summary, the results of cancer clinical trials to test the same regimen may differ for a variety of reasons, including differences related to ethnicity. FACS, LC00-03, and S0003 were prospectively designed to facilitate a comparison of patient outcomes and pharmacogenomic results, in a setting where joint clinical trials sponsored by the US National Cancer Institute were not possible. Our

results suggest that global clinical trials (ie, those conducted internationally) should be carefully designed and conducted to account for potential genetic differences in the patient populations studied. This common-arm approach provides a model for the prospective study of population-related pharmacogenomics in which ethnic differences in antineoplastic drug disposition are anticipated.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

Employment or Leadership Position: None **Consultant or Advisory Role:** David R. Gandara, Bristol-Myers Squib (C) **Stock Ownership:** None **Honoraria:** Kaoru Kubota, sanofi-aventis, Eli Lily, Chugai **Research Funding:** David R. Gandara, Bristol-Myers Squib; Charles Arthur Coltman Jr, National Cancer Institute **Expert Testimony:** None **Other Remuneration:** None

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Financial support: Charles Arthur Coltman Jr

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Serum Osteopontin Levels are Highly Prognostic for Survival in Advanced Non-small Cell Lung Cancer

Results from JMTO LC 0004

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Background: The Japan-Multinational Trial Organization (JMTO) lung cancer (LC) 0003 was a prospective randomized phase III trial investigating advanced non-small cell lung cancer comparing paclitaxel (P) plus carboplatin (C) versus vinorelbine (V), gemcitabine (G) followed by docetaxel (D). This trial was conducted with Southwest Oncology Group (SWOG) 0003 using a common arm of PC. An analysis of SWOG 0003 samples showed that low osteopontin (OPN) plasma levels were highly prognostic for a better outcome. We performed an independent investigation to validate these results using samples from Japanese patients enrolled in the JMTO LC 0004, a correlative study associated with JMTO LC 0003.

Methods: A total of 20 ml of blood was collected before treatment from patients enrolled in JMTO LC 0003. Serum concentrations of OPN and basic fibroblast growth factor (bFGF) were measured by enzyme-linked immunosorbent assay. Effects of OPN and bFGF levels on tumor response, progression-free survival (PFS), and overall survival (OS) were examined.

Results: Seventy-one samples were obtained, including 32 specimens from the PC arm and 39 from the VGD arm. There were no significant relationships between either OPN or bFGF levels with patient characteristics. In an analysis of clinical outcome, low OPN levels correlated with better OS and progression-free survival (hazard ratio [HR] = 0.57; 95% confidence interval [CI], 0.33–0.97; p = 0.037, HR = 0.42; 95% CI, 0.25–0.70; p = 0.001, respectively) and high bFGF levels correlated with better OS (HR = 0.53; 95% CI, 0.31–0.90; p = 0.018).

Conclusion: Consistent with the findings from SWOG 0003, low OPN serum levels were significantly associated with a favorable prognosis in the JMTO LC 0004. Additionally, high bFGF levels were associated with improved survival.

Key Words: Non-small cell lung cancer, Osteopontin, bFGF, Correlative study.

(*J Thorac Oncol.* 2009;4: 1104–1110)

Lung cancer (LC) is the most common cause of death due to cancer in Japan and in the United States.[1,2] Non-small cell LC (NSCLC) accounts for about 80 to 85% of LC histology in both countries,[3] and most patients with NSCLC have either locally advanced or metastatic disease at the time of diagnosis. Because currently available treatments still provide only a modest increase in overall survival (OS) for patients with advanced NSCLC, identification of prognostic and/or predictive biomarkers would be important. Global clinical trials have been conducted, and international efforts to develop predictive/prognostic biomarkers should also be made to improve clinical outcome in patients with advanced NSCLC.

A prospective randomized phase III trial conducted for advanced NSCLC compared paclitaxel (P) plus carboplatin (C) versus vinorelbine (V), gemcitabine (G) followed by docetaxel (D) in the Japan-Multinational Trial Organization (JMTO).[4] A total of 401 patients were enrolled and 393 were eligible. This trial was conducted with Southwest Oncology Group (SWOG) 0003 using a "common arm" of PC. The common arm concept was designed to prospectively evaluate Japanese and U.S. populations using equivalent patient eligibility criteria, staging, and treatment.[5] SWOG 0003 was a phase III trial investigating PC with or without tirapazamine in advanced NSCLC. A correlative study conducted on specimens from SWOG 0003 showed that low osteopontin (OPN) plasma levels were associated with a better survival regardless of the treatment arm. They concluded that OPN plasma

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Disclosure: The authors declare no conflicts of interest.

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ISSN: 1556-0864/09/0409-1104

levels can be considered as a prognostic biomarker in chemotherapy-treated patients with advanced NSCLC.[6]

OPN is a phosphorylated acid glycoprotein with a diverse range of biologic activities.[7] Secreted OPN interacts with members of the integrin family and variants of CD44, stimulating a variety of downstream processes associated with tumor progression or cellular transformation.[8–10] OPN induces an expression of urokinase-type plasminogen activator and increases the cell migration and adhesion, contributing to cellular transformation and metastasis.[11–14] Through this process, OPN is associated with angiogenesis, leading to a more aggressive tumor phenotype. Moreover, OPN was shown to be associated with clinical prognosis in various tumor types including LC.[15,16]

On the other hand, basic fibroblast growth factor (bFGF) is a proangiogenic growth factor, which is encoded by a single copy gene and has several isoforms ranging in size from 18 to 24 kd, including a heparin-binding domain.[17] It acts as a mitogenic and chemotactic stimulant for endothelial cells and can synergize with vascular endothelial growth factor to promote tumor angiogenesis. Overexpression of bFGF in LC is reported to be closely involved in cancer proliferation and is associated with prognosis.[18,19]

In JMTO LC 0004 (the correlative study of JMTO LC 0003; Ref. 4), we elected to study the prognostic value of OPN and bFGF in our patient population. We hypothesized that serum levels of OPN and bFGF would correlate with patient outcomes. Here, we report results demonstrating that OPN concentrations were significantly associated with prognosis in patients with NSCLC, consistent with the results from SWOG 0003.

PATIENTS AND METHODS

Study Population and Sample Collection

Blood specimens were obtained from consenting patients with histologically or cytologically confirmed stage IIIB (positive pleural effusion) or stage IV disease (no brain metastases) enrolled in JMTO LC 0003. Approximately 20 ml of peripheral blood was collected before treatment from patients electing to enroll in JMTO LC 0004. Serum was isolated and stored in aliquots at −80°C.

Measurement of Serum OPN and bFGF Concentrations

Serum was separated from peripheral blood by microcentrifuge at 3000 rpm at room temperature. Serum concentrations of OPN and bFGF were measured using the Quantikine Human OPN immunoassay kit (DOST00; R&D systems, Minneapolis, MN) according to the manufacturer's instructions. Briefly, 1:25 diluted samples were incubated in OPN or bFGF antibody-precoated plates for 2 hours at room temperature. After washing, 200 μl of labeled OPN or bFGF conjugate solution was added to each well and incubated for 2 hours at room temperature. After washing, a substrate solution containing tetramethylbenzidine was used as a coloring agent. Measurements were taken on a plate reader (Model 550 Microplate Reader; Bio-Rad Laboratories, Hercules, CA) at 450 nm with wavelength correction at 550 nm.

Statistical Analysis

The relationships between OPN/bFGF levels, and patients characteristics were examined using the χ^2 test, including treatment arm, age, smoking status, histology, clinical stage, performance status, weight loss, and lactate dehydrogenase. Effects of OPN and bFGF levels on tumor response were examined using the logistic regression analysis. Progression-free survival (PFS) and OS curves were calculated using the Kaplan-Meier method and analyzed by the log-rank test. Statistical analyses were done using SAS version 9.1 (SAS Institute, Cary, NC).

RESULTS

Serum OPN and bFGF Levels of Patients with NSCLC in LC 0004

Seventy-one samples were obtained, including 32 specimens from the PC arm and 39 from the VGD arm. Serum OPN levels of 67 patients ranged from 17.5 to 300.4 ng/ml, with a median value of 69.0 ng/ml. Serum bFGF level in 71 patients ranged from 0.3 to 133.1 ng/ml, with a median value of 14.9 ng/ml. No correlation was observed between serum levels of OPN and bFGF (Spearman's $r = 0.264$).

Patient Characteristics by Median Levels

We analyzed the association between patient characteristics and tumor marker status stratified at the median (Tables 1, 2). Smokers trended toward having higher OPN levels ($p = 0.083$); however, no other relationships were observed.

Response

Patients were dichotomized at the median serum concentrations of OPN and bFGF and were examined for relationships with tumor response. The response rate was 18% (6 of 34) in patients with high OPN levels and 33% (11 of 33) in those with low OPN levels. In patients with high bFGF levels, response rates were 25% (9 of 36) compared with 29% (10 of 35) in those with low bFGF levels. Differences in response rates between high and low biomarker concentrations were not significant (OPN: OR = 0.43, 95% confidence interval [CI]: 0.14–1.34, $p = 0.145$; bFGF: OR = 0.83, 95% CI: 0.29–2.39, $p = 0.734$).

Serum Levels of OPN and bFGF are Prognostic for Patient Survival

The relationship between OPN and bFGF and patient survival was investigated. Kaplan-Meier plots for OS and PFS are shown in Figures 1 and 2, respectively. Patients were dichotomized according to median serum levels of OPN and bFGF. Those with OPN levels below the median showed a significantly favorable OS and PFS (hazard ratio [HR] = 0.57; 95% CI: 0.33–0.97; $p = 0.037$; HR = 0.42; 95% CI: 0.25–0.70; $p = 0.001$, respectively; Figures 1*A*, *B*). When examined as a continuous variable, OPN levels also had a significant association with both OS and PFS (HR = 1.006; 95% CI: 1.001–1.011; $p = 0.021$; HR = 1.008; 95% CI: 1.003–1.013; $p = 0.002$, respectively).

Patients with bFGF levels above the median showed significantly improved survival compared with those with

TABLE 1. Patient Characteristics by Osteopontin

	Osteopontin <69 ng/ml (n = 33), n (%)	Osteopontin >69 ng/ml (n = 34), n (%)	p
Treatment			
PC	16 (48)	17 (50)	1.000
VGD	17 (52)	17 (50)	
Age (yr)			
Median	66	63	0.198
Range	40–79	51–79	
Sex			
Male	21 (64)	25 (74)	0.437
Female	12 (36)	9 (26)	
Smoking			
Never smoker	15 (45)	7 (21)	0.083
Former smoker	9 (27)	11 (32)	
Current smoker	9 (27)	16 (47)	
Histology			
Squamous cell	8 (24)	7 (21)	0.321
Adenocarcinoma	25 (76)	24 (70)	
Other	0 (0)	3 (9)	
Stage			
IIIB	7 (21)	11 (32)	1.000
IV	26 (79)	23 (68)	
Performance status			
0	7 (21)	11 (32)	0.410
1	26 (79)	23 (68)	
Weight loss			
<5%	26 (79)	29 (85)	0.539
>5%	7 (21)	5 (15)	
Lactate dehydrogenase			
Normal	27 (82)	28 (82)	1.000
Abnormal	6 (18)	6 (18)	

PC, paclitaxel (P) plus carboplatin (C); VGD, vinorelbine (V), gemcitabine (G) followed by docetaxel (D).

TABLE 2. Patient Characteristics by bFGF

	bFGF <14.9 ng/ml (n = 35), n (%)	bFGF >14.9 ng/ml (n = 36), n (%)	p
Treatment			
PC	18 (51)	14 (39)	0.0344
VGD	17 (49)	22 (61)	
Age (yr)			
Median	66	65	0.607
Range	51–76	48–79	
Sex			
Male	28 (80)	25 (69)	0.415
Female	7 (20)	11 (31)	
Smoking			
Never smoker	10 (29)	10 (28)	0.793
Former smoker	11 (31)	9 (25)	
Current smoker	14 (40)	17 (47)	
Histology			
Squamous cell	10 (29)	5 (14)	0.294
Adenocarcinoma	23 (66)	28 (78)	
Other	2 (6)	3 (8)	
Stage			
IIIB	9 (26)	8 (28)	0.786
IV	26 (74)	28 (72)	
Performance status			
0	8 (23)	11 (31)	0.594
1	27 (77)	25 (69)	
Weight loss			
<5%	30 (86)	29 (81)	0.753
>5%	5 (14)	7 (19)	
Lactate dehydrogenase			
Normal	24 (69)	31 (86)	0.094
Abnormal	11 (31)	6 (14)	

bFGF, basic fibroblast growth factor; PC, paclitaxel (P) plus carboplatin (C); VGD, vinorelbine (V), gemcitabine (G) followed by docetaxel (D).

levels below the median (HR = 0.53, 95% CI: 0.31–0.90, p = 0.018; Figure 2*A*). However, this association did not achieve significance for PFS (p = 0.192; Figure 2*B*).

The multiparametric analysis using both markers is shown in Figure 3. The cases were stratified according to OPN and bFGF levels into four groups: group 1, OPN less than median level/bFGF more than median level; group 2, OPN less than median level/bFGF less than median level; group 3, OPN more than median level/bFGF more than median level; and group 4, OPN more than median level/bFGF less than median level. Assuming group 4 as a reference, the patients of group 1 had significantly better OS and PFS (OS: HR = 0.21, 95% CI: 0.09–0.50, $p < 0.001$; PFS: HR = 0.23, 95% CI: 0.10–0.53, $p < 0.001$).

DISCUSSION

We demonstrated that low OPN serum levels were significantly associated with a favorable prognosis in the Japanese patients, which is consistent with findings from SWOG 0003 in the U.S. population.[6] In addition, high bFGF levels were also associated with improved patient survival.

JMTO LC 0004, a correlative study of JMTO LC 0003, was originally designed to assess β-tubulin mutations and methylation of DNA repair gene in the patient serum as biomarkers. However, the former was determined to be insignificant,[20,21] and in the latter, we failed to detect sufficient tumor DNA in the serum for analysis. We had planned to keep patient specimens for future analysis as a secondary end point. The JMTO LC 0003 was conducted with SWOG 0003 using a common arm of PC. As for ethnicity, there was a marked difference between the two trials. Exclusively, our study included Japanese patients, whereas in SWOG, most patients were African American.

For collaboration with SWOG 0003, we performed two additional studies using the stock samples approved by the JMTO Institutional Review Board. We previously collaborated on pharmacogenomic studies of patient samples analyzed from both trials. Gandara et al. found significant differences in survival and toxicity between the two populations. The Japanese patients with advanced NSCLC lived longer and had more severe side effects compared with the U.S. population. Median survival time was 14 months in the

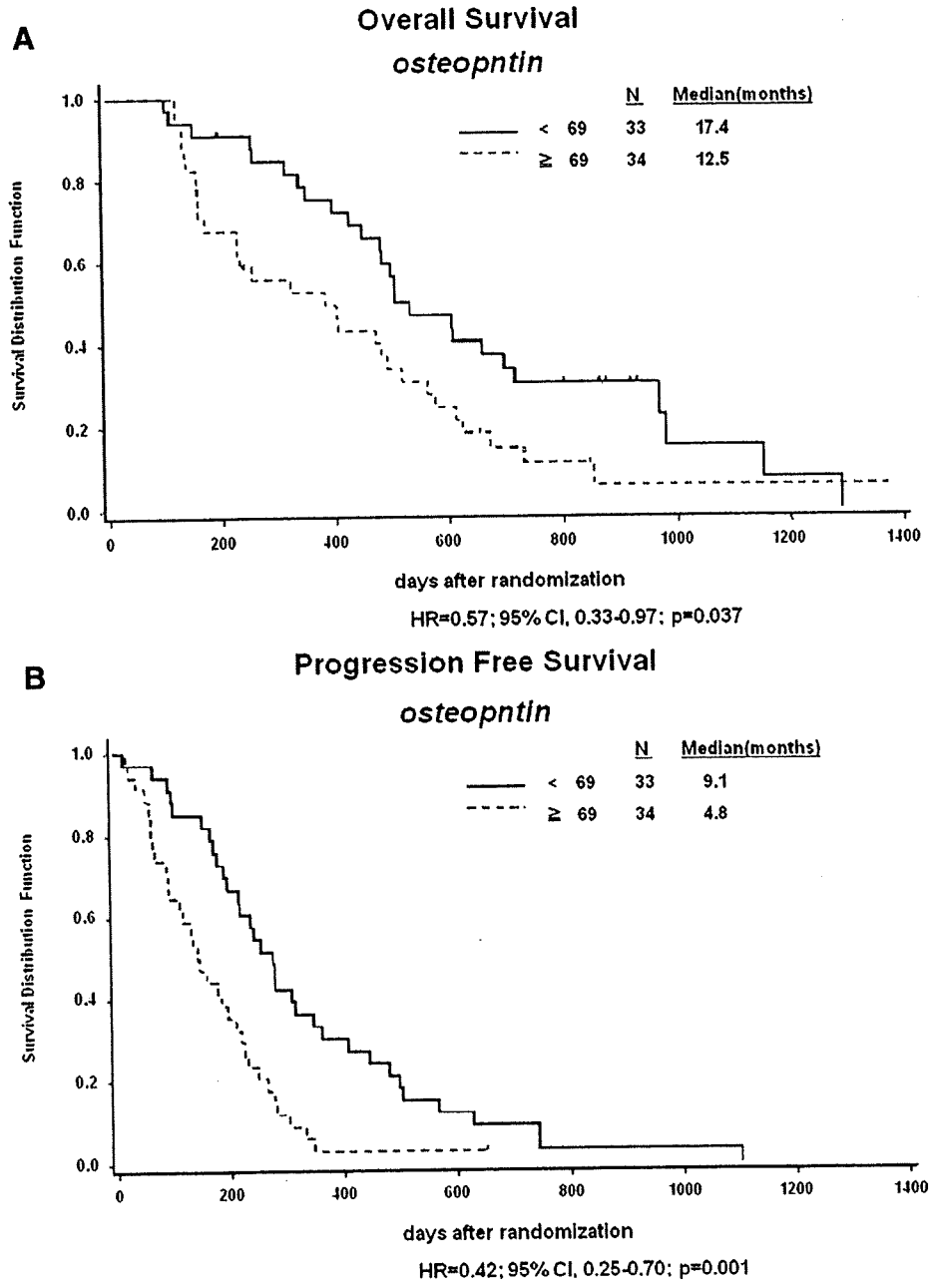


FIGURE 1. Overall survival and progression-free survival by serum OPN level. *A*, Overall survival (Kaplan-Meier) plot showing patients divided into higher or lower than the median serum concentration of OPN (69 ng/ml). Patients with lower OPN levels (*solid line*) showed significantly better overall survival compared with those with higher levels (*dotted line*). *B*, Patients with lower OPN levels (*solid line*) had significantly better progression-free survival compared with those with higher levels (*dotted line*; $p < 0.001$). OPN, osteopontin.

Japanese patients, whereas it was 9 months in the U.S. patients, and neutropenia for toxicity was observed in 70% of Japanese patients compared with 38% in the U.S. patients. Significant differences in allelic distribution for genes involved in paclitaxel disposition and DNA repair were observed between Japanese and U.S. patients.[5]

Additional analysis of SWOG 0003 samples conducted previously showed that OPN was a prognostic marker, independent of treatment arm.[6] We then sought to determine whether these results would be recapitulated in Japanese patients from the JMTO LC 0004. In this study, we found OPN to be a useful and prognostic marker in patients with NSCLC independent of ethnicity, despite the clinical and pharmacogenomic differences observed between the populations mentioned earlier.

Consistent with the SWOG study, we found that the patients with low OPN had a significantly better OS and PFS (HR = 0.57; 95% CI: 0.33–0.97; $p = 0.037$; HR = 0.42; 95% CI: 0.25–0.70; $p = 0.001$, respectively) when dichotomized at the median and as a continuous variable. OPN has been reported to be involved in tumor invasion and metastasis. High OPN serum levels have been correlated to increased invasion in solid tumors,[22] which is suggested to predict worse prognosis.

In the treatment response rate, OPN levels were also a significant predictive marker in the SWOG study, but we

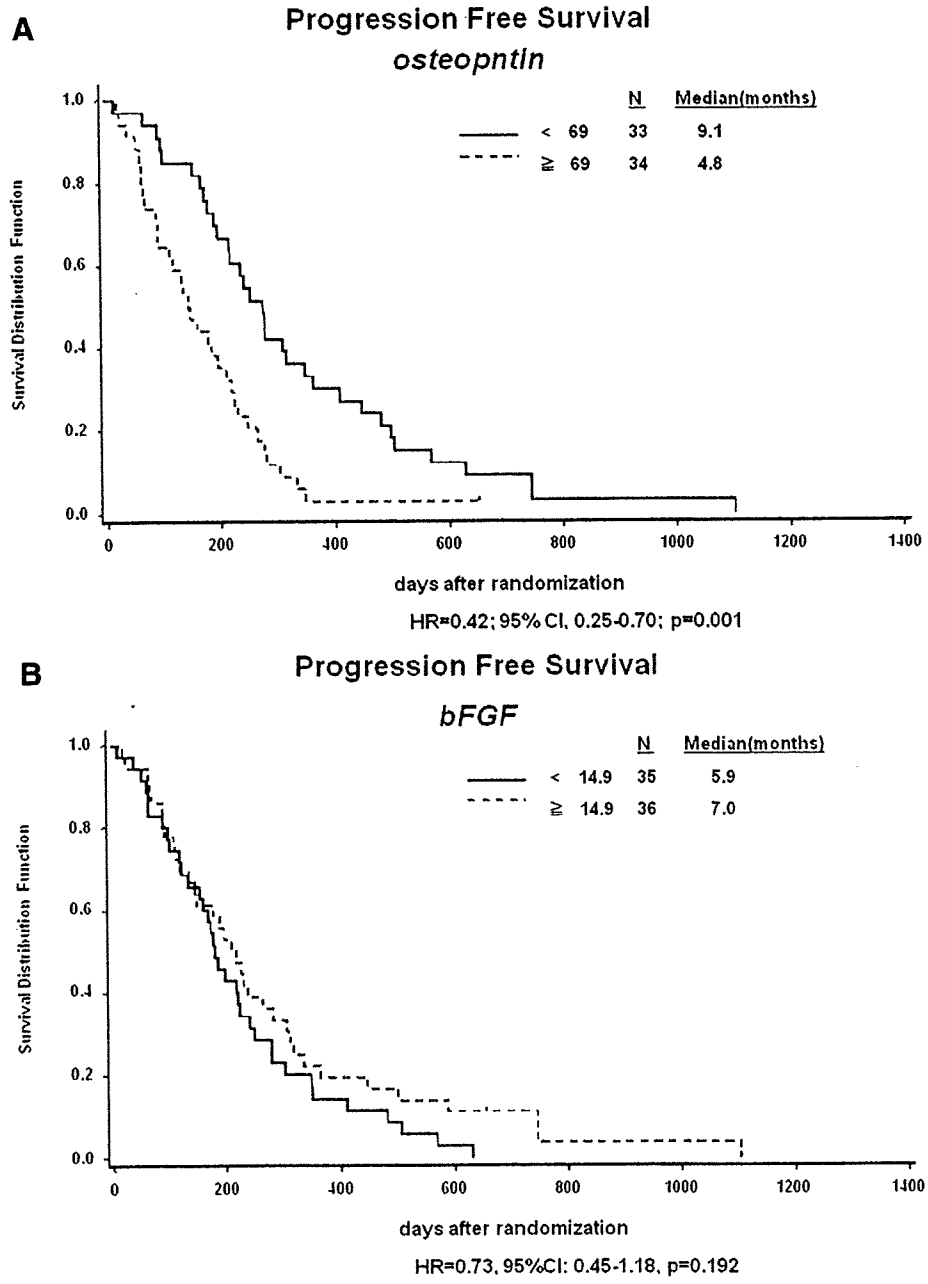
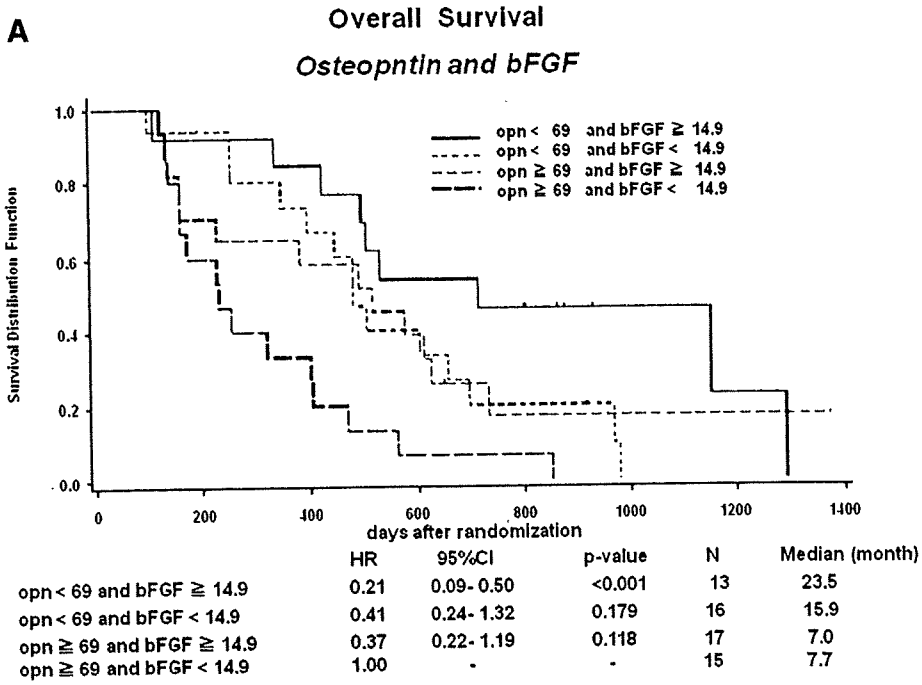


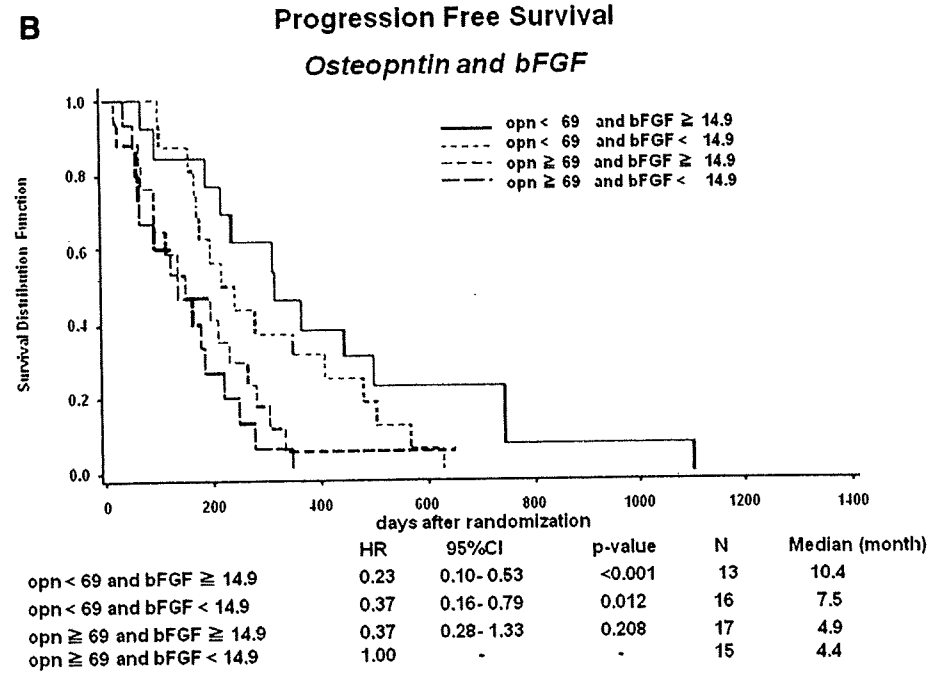
FIGURE 2. Overall survival and progression-free survival by bFGF serum level. *A*, Overall survival (Kaplan-Meier) plot showing patients divided into higher or lower than the median serum concentration of bFGF (14.9 ng/ml). Patients with higher bFGF levels (*dotted line*) had significantly better overall survival compared with those with lower levels (*solid line*; $p = 0.03$). *B*, There was no significant association between bFGF level and progression-free survival. bFGF, basic fibroblast growth factor.

could not confirm these results. It was reported that PFS was significantly associated with response rate in LC patients,[23] and the significant relationship between OPN level and PFS in this study may indicate that our lack of statistical significance is perhaps because of the limited sample size. However, we cannot rule out the different types of drugs used in the experimental arms in the two trials.

In this study, we also found that serum bFGF levels were a prognostic factor in patients with NSCLC. The low OPN/high bFGF group had the most favorable survival in a multiparametric model, suggesting that adding bFGF analysis enhances the prognosis value of OPN. Previously, we reported that bFGF showed no correlation with survival in 60 patients with stage I–IV NSCLC. The difference in the results is partly because of the clinical stage of the patients examined. To date, there have been four studies on the prognostic value of circulating bFGF; three studies reported a negative prognostic impact, whereas one indicated bFGF as a favorable prognostic factor.[24] Yiangou et al.[25] reported that patients with high expression of bFGF had better outcomes than patients with low expression in breast cancer. Although bFGF is a potent mitogenic factor, they suggested that bFGF contributes to the maintenance of differentiation of the duct rather than cell proliferation in breast cancer. Also, elevated levels of serum bFGF



	HR	95%CI	p-value	N	Median (month)
opn < 69 and bFGF ≧ 14.9	0.21	0.09-0.50	<0.001	13	23.5
opn < 69 and bFGF < 14.9	0.41	0.24-1.32	0.179	16	15.9
opn ≧ 69 and bFGF ≧ 14.9	0.37	0.22-1.19	0.118	17	7.0
opn ≧ 69 and bFGF < 14.9	1.00	-	-	15	7.7



	HR	95%CI	p-value	N	Median (month)
opn < 69 and bFGF ≧ 14.9	0.23	0.10-0.53	<0.001	13	10.4
opn < 69 and bFGF < 14.9	0.37	0.16-0.79	0.012	16	7.5
opn ≧ 69 and bFGF ≧ 14.9	0.37	0.28-1.33	0.208	17	4.9
opn ≧ 69 and bFGF < 14.9	1.00	-	-	15	4.4

FIGURE 3. The multiparametric model showed that the patients of group 1 had significantly better OS and PFS compared with group 4 patients (OS: HR = 0.21, 95% CI: 0.09–0.50, $p < 0.001$; PFS: HR = 0.23, 95% CI: 0.10–0.53, $p < 0.001$). Patients were subdivided into four groups based on OPN and bFGF serum levels: group 1, OPN less than median level/bFGF more than median level; group 2, OPN less than median level/bFGF less than median level; group 3, OPN more than median level/bFGF more than median level; and group 4, OPN more than median level/bFGF less than median level. OPN, osteopontin; bFGF, basic fibroblast growth factor; OS, overall survival; PFS, progression-free survival.

might contribute to abundant vascular bed formation in LC and more anticancer drugs might reach cancer cells.[26] Given these suggestions, improved clinical outcome might be shown in the patients with high bFGF. However, it is still controversial as to whether bFGF is predictive of outcome in patients treated with chemotherapy. These results need to be confirmed by further examination.

Limitations of the current study include a small sample size. JMTO LC 0004 is a correlative study and had a protocol different from LC 0003. Not all the investigators participated in it, which might result in the low percentage of patient samples analyzed. Another problem in this study is a long period for accrual for the clinical trial; we needed 4 years to complete the trial and the end point had to be changed through scientific advancement. This study highlights the importance of collecting and retaining patient samples for future research.

In conclusion, consistent with findings from SWOG 0003, low OPN serum levels were significantly associated with a favorable prognosis in the JMTO LC 0004. OPN

will be a useful biomarker to predict prognosis in patients with NSCLC to be tested in future multinational clinical trials.

ACKNOWLEDGMENTS

We thank Dr. David Gandara, Davis Cancer Center, University of California, and Dr. Sato, Division of Molecular Biology, Tottori University, for their review. We also thank Dr. Kubo, NHO Kinki-chuo Chest Medical Center, and Dr. Takada, Kinki University Sakai, for their kind advice on the preparation of the manuscript.

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J Cancer Res Clin Oncol (2009) 135:313–318
DOI 10.1007/s00432-008-0464-5

ORIGINAL PAPER

EGFR R497K polymorphism is a favorable prognostic factor for advanced lung cancer

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Received: 19 May 2008 / Accepted: 10 August 2008 / Published online: 23 August 2008

Abstract
Introduction It has been reported that the R497K polymorphism of the epidermal growth factor receptor (*EGFR*) gene has attenuated functions in ligand binding, tyrosine kinase activation, and growth stimulation. On other hand, *EGFR* gene mutations at kinase domain in non-small cell lung cancer (NSCLC) have been examined for their ability to predict sensitivity to gefitinib or erlotinib.
Materials and methods We investigated the *EGFR* mutations and/or R497K polymorphism statuses in 225 surgically treated NSCLC cases. 192 adenocarcinoma cases were included. The presence or absence of *EGFR* polymorphism of exon 13 was analyzed by PCR–RFLP method.
Results *EGFR* mutations at kinase domain were found from 95 of 225 lung cancer patients. In 86.2% of patients, homo- or heterozygous Lys497 allele was present. No correlation existed between R497K *EGFR* genotype and clinico-pathological features, such as gender, smoking status, and pathological subtypes.
Conclusions *EGFR* mutation status was not correlated with R497K*EGFR* genotype of lung cancers. In node-negative patients, R497K*EGFR* genotype was not correlated with disease outcome. In node-positive patients, however, R497K *EGFR* was significantly associated with better overall survival. This association was attributable to neo-adjuvant or adjuvant chemotherapy. In 46 total gefitinib treated NSCLC patients, the prognosis was not different between the *EGFR* wild type (GG) patients and AG+AA patients. R497K*EGFR* polymorphism might be associated with favorable prognosis of advanced lung cancers and correlated with chemosensitivity.

Keywords EGFR · Lung cancer · Polymorphism · R497K

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Introduction

Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior, and lack of major advancements in treatment strategy (Ginsberg et al. 1993). There are much accumulated evidences that epidermal growth factor receptor (*EGFR*) and its family member are strongly implicated in the development and progression of numerous human tumors, including lung cancer (Nicolson et al. 2001; Onn et al. 2004). The *EGFR* tyrosine kinase inhibitor, gefitinib, was approved in Japan for the treatment of non-small cell lung cancer (NSCLC) since 2002. In 2004, two reports have shown that *EGFR* mutation statuses at tyrosine kinase (TK) domain in NSCLC patients were correlated with the clinico-pathological features related to good response to gefitinib (Paez et al. 2004; Lynch et al. 2004). *EGFR* mutations in lung cancer have been correlated with clinical response to gefitinib therapy in vivo and in vitro (Paez et al. 2004; Lynch et al. 2004; Pao et al. 2004). Genomic

profiling of the EGFR signaling is also helpful in identifying lung cancer patients who are at risk of tumor recurrence and those who are more likely to benefit from chemoradiation therapy. For example, the NSCLC patients with more than 35 (CA)n repeats in *EGFR* intron 1 polymorphism had a significantly longer overall survival than the patients with the 35 or fewer (CA)n alleles, who received radiation (RT; 50.4 Gy) or RT concurrent with chemotherapy (CT; four cycles of cisplatin plus etoposide) (Dubey et al. 2006; Keller et al. 2000). *EGFR* intron 1 and −216G/T polymorphisms influenced clinical outcomes in gefitinib-treated NSCLC patients (Liu et al. 2008). A polymorphic variant *EGFR* arising from a single nucleotide change (G–A) leading to an arginine (Arg) to lysine (Lys) substitution in codon 497 (R497K) in the extracellular domain of EGFR has been identified (Moriai et al. 1994). This polymorphism alone or in combination with another polymorphism in the same gene is associated with a lower recurrence of tumor in rectal cancer patients treated with chemoradiation (Zhang et al. 2005). To determine this *EGFR* polymorphism status and correlation with clinicopathological features in Japanese lung carcinoma, we investigated *EGFR* gene status by PCR–RELP method and direct sequencings. The findings were compared to the clinicopathologic features of lung cancer.

Materials and methods

Patients and samples

The study group included 206 lung cancer patients who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 1997 and 2005. Fifty eight patients were treated with platinum-based neoadjuvant or adjuvant chemotherapy. Twenty seven patients were treated with gefitinib for their recurrence of lung cancer after they had undergone surgery. We have also investigated *EGFR* R497K status for 19 NSCLC patients who had treated with gefitinib for their recurrence of lung cancer after undergone surgery at the National Hospital Organization, Kinki-chuo Chest Medical Center. The lung tumors were classified according to the general rule for clinical and pathological record of lung cancer in Japan, as well as WHO classification. All tumor samples were immediately frozen and stored at −80°C until assayed.

The clinical and pathological characteristics of the 225 lung cancer patients were as follows; 132 (58.6%) were male and 93 were female. One hundred and ninety two were diagnosed as adenocarcinoma, and 33 were diagnosed as other types of carcinoma (20 squamous cell carcinomas, eight adenosquamous carcinomas and five large cell carcinomas). One hundred and twenty five (55.6%) were smoker (current smoker or ever smoker) and 100 were non-smoker. Written informed consent was obtained from the patients, and the institutional ethics committee of the Nagoya City University approved the study.

PCR assays for *EGFR* polymorphism

Genomic DNA was extracted using Wizard SV Genomic DNA purification Systems (Promega) according to the manufacturers' instructions. *EGFR* mutation statuses at kinase domain were investigated using TaqMan PCR assay (Applied Biosystems). The sequences of 13 allele-specific TaqMan MGB probes and primer sets used in the TaqMan PCR assay were already shown (Endo et al. 2005). The results of TaqMan PCR assays were already reported. The R497K *EGFR* (G–A) polymorphism was examined by PCR–RELP method as described previously (Zhang et al. 2005; Wang et al. 2007). Briefly, the PCR reactions were performed using LA-Taq kit (Takara Bio Inc, Shiga, Japan) in a 50 μl reaction volume. The primer sequences for *EGFR* gene at exon 13 were as follows: the forward primer, 5′-TGCTGTGACCCACTCTGTCT-3′ and the reverse primer, 5′-CCAGAAGGTTGCACTTGTCC-3′. The cycling conditions were as follows: initial denaturation at 95°C for 3 min, followed by 35 cycles at 94°C for 60 s, 59°C for 60 s, 72°C for 60 s. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA), and then digested by BstN1 restriction enzyme (New England Biolabs) at 60°C for 16 h. These samples were separated on 4% ethidium bromide-stained agarose gels. In some cases, direct sequencing were performed and analyzed by BLAST and chromatograms by manual review.

Statistical analysis

Statistical analyses were done using the Mann–Whitney *U* test for unpaired samples and Wilcoxon's singed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and χ^2 test. The overall survival of lung cancer patients was examined by the Kaplan–Meier methods, and differences were examined by the Log-rank test. All analysis was done using the Stat-View software package (Abacus Concepts Inc. Berkeley, CA), and was considered significant when the p value was less than 0.05.

Results

EGFR gene mutation status

Of 225 patients, in exon 19, 51 patients had the deletion type mutation. In exon 18 or exon 21, 39 patients had the

missense point mutations (1 G719S, 3 G719C, 34 L858R and 1 L861Q). Five patients had exon 20 insertion mutations (Sasaki et al. 2007). Of these 95 patients, 34 were male and 61 were female. Sixty seven were non-smokers and 28 were smokers. Ninety two patients had adenocarcinoma and three had adenosquamous cell carcinoma. Thus *EGFR* mutation statuses at exon 18–21 were significantly correlated with gender ($p < 0.0001$), tobacco-smoking ($p < 0.0001$), and pathological subtypes (adenocarcinoma vs. non-adenocarcinoma, $p < 0.0001$). Of 206 patients from Nagoya City University, 97 (51.5%) were stage I. There was a higher *EGFR* mutation in stage I (51/97, 28.4%) than in stage II–IV (33/89, 19.7%, $p = 0.0235$).

EGFR polymorphism at exon 13

Using the PCR–RFLP assay, a sequence difference in exon 13 (R497K) was found in tumors that defined in the *EGFR* gene. Example of the *EGFR* gene analyzed by PCR–RELP method was shown in Fig. 1. Same codon 497 polymorphism of *EGFR* was found in both DNAs isolated from several lung cancer samples and adjacent peripheral blood samples. Several samples were also confirmed by direct sequencing (Fig. 2). Of 225 patients, 194 patients had the *EGFR* polymorphism (80 AA and 114 GA), 117 were male and 77 were female, 110 were non-smokers and 84 were smoker, and 166 patients had adenocarcinoma and 28 had other types of lung cancers. The R497K polymorphism did not correlate with gender ($p = 0.2410$), smoking status

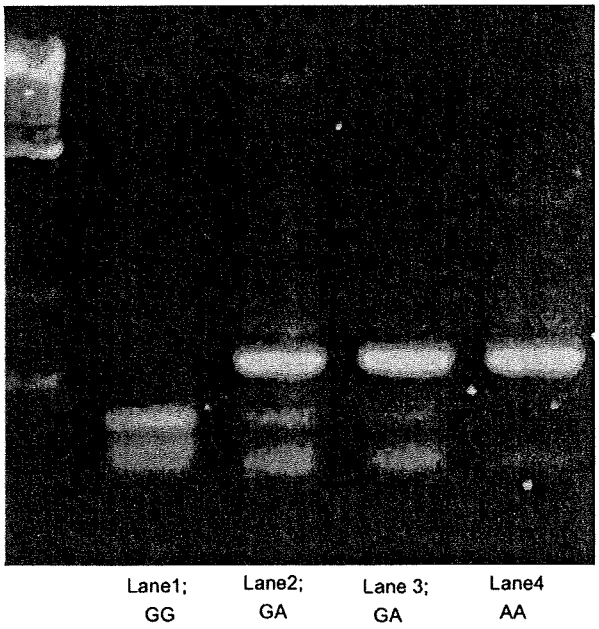

Fig. 1 Representative PCR–RELP patterns of different EGFR codon 497 status. PCR products after being digested by BstN1 were separated by agarose gel electrophoresis

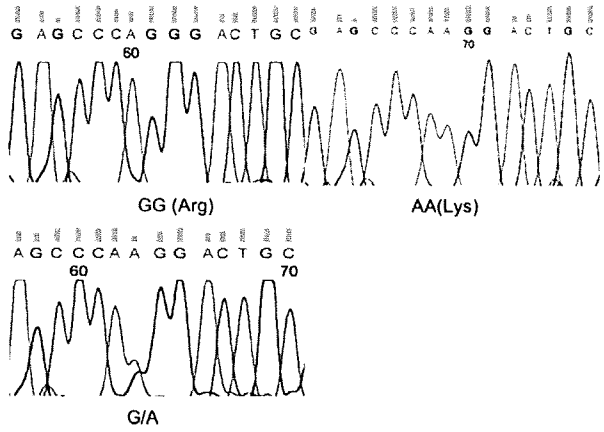

Fig. 2 The sequence results of *EGFR* exon 13. *Left upper* wild type (GG). *Right upper* heterozygous change (GA). *Left lower* homozygous change (AA)

Table 1 Clinico-pathological data of 225 lung cancer patients

Factors	EGFR		*p*-value
	GG	GA+AA	
	Patients	Patients	
Mean age (years) 63.2 ± 10.3	63.4 ± 10.0	62.0 ± 12.0	0.6685
Gender			
Male	15 (48.4%)	117 (60.3%)	0.2410
Female	16 (51.6%)	77 (49.7%)	
Smoking			
Non-smoker	16 (51.6%)	84 (43.3%)	0.4387
Smoker	15 (48.4%)	110 (56.8%)	
Pathological subtype			
Adeno	26 (83.9%)	166 (85.6%)	0.7865
Others	5 (16.1%)	28 (14.4%)	
EGFR mutation			
Positive	14 (45.2%)	81 (41.8%)	0.5566
Negative	17 (54.8%)	113 (58.2%)	
Age			
≤60	12 (38.7%)	72 (39.1%)	>0.9999
>60	19 (61.3%)	112 (60.8%)	
Pathological stages			
I	10 (35.7%)	96 (53.9%)	0.1073
II	4 (14.3%)	29 (16.3%)	
III–IV	14 (50.0%)	53 (29.8%)	
Lymph node metastasis			
Negative	14 (50.0%)	118 (66.3%)	0.1366
Positive	14 (50.0%)	60 (33.7%)	

**EGFR* epidermal growth factor receptor, *Smoker* current smoker or ever smoker, *Adeno* adenocarcinoma

($p = 0.4387$), pathological subtypes ($p = 0.7865$), and *EGFR*-TK mutation status of lung cancer ($p = 0.5566$) (Table 1). Major components of adenocarcinomas with

R497K were as follows; acinar 58.3%, solid 25.0%, and papillary 12.5%. Major components of adenocarcinomas with wild type (Lys/Lys) were as follows; acinar 40.0%, papillary 40.0%, and solid 20.0%. Thus polymorphism status did not correlated with the major components of adenocarcinomas. No significant association between R497K *EGFR* genotype and patient outcome was seen for the 206 patients from Nagoya City University ($p = 0.1121$). Pathological stages ($p < 0.0001$) but not gender ($p = 0.0696$) was a prognostic factor. In node-negative patients, 119 (28 were dead) were R497 K*EGFR* and 14 (three were dead) were wild type *EGFR*. Thus *EGFR* genotype was not correlated with disease outcome (Log-rank test $p = 0.8882$) (Fig. 3). In node-positive patients, however, 59 (33 were dead) were R497K *EGFR* and 14 (12 were dead) were wild type. Thus R497K *EGFR* was significantly associated with better overall survival (Log-rank test, $p = 0.0072$) (Fig. 4). In this cohort, pathological stage (stage II, $n = 17$ vs. stage III–IV, $n = 56$, $p = 0.2932$) or gender (male, $n = 41$ vs. female, $n = 32$, $p = 0.7957$) was not a prognostic factor. Multi-variate analysis showed that R497K status was a prognostic factor ($p = 0.0104$, relative risk 2.4, 1.229–4.689). We also compared associations between *EGFR* polymorphism status and patient outcome who were treated with platinum-based adjuvant or neo-adjuvant chemotherapy who had undergone surgery. The overall survival of 58 lung cancer patients with follow-up through March 1, 2008 was studied in reference to the *EGFR* polymorphism status. Ten were wild type (eight were dead) and 48 were R497K (23 were dead). The prognosis was significantly worse in *EGFR* wild type than in *EGFR* R497K polymorphism ($p = 0.0038$) (Fig. 5). In this cohort, pathological stages (stage I, $n = 11$, stage II, $n = 14$, stage III–IV, $n = 33$, $p = 0.0445$) but not gender (male, $n = 42$ vs. female, $n = 16$, $p = 0.9103$) was a prognostic factor. However, multi-variate analysis showed none of them was a prognostic factor.

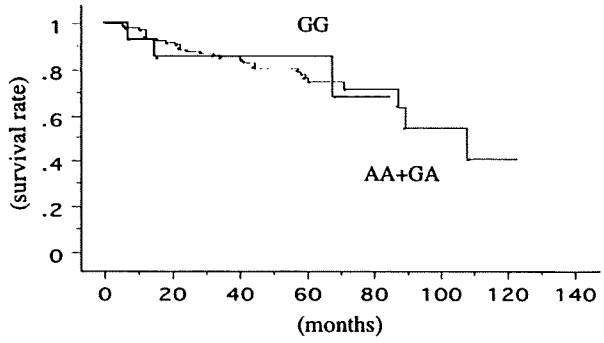

Fig. 3 The overall survival of node-negative lung cancer patients was studied in reference to the *EGFR* (R497K) status. There was no difference of survival between the patient with EGFR wild type (GG) ($n = 14$, 3 were dead) and the patient with R497K *EGFR* (GA or AA) ($n = 119$, 28 were dead) (Log-rank test, $p = 0.8882$)

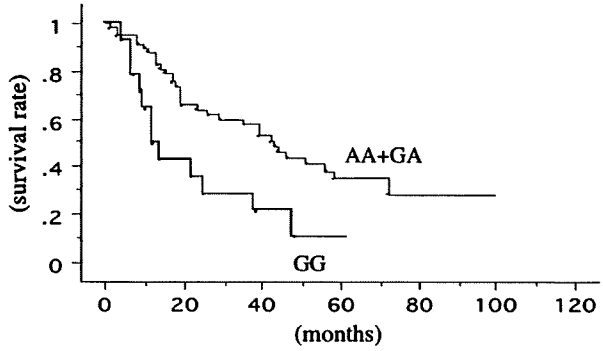

Fig. 4 The overall survival of node-positive lung cancer patients was studied in reference to the *EGFR* (R497K) status. The patients with EGFR wild type (GG) ($n = 14$, 12 were dead, median follow up = 21.7 months) had significantly worse prognosis than the patients with R497K *EGFR* (GA or AA) ($n = 59$, 33 were dead, median follow up = 42.7 months) (Log-rank test, $p = 0.0072$) (relative risk 2.4, 1.229–4.689)

Relationship between clinical courses of lung cancer patients treated with gefitinib and *EGFR*

The overall survival of gefitinib treated lung cancer patients from Nagoya City University, with follow-up through March 1, 2008, was studied in reference to the *EGFR* polymorphism status. Of 206 patients from Nagoya City University, 27 were treated with gefitinib therapy. Total 46 gefitinib treated patients were investigated the R497K polymorphism statuses. In this analysis, 38 patients had *EGFR* polymorphism (AG or GG). The prognosis after gefitinib therapy was not significantly different between *EGFR* wild type patients (GG, 5/8 were dead) and *EGFR* polymorphism patients (AG+GG; 28/38 were dead) ($p = 0.3100$) (Fig. 6).

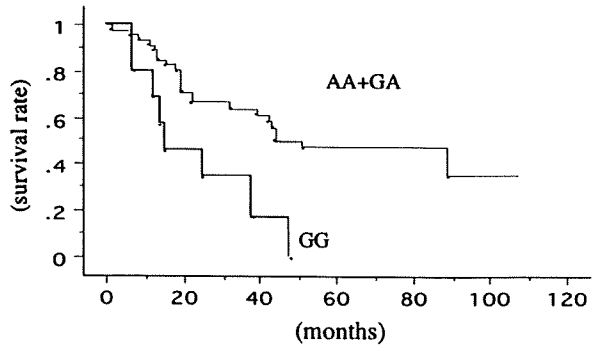

Fig. 5 The overall survival of adjuvant or neo-adjuvant chemo-untreated lung cancer patients was studied in reference to the *EGFR* (R497K) status. The patients with EGFR wild type (GG) ($n = 10$, 8 were dead, median follow up = 23.7 months) had significantly worse prognosis than the patients with R497K *EGFR* (GA or AA) ($n = 48$, 23 were dead, median follow up = 55.1 months) (Log-rank test, $p = 0.0038$)

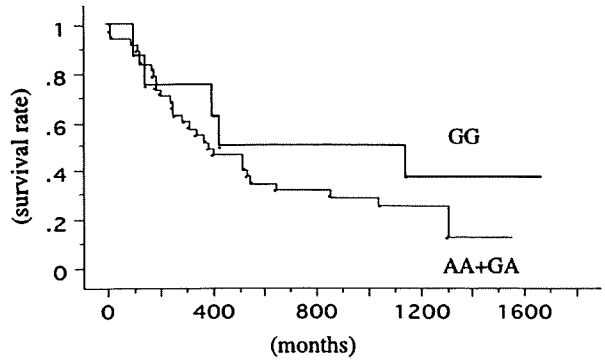

Fig. 6 The overall survival of 46 gefitinib untreated lung cancer patients was studied in reference to the *EGFR* (R497K) status. There was no difference of survival between the patients with EGFR wild type (GG) (n = 8, 5 were dead) and the patients with R497K *EGFR* (GA or AA) (n = 38, 28 were dead) (Log-rank test, p = 0.3100)

Discussion

In the present study, we showed that the R497 polymorphism of EGFR in node-positive lung cancer patients who received curative surgery might account for a longer overall survival. Moreover, this polymorphism was shown to correlate with a better prognosis after platinum-based adjuvant treatment. Although the underlying mechanisms remain unclear, an attenuated ligand interaction and consequential signal transduction might be the main reason for the subopitimal function of this receptor variant (Moriai et al. 1994).

The quantification of certain intratumoral molecules involved in the targeting or metabolism of specific chemotherapeutic agents may be valuable in predicting their efficacies or toxicities in cancer patients. For example, patients with a higher intratumoral level of excision repair cross complementation group 1 (ERCC1), an enzyme involved in nucleotide excision repair, may have a higher resistance to cisplatin-based adjuvant therapy in NSCLC (Olaussen et al. 2006). Moreover, NSCLC patients with a higher class III beta tubulin may have a higher resistance to taxane chemotherapy (Dumontet et al. 2005).

In this report, the R497K *EGFR* SNP(exon 13) is not associated with somatic *EGFR*-TK mutation. Approximately 563 *EGFR*-SNPs have been identified in human genome according to the National Cancer for Biotechnology information database. However, there are few studies examining associations between *EGFR* SNPs and human disease (Shintani et al. 1999; Kang et al. 2005; Fukushima et al. 2006; Zhang et al. 2006; Wang et al. 2007; Liu et al. 2008). In this study, we detected a polymorphism in exon 13 of the *EGFR*-extracellular domain, which changed amino acid Arg (R) to Lys (K), and the K allele seems to decrease the activity of EGFR (Moriai et al. 1994). Previous reports suggested that *EGFR* R497K polymorphism was weakly associated with gefitinib response (Liu et al. 2007). However, in our Japanese cohort, *EGFR* R497K was not associated with response to gefitinib. Although the survival curve of R497K showed higher than *EGFR* wild type (G/G) in our data, the larger number would help to determine the correlation between the R497K polymorphism and gefitinib sensitivity.

Previous report showed that patients with 497 Arg/Arg genotype tended to have a higher risk of local recurrence in chemo-treated rectal cancer patients (Zhang et al. 2005; Brandt et al. 2006). The patients with Arg/Arg genotype showed the highest risk of disease-specificity mortality and none of the patients with the Lys/Lys genotype died throughout the follow-up period of head and neck cancer treated with chemoradiation (Bandres et al. 2007). The mechanism through which the variant human EGFR R497K may account for lower local failures after chemotherapy is unknown (Zhang et al. 2005). A study with Chinese hamster ovary cells, the variant EGFR 497K cell line, showed an attenuated growth response to EGF and transforming growth factor-α, and a reduced induction of the proto-oncogenes fos, jun, and myc (Moriai et al. 1994). It was suggested that the amino acid substitution in the extracellular domain might modulate ligand binding and transmembrane signaling to the intracellular domain (Zhang et al. 2005). Thus, variant EGFR receptor may be less efficient in the recruitment of intracellular substrates and/or cause downstream activation of alternative signaling pathways with decreased proto-oncogene induction or growth stimulation, affecting chemosensitivity. Shintani et al. (1999) demonstrated that another *EGFR*-SNP at position 2073 was correlated with truncated *EGFR* transcription, which might interfere with EGFR three-dimensional structure and EGFR expression.

In summary, R497 polymorphism of EGFR in node-positive lung cancer patients had a better overall survival. R497K*EGFR* polymorphism might be associated with favorable prognosis of advanced lung cancers.

Acknowledgments The authors would like to thank Mrs. Emi Sugiyama for his excellent technical assistances. This work was supported by AstraZeneca Research Grant 2004, Grand-in-Aid for Research in Nagoya City University (2006), and Grants-in-Aid for Scientific Research, Japan Society for the Promotion of Science (JSPS) (Nos, 19390367, 18390381,18659407).

Conflict of interest statement None declared.

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EUROPEAN JOURNAL OF CANCER 45 (2009) 1950–1958

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Pretreatment neutrophil count as an independent prognostic factor in advanced non-small-cell lung cancer: An analysis of Japan Multinational Trial Organisation LC00-03

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ARTICLE INFO

Article history:
Received 3 December 2008
Received in revised form 14 January 2009
Accepted 16 January 2009
Available online 21 February 2009

Keywords:
Prognostic factors
Neutrophil count
Non-small-cell lung cancer

ABSTRACT

We examined the impact of pretreatment neutrophil count on survival in patients with advanced non-small-cell lung cancer (NSCLC). A total of 388 chemo-naïve patients with stage IIIB or IV NSCLC from a randomised controlled trial were evaluated. The effects of pretreatment peripheral blood neutrophil, lymphocyte and monocyte counts and neutrophil–lymphocyte ratio on survival were examined using the proportional hazards regression model to estimate hazard ratios after adjustment for covariates. The optimal cut-off value was determined by proportional hazards regression analysis with the minimum P-value approach and shrinkage procedure. After adjustment for prognostic factors, the pretreatment elevated neutrophil count was statistically significantly associated with short overall ($P = 0.0008$) and progression-free survival ($P = 0.024$), whereas no association was found between prognosis and lymphocyte or monocyte count. The cut-off value selected for neutrophil count was 4500 mm^{-3} (corrected hazard ratio, 1.67; 95% confidence interval (CI), 1.09–2.54). The median survival time was 19.3 months (95%CI, 16.5–21.4) for the low-neutrophil group (<4500 mm^{-3}, $n = 204$) and was 10.2 months (95%CI, 8.0–12.3) for the

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0959-8049/$ - see front matter
doi:10.1016/j.ejca.2009.01.023

high-neutrophil group (≥4500 mm^{-3}, $n = 184$). We confirmed that pretreatment elevated neutrophil count is an independent prognostic factor in patients with advanced NSCLC receiving modern chemotherapy. Neutrophil count is easily measured at low cost, and it may be a useful indicator of patient prognosis.

1. Introduction

The prognosis for patients with advanced non-small-cell lung cancer (NSCLC) (TNM stage IIIB with a positive pleural effusion, or stage IV) has improved with recent advances in systemic chemotherapy, but still remains poor, with a median overall survival time between 4 and 15 months.[1] Prognostic factors identified in previous studies include tumour stage, performance status (PS), weight loss, sex, plasma lactate dehydrogenase (LDH) level and the presence of bone, liver or skin metastases.[2] Although novel immunological and histological biomarkers have been identified, these are often time-consuming to measure, and this is not part of the standard practice.

It is now evident that inflammatory cells in the tumour microenvironment have significant effects on tumour development.[3–6] Elevation in the pretreatment neutrophil count has been proposed as a prognostic factor for poor survival in patients with metastatic renal cell carcinoma,[7–9] and elevated neutrophil, monocyte or leucocyte count has been associated with poor survival in patients with metastatic melanoma.[10,11] A high-neutrophil–lymphocyte ratio may be related to poor prognosis in patients with colorectal cancer[12] and in those with advanced gastric cancer.[13] The European Lung Cancer Working Group found that the high-neutrophil count was an independent prognostic factor for poor survival in patients with unresectable advanced NSCLC[14] and in those with small-cell lung cancer.[15] A retrospective study found that neutrophil count was of prognostic value in patients with lung cancer.[16]

The aim of this study was to examine and confirm the impact of pretreatment peripheral blood neutrophil, monocyte and lymphocyte counts on overall and progression-free survival in a well-defined population of patients with advanced NSCLC being treated with regimens using newer chemotherapeutic agents in a randomised controlled clinical trial.

2. Patients and methods

2.1. Study population

A total of 401 chemo-naïve NSCLC patients with stage IIIB with pleural effusion or stage IV without brain metastasis, who had Eastern Cooperative Oncology Group (ECOG) PS of 0 or 1, were enrolled from 45 institutions in Japan between March 2001 and April 2005 into Japan Multinational Trial Organisation LC00-03[17] (registered with ClinicalTrials.gov identifier NCT00079287). Patients underwent one of two treatment regimens: intravenous vinorelbine (25 mg/m^2) plus gemcitabine (1000 mg/m^2) on days 1 and 8 every 21 d for three cycles, followed by intravenous docetaxel (60 mg/m^2) on day 1 every 21 d for three cycles [VGD arm, $n = 196$] versus intravenous paclitaxel (225 mg/m^2) and carboplatin (area under the curve = 6) for 3 h on day 1, every 21 d for six cycles [PC arm, $n = 197$]). As there were no significant differences between treatment groups in terms of either overall (hazard ratio: 0.996, $P = 0.974$) or progression-free survival (hazard ratio: 0.966, $P = 0.742$), the combined data from the two arms were analysed in this study. Of 393 eligible patients, information regarding pretreatment neutrophils in peripheral blood was not available for five patients. Thus, data from 388 patients were included in the present study.

2.2. Statistical analysis

Overall survival was defined as the time from randomisation until death from any cause, and progression-free survival was defined as the time from randomisation until objective tumour progression or death. Survival curves were estimated with the Kaplan–Meier method. Associations between the factors and the prognosis were examined with the log-rank test in univariate analyses. The prognostic impact of pretreatment peripheral blood neutrophil, lymphocyte and monocyte counts, and neutrophil–lymphocyte ratio were examined using the proportional hazards regression model to estimate hazard ratios after adjustment for covariates without variable selection. Optimal cut-off points for continuous variables were selected using the minimum P-value approach with correction of the P-value.[18] The corrected hazard ratio and its 95% confidence interval (CI) were estimated using a shrinkage procedure with bootstrap resampling.[19] All statistical analyses were done using SAS version 9.1 (SAS Institute, Cary, NC).

3. Results

3.1. Patients' characteristics

Of 388 patients, 276 patients had died, and the median follow-up time for the 112 surviving patients was 567 d (range: 70–1711 d). The characteristics of the 388 patients (276 men [71%], 112 women [29%], median age 65 years [range, 33–81 years]) included in the present study are shown in Table 1. Median pretreatment counts of neutrophils, lymphocytes and monocytes were 4304 mm^{-3}, 1386 mm^{-3} and 404.2 mm^{-3}, respectively. Spearman's rank correlations were 0.351 for neutrophils and monocytes, 0.034 for neutrophils and lymphocytes and 0.352 for monocytes and lymphocytes.

3.2. Relationship between pretreatment neutrophil, lymphocyte and monocytes counts and survival

In univariate analyses, pretreatment elevated counts of neutrophils were statistically significantly associated with short

Table 1 – Baseline patients characteristics (n = 388).

Characteristics	No.	%
Age, years, median (range)	65 (33–81)	
Sex		
Male	276	71
Female	112	29
Smoking history		
Non-smokers	96	25
Former smokers	107	28
Current smokers	168	43
Unknown	17	4
Stage		
IIIB	68	18
IV	320	82
Histologic type		
Squamous cell	76	20
Adenocarcinoma	274	70
Others	38	10
ECOG performance status		
0	154	40
1	234	60
Weight loss (from 6 months before enrolment)		
<5%	317	82
≥5%	71	18
LDH		
Normal (<ULN)	279	72
High (≥ULN)	109	28
Bone metastases		
No	280	72
Yes	108	28
Liver metastases		
No	357	92
Yes	31	8
Skin metastases		
No	379	98
Yes	9	2
Neutrophils, mm^{-3}, median (range)	4304 (205–17,100)	
Lymphocytes, mm^{-3}, median (range)	1386 (243–4200)	
Monocytes, mm^{-3}, median (range)[a]	404.2 (0–1620)	
Red blood cells, $\times 10^4$ mm^{-3}, median (range)	420 (286–579)	
Platelets, $\times 10^4$ mm^{-3}, median (range)[b]	26 (11–380)	

ULN: upper limit of normal.
a One missing value.
b Two missing values.

overall (Fig. 1A, $P < 0.0001$) and progression-free survival (Fig. 1B, $P = 0.0001$). Although lymphocyte count did not correlate with survival, there were significant relationships between high-neutrophil–lymphocyte ratio and short overall ($P < 0.0001$) and progression-free survival ($P = 0.005$). The elevated monocyte count was also significantly associated with short overall survival ($P = 0.004$), and was moderately related to short progression-free survival ($P = 0.052$). We selected sex, smoking history, stage, ECOG PS, weight loss, plasma LDH and the presence of bone, liver or skin metastases as the known pretreatment prognostic factors.[2,14] Adjusted hazard ratios for the relationship between pretreatment neutrophil, lymphocyte and monocyte counts and neutrophil–lymphocyte ratio and overall and progression-free survival after adjustment for the known prognostic factors are shown in Table 2. There was a statistically significant association between elevated neutrophil count and short overall ($P = 0.0008$) and progression-free survival ($P = 0.024$), and between high-neutrophil–lymphocyte ratio and short overall ($P = 0.011$) and progression-free survival ($P = 0.040$), whereas no association was found between lymphocyte or monocyte count and prognosis. The relationship between neutrophil count and both overall and progression-free survival was linear, whereas the relationship between neutrophil–lymphocyte ratio and overall survival was to some degree non-linear.