Table 3 Incidence of EGFR mutations in surgically resected specimens

		All cases		Adenocarcinoma		Non-smokers	
Author	Country	Total N	Mutation N (%)	Total N	Mutation N (%)	Total N	Mutation N (%)
Western areas							
Shigematsu	USA	80	11 (14)	44	11 (25)	26	7 (27)
Pao	USA	96	11 (11)	72	11 (15)	15	7 (47)
Yang	USA	219	26 (12)	164	25 (15)	34	12 (35)
Marchetti	Italy	860	39 (5)	375	39 (10)	103[a]	23 (22)
	Subtotal	1255	87 (7)	655	86 (13)	75	26 (35)
Asian areas							
Shigematsu	Japan	263	71 (27)	154	67 (44)	78	47 (60)
Kosaka	Japan	277	111 (40)	224	110 (49)	112[a]	76 (68)
Tokumo	Japan	120	38 (32)	82	37 (45)	36	25 (69)
Sasaki	Japan	95	35 (37)	71	32 (45)	36	25 (69)
Shigematsu	Taiwan	93	32 (34)	55	31 (56)	55	27 (49)
Qin	China	41	10 (24)	17	7 (41)	21	6 (29)
Soung	Korea	153	30 (20)	69	26 (38)	54	25 (46)
Shigematsu	Others	361	107 (30)	214	102 (48)	135	76 (56)
	Subtotal	1403	434 (31)	886	412 (47)	415	231 (56)
Other areas							
Shigematsu	Australia	83	6 (7)	36	5 (14)	7	4 (57)
Shigematsu	Others	158	13 (8)	75	12 (16)	31	9 (29)
	Subtotal	241	19 (8)	111	17 (15)	38	13 (34)
	Toatl	2899	540 (19)	1652	515 (31)	528	270 (51)

[a]Including only patients with adenocarcinoma histology.

INTERSTITIAL LUNG DISEASE ASSOCIATED WITH GEFITINIB AND ERLOTINIB

The frequencies of grades 3–4 common toxicities after the administration of gefitinib, including diarrhoea, skin rash, and elevated liver transaminase levels, have been similar among study populations, but the incidence of severe interstitial lung disease (ILD) associated with the administration of gefitinib differs between patients in Japan and those in other countries. In the IDEAL studies, two Japanese patients developed grades 3–4 ILD (2%), whereas no patients outside of Japan experienced ILD (Fukuoka *et al*, 2003; Kris *et al*, 2003). A retrospective study of 1976 consecutive patients treated with gefitinib at 84 institutions showed that the incidence of ILD was 3.5% and the mortality rate was 1.6%. Several risk factors for the development of gefitinib-induced ILD were identified in the Japanese population: a history of pulmonary fibrosis, a history of smoking, a poor performance status, and a male sex (Ando *et al*, 2006). A similar incidence of ILD (4.6%) was also noted in association with erlotinib chemotherapy in Japanese phase II trials (Tamura *et al*, 2007).

The association between ILD and anticancer treatment is a major topic in Japan because (1) the diagnosis of ILD can be difficult and a consensus among physicians is sometimes not reached, (2) the risk factors for ILD have not been fully established, 3) an effective treatment for ILD has not been established and the condition is often fatal, and (4) the low frequency of this complication makes it difficult to conduct pertinent clinical trials. Gefitinib-induced ILD seems to be more common among Japanese patients than among other patients, but the reasons for this ethnic difference are totally unknown.

CONCLUSION

The findings discussed here suggest that considerable variations in the toxicity and efficacy of anticancer agents may exist among patients of different ethnicities. Although research into these differences has just begun, these studies suggest that possible pharmacogenomic and tumour genetic differences associated with individual responses to anticancer agents should be carefully considered when conducting global clinical trials.

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ADVANCES IN DRUG DEVELOPMENT

Current Developments in Oncology Drug Research

Section Editor: Mark J. Ratain, MD

Population Differences in the Use of EGFR-targeted Agents

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H&O What do we currently know about epidermal growth factor receptor (EGFR)-targeted therapies and population differences?

NS EGFR-targeted therapies can largely be divided into 2 categories: EGFR small molecule tyrosine kinase inhibitors (TKIs) and antibodies.

First of all, we know that the response rates for EGFR TKIs such as gefitinib (Iressa, Astrazeneca) and erlotinib (Tarceva, Osi Phamaceuticals) are significantly higher in Asians, females, adenocarcinomas, and non-smokers. Survival rates are also better than in the total population. As for the toxicity profiles, incidences of pulmonary toxicities are higher in males, smokers, and squamous cell carcinomas. Within the Asian population, we currently know that the frequency of interstitial pneumonia is significantly higher in Japanese patients than in the Chinese and Korean population.

Secondly, there are the antibodies such as cetuximab (Erbitux, Imclone). This year at the American Society of Clinical Oncology meeting, the results from an interesting FLEX study—a randomized, multicenter, phase III investigation that compared cetuximab in combination with cisplatin/vinorelbine versus cisplatin/vinorelbine alone in advanced non-small cell lung cancer patients—were presented. There were very few data pertaining to the Asian population, but when the researchers divided data of the Caucasians and the Asians, the results seemed to be better in Caucasians. In the Asian population, there was no difference in survival rates; I think this is because the majority of Asian people receive small-molecule EGFR TKIs after antibody treatment, a factor that may confuse the survival results.

H&O How are these differences explained by EGFR and K-Ras mutation rates in certain populations?

NS About 30–40% of Asians are said to have an EGFR mutation. In Caucasians, the reported mutation rate is less than 10%. This corresponds with study results that show the same type of difference—a higher response and survival rates in the Asian population to EGFR inhibitor therapy—between the 2 populations. We currently do not know much about the mutation rates in other populations such as blacks, hispanics, etc., although it is said that in the hispanic population, the mutation rate seems to be very low.

In the European Society of Medical Oncology (ESMO) meeting this September, Professor Tony Mok from the Chinese University of Hong Kong presented results from the IRESSA Pan-Asia Study (IPASS), which clearly showed that EGFR mutation is related to response and survival.

The IPASS study, of which I was one of the co-workers, was an open label, randomized, parallel-group trial that tested gefitinib versus carboplatin/paclitaxel (carbo/paclitaxel) as first line treatment in a selected population of patients from Asia. It included 1,217 Asian people whose tumors were of adenocarcinoma histology, who had not

received prior chemotherapy, and who were non smokers or light smokers. Japanese people were about 20% of the participants; Chinese were about 30%; the rest were from other Asian countries. The aim of the trial was to demonstrate that gefitinib was non inferior to carbo/paclitaxel doublet chemotherapy.

Subjects were randomized (about 600 subjects in each arm) to gefitinib or carbo/paclitaxel (ie, standard chemotherapy). The primary endpoint was progression-free survival (PFS).

Results showed that the gefitinib group had superior PFS and higher tumor response compared with intravenous carbo/paclitaxel chemotherapy in the overall population. However, although the PFS in the gefitinib group was significantly better, we noticed that the 2 curves for gefitinib and carbo/paclitaxel crossed at 5–6 months. Interestingly, during the first 5–6 months, the carbo/paclitaxel group was doing better, but after that point, the gefitinib group showed better PFS. These were 2 very strange curves. Statistically, when we analyzed the differences using the Cox proportional hazard model, there was a significant difference between the 2 groups, overall favoring gefitinib. However, there is really no consensus as to whether crossed curves can be analyzed by the Cox proportional hazard model.

Also noteworthy was that among the 1,217 patients, about one-third were anaylized by biomarkers such as EGFR mutation, EGFR amplification, and EGFR expression. We found that in patients with EGFR mutation, gefitinib did significantly better than carbo/paclitaxel. However, in patients with the wild-type EGFR, the PFS of the carbo/paclitaxel group was significantly better than that of the gefitinib group. This was a very interesting observation.

As you know, patients who have an EGFR mutation do not have a K-Ras mutation, and vice versa. One might therefore speculate that, in a sense, K-Ras mutation is inversely associated with the efficacy of EGFR-targeted therapy, but the truth is that there is not enough data in lung cancer. In colon cancer, if the EGFR is mutated, anti-EGFR antibodies such as cetuximab are not effective. In lung cancer, we do not have much data mainly because K-Ras mutation rate is not very high.

H&O Have there been studies investigating the differences within the Asian population (ie, Japanese, Korean, Chinese, etc.)?

NS This is a difficult question because we have very few data. We do know that the mutation rates of the Japanese and Koreans are nearly the same—around 30–40%. At present, we do not have sufficient data on the mutation rates of the Chinese and other Asian countries, so we have not been able to make a complete comparison yet.

H&O What technology is there to detect EGFR mutation, and how reasonable is it to use it to predict EGFR TKI efficacy?

NS Some claim that other biomarkers such as EGFR amplification and fluorescence in situ hybridization (FISH) could also be indicators; but in my mind, they are not very reliable. I believe that EGFR mutation is the most reliable predictor we currently know. And reliability here depends on the number of samples; we need to get enough samples to analyze. How we detect mutation is a separate issue—a technical problem. I think that if we use copy numbers of the EGFR for amplification parameters, it would be reasonably reliable because it is very quantitatvie.

The problem with FISH results is that they contain 2 elements. FISH positive includes EGFR amplification and high polysomy. However, EGFR amplification is closely correlated to mutation whereas high polysomy does not show any correlation.

When studies include both, the end analysis may be very complicated. This is the case with the majority of the data from the University of Colorado Cancer Center or from Dr. Federico Cappuzzo at the Istituto Clinico Humanitas IRCCS in Italy, who sees FISH technology to be the best method for patient selection when the main endpoint is survival. But I think the mix of 2 different kinds of FISH data is very difficult for us to interpret. Even in the IPASS trial, analysis of survival based on FISH positivity showed a similar tendency but the analysis based on EGFR mutation was much more clear.

I also think that clinical factors such as nonsmoking, females, adenocarcinomas, etc. are related to these EGFR mutations. So at this point, I believe that EGFR mutation is most highly predictive. If patients have the mutation, nearly 80% of them will respond.

H&O Should EGFR TKIs be included in the initial therapy for patients with EGFR mutation?

NS This is a crucial question. And as was evident from my results at the ESMO meeting this year, we can conclude that for patients with EGFR mutation, the first choice of therapy could be gefitinib. For patients without EGFR mutation, chemotherapy should be chosen as the first choice of therapy. But, the IPASS data are for PFS and not overal survival (OS). We still need to wait for OS data, and it will take some time.

But I think the important thing is to focus on the primary endpoint of a clinical trial. If the primary endpoint is OS, it is rather easy for us to interpret the results. If

the primary endpoint is PFS or time to treatment failure (TTF), it is rather difficult to make hard conclusions. PFS and TTF are not that accurate, making them softer endpoints, which do not directly relate to patient benefit.

H&O What sort of studies do you think are necessary to investigate this topic further? Are there any ongoing that are noteworthy?

NS Right now, there are talks of 2 randomized Japanese trials: one by researchers at Tohoku University and the other by the West Japan Oncology Group (WJOG). The study designs are very similar; both are testing gefitinib versus platinum doublet in EGFR-mutated patients. The Tohoku group is testing carbo/paclitaxel, whereas the WJOG group is testing cisplatin plus docetaxel, for their chemotherapy arm. The primary endpoint is PFS. Both trials are currently accruing patients.

However, the IPASS data has heavily influenced these clinical trials because they have already shown that PFS in EGFR-mutated patients is significantly better in the gefitinib group than in the chemotherapy group. So the question whether to continue these 2 randomized trials has become an ethical one, and still remains unanswered.

Problems in the current diagnostic standards of clinical N1 non-small cell lung cancer

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ABSTRACT

Background: Although clinical N1 (cN1) non-small cell lung cancer (NSCLC) is considered to be locoregional, the postoperative outcome is disappointing, with a 5 year survival of less than 50%. One possible reason may be that cN1disease diagnosed by current standard imaging modalities often contains unexpected N2 disease. This study was conducted to evaluate the surgical and pathological results of patients with cN1 NSCLC.

Methods: Among 1782 patients with NSCLC who underwent intended curative resection from 1993 to 2003, 143 patients were identified as having cN1 disease and were enrolled in this study. The clinicopathological records and CT films of each patient were retrospectively reviewed to identify predictors for pN2–3 disease.

Results: The pathological nodal status was pN0 in 23% (n = 33), pN1 in 47% (n = 67) and pN2–3 in 30% (n = 43) of patients. Patients with pN2–3 showed a significantly worse 5 year survival rate of 38% compared with patients with pN0 (68%) and pN1 (60%) (p = 0.017 and 0.007, respectively). Multivariate analysis showed that adenocarcinoma histology was a significant predictor for pN2–3 disease (OR 3.312, 95% CI 1.439 to 7.784; p = 0.005). The presence of N1 node separate from the main tumour on CT scans tended to predict pN2–3 disease although this did not reach statistical significance (OR 2.103, 95% CI 0.955 to 4.693; p = 0.066). Pathological N2–3 disease was found in 53% of patients with adenocarcinoma with a separate N1 pattern and in only 12% of patients with non-adenocarcinoma with a continuous N1 pattern.

Conclusions: The diagnosis of N1 status by contrast enhanced CT scans is unsatisfactory with a high rate of unexpected pN2 disease. To avoid infertile lung resection, patients with CT diagnosed N1 adenocarcinoma, especially with a separate N1 pattern on CT, should be considered for additional invasive node biopsy modalities, including mediastinoscopy.

Clinical N1 (cN1) disease of non-small cell lung cancer (NSCLC) represents the subset suggestive of ipsilateral hilar and/or intrapulmonary lymph node metastasis.[1] Although cN1 disease is considered to be locoregional, surgical resection often fails to cure patients with cN1. Mountain reported that the postoperative 5 year survival rates of patients with clinical T1N1, T2N1 and T3N1 were 34%, 24% and 9%, respectively.[2] One possible explanation for the disappointing outcome is that patients with cN1 often have clinically occult mediastinal lymph node metastases (N2), which is a sign of systemic disease.

Contrast enhanced CT scan is the most widely available and most commonly used non-invasive method to evaluate lymph node status for patients with NSCLC, in spite of the introduction of positron emission tomography (PET) scans.[3] Many studies have reported the diagnostic accuracy of CT scans for N2 disease. A meta-analysis of 20 studies involving 3438 patients demonstrated that CT had 57% sensitivity and 82% specificity for mediastinal node staging.[4] We previously reported that 17% of patients with clinical N0 NSCLC had pN2 disease.[5] Although it is very likely that the cN1 patient cohort includes more clinically undetectable N2 patients, few reports have evaluated pathological stage distribution of patients with CT diagnosed clinical N1 NSCLC.[6]

To reveal the problems in the current diagnostic standards of cN1 NSCLC, we retrospectively evaluated the surgical and pathological results of this cohort. Furthermore, we attempted to identify clinical and radiological characteristics of patients with cN1 to predict mediastinal lymph node metastases in order to help select patients who would benefit from additional lymph node staging modalities.

METHODS

Patient population

We reviewed the medical records of 1782 consecutive patients with NSCLC who underwent intended curative surgical resection at the National Cancer Centre Hospital East, Chiba, Japan, from January 1993 to June 2003. Among them, a total of 159 patients with cN1 disease were identified. Data collection and analyses were approved and the need for obtaining informed consent from each patient was waived by the institutional review board in March 2005. We excluded 16 patients from this study for the following reasons: eight patients underwent preoperative induction therapy, three patients underwent limited resection without systemic hilar and mediastinal lymph node dissection, three patients underwent mediastinoscopy to rule out cN2 disease and two patients had synchronous metastatic disease (M1). Subsequently, 143 (8%) patients were enrolled in this retrospective study. The clinical characteristics of these patients are shown in table 1. There were 114 men and 29 women with a median age of 64 years (range 25–83). Eighty-five (59%) patients complained of at least one symptom on their first visit to our outpatient clinic. The most prevalent symptom was cough in 42 patients, followed by haemoptysis in 31 patients. Before surgery, all patients underwent a thorough staging with chest roentogenography, chest and upper abdominal contrast enhanced CT, bone scintigraphy and MRI or CT scan of the whole brain. The most common T factor was T2 (61%), reflecting

Table 1 Patient characteristics

Characteristic	No of patients	% of patients
Patients enrolled	143	
Age (y)		
Median	64	
Range	25–83	
Sex		
Male	114	80
Female	29	20
Symptomatic patients	85	59
Present and past smokers	121	85
CEA level (ng/ml)		
Median	6.2	
Range	0.7–347.7	
Tumour location		
RUL/RML/RLL	32/8/46	22/6/32
LUL/LLL	34/23	24/16
Clinical T status		
T1/T2/T3/T4	20/88/27/8	14/61/19/6
Resection type		
Pneumonectomy	42	29
Bilobectomy	35	25
Lobectomy	66	46
Histological type		
Squamous cell carcinoma	69	48
Adenocarcinoma	54	38
Large cell carcinoma	10	7
Adenosquamous carcinoma	6	4
Other*	4	3

*Two atypical carcinoids, one pleomorphic carcinoma and one giant cell carcinoma. CEA, carcinoembryonic antigen; LLL, left lower lobe; LUL, left upper lobe; RLL, right lower lobe; RML, right middle lobe; RUL, right upper lobe.

the mean tumour size on CT scans (4.6 (SD 1.9) cm). Squamous cell carcinoma was the most frequent histology (48%, 69/143). Bronchoscopy was done in selected patients to evaluate cancer extension in the bronchial tree. We did not routinely use PET scan for preoperative staging. We did not perform mediastinoscopy or thoracoscopy for patients with cN1. All patients underwent anatomical lung resection (at least lobectomy) with systemic hilar and mediastinal lymph node dissection, as described previously.[7] The number of dissected lymph nodes ranged from 4 to 70, with a mean of 30. Resected specimens were examined histologically, and their histological type was determined according to the World Health Organization International Histological Classification of Tumours.[8] The pathological stages were determined based on the TNM classification of the International Union Against Cancer.[1] After surgery, patients were scheduled to visit our outpatient clinic at 3–6 month intervals for 5 years. The median follow-up period was 5.1 years (range 7 months to 11 years).

Preoperative evaluation and analysis of N1 status

Dynamic incremental scanning was performed on X-Vigour or Aquilion CT equipment (Toshiba; Tokyo, Japan) after a bolus injection of 100 ml of contrast material using an automatic injector, and 10 mm thick contiguous CT sections were reconstructed. All CT films were interpreted by at least two experienced thoracic radiologists who were blinded to the other clinical information. Clinical N1 disease status was diagnosed when one or more lymph nodes in the N1 region were larger than 1.0 cm in the shortest axis, in accordance with the general consensus of the upper size limit for normal mediastinal lymph nodes. The N1 node stations were designated according to the lymph node map by Naruke and colleagues[7]: No 10 hilar along the main bronchus, No 11 interlobar, No 12 lobar, No 13 segmental bronchial and No 14 intrapulmonary lymph nodes. As our series was obtained over a 10 year period and not evaluated with identical CT equipments and radiologists, we retrospectively reviewed preoperative chest CT scans and confirmed that all 143 enrolled patients truly satisfied cN1 diagnosis criteria. During the review process, additional radiological findings were collected for the purposes of the current study. They included the presence or absence of atelectasis and obstruction pneumonia and cN1 patterns. We defined two cN1 patterns according to the relationship between the main tumour and N1 node depicted on CT. We defined "continuous N1" as an N1 node directly involving the main tumour and "separate N1" as an N1 node separate from the main tumour (fig 1). N1 node connected to the main tumour only by pre-existing normal bronchovascular structure was classified as separate N1.

Statistical analysis

We analysed categorical variables using Pearson's χ^2 test. Survival rates were estimated using the Kaplan–Meier method, and survival curves were compared by log rank tests. The length of survival was defined as the interval between the day of surgical resection and the date of either death or the last follow-up. An observation was censored at the last follow-up when the patient was alive or lost to follow-up. Univariate logistic regression analysis was performed to identify factors predicting pN2–3 disease among the cN1 population. Multivariate logistic regression analysis was carried out by using significant predictors in univariate analysis to clarify which factor was associated with a higher risk of pN2–3 disease. Cox proportional

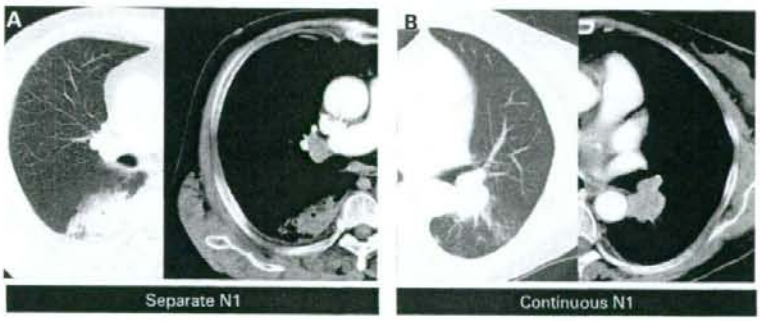


Figure 1 (A) "Separate N1", in which the main tumour and suspected N1 node are apart from each other. (B) "Continuous N1", which forms a single mass with the main tumour.

Table 2 N1 location depicted on CT according to tumour location

N1 station depicted on CT	Tumour location RUL (n = 32)	RML (n = 8)	RLL (n = 46)	LUL (n = 34)	LLL (n = 23)	Total (%) (n = 143)
No 10	14	1	13	4	2	34 (24)
No 11	22	8	40	28	18	116 (81)
No 12	1	1	3	7	2	14 (10)
No 13	0	0	0	0	1	1 (1)
No 14	0	0	0	0	0	0

LLL, left lower lobe; LUL, left upper lobe; RLL, right lower lobe; RML, right middle lobe; RUL, right upper lobe.

hazards regression analysis was used to determine the relationship between survival and pathological nodal status. Differences were considered to be statistically significant when $p<0.05$. All statistical analyses were performed using software packages (JMP, release 5.0; SAS Institute Inc, Cary, North Carolina, USA and GraphPad Prism, release 4.03; GraphPad Software Inc, San Diego, California, USA).

RESULTS

Diagnostic reliability of cN1 status by CT scans

The pathological lymph node status of the 143 patients with cN1 was N0 in 33 (23%), N1 in 67 (47%), N2 in 40 (28%) and N3 in three (2%) patients. In 30% of patients with cN1 (n = 43), lymph node status was upstaged to pN2–3. Among 40 patients with cN1/pN2, six (15%) had metastases in multiple N2 stations. The locations of N1 lymph nodes depicted on CT scans are shown in table 2. The interlobar (No 11) node was detected most frequently (81%), followed by the hilar (No 10) node in 24%. The lobar (No 12), segmental bronchial (No 13) and intrapulmonary (No 14) nodes were depicted in only 15 (10%) patients.

Predictors for pN2–3 disease among patients with cN1

Table 3 shows the relationship between preoperative clinicoradiological characteristics and proportion of pN2–3 disease among patients with cN1 NSCLC. In univariate logistic regression analysis, we found statistically significant associations between the probability of pN2–3 disease and female gender (odds ratio (OR) 2.74; 95% confidence interval (CI) 1.18 to 6.35; $p = 0.019$), adenocarcinoma histology (OR 5.27; 95% CI 2.28 to 12.17; $p<0.0001$) and separate N1 pattern on CT scans (OR 2.75; 95% CI 1.32 to 5.76; $p = 0.0072$). Multivariate logistic regression analysis indicated that adenocarcinoma histology (OR 3.312; 95% CI 1.439 to 7.784; $p = 0.005$) was the highest risk factors (triple risk compared with non-adenocarcinoma) for pN2–3 disease. Although this did not reach statistical significance, separate N1 pattern on CT scans was also associated with pN2–3 disease (OR 2.103; 95% CI 0.955 to 4.693; $p = 0.066$) (table 4). Pathological N2–3 disease was found in 50% of patients with adenocarcinoma and 42% of patients with a separate N1 pattern on CT. In 34 patients with adenocarcinoma with a separate N1 pattern, 18 (53%) had pN2–3 status. In contrast, in 58 patients with non-adenocarcinoma with a continuous N1 pattern, only seven (12%) had pN2–3 disease (table 5).

Treatment for patients with cN1

As shown in table 1, it is remarkable that about half of the enrolled patients with cN1 underwent extensive lung resection greater than lobectomy; pneumonectomy was performed in 42 (29%) patients and bilobectomy in 35 (24%). Bronchoplastic procedures were performed in 16 (11%) patients.

Table 3 Univariate logistic model predicting pN2–3 disease among patients with cN1 non-small cell lung cancer

Chinicoradiological characteristics	n	Proportion of pN2–3 disease (95% CI)	OR (95% CI)	p Value
Sex				
Male	114	0.25 (0.18–0.34)		
Female	29	0.48 (0.31–0.66)	2.74 (1.18–6.35)	0.019
CEA (ng/ml)				
≤5	59	0.27 (0.17–0.40)		
>5	84	0.32 (0.23–0.43)	1.27 (0.61–2.65)	0.52
Histology				
Squamous cell carcinoma	69	0.16 (0.09–0.27)		
Adenocarcinoma	54	0.50 (0.37–0.63)	5.27 (2.28–12.17)	<0.001
Other carcinoma	20	0.25 (0.11–0.48)	1.76 (0.53–5.83)	0.36
Tumour location				
Upper/middle lobe	74	0.26 (0.17–0.37)		
Lower lobe	69	0.35 (0.25–0.47)	1.54 (0.75–3.17)	0.24
N1 involvement pattern on CT				
Continuous N1	78	0.21 (0.13–0.31)		
Separate N1	65	0.42 (0.30–0.54)	2.75 (1.32–5.76)	0.0072
Atelectasis or obstruction pneumonia on CT				
No	116	0.33 (0.25–0.42)		
Yes	27	0.19 (0.08–0.38)	0.47 (0.16–1.33)	0.15

CEA, carcinoembryonic antigen.

Table 4 Multivariate logistic model revealing predictors associated with pN2–3 disease among patients with cN1 non-small cell lung cancer

Variables (vs standard)	OR (95% CI)	p Value
Sex		
Female (vs male)	1.612 (0.617–4.154)	0.324
Histology		
Adenocarcinoma (vs non-adenocarcinoma)	3.312 (1.439–7.784)	0.005
N1 involvement pattern on CT		
Separate N1 (vs continuous N1)	2.103 (0.955–4.693)	0.066

Pneumonectomy was done mainly for patients with squamous cell carcinoma (squamous cell carcinoma in 26 (62%), adenocarcinoma in 12 (29%) and adenosquamous carcinoma in four (9%)). Radiotherapy was performed postoperatively in seven patients: six for their positive surgical margins and one with pT1N1M0 moderately differentiated squamous cell carcinoma for adjuvant purposes. Platinum based adjuvant chemotherapy was given to only two patients with cN1/pN2 because it was not the standard of care in our practice until January 2004 when the International Adjuvant Lung Cancer Trial results were published.[9]

Prognosis of patients with cN1

During the follow-up period, 65 (45%) patients developed recurrence. Median time from resection to recurrence was 316 days (range 11–2732). Table 6 shows number of patients with recurrence and their initial recurrence sites according to pathologic N status. The cN1/pN2–3 population developed approximately twice as many recurrent diseases as the cN1/pN0–1 populations. Recurrent disease arising in the cN1/pN2–3 population was almost distant recurrences (29/32, 91%). Two patients with cN1/pN2 who received adjuvant chemotherapy developed multiple distant recurrences and died at 2.7 and 8.3 years, respectively. The cumulative overall 1, 3 and 5 year survival rates of patients with cN1 were 83%, 63% and 55%, respectively, with a median survival time of 7.4 years (fig 2). When we stratified survival according to pathological lymph node status, cumulative overall 1, 3 and 5 year survival rates for patients with cN1/pN0 (n = 33) were 88%, 75% and 68%, respectively, and those for patients with cN1/pN1 (n = 67) were 85%, 67% and 60%, respectively. Patients with upstaged (pN2–3) nodal status (n = 43) showed significantly worse survival than patients with pN0 and pN1 (p = 0.017 and 0.0068, respectively). Hazard ratios for pN2–3 disease relative to pN0 and pN1 were 2.19 (95% CI 1.15 to 4.38; p = 0.017) and 2.06 (95% CI 1.20 to 3.54; p = 0.0093), respectively. The cumulative overall 1, 3 and 5 year survival rates of patients with cN1/pN2–3 were 67%, 44% and 38%, respectively, with a median survival time of 2.7 years (fig 3).

DISCUSSION

Lymph node status is one of the most important determinants in diagnosing surgical respectability for patients with NSCLC.[2] For patients with N0 and N1 disease, the standard of care is local treatment, mostly surgical resection, whereas patients with N2 and N3 are usually considered to be candidates for systemic therapy.[10] As cN1 disease is a borderline subset for which different treatment strategies are considered, accurate preoperative diagnosis of N1 status is essential.[6] Although overestimating a patient with pN0 as cN1 does not alter the treatment strategy, underestimating a patient with pN2–3 as cN1 can result in an incorrect treatment strategy and thus should be avoided.

Table 5 pN2–3 disease rate among patients with cN1 non-small cell lung cancer

cN1 patient with	pN2–3 disease rate (%)
Adenocarcinoma and separate N1 on CT	53
Adenocarcinoma and continuous N1 on CT	45
Non-adenocarcinoma and separate N1 on CT	29
Non-adenocarcinoma and continuous N1 on CT	12

In our series, N1 status was correctly diagnosed in only 47% of the CT diagnosed cN1 population. Thirty per cent of patients with cN1 had pN2–3 disease, and 23% had pN0. Our results were fairly consistent with those reported previously by Watanabe and colleagues.[6] They evaluated clinicopathological correlates of nodal status for 135 patients with cN1 diagnosed in a similar manner to us, and reported that the pathological nodal status was pN0 in 19%, pN1 in 44% and pN2–3 in 37%.[6] N1 diagnosis by contrast enhanced CT was concluded to be inaccurate.

The high frequency of upstaging to pN2–3 among the cN1 population might be explained by the following. Firstly, the majority of the CT detected N1 nodes were located around the hilum. As shown in table 2, enlargement of interlobar (No 11) and hilar (No 10) nodes was detected in 81% and 24% of our study population, respectively. However, lobar (No 12), segmental (No 13) and intrapulmonary (No 14) node involvement was evident in only 15 (10%) patients. Peripheral lymph nodes are usually small, and are rarely enlarged more than 1 cm, even when metastatic. Tumour cells in the N1 nodes located downstream in the pulmonary lymphatic system (ie, the nodes around the hilum compared with the peripheral nodes) are more likely to pass into the mediastinum. Secondly, some mediastinal nodes adjacent to the hilum might be misdiagnosed as N1. The anatomical border between the hilar (No 10) and tracheobronchial (No 4) or subcarinal (No 7) nodes is controversial,[7 11 12] and the diagnostic criteria for hilar nodes on CT scans have not yet been clearly established. In our series, among 34 patients with No 10 node enlargement on CT scans, 14 (41%) revealed metastatic nodes in the No 4 or 7 station. Consequently, CT diagnosed N1 disease may represent "advanced" N1 disease that behaves similar to limited N2 disease.

The cN1/pN2–3 population in our series had a significantly poor prognosis, as shown in fig 3. The optimal treatment for N2 disease has not been established: surgery followed by adjuvant

Table 6 Recurrence pattern of patients with cN1 according to pathological nodal status

	pN0 (n = 33)	pN1 (n = 67)	pN2–3 (n = 43)
Patients with recurrence	10 (30)	23 (34)	32 (74)
Initial recurrence site			
Local (only)	4 (12)	9 (13)	3 (7)
Brain	3 (9)	5 (7)	4 (9)
Bone	1 (3)	0	6 (14)
Lung	0	5 (7)	3 (7)
Liver	0	0	2 (5)
Adrenal gland	0	0	2 (5)
Cervical nodes	0	0	1 (2)
Small bowel	0	1 (1)	0
Multiple organs	2 (6)	3 (4)	11 (26)

*Data are presented as number of patients (%).

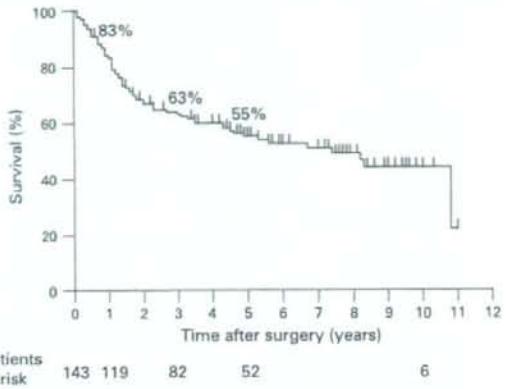
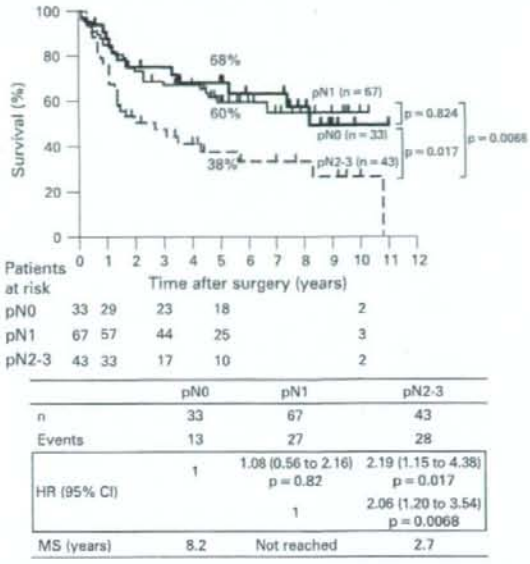


Figure 2 Survival curve for patients with cN1 disease (n = 143). The 1, 3 and 5 year cumulative overall survival rates were 83%, 63% and 55%, respectively.



	pN0	pN1	pN2-3
n	33	67	43
Events	13	27	28
HR (95% CI)	1	1.08 (0.56 to 2.16) p = 0.82	2.19 (1.15 to 4.38) p = 0.017
		1	2.06 (1.20 to 3.54) p = 0.0068
MS (years)	8.2	Not reached	2.7

Figure 3 Survival curves for patients according to pathological N status. There was no statistically significant difference between the pN0 and pN1 groups (p = 0.824, log rank test). Patients with pN2–3 disease showed a significantly worse survival than the pN0 and pN1 groups (p = 0.017 and 0.0068, respectively, log rank test). Five year survival rates for pN0, pN1 and pN2–3 disease were 68%, 60% and 38%, respectively. Hazard ratio (HR) with 95% CI and median survival (MS) for each pathological status are shown.

chemotherapy, induction therapy followed by resection or chemoradiotherapy. In clinical practice, treatment may depend on physical characteristics and the condition of the individual patient. N2 disease detection before thoracotomy may change the treatment strategy from primary surgery to other treatments. The high frequency and poor prognosis of the cN1/pN2–3 disease population prompted us to identify clinical and radiological factors predicting occult N2 disease. Adenocarcinoma histology, the statistically significant predictor (p = 0.005), predicted pN2 disease in 50% of cases. Separate N1 pattern on CT predicted pN2 in 42%, although it did not reach statistical significance (p = 0.066). When both factors were combined, pN2 status was predictive in 53% of patients. Several authors have indicated, from a prognostic point of view, that metastatic involvement of N1 node apart from the primary tumour should be distinguished from direct invasion.[13 14] Separate N1 appearance on CT may represent distinct tumour metastases and may also predict more frequent occult mediastinal node metastases.

Patients with adenocarcinoma, especially with a separate N1 pattern on CT, may need additional imaging modalities to rule out N2 disease for appropriate treatment strategy.[6] Fluorodeoxyglucose-PET scan, which is based on tumour physiology, has a higher diagnostic accuracy than CT scan for mediastinal staging. A recent meta-analysis reported median sensitivity and specificity of 85% and 90%, respectively, by PET scan (vs 61% and 79%, respectively, for CT scan).[15] Integrated PET/CT, which combines the functional information of PET with the anatomical precision of CT, has been found to have an even higher diagnostic accuracy than either CT or dedicated PET, with a reported sensitivity of 89% and specificity of 94% and an overall diagnostic accuracy of 93%.[16] Adding PET or PET/CT study to CT might enable the detection of occult N2 disease among the cN1 population. However, negative PET findings dose not necessarily exclude N2 disease, and pathological confirmation of positive PET findings is commonly required to exclude false positive findings.[17] In the current series, 13 (9%) of 143 patients with cN1 underwent dedicated PET, and N2 disease was proven after surgery in two (15%) patients. Cerfolio *et al* recently reported that seven (41%) of 17 patients with cN1 in whom N2 disease was ruled out by PET/CT were subsequently diagnosed with N2 by mediastinoscopy or endoscopic ultrasound with fine needle aspiration (EUS-FNA).[18] For patients with CT diagnosed N1, PET and PET/CT imaging on N2 disease detection may be limited, and invasive biopsy modalities may need to be considered. Mediastinoscopy is considered to be the "gold standard" for examining whether there is N2 disease, and several authors concluded that all patients with NSCLC considered for surgery should undergo mediastinoscopy, irrespective of mediastinal lymph node sizes.[19 20] However, we believe the recommendation is excessive to be indicated for all patients with cN1. Mediastinoscopy is highly reliable with a sensitivity of 81% and a false negative rate of less than 10%.[21] But it is also costly, requires general anaesthesia and has a complication rate of 0.5–2.5%.[22 23] The two predictive factors (adenocarcinoma histology and separate N1 status on CT scans) that we have demonstrated in the present study might be helpful in selecting candidates for mediastinoscopy.

EUS-FNA and endobronchial ultrasound transbronchial needle aspiration (EBUS-TBNA) have recently been reported as useful minimally invasive node biopsy modalities. Well experienced endoscopists can access lymph nodes greater than 5 mm in size.[24 25] Both procedures can be performed using moderate sedation with high sensitivity and specificity over 90%.[24–26] EUS-FNA also has the advantage of accessibility to subaortic (No 5), paraoesophageal (No 8) and pulmonary ligament (No 9) stations, which are not accessible by mediastinoscopy. By combining EUS-FNA and EBUS-TBNA, the majority of mediastinal lymph nodes could be accessible with minimal invasiveness.[27] EUS/EBUS biopsy might be a good

alternative in detecting unsuspected N2 disease among the cN1 population.

It is remarkable that more than half of our patients with cN1 underwent extensive resection of lung parenchyma: pneumonectomy in 29% and bilobectomy in 25% of patients. Several investigators have conducted randomised trials comparing induction chemotherapy followed by surgery with surgery alone in patients with stage IB or stage II NSCLC. Induction therapy is meaningful if it induces pathological downstaging, avoids extensive resection and increases the chance of organ sparing surgery. However, a recent multicentre phase III study by the French Thoracic Group targeting early stage NSCLC, including cN1 diseases, could not significantly decrease the overall pneumonectomy rate: 55.7% in surgery alone versus 48.6% in induction chemotherapy followed by surgery groups ($p = 0.30$).[36] Further studies are necessary to clarify the role of neoadjuvant chemotherapy for patients with cN1. In the current standard, we have to evaluate patients with cN1 whether or not they have enough cardiopulmonary reserve tolerable to extensive lung resection.[6]

In conclusion, cN1 diagnosis by CT, the current standard staging modality for NSCLC, is not satisfactorily accurate. Approximately 30% of patients with cN1 had pN2–3 disease, and they may have benefited better from a treatment strategy other than primary surgical resection if N2 status was proven before thoracotomy. Patients with clinical N1, especially with adenocarcinoma and possibly with a separate N1 appearance on CT, should be considered for additional invasive node biopsy modalities, including mediastinoscopy. Because extensive lung resection is often required for patients with cN1, careful cardiopulmonary function examination is needed to reduce perioperative complications.

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Competing interests: None.

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Predominant Infiltration of Macrophages and CD8$^+$ T Cells in Cancer Nests Is a Significant Predictor of Survival in Stage IV Nonsmall Cell Lung Cancer

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BACKGROUND. The purpose of this study was to investigate whether tumor-infiltrating immune cells in biopsy specimens can be used to predict the clinical outcome of stage IV nonsmall cell lung cancer (NSCLC) patients.

METHOD. The authors performed an immunohistochemical study to identify and count the number of CD68$^+$ macrophages, c-kit$^+$ mast cells, and CD8$^+$ T cells in both cancer nests and cancer stroma in pretreatment biopsy specimens obtained from 199 patients with stage IV NSCLC treated by chemotherapy, and then analyzed for correlations between the number of immune cells and clinical outcome, including chemotherapy response and prognosis.

RESULTS. There was no correlation between the number of immune cells in either cancer nests or stroma and chemotherapy response. Patients with more tumor-infiltrating macrophages in cancer nests than in cancer stroma (macrophages, nests > stroma) had significantly better survival than nests < stroma cases median survival time (MST 440 days vs 199 days; $P < .0001$). Patients with more tumor-infiltrating CD8$^+$ T cells in cancer nests than in cancer stroma (CD8$^+$ T cells: nests > stroma) showed significantly better survival than in nests < stroma cases (MST 388 days vs 256 days; $P = .0070$). The proportion of tumor-infiltrating macrophages or CD8$^+$ T cells between cancer nests and stroma became independent prognostic factors in the multivariate analysis. Neither the number of mast cells in nests nor in stroma correlated with the clinical outcome.

CONCLUSIONS. Evaluation of the numbers of macrophages and CD8$^+$ T cells in cancer nests and stroma are useful biomarkers for predicting the prognosis of stage IV NSCLC patients treated with chemotherapy, but could fail to predict chemotherapy response. ***Cancer*** **2008;113:1387–95.** © *2008 American Cancer Society.*

KEYWORDS: stage IV, nonsmall cell lung cancer, macrophage, CD8$^+$ T cell, mast cell.

Lung cancer is the leading cause of cancer deaths throughout the world, and nonsmall cell lung cancer (NSCLC) accounts for approximately 80% of lung cancer. The prognosis of NSCLC is poor, and patients with advanced NSCLC are candidates for systemic chemotherapy.[1] During the 1990s, 5 new drugs became available for the treatment of metastatic NSCLC: paclitaxel, docetaxel, vinorelbine, gemcitabine, and irinotecan. Each of them has since been evaluated in combination regimens with cisplatin or carboplatin, and the median survival time of patients with metastatic NSCLC treated with such regimens is approximately 8 to 10 months.[2,3] However, some patients with metastatic NSCLC exhibit long-term

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survival, and their tumors progress slowly after chemotherapy, or even in the absence of treatment.[4]

Tumor cells are surrounded by infiltrating inflammatory cells, such as lymphocytes, neutrophils, macrophages, and mast cells. Infiltration of $CD8^+$ T cells has been shown to be an important phenomenon for a specific immune response in several tumor systems, and $CD8^+$ T cells have been reported to play an important suppressive role in cancer progression, including ovarian cancer, esophageal cancer, pancreatic cancer, bile duct cancer, gallbladder cancer, and colorectal cancer.[5-12] Immunologists have long considered the presence of tumor-infiltrating macrophages as evidence of a host response against the growing tumor, and the presence of tumor-infiltrating macrophages has been reported to be associated with anticancer immunomechanisms of the tumor-bearing host and a favorable prognosis. However, recently, tumor-associated macrophage infiltration has been found to be correlated with angiogenesis and an unfavorable prognosis in several kinds of cancer, including gastric cancer, endometrial cancer, and breast cancer.[13-15] Furthermore, it has been reported that mast cells produce many angiogenic factors and a variety of cytokines, including transforming growth factor-beta, tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), fibroblast growth factor-2, and vascular endothelial growth factor, which are implicated in both normal and tumor-associated neoangiogenesis.[16] In fact, mast cell density has been reported to be highly correlated with the extent of both normal and pathologic angiogenesis, such as the angiogenesis observed in chronic inflammatory diseases and tumors, including gastric cancer and endometrial cancer.[17,18]

Tumor-infiltrating immune cells are thought to play important roles in disease progression and therapeutic efficacy. The effect of chemoradiotherapy has been found to be correlated with the presence of $CD8^+$ T cells in esophageal cancer,[11] and cervical cancer patients with T-cell infiltration showed improved local response to radiation therapy.[19]

In the current study, we evaluated the status of tumor-infiltrating immune cells in tumor biopsy specimens obtained from stage IV NSCLC patient and analyzed the numbers of immune cells and clinical outcome, including chemotherapy response and prognosis, for correlations.

MATERIALS AND METHODS

Patients and Tissue Specimens

The tumor specimens analyzed in this study were obtained from a total of 199 patients with stage IV NSCLC who received platinum-based combination chemotherapy (cisplatin plus paclitaxel, docetaxel, gemcitabine, vinorelbine, or irinotecan, or carboplatin plus paclitaxel), which is considered to be the standard regimen[20,21] at the National Cancer Center Hospital East in Kashiwa, Chiba, Japan, between January 1996 and December 2004, with performance status (PS) 0 or 1 on the Eastern Cooperative Oncology Group scale. Of the 199 patients, 184 had died by the time of the analysis. All patients had adequate tumor biopsy specimens obtainable before chemotherapy and were analyzed in this study. The tumor specimens were obtained by bronchoscopy in 152 patients, and by percutaneous needle biopsy in 47 patients. The histological classification was based on a World Health Organization report. Clinical staging was based on an initial evaluation consisting of a clinical assessment, chest radiography, computed tomography of the chest and abdomen, computed tomography or magnetic resonance imaging of the brain, and bone scintigraphy. The current International Staging System was used to stage clinical disease.[22] All target lesions were evaluated for response by computed tomography or magnetic resonance imaging after completion of the first-line chemotherapy, and all patients underwent tumor biopsy and chemotherapy, after obtaining informed consent in accordance with institutional guidelines.

Immunohistochemistry and Cell Counts

All specimens were fixed in 10% formalin and paraffin embedded. Four-micrometer-thick sections were mounted on silanized slides and deparaffinized with xylene and ethanol. To retrieve the antigen for macrophages, sections were pretreated in 0.05% trypsin and incubated for 20 minutes at 37°C in a humidity chamber. Endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol for 15 minutes. We used mouse antihuman CD68 antibody (Dako, Kyoto, Japan) to detect macrophages, mouse antihuman CD8 antibody (Novocastra, Newcastle, UK) to detect T cells, and mouse antihuman c-kit antibody (Dako) to detect mast cells; immunostaining was performed with Envision (Dako). To retrieve the antigen for CD8 and c-kit, sections were immersed in 10 mM citric buffer solution (pH 6.0) and heated to 95°C by exposure to microwave irradiation for 20 minutes.

We performed an immunohistochemical study to identify and count the number of $CD68^+$ macrophages, $c\text{-}kit^+$ mast cells, and $CD8^+$ T cells and confirmed that cancer cells and mesenchymal cells such as endothelial cells were not immunostained with these antibodies.

Immunostained cells counts were blinded to the patients' clinical data. Macrophages, $CD8^+$ T cells,

and mast cells in the specimen were counted in 2 locations: in the "cancer nests" and in the "cancer stroma." Cancer nests were defined as "cancer nests without fibroblasts and vasculatures" and cancer stroma as "connective tissues surrounding cancer nests without any cancer cells." Every biopsy specimen had both cancer nest and stroma, and it was possible to distinguish these lesions. We counted $CD68^+$ round cells as macrophages, c-kit^+ round cells as mast cells, and $CD8^+$ round cells as T cells. By using a high-power microscopic field (×400; 0.0625 mm^2), we separately counted the number of macrophages, $CD8^+$ T cells, and mast cells in each 2 most intensive areas. Two pathologists (O.K. and G.I) reviewed all slides and counted the cells.

Statistical Analysis

Statistical analysis was performed using the Scientific Package for Social Sciences (SPSS, Chicago, Ill) software. We used median values to calculate category correlations between macrophages, $CD8^+$ T cells, mast cells, and survival rate by the Kaplan-Meier method, and performed univariate analyses by means of log-rank test. The chi-square test was used to test for relationships between categorical variables. Multivariate analysis was performed by means of the Cox proportional hazards model. Student *t* test was used to test for correlation between macrophage counts, $CD8^+$ T cell counts, mast cell counts and response to first-line chemotherapy. We evaluated test results as significant if the *P* value was $P < .05$.

RESULTS

Patient Characteristics

The clinicopathological characteristics of all patients are listed in Table 1. Their median age at the time of diagnosis was 62 years (range, 39 years-79 years), and 139 of the 199 patients were men. There were 134 patients with adenocarcinoma, 41 patients with squamous cell carcinoma, and 24 patients with NSCLC that could not be specified by biopsy specimen.

Macrophages, Mast Cells, and $CD8^+$ T Cells, in Cancer Nests and Cancer Stroma

Macrophages were observed in cancer nests (Fig. 2A) in 194 of the 199 tumors, and the mean number was 18.0 ± 2.4 (median, 13; range, 0-76). Macrophages were also observed in cancer stroma (Fig. 2B) in 195 of the 199 tumors, and the mean number was 15.3 ± 1.9 (median, 12; range, 0-105). Mast cells were observed in cancer nests (Fig. 2C) in 149 tumors and in the cancer stroma (Fig. 2D) in 158 tumors, and the mean number was 4.5 ± 0.8 (median, 2; range, 0-52), and 5.4 ± 0.8 (median, 3; range, 0-28), respectively. $CD8^+$ T cells were observed in cancer nests (Fig. 2E) in 197 tumors, and the mean number was 16.9 ± 2.2 (median, 12; range, 0-89). $CD8^+$ T cells were observed in the cancer stroma (Fig. 2F) in 198 tumors, and the mean number was 15.7 ± 1.8 (median, 13; range, 0-88).

TABLE 1
Patient Characteristics and Response to First-Line Chemotherapy

	Patients (N = 199)	
	No.	%
Age		
Median, y (range)	62 (39-79)	
<70 y	158	79.3
≥70 y	41	20.6
Sex		
Women	60	30.1
Men	139	69.8
Histological diagnosis		
Adenocarcinoma	134	67.3
Squamous cell carcinoma	41	20.6
NSCLC	24	12
ECOG performance status		
0	44	22.1
1	155	77.8
Smoking history		
<20 pack years	73	36.6
≥20 pack years	126	63.3
Median survival time, d (range)	317 (19-1969)	
Response to first-line chemotherapy		
PR	53	26.6
SD	95	47.7
PD	51	25.6

NSCLC indicates nonsmall cell lung cancer; ECOG, Eastern Cooperative Oncology Group; PR, partial response; SD, stable disease; PD, progressive disease.

Relationships between the number of infiltrating macrophages, mast cells, $CD8^+$ T cells, and clinicopathological variables

The numbers of infiltrating macrophages, mast cells, and $CD8^+$ T cells were divided into 2 groups at the median value. The relationships between these groups in cancer nests or stroma and the individual clinicopathological characteristics (sex, age, smoking history, PS, histological type) were examined by the chi-square test. More macrophages were present in cancer nests in the nonadenocarcinomas than in the adenocarcinomas (data not shown).

Correlations between the numbers of macrophages, $CD8^+$ T cells, mast cells, and first-line chemotherapy response

We analyzed the number of macrophages, mast cells, and $CD8^+$ T cells in cancer nests and stroma and

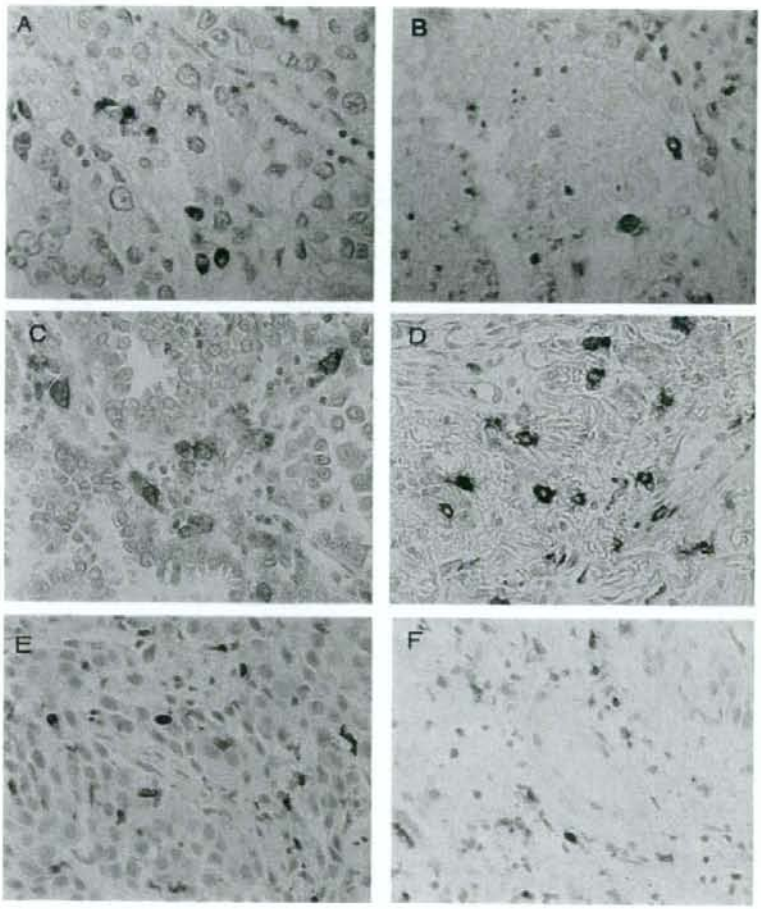

FIGURE 1. Typical photographs are shown of the results of immunostaining for the presence of $CD68^+$ macrophages in (A) cancer nests and in (B) stroma, C-kit^+ mast cells in (C) cancer nests and in (D) stroma, and $CD8^+$ T cells in (E) cancer nests and in (F) stroma.

first-line chemotherapy response for correlations by Student *t* test (Table 2), but the results showed no correlations between numbers of any of the infiltrating immune cells and response to first-line chemotherapy.

Correlations between the numbers of tumor-infiltrating macrophages, mast cells, and $CD8^+$ T cells and patient survival

Kaplan-Meier survival analyses and the log-rank test were performed to compare survival with the number of infiltrating cells (Fig. 2, Table 3). Patients with more macrophages in cancer nests than the median value had the same survival rate as patients with fewer macrophages. By contrast, patients with more macrophages in the cancer stroma had significantly poorer survival than those with fewer macrophages ($P = .0001$). The median survival time was 243 days in the group with higher numbers of macrophages in the stroma, versus 391 days in the group with fewer macrophages in the stroma (1-year survival rate, 33.9% and 55.2%, respectively). Patients were divided into 2 groups, according to the distribution of infiltrating macrophages; a High Nests Macrophage (HNM) group, in which the number of macrophages in the cancer nests was higher than in the cancer stroma (macro-

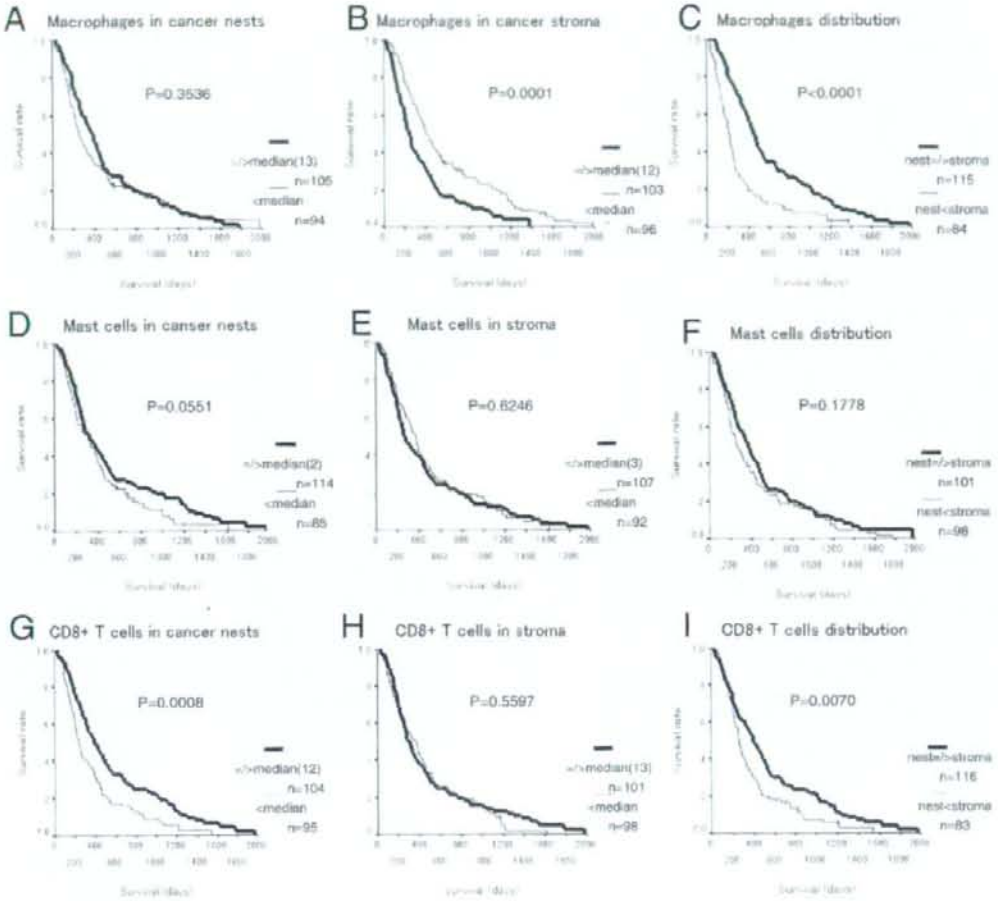

FIGURE 2. Kaplan-Meier analysis of overall survival is shown according to the level of infiltration by macrophages, mast cells, and CD8$^+$ T cells in cancer nests (A), (D), (G) and stroma (B), (E), (H) and their distribution (C), (F), (I). Data were dichotomized at the median value for each parameter (A), (B), (D), (E), (G), (H) and the distribution of infiltrating macrophages, mast cells, and CD8$^+$ T cells (C), (H), (I).

phages, nests > stroma) and a Low Nests Macrophage (LNM) group (nests < stroma). The HNM group had significantly better survival than the LNM group ($P < .0001$) (Fig. 2C). Median survival time was 440 days in the HNM group versus only 199 days in the LNM group, and the 1-year survival rate was 60.8% and 21.4%, respectively. Although mast cells in the cancer nests have been reported to contribute to a favorable outcome,[23] there was no significant relationship with patient survival in this study (Fig. 2D-F). Figure 2G-I shows the relation between the number of CD8$^+$ T cells and patient survival; there was a positive association between survival and the number of CD8$^+$ T cells in cancer nests (Fig. 2G, $P = .0008$). Median survival was 388 days in the group with the higher number of CD8$^+$ T cells in the cancer nests, versus 242 days in the group with the lower number (1-year survival rate, 52.8% and 34.3%, respectively). According to the distribution of infiltrating CD8$^+$ T cells, patients in the High Nests CD8$^+$ T cell (HNT) group, in which the number of tumor-infiltrating CD8$^+$ T cells was higher in the cancer nests than in the cancer stroma (nests > stroma), had significantly better survival than those in the Low Nests CD8$^+$ T cell (LNT) (nests < stroma) group ($P = .0070$) (Fig. 2I). Median survival time was 440 days in the HNT group, versus

TABLE 2
Correlations Between Immune Cells and Response to First-Line Chemotherapy

Parameter	t	95% CI	P*
Macrophages in cancer nests	−0.577	−7.173-3.946	.556
Macrophages in cancer stroma	0.119	−4.094-4.617	.905
Mast cells in cancer nests	−0.413	−2.310-1.512	.680
Mast cells in cancer stroma	1.476	−0.427-2.929	.143
$CD8^+$ T cells in cancer nests	−1.045	−7.114-2.201	.298
$CD8^+$ T cells in cancer stroma	−0.586	−5.162-2.807	.559

CI indicates confidence interval.
* Student *t* test.

only 199 days in the LNT group, and 1-year survival rate was 53.4% and 31.3%, respectively.

We then classified the patients into 4 groups according to macrophage and $CD8^+$ T cell distribution: 1) the HNM and HNT group (macrophages, nests > stroma; $CD8^+$ T cells, nests > stroma), 2) the HNM and LNT group (macrophages, nests > stroma; $CD8^+$ T cells, nests < stroma), 3) the LNM and HNT group (macrophages, nests < stroma; $CD8^+$ T cells, nests > stroma), and 4) the LNM and LNT group (macrophages, nests < stroma; $CD8^+$ T cells, nests < stroma). Median survival time was 495 days in the HNM and HNT group, versus only 196 days in the LNM and LNT group, and the 1-year survival rate was 68.4% and 12.5%, respectively. Median survival time was 342 days, and 1-year survival rate was 45.0% in the HNM and LNT group; median survival time was 221 days, and the 1-year survival rate was 27.2% in the LNM and HNT group. Patients in the HNM and HNT group had significantly better survival than patients in the other groups (Fig. 3, Table 3)

Multivariate Regression Analysis of Survival in NSCLC Patients

As immune cells in cancer nests and cancer stroma would have different biological activity in regard to tumor progression, it would be meaningful to evaluate immune cell distribution. Considering that the distributions of macrophages in cancer nests and cancer stroma may impact clinical outcome, multivariate analysis of macrophage and $CD8^+$ T cell distribution and clinicopathological predictors of survival was performed by means of the Cox proportional hazards model (Table 4), and both macrophage distribution ($P < .001$) and $CD8^+$ T cell distribution ($P = .040$) emerged as independent favorable prognostic indicators. Smoking status also emerged as an independent prognostic indicator ($P = .033$).

TABLE 3
Overall Survival

		Survival, d		
Groups	No.	Median	95% CI	P
Macrophages in cancer nests				.3536
<Median	94	248	192-304	
≥Median	105	376	299-453	
Macrophages in stroma				.0001
<Median	96	391	307-475	
≥Median	103	243	206-280	
Macrophage distribution				<.0001
Nests < stroma	84	199	178-220	
Nests > stroma	115	440	370-505	
Mast cells in cancer nests				.0551
<Median	85	307	201-413	
≥Median	114	317	230-404	
Mast cells in stroma				.6246
<Median	92	366	301-431	
>Median	107	259	200-318	
Mast cell distribution				.1778
Nests < stroma	98	250	188-324	
Nests ≥ stroma	101	370	304-436	
$CD8^+$ T cells in cancer nests				.0008
<Median	95	242	199-285	
≥Median	104	388	290-486	
$CD8^+$ T cells in stroma				.5597
<Median	98	358	237-479	
≥Median	101	297	247-347	
$CD8^+$ T cell distribution				.0070
Nests < stroma	83	256	224-288	
Nests ≥ stroma	116	388	316-460	

CI indicates confidence interval.
*Log-rank test.

DISCUSSION

This is the first study to investigate the relationship between the number of tumor-infiltrating macrophages, mast cells, and $CD8^+$ T cells in tumor biopsy specimens and the clinical outcome of patients with stage IV NSCLC. Patients with higher numbers of tumor-infiltrating macrophages and $CD8^+$ T cells in cancer nests than in cancer stroma had significantly better survival. These factors were also independent prognostic factors in multivariate analysis.

Immunologists have long considered the presence of tumor-infiltrating immune cells as evidence of a host response against the growing tumor. However, recently reports have shown that macrophages and mast cells in cancer stroma secrete several growth factors and proteases involved in angiogenesis, thereby promoting cancer progression. An experimental study has demonstrated that interaction between lung cancer cells and macrophages promotes the invasiveness and matrix-degrading activity of cancer cells,[24] and infiltration by these cells has been reported to be

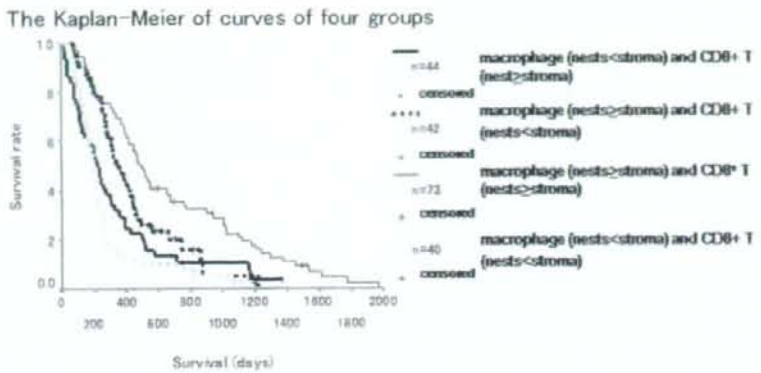

FIGURE 3. Kaplan-Meier analysis of overall survival is shown according to distribution in 4 groups of macrophages and $CD8^+$ T cells. Patients whose tumors contained macrophages in the nest and more $CD8^+$ T cells in the nest had significantly better survival (macrophages, nest > stroma; $CD8^+$ T cells, nest > stroma) than patients with macrophages nest > stroma and $CD8^+$ T cells nest < stroma ($P = .0070$), patients with macrophages nest < stroma and $CD8^+$ T cells nest > stroma ($P = .0010$), and patients with macrophages nest < stroma and $CD8^+$ T cells: nest < stroma ($P < .0001$).

TABLE 4
Multivariate Cox Proportional Hazards Analysis of Overall Survival

Parameter	Hazard Ratio	95% CI	*P*
Age (<70 y vs ≥70 y)	1.093	0.740-1.613	.655
Sex (men vs women)	1.166	0.772-1.760	.897
PS (0 vs 1)	1.41	0.971-2.049	.071
Smoking (< pack years vs > pack years)	1.561	1.037-2.348	.033
Histology (adeno vs nonadeno)	1.031	0.742-1.432	.856
Macrophage distribution (nests < stroma vs nests > stroma)	0.439	0.320-0.602	<.001
$CD8^+$ T cells distribution (nests < stroma vs nests > stroma)	0.723	0.530-0.985	.040

CI indicates confidence interval; PS, performance status; adeno, adenocarcinoma.

associated with an unfavorable outcome in several kinds of cancers.[25-27] Conversely, macrophages in cancer nests produce cytotoxic cytokines, such as IL-1α, IL-1β, IL-6, and TNF-α, which may protect against tumor progression.[28] Considering the results of this study showing that the distributions of macrophages in cancer nests and cancer stroma impacted outcome of stage IV NSCLC, the macrophages in cancer nests and cancer stroma may have different biological activity in regard to tumor progression. Welsh et al demonstrated that higher numbers of macrophages in cancer stroma and lower numbers of macrophages in cancer nests were unfavorable prognosis factors in surgically resected NSCLC,[23] and their findings are in part consistent with the results of our study. No relationship between the numbers of macrophages in cancer nests and patient survival was found in our study. This can be explained by the difference between the specimens from operable cases of NSCLC (stage I-III) and stage IV cases.

$CD8^+$ T cells with cytotoxic activity play an important role in antitumor immunity. $CD8^+$ T cells can circumvent many of the barriers inherent in cancer-induced stroma, while optimizing T-cell specificity, activation, homing, and antitumor function.[29] The presence of tumor-infiltrating $CD8^+$ T cells has previously been reported to be associated with a favorable outcome, the same as in our own study.[5-12,30] Patients in the HNM and HNT group had significantly better survival (median survival was 495 days; 1-year survival rate was 68.5%) than patients in the HNM group (median survival, 440 days; 1-year survival rate, 60.8%; Fig. 2C) and patients in the HNT group (median survival, 388 days; 1-year survival rate, 53.4%; Fig. 2I). There were also many long-term survivors in the HNM and HNT group, which notably had a 3-year survival rate of 19.1%. Because aggregation of tumor-infiltrating macrophages in cancer nests has been reported to have a beneficial effect by activating cytotoxic T cells,[31] the macrophages and $CD8^+$ T cells in cancer nests should exert synergistic antitumor effects. Infiltration of $CD8^+$ T cells in gastric carcinoma is actually directly correlated with macrophage infiltration, suggesting that macrophages play an important part in the activation of T cells and subsequent tumor cell destruction.[31]

Whether there is any correlation between the presence of tumor-infiltrating mast cells and cancer progression is a matter of controversy. In previous studies, mast cells were found to have antitumor

functions, including serving as natural cytotoxic effectors[32,33] and antitumor compounds,[34] and to be a favorable prognostic factor in surgically resected NSCLC, breast cancer, and colorectal cancer.[35-37] Although mast cells produce histamine, basic fibroblast growth factor, heparin, chymase, and tryptase, which have been shown to promote cancer progression, including in surgically resected NSCLC, gastric cancer, and endometrial cancer,[18,38] no significant relation to survival was found in this study.

Accumulation of immune cells in tumor tissue either before or during chemoimmunotherapy has been reported to be associated with a better clinical response and improved survival.[39-41] The effect of chemoradiotherapy in esophageal cancer is correlated with the number of $CD8^+$ T cells in the tumor of each patient, and the patterns of gene expressions for T cell activation and for tumor vessel formation may become good markers for identifying potential long-term survivors.[11] However, in the present study, no correlations between numbers of macrophages, $CD8^+$ T cells, or mast cells and response to first-line chemotherapy were found (Table 2). The results of our study suggested that patients with a favorable or unfavorable prognosis could be identified by the status of tumor-infiltrating macrophages and $CD8^+$ T cells in tumor biopsy specimens before receiving chemotherapy regardless of chemotherapy response. Cancer patients can mount cellular immune responses against their own tumor cells, and hosts can respond to a large compendium of tumor-associated antigens and epitopes. The natural immune system within the cancer microenvironment may affect its ability to control malignant disease beyond the response to chemotherapy. The only treatment currently available for metastatic NSCLC is chemotherapy, but patients with a poor prognosis, and patients with a predominant distribution of macrophages and $CD8^+$ T cells in the cancer stroma, require some additional therapy to prolong life. For example, elimination of macrophages from the cancer stroma or transfer of $CD8^+$ T cells to cancer nests might be beneficial in prolonging the life of stage IV NSCLC patients in these unfavorable groups.

In conclusion, we found that predominant distribution of macrophages and/or $CD8^+$ T cells in cancer nests as opposed to cancer stroma was correlated with a favorable prognosis in stage IV NSCLC patients. Patients with advanced NSCLC require additional therapy, because the response rate to chemotherapy has been poor (only 30%-40%), and the median survival time of patients with metastatic NSCLC is approximately 8 months to 10 months.[20,21] The results of our study indicate the possibility of using macrophages and $CD8^+$ T cells to treat advanced NSCLC in the future.[42] Decreasing the number of tumor-associated macrophages in the tumor stroma in an animal model of breast cancer effectively altered the tumor microenvironment involved in tumor angiogenesis and progression and markedly suppressed tumor growth and metastasis.[43]

Thus, a more accurate insight into the role of macrophages and $CD8^+$ T cells in tumors and consideration of the local microenvironment in regulating the functions of these cells is needed and has important implications for the design of future clinical trials of adjuvant therapy, as well as for our understanding of the immunopathobiology of stage IV NSCLC.

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