had Eastern Cooperative Oncology Group performance status of 0 and 45 (82%) patients had stage IV disease. The predominant histology type was adenocarcinoma (67%).

Response and Survival. Among all 55 eligible patients, 1 had a complete response and 25 had a partial response. Thus, the overall response rate was 47% (95% CI, 34–61%). Because one ineligible patient had a partial response, the overall response of all registered 56 patients was 48% (95% CI, 35–62%). The responding patients were classified in terms of the items shown in Table 2. There was no statistically significant difference in the response rates between the items compared. The median response duration was 4.2 months.

The median follow-up period was 28 months (range, 20–33 months). As shown in Fig. 1, median survival time of the 55 eligible patients was 11 months and the 1-year and 2-year survival rates were 45% (95% CI, 32–59%) and 17% (95% CI, 6–27%), respectively.

Adverse Events. The adverse events observed throughout the treatment of the 55 eligible patients are shown in Table 3. Among the hematologic adverse event, grade 3/4 neutropenia and anemia was observed in 29 and 22% of the patients, respectively. However, grade 3 thrombocytopenia was observed in only one patient (2%), and no patient had grade 4 thrombocytopenia. Among the observed nonhematologic adverse events, no grade 4 level was observed. There were no unexpected toxicities.

Table 2 Patient characteristics in relation to the response

Characteristics	No. of patients	Response				Response rate (%)
		CR	PR	NC	PD	
All	55	1	25	23	6	47
Gender						
Male	41	1	20	15	5	51
Female	14	0	5	8	1	36
Stage						
IIIB	10	0	4	5	1	40
IV	45	1	21	18	5	49
Histology						
Adenocarcinoma	37	0	15	17	5	41
Squamous cell carcinoma	14	1	7	5	1	57
Others	4	0	3	1	0	75

Abbreviations: CR, complete response; PR, partial response; NC, no change; PD, progressive disease.

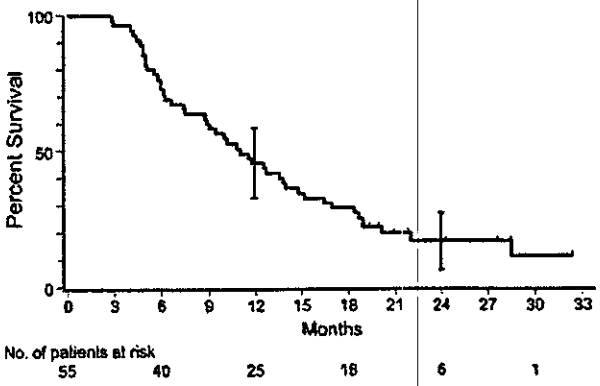

Fig. 1 Overall survival. Each *tick* represents a patient who is alive. The *bars* represent the 95% confidence interval of the survival rate at 1 year and 2 years after treatment.

Table 3 Hematologic and nonhematologic toxicities

Toxicity	Grade				Frequency of 3 or 4 (%)
	1	2	3	4	
Leukopenia	8	18	2	1	6
Neutropenia	7	13	13	3	29
Anemia	14	24	10	2	22
Thrombocytopenia	28	4	1	0	2
Aspartate aminotransferase	7	0	1	0	2
Alanine aminotransferase	6	1	1	0	2
Creatinine	9	1	1	0	2
Anorexia	21	15	7	0	13
Vomiting	14	3	4	0	7
Diarrhea	12	3	4	0	7
Stomatitis	12	2	0	0	0
Dermatitis	13	0	0	0	0

Compliance. A range of 1 to 12 treatment cycles were administered (1 cycle, 6 patients; 2 cycles, 18 patients; 3 cycles, 5 patients; 4 cycles, 12 patients; >4 cycles, 14 patients). The reasons for only one cycle of treatment were progressive disease in 4 patients and adverse events in 2 patients. The dose of S-1 was reduced in 8 patients because of adverse events including myelosuppression in 4 patients, gastrointestinal toxicity in 2 patients, glycemia in 1 patient, and dermatitis in 1 patient. A total of 197 cycles were given to the 55 patients. Sixty-nine (49%) of 142 treatment cycles excluding the first cycle was given at 4-week interval, 58 (40%) were at a 5-week interval, and 15 (11%) were at a >5-week interval.

DISCUSSION

Because the half-life of 5-FU is as short as 5 to 20 minutes (13) and the antitumor activity of 5-FU is time dependent, the continuous intravenous administration of 5-FU is considered to be appropriate rather than a bolus intravenous injection of 5-FU. In fact, a meta-analysis of six randomized trials in patients with colorectal cancer showed that the response rate was clearly higher for continuous infusion of 5-FU over 5 consecutive days than for weekly bolus injection of 5-FU (14). Although NSCLC has also been reported not to respond to a bolus injection of 5-FU (15), whether or not continuous treatment with 5-FU is effective for NSCLC remains unclear. However, studies have shown that a combination of cisplatin and protracted intravenous injection of 5-FU is effective for NSCLC (16). In prior trials, we used this combination chemotherapy with daily oral administration of UFT in place of the protracted intravenous injection of 5-FU which negatively affects the quality of life of a patient for advanced NSCLC (9–11).

The combination chemotherapy of cisplatin and 5-FU has been proven to have synergic antitumor effect in many experimental and clinical studies (17, 18). However, the optimal sequence for the administration of these drugs has yet to be determined. The sequence of cisplatin followed by 5-FU has been shown to be more cytotoxic than the reverse succession in *in vitro* and *in vivo* studies (19, 20) whereas the sequence of 5-FU followed by cisplatin has been proven to have a greater

antitumor activity than the opposite order of administration in tumor-bearing animals (21). Therefore, in our prior trials using UFT, we designed a treatment regimen that is thought to be a compromise solution between the present conflicting experimental data; namely, a daily administration of UFT from day 1 to 14 or 21 and a bolus infusion of cisplatin on day 8 (9, 10).

In the present study with S-1, the treatment modality was determined based on the UFT trials (9, 10) and phase I/II trial of S-1 combined with cisplatin in patients with advanced gastric cancer (22). The dose of cisplatin was decreased from 80 mg/m^2 in prior UFT trial to 60 mg/m^2 in the present trial because phase I trial indicated that 60 mg/m^2 of cisplatin on day 8 was the recommended dose when it was combined with daily administration of S-1 from day 1 for 3 weeks (22). Concerning the dose of cisplatin in combination chemotherapy in NSCLC patients, the effect of the dosage on survival has not yet been clearly elucidated. Klastersky *et al.* (23) reported the median survival time of patients who received vindesine plus combination chemotherapy consisting of either 60 or 120 mg/m^2 of cisplatin to be 7.6 and 6.4 months, respectively, and no overall survival difference between the two groups was observed ($P = 0.138$). On the other hand, the incidence of adverse events was significantly higher in the 120-mg dose than that in 60-mg dose.

Although a comparison between the present S-1 trial and the prior UFT trial with 108 patients (10) has limitation because of different trials, the response rate and survival seems to be favorable in the present trial despite the fact that proportion of stage IV patients in the present trial was higher than that in the UFT trials (82% *versus* 68%). The response rate and median survival time was 47% and 11.2 months in the present study and 29% and 10 months in the UFT trial, respectively. The frequency of severe adverse events in the both trials was similarly low.

The standard chemotherapy regimen for NSCLC is considered to be a platinum-based two-drug combination chemotherapy that uses paclitaxel, docetaxel, gemcitabine, or vinorelbine. The response rate and median survival time in the recent phase III trials that use these combination chemotherapies have been reported to be 17 to 28% and 7 to 9 months, respectively. Grade 3 or 4 hematologic and nonhematologic adverse events were observed in 57 to 76% (neutropenia) and 4 to 35% (vomiting), respectively (24, 25). In the present study with S-1 and cisplatin, the incidence of those adverse events seems to be lower than the above mentioned data. In addition, the antitumor mechanism is different from those agents. On the basis of these observations, we plan to conduct a randomized trial comparing the present combination chemotherapy with standard platinum-based two-drug combination chemotherapy regarding survival and the quality of life.

ACKNOWLEDGMENTS

We thank Brian Quinn for critical review and Yumiko Oshima for help in preparing the report.

REFERENCES

1. Shirasaka T, Shimamoto Y, Fukushima M. Inhibition by oxonic acid of gastrointestinal toxicity of 5-fluorouracil without loss of its antitumor activity in rats. Cancer Res 1993;53:4004–9.

2. Tatsumi K, Fukushima M, Shirasaka T, Fujii S. Inhibitory effects of pyrimidine, barbituric acid and pyridine derivatives on 5-fluorouracil degradation in rat liver extracts. Jpn J Cancer Res 1987;78:748–55.

3. Shirasaka T, Shimamato Y, Ohshimo H, et al. Development of a novel form of an oral 5-fluorouracil derivative (S-1) directed to the potentiation of the tumor selective cytotoxicity of 5-fluorouracil by two biochemical modulators. Anticancer Drugs 1996;7:548–57.

4. van Groeningen CJ, Peters GJ, Schornagel JH, et al. Phase I clinical and pharmacokinetic study of oral S-1 in patients with advanced solid tumors. J Clin Oncol 2000;18:2772–9.

5. Yamada Y, Hamaguchi T, Goto M, et al. Plasma concentrations of 5-fluorouracil and F-beta-alanine following oral administration of S-1, a dihydropyrimidine dehydrogenase inhibitory fluoropyrimidine, as compared with protracted venous infusion of 5-fluorouracil. Br J Cancer 2003;89:816–20.

6. Kawahara M, Furuse K, Segawa Y, et al. Phase II study of S-1, a novel oral fluorouracil, in advanced non-small-cell lung cancer. Br J Cancer 2001;85:939–43.

7. Fujii S, Kitano S, Ikenaka K, Shirasaka T. Effect of coadministration of uracil or cytosine on the anti-tumor activity of clinical doses of 1-(2-tetrahydrofuryl)-5-fluorouracil and level of 5-fluorouracil in rodents. Gann 1979;70:209–14.

8. Keicho N, Saijo N, Shinkai T, et al. Phase II study of UFT in patients with advanced non-small cell lung cancer. Jpn J Clin Oncol 1986;16: 143–6.

9. Ichinose Y, Takanashi N, Yano T, et al. A phase II trial of oral tegafur and uracil plus cisplatin in patients with inoperable nonsmall cell lung cancer. Cancer (Phila) 1995;75:2677–80.

10. Ichinose Y, Yosimori K, Yoneda S, Kuba M, Kudoh S, Niitani H. UFT plus cisplatin combination chemotherapy in the treatment of patients with advanced nonsmall cell lung carcinoma: a multiinstitutional phase II trial. For the Japan UFT Lung Cancer Study Group. Cancer (Phila) 2000;88:318–23.

11. Saito J, Nakai Y, Saijo Y, et al. A phase II trial of oral UFT plus cisplatin (CDDP) in patients with non-small cell lung cancer (NSCLC). Lung Cancer 2001;31:285–93.

12. Miller AB, Hoogstraten B, Staquet M, Winkler A. Reporting results of cancer treatment. Cancer (Phila) 1981;47:207–14.

13. Iyer L, Ratain MJ. 5-Fluorouracil pharmacokinetics: causes for variability and strategies for modulation in cancer chemotherapy. Cancer Investig 1999;17:494–506.

14. Meta-analysis Group In Cancer. Efficacy of intravenous continuous infusion of fluorouracil compared with bolus administration in advanced colorectal cancer. J Clin Oncol 1998;16:301–8.

15. Crawford J, O'Rourke M, Schiller JH, et al. Randomized trial of vinorelbine compared with fluorouracil plus leucovorin in patients with stage IV non-small-cell lung cancer. J Clin Oncol 1996;14:2774–84.

16. Heim W, Wampler GL, Lokich JJ, et al. A study of infusional cisplatin and infusional fluorouracil for locally advanced or metastatic non-small-cell lung cancer: a Mid-Atlantic Oncology Program study. J Clin Oncol 1991;9:2162–6.

17. Decker DA, Drelichman A, Jacobs J, et al. Adjuvant chemotherapy with cis-diamminodichloroplatinum II and 120-hour infusion 5-fluorouracil in stage III and IV squamous cell carcinoma of the head and neck. Cancer (Phila) 1983;51:1353–5.

18. Kemeny N, Israel K, Niedzwiecki D, et al. Randomized study of continuous infusion fluorouracil versus fluorouracil plus cisplatin in patients with metastatic colorectal cancer. J Clin Oncol 1990;8:313–8.

19. Scanlon KJ, Newman EM, Lu Y, Priest DG. Biochemical basis for cisplatin and 5-fluorouracil synergism in human ovarian carcinoma cells. Proc Natl Acad Sci USA 1986;83:8923–5.

20. Shirasaka T, Shimamoto Y, Ohshimo H, Saito H, Fukushima M. Metabolic basis of the synergistic antitumor activities of 5-fluorouracil and cisplatin in rodent tumor models in vivo. Cancer Chemother Pharmacol 1993;32:167–72.

21. Kuroki M, Nakano S, Mitsugi K, et al. In vivo comparative therapeutic study of optimal administration of 5-fluorouracil and cisplatin using a newly established HST-1 human squamous-carcinoma cell line. Cancer Chemother Pharmacol 1992;29:273–6.

22. Koizumi W, Tanabe S, Saigenji K, et al. Phase I/II study of S-1 combined with cisplatin in patients with advanced gastric cancer. Br J Cancer 2003;89:2207–12.

23. Klastersky J, Sculier JP, Ravez P, et al. A randomized study comparing a high and a standard dose of cisplatin in combination with etoposide in the treatment of advanced non-small-cell lung carcinoma. J Clin Oncol 1986;4:1780–6.

24. Kelly K, Crowley J, Bunn PA Jr, et al. Randomized phase III trial of paclitaxel plus carboplatin versus vinorelbine plus cisplatin in the treatment of patients with advanced non–small-cell lung cancer: a Southwest Oncology Group trial. J Clin Oncol 2001;19:3210–8.

25. Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med 2002;346:92–8.

A Clinicopathological Study of Resected Adenocarcinoma 2 cm or Less in Diameter

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Background. The biological behavior of small adenocarcinoma is different in each patient and these are especially enormous differences when evaluating solid tumors and nonsolid tumors.

Methods. A total of 159 adenocarcinomas 2 cm or less in diameter were studied. Several clinicopathological factors were retrospectively analyzed.

Results. The diameter of the primary tumors was less than 1 cm in 47 patients, 1–1.5 cm in 49 patients, and 1.5–2 cm in 63 patients, respectively. Almost all patients (147) were pathologic N0 and there were 12 node-positive patients (7.5%). Lymph-node involvement was observed in 1 patient with a tumor diameter measuring less than 1 cm and in 11 patients with a tumor diameter measuring 1–2 cm. According to Noguchi' s classification, 33 patients belonged to class A or B, 71 patients belonged to class C, and 55 patients belonged to class D, E, or F. The ratio of ground-glass opacity (GGO) area in the main tumor in high resolution computed tomography was classified into two groups with a threshold of 50%. There were 44 patients with a GGO ratio of equal to or greater than 50%, none of which indicated lymph-node metastasis or tumor recurrence during follow-up (5-year survival = 100%). On the contrary among 115 patients with a GGO ratio less than 50%, lymph-node involvement was indicated in 12 patients (10.4%) and the 5-year survival rate was 83.9%.

Conclusions. The biological malignancy of small adenocarcinomas might be accurately evaluated by the proportion of GGO area as well as the Noguchi classification.

(Ann Thorac Surg 2004;78:1011–6)

Lung cancer is the greatest cause of cancer-related death in the world because most lung cancers are detected at a late stage and curative treatment is not an option. Nevertheless a cure rate of greater than 70% was obtained in completely resected patients of stage I cancer [1]. Prevention and early detection are thus essential with regard to the reduction of lung cancer mortality. Adenocarcinoma is the most common type of lung cancer arising from the peripheral lung parenchyma. Chest x-ray surveys have been considered useful for early detection. However if the lesions are located in a "dead angle" on the chest roentgenogram film, such as behind the aorta or heart, abnormalities may be overlooked. Bronchioloalveolar carcinoma (BAC) seldom reveals abnormalities on chest roentgenogram because it grows without destroying alveolar structure [2]. Helical computed tomography (CT) screening has greatly increased the sensitivity of cancer detection compared with that of conventional chest roentgenogram screening [3–7]. A prospective randomized trial comparing the lung cancer mortality rate of a CT screening group with that of a conventional chest roentgenogram screening group has been conducted by the National Cancer Institute [8]. In this respect, the biggest issue facing thoracic surgeons is the treatment strategy for small cancers detected by CT screening, including the possibility of limited resection. BAC is known to exhibit a relatively nonaggressive nature, therefore a favorable outcome can be expected after curative operation [2, 9–12]. However patients with solid images on chest CT tend to have invasive adenocarcinomas and their survival is definitely worse than that of BAC [9–11]. Pathologic classification of the tumor is essential regarding the evaluation of the aggressiveness of each patient [2] but postoperative pathologic findings cannot exhibit a strong impact on the choice of treatment.

There are several reports indicating that the ratio of the size of ground-glass opacity (GGO) and that of consolidation on high resolution CT (HRCT) is strongly related to the stage and prognosis of the cancer [10, 13–15]. Lung cancers with a large GGO component tend to be BAC or minimally invasive adenocarcinomas that exhibit favorable prognoses [10, 13–15]. If a definition of peripheral early cancer could be established, it would be useful with regard to selecting optimal treatment for individual patients. For this purpose we retrospectively analyzed clinicopathological features of adenocarcinomas with a diameter of 2 cm or less resected in our hospital between 1997–2002.

Patients and Methods

Patients

A total of 983 lung cancer operations were performed from January 1997 to December 2002 at the Department

Accepted for publication March 15, 2004.

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Published by Elsevier Inc

0003-4975/04/$30.00
doi:10.1016/j.athoracsur.2004.03.048

Table 1. Patient Characteristics

Character	
Age	
Average	63
Minimum	40
Maximum	84
Sex	
Male	67
Female	92
Smoking habit	
Non-smoker	89
Smoker	70
Operative procedure	
Lobectomy	112
Segmantectomy	20
Wedge resection	27

of Thoracic Surgery, Tokyo Medical University Hospital (Tokyo, Japan). Among these, there were 168 patients with peripheral adenocarcinomas less than 2 cm in diameter as well as a total of 159 patients who had undergone high-resolution computed tomography (HRCT) and in whom complete records were available for study (Table 1). There were 67 men and 92 women ranging in age from 40–84. There were 89 nonsmokers and 70 smokers. The primary lesions were detected by chest x-ray in 115 patients: detection was determined by mass survey or private general check-up in 81 patients, follow-up for other diseases in 18 patients, and respiratory symptoms in 16 patients. The other 44 patient's lesions were detected by chest CT performed by mass survey program or private general check-up.

All patients underwent a physical examination and blood examination, respiratory function test, electrocardiogram, and chest radiography. Also, all patients received helical CT of the chest preoperatively with 10-mm thick continuous sections. HRCT images with 1–2 mm slices of the primary tumors were then performed to obtain the precise findings of GGO and consolidation of the tumors. Histologic typing was diagnosed based upon the classification of the World Health Organization (WHO) and we also classified all of the patients into six subtypes using the Noguchi classification. The staging of patients was determined by the thoracic wall, node involvement, and metastases (TNM) classification of the International Union Against Cancer (UICC).

Lobectomy combined with systemic mediastinal lymph-node dissection was performed in 112 patients and limited surgery was performed in 47 patients. Of these 47 patients, 37 received intentionally limited operation because of the nonaggressive appearance on HRCT and the remaining 10 patients because of impaired condition. Segmentectomy with mediastinal sampling was performed in 27 patients and wedge resection without nodal dissection was performed in 20 patients. All patients that underwent wedge resection indicated pure GGO or enormously GGO-dominant findings on HRCT as well as being clinically node negative.

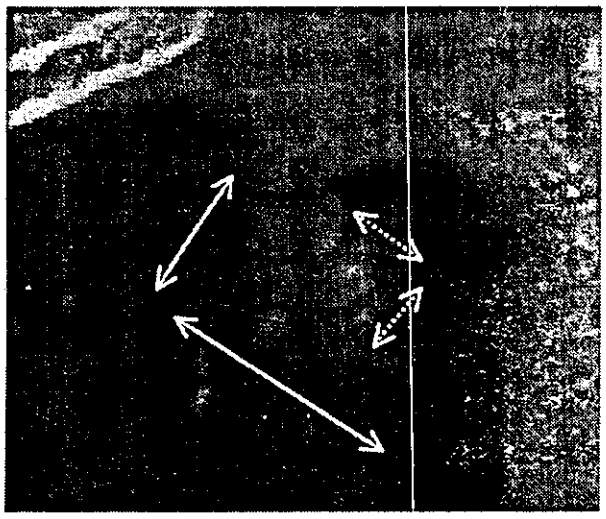

Fig 1. Thin section computed tomographic scan of lung cancer depicting solid attenuation and ground-glass opacity (GGO). The largest area of tumor (solid line) *and solid attenuation* (dotted line) *were decided based on this film. The proportion of GGO area to the entire tumor was defined; GGO ratio = (maximum GGO − maximum consolidation)/maximum GGO. Max GGO* (solid arrow); *Max consolidation* (dotted arrow).

CT Findings

In this study the ratio of the size of solid attenuation to that of GGO was extensively analyzed. GGO was defined as a hazy increase in lung attenuation without obscuration of the underlying vascular marking. At least two experienced chest surgeons and radiologists reviewed the hard-copy films of HRCT and determined the maximal area of GGO and tumor. Discrepancies between reviewers were resolved by consensus. The ratio area of GGO to the area of primary tumor was calculated as illustrated in Figure 1. Patients were divided into two groups: those with a GGO ratio greater than 50% and those with a GGO ratio less than 50%.

Pathology

Resected lungs were fixed in formalin and stained by hematoxylin and eosin staining in a routine manner and also stained with elastica van Gieson. Experienced pathologists diagnosed the subtypes of primary tumors according to the Noguchi classification as well as the nodal status. The Noguchi classification is presented in Table 2. Types A and B are considered to be noninvasive cancers and types D, E, and F are considered to be invasive cancer.

Statistics

We examined the relation of the proportion of GGO area to maximal tumor size, stage, Noguchi classification, and other prognostic factors. The χ^2 test using StatView 5.0 (SAS Institute Inc., Cary, NC) was performed and the differences were considered to be statistically significant

Table 2. Tumor Size and Nodal Status

Tumor Size	N0	N1	N2
1.0 cm or less (n = 47)	46	0	1
1.0–1.5 cm (n = 49)	46	1	2
1.5–2.0 cm (n = 63)	55	2	6

when the p value was less than 0.05. All patients were periodically examined and the average length of follow-up was 40 months. The 5-year survival curve was obtained using the Kaplan–Meier method.

Results

A total of 159 patients were studied. The size was classified into three categories: 1 cm or less, 1–1.5 cm, and 1.5–2 cm. There were 47, 49, and 63 patients, respectively. There were 147 pathologic N0 patients and lymph-node metastasis was recognized in 12 patients (7.5%); N1 in 3 patients and N2 in 9 patients. Table 3 lists the rate of lymph-node involvement according to tumor size. Lymph-node involvement was not indicated in 98% of patients who had a tumor size of 1 cm or less, however even in patients with tiny tumors, 2% indicated N2 disease. In patients who had a tumor size of 1 and 1.5 cm, 94% indicated no metastasis but 6% were either N1 or N2. In patients who had a tumor size of 1.5 and 2 cm, lymph-node involvement was recognized in 13%.

In this study the proportion of the size of GGO to that of the tumor was extensively analyzed. We divided patients into two categories according to how much of the lesion consisted of GGO findings. According to these criteria, 44 tumors consisted of greater than 50% of GGO and 115 tumors consisted of less than 50% of GGO. Patients with a GGO ratio of greater than 50% indicated no lymph-node metastases. On the contrary all node-positive patients indicated a GGO ratio of less than 50% (Table 3). The relationship between the proportion of GGO area on HRCT and the Noguchi classification is indicated in Table 4.

Twenty-five out of 44 patients (76%) of types A and B indicated a GGO component of greater than 50% on HRCT. Seventeen out of 71 patients (24%) of type C indicated greater than 50% GGO and the remaining 54 patients (76%) indicated less than 50% GGO. Fifty three out of 55 patients (96%) of types D, E, and F tumors indicated less than 50% GGO. A favorable correlation between CT findings and the Noguchi classification was recognized.

Table 3. GGO Area and T,N Status

GGO%	$T \leqq 1$	$1 < T \leqq 5$	$1.5 < T \leqq 2$	
50 ↑	18	16	10	44
50 ↓	29 (1)[a]	33 (3)[a]	53 (8)[a]	115 (12)[a]

[a] The number in parentheses corresponds to the number of node-positive cases.

GGO = ground-glass opacity.

Table 4. GGO Area and Noguchi Classification

GGO%	A, B	C	D, E, F	
50 ↑	25	17	2	44
50 ↓	8	54	53	115

GGO = ground-glass opacity.

The relationship between representative clinicopathological factors and the proportion of GGO area is indicated in Table 5. According to the χ^2 test, the ratio of GGO area to that of the tumor is related to the tumor size ($p = 0.0135$) and pathologic stage ($p = 0.04$). In particular a significant relationship was obtained regarding the pathologic features including Noguchi classification ($p = 0.0001$), vascular invasion, and lymphatic invasion .

Patients were followed-up in the outpatient clinic and periodically received blood examinations, chest roentgenogram, and chest CT. The median follow-up period for all patients was 40 months. The overall 5-year survival rate of patients studied was 88.0% (Fig 2), but it was 96.7% in patients with tumors less than 1 cm in diameter, 81.6% in patients with tumors between 1 and 1.5 cm, and 84.4% in patients with tumors between 1.5 and 2 cm (Fig 3).

The 5-year survival rate according to how much of the lesion consisted of GGO findings was also analyzed. In patients with tumors greater than 50% GGO, a 100% 5-year survival rate was obtained, but in patients with tumors less than 50% GGO an 83.9% 5-year survival rate was obtained (Fig 4)

The survival rate according to the Noguchi classification is illustrated in Figure 5. A 100% 5-year survival rate was obtained in types A and B, 97.4% in type C, and 67.1% in types D, E, and F, respectively, which was statistically lower than the results of types A, B, and C.

Comment

Because of the increasing widespread application of helical CT, the detected number of small lung peripheral nodules has enormously increased [3–7]. In addition the size of peripheral type adenocarcinomas has been smaller on average when they were detected. This has raised several issues: discerning how to discriminate

Table 5. Relationship Between Prognostic Factors and GGO Ratio on HRCT

Prognostic Factor	χ^2	p Value
Gender	0.162	0.687
Tumor size	8.616	0.0135
p stage		
I or II–IV	4.168	0.0412
Noguchi classification		
A, B, C or DEF	14.442	0.0001
Vascular invasion	6.76	0.0093
Lymphatic invasion	5.326	0.0206

GGO = ground-glass opacity; HRCT = high resolution computed tomography.

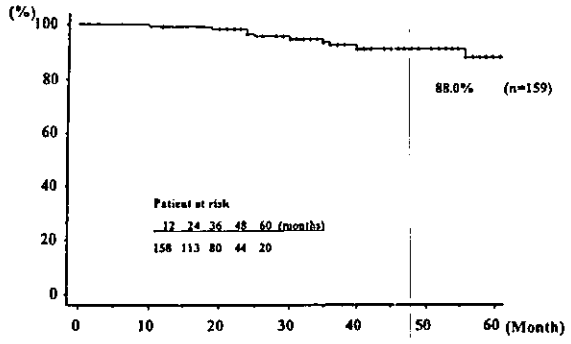

Fig 2. Five-year survival rate of adenocarcinoma less than or equal to 2 cm was 88.0%.

malignant from benign nodules, the usefulness of CT screening in diminishing lung cancer mortality, the optimal intervention in patients who have small nodules, and so on [16, 17]. The management of small cancers is a particular concern of thoracic surgeons, because some of these small cancers might be managed appropriately by limited resection. As previously reported adenocarcinoma tends to metastasize to the regional lymph nodes even if small in size. Nearly 20% of adenocarcinomas less than 2 cm in diameter were reported to be node positive and 5% of adenocarcinomas less than 1 cm were also considered as N1 or N2 disease [18–20]. The Lung Cancer Study Group failed to demonstrate positive results with regard to limited resection for clinical T1 lung cancers. The limited surgery group indicated a local recurrence rate of 5–6 times higher than the lobectomy group [21]. Thus lobectomy and locoregional lymph-node dissection have been recommended as standard lung cancer procedures. However if peripheral early cancer is properly defined, such patients could be managed by lesser resection, which would be useful with regard to decreasing the operative mortality and morbidity as well as enhancing the performance status of the patients.

In our study 12 out of 159 patients (7.5%) exhibited lymph-node metastasis and even tumors measuring 1 cm or less indicated lymph-node metastasis in 2% of patients. The 5-year survival rate did not indicate a statistically significant difference between the three groups

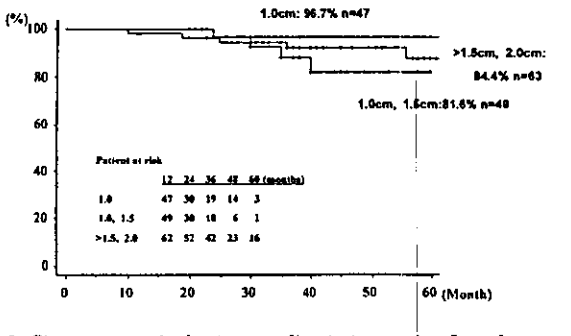

Fig 3. Five-year survival rate according to tumor size. Less than or equal to 1 cm = 96.7%, 1.0–1.5 cm = 81.6%, 1.5–2.0 cm = 84.4%.

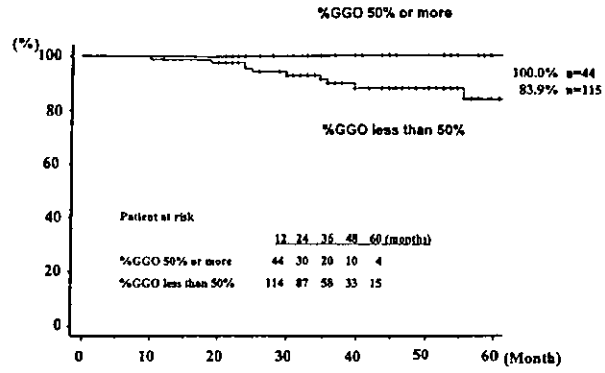

Fig 4. Five-year survival rate according to the proportion of ground-glass opacity (GGO) area. A GGO dominant patient indicated a 100% 5-year survival, whereas patients exhibiting a GGO area less than 50% indicated an 83.9% 5-year survival.

according to tumor size in this study. There are reports that 5%–8% of such tiny adenocarcinomas indicated lymph-node metastasis [18, 22]. Kondo reported 57 adenocarcinomas measuring 1 cm or less, none of which indicated lymph-node metastasis, and 49 revealed BAC without destructive growth that were categorized as nonaggressive tumors [23]. This demonstrates that the indications of limited surgery cannot be determined by size alone. In our study, 47 patients received limited resection. Out of these, mediastinal lymph node or sampling were performed in 20 patients and the rest of 27 patients received wedge resection without nodal dissection. Of these 27 patients stage migration may occur because nodal status was not evaluated pathologically. However these patients indicated pure GGO or overwhelmingly dominant GGO findings on chest CT as well as being clinically node negative. Such patients have been reported to be free from lymph-node metastasis [10, 12–15, 20] and recurrence was not observed in any of these patients by chest CT examination during follow-up.

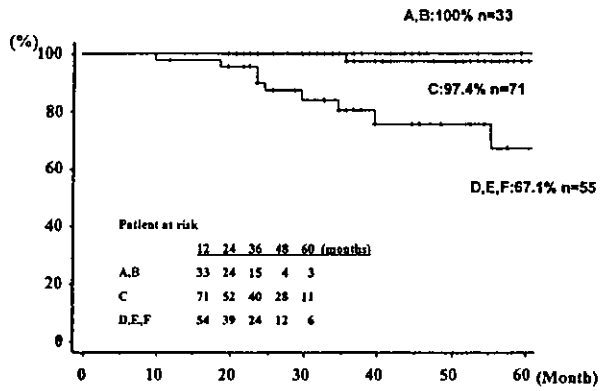

Fig 5. Five-year survival rate according to the Noguchi classification. Noguchi A, B indicated a 100% 5-year survival, type C indicated a 97.4% 5-year survival, and types D, E, and F, indicated a 67.1% 5-year survival, respectively.

Therefore we classified these patients as N0 in this study. Noguchi classified small adenocarcinomas into six categories (types A–F) and this classification indicated a favorable correlation with the biologically aggressive nature of the tumor [2]. Types A and B are localized BAC with or without foci indicating a collapse of alveolar structures that are recognized to be noninvasive. Types D, E, and F are poorly differentiated, tubular, papillary type, respectively, and are invasive. Pathologic analysis revealed that all type A and B patients were N0, however 25%–56% of type D, E, and F patients indicated lymph-node metastasis [2]. Many thoracic surgeons postulated that certain types of adenocarcinomas might be candidates for limited resection and have sought for criteria of "peripheral early cancer." The Noguchi classification is useful with regard to evaluating the aggressive nature in individual patients, but this criteria is based on postoperative pathologic findings and could not have a strong impact on the choice of treatment. Therefore we require criteria that are available preoperatively to define early minimally invasive cancers.

Increased amounts of collagenization or hyalinization microscopically detected in the central fibrotic focus in adenocarcinoma have been reported to influence the prognosis and the smaller the central fibrosis, the more favorable the prognosis [24, 25]. Suzuki reported that central fibrosis in a tumor corresponds to consolidation on HRCT. Thus the ratio of the area of GGO and that of consolidation seems to be strongly related to nodal status and stage [25].

In our study there were 12 N1 or N2 out of 159 patients, in all of whom the proportion of the area of GGO to the entire tumor was less than 50%. All patients with a ratio of GGO greater than 50% survived without recurrence during the follow-up period, although patients with GGO less than 50% indicated an 83.9% 5-year survival rate. The proportion of the GGO area correlates well with the Noguchi classification [26]. There were 33 Noguchi type A and B patients, 25 of which indicated a GGO area of greater than 50% and 8, of which indicated a GGO area of less than 50%. As for type D, E, and F patients, 53 out of 55 indicated a low GGO% and only 2 patients belonged to the high GGO ratio group. A statistically significant correlation was obtained between GGO% and Noguchi classification but types A and B could be completely diagnosed by HRCT findings as they should be the suitable indication of limited surgery. The 5-year survival rate of the high GGO group was 100% and the 5-year survival rate of the low GGO group was 83.9%. Similar results were obtained by Matsuguma who compared the preoperative HRCT findings with pathologic results in 96 patients who underwent surgical resection because of stage Ia cancers [14]. They determined that patients in whom the proportion of GGO to the whole tumor on CT was equal to or greater than 50% exhibited no nodal metastasis or postoperative recurrence. Small cancers with a high GGO ratio might be candidates for limited resection and a large multicenter study is necessary to confirm this postulate.

Limited resection has mostly been performed on patients with poor pulmonary reserve. Intentional limited surgery has not been common, particularly because lobectomy has been considered to be the standard treatment, which was confirmed by a randomized trial of the Lung Cancer Study Group [21]. However some successful results regarding limited surgery for T1 N0 tumors were published by Yamato who proposed limited resection for BAC by employing intraoperative pathological examination to confirm the absence of nodal metastasis [27]. They planned to convert limited resection to lobectomy if some invasive signs were recognized by frozen section. Tsubota performed extended segmentectomy for 55 patients with peripheral cancers measuring less than 2 cm in diameter and only 1 patient locally recurred in whom N2 disease was not indicated during operation [28]. Nakata performed thoracoscopic wedge resection for 33 pure GGO patients with tumors measuring less than 1 cm and no recurrence or metastasis was indicated during the follow-up period [12]. However well-differentiated adenocarcinomas or GGO-dominant tumors are considered to be indolent and slow-growing, therefore a long-term observation period is necessary to evaluate whether limited surgery could be an alternative to lobectomy.

In this study the ratio of GGO and consolidation on chest CT allows for the evaluation of the aggressive nature of small adenocarcinomas. However further investigation is required in this area, especially to characterize GGO on HRCT. Also genomic or proteomic studies are necessary to provide the clues to discriminate tumors with an indolent nature from those with an aggressive nature. Comprehensive research including pathology and molecular analysis will alter the conventional method of management regarding tiny cancers, which will be of great importance in daily practice.

The authors are indebted to Professor James Patrick Barron and Assistant Professor Raoul Breugelmans of the International Medical Communications Center at Tokyo Medical University (Tokyo, Japan) for their review of this manuscript.

References

1. Naruke T, Goya T, Tsuchiya R, et al. Prognosis and survival in resected lung carcinoma based on the new international staging system. J Thorac Cardiovasc Surg 1988;96:440–7.
2. Noguchi M, Morikawa A, Kawasaki M, et al. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. Cancer 1995;75:2844–52.
3. Henschke C, McCauley D, Yakelevitz D, et al. Early Lung Action Project. Overall design and findings from baseline screening. Lancet 1999;354:99–105.
4. Sobue T, Moriyama N, Kaneko M, et al. Screening for lung cancer with low-dose helical computed tomography: anti-lung cancer association project. J Clin Oncol 2002;20:911–20.
5. Sone S, Takashima S, Li F, et al. Mass screening for lung cancer with mobile spiral computed tomography scanner. Lancet 1998;351:1242–5.
6. Kaneko M, Eguchi K, Ohmatsu H, et al. Peripheral lung cancer: screening and detection with low-dose spiral CT versus radiography. Radiology 1996;201:798–802.
7. Nawa T, Nakagawa T, Kusano S, et al. Lung cancer screening using low-dose spiral CT- results of baseline and one-year follow-up studies. Chest 2002;122:15–20.

8. Marcus PM. Lung cancer screening: an update. J Clin Oncol 2001;19(Suppl 18):83S–6.
9. Suzuki K, Asamura H, Kondo H, et al. Clinical predictors of minimally invasive peripheral adenocarcinoma of the lung: possible indications for limited surgical resection. Lung Cancer 2000;29:142.
10. Suzuki K, Asamura H, Kusumoto M, et al. "Early" peripheral lung cancer: Prognostic significance of ground glass opacity on thin-section computed tomographic scan. Ann Thorac Surg 2002;74:1635–9.
11. Higashiyama M, Kodama K, Yokouchi H, et al. Prognostic value of bronchiolo-alveolar carcinoma component of small lung adenocarcinoma. Ann Thorac Surg 1999;68:2069–73.
12. Nakata M, Sawada S, Saeki H, et al. Prospective study of thoracoscopic limited resection for ground-glass opacity selected by computed tomography. Ann Thorac Surg 2003; 75:1601–6.
13. Aoki T, Tomoda Y, Watanabe H, et al. Peripheral lung adenocarcinoma correlation of thin-section CT findings with histologic prognostic factors and survival. Radiology 2001; 220:803–9.
14. Matsuguma H, Yokoi K, Anraku M, et al. Proportion of ground grass opacity on high-resolution computed tomography in clinical T1N0M0 adenocarcinoma of the lung: A predictor of lymph node metastasis. J Thorac Cardiovasc Surg 2002;124:278–84.
15. Takashima S, Li F, Maruyama Y, et al. Discrimination of subtypes of small adenocarcinoma in the lung with thin-section CT. Lung Cancer 2002;36:175–82.
16. Warner E, Mulshine J. Surgical considerations with lung cancer screening. J Surg Oncol 2003;84:1–6.
17. Rusch V. High-resolution computed tomography in clinical T1N0M0 adenocarcinoma of the lung. J Thorac Cardiovasc Surg 2002;124:221–2.
18. Asamura H, Nakayama H, Kondo H, et al. Lymph node involvement, recurrence, and prognosis in resected small, peripheral, non-small-cell lung carcinomas: are these carcinomas candidates for video-assisted lobectomy? J Thorac Cardiovasc Surg 1996;111:1125–34.
19. Konaka C, Ikeda N, Hiyoshi T, et al. Peripheral non-small cell lung cancers 2.0 cm or less in diameter: proposed criteria for limited pulmonary resection based upon clinicopathological presentation. Lung Cancer 1998;21:185–91.
20. Suzuki K, Nagai K, Yoshida J, et al. Predictors of lymph node and intrapulmonary metastasis in clinical stage IA non-small cell lung carcinoma. Ann Thorac Surg 2001;72:352–6.
21. Lung Cancer Study Group. Randomized Trial of lobectomy versus limited resection for T1N0 non-small cell lung cancer. Ann Thorac Surg 1995;60:615–23.
22. Yoshida J, Nagai K, Yokose T, et al. Primary peripheral lung carcinoma smaller than 1 cm in diameter. Chest 1998;114: 710–2.
23. Kondo D, Yamada K, Kitayama Y, et al. Peripheral lung adenocarcinomas. 10 mm or less in diameter. Ann Thorac Surg 2003;76:350–5.
24. Shimosato Y, Suzuki A, Hashimoto T, et al. Prognostic implications of fibrotic focus (scar) in small peripheral lung cancers. Am J Surg Pathol 1980;4:365–73.
25. Suzuki K, Yokose T, Yoshida J, et al. Prognostic significance of size of central fibrosis in peripheral adenocarcinoma of the lung. Ann Thorac Surg 2000;69:893–7.
26. Ikeda N, Tsuboi M, Hiyoshi T, et al. A clinicopathological study of resected adenocarcinoma less than 2 cm in size. Lung Cancer 2003;41:S51.
27. Yamato Y, Tsuchida M, Watanabe T, et al. Early results of a prospective study of limited resection for bronchioloalveolar adenocarcinoma of the lung. Ann Thorac Surg 2001;71:971–4.
28. Tsubota N, Ayabe K, Doi O, et al. Ongoing prospective study of segmentectomy for small lung tumors. Ann Thorac Surg 1998;66:1787–90.

Genome-wide cDNA microarray screening to correlate gene expression profile with survival in patients with advanced lung cancer

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Received November 24, 2003; Accepted January 22, 2004

Abstract. We conducted a study using cDNA microarray analysis to determine whether expression levels of genes in tumors were correlated with survival after chemotherapy. Between September 2000 and December 2001, 47 patients were registered in the study. Eighteen patients had small cell lung cancer (SCLC), and the others had non-small cell lung cancer (NSCLC). All patients except three received platinum-based chemotherapy. Transbroncheal biopsy specimens of tumors were obtained before chemotherapy. The expression levels of 1176 genes in tumor specimens were analyzed using the Atlas™ Human Cancer 1.2 Array. The expression levels of three genes, G1/S-specific cyclin, type II cGMP-dependent protein kinase and hepatocyte growth factor-like protein, were significantly correlated with survival ($p<0.01$). Ten of the 47 patients who showed an elevated expression level of one or more of the three genes had a significantly increased chance of survival ($p=0.0056$). In conclusion, some survival-related genes were detected in the tumor tissue of lung cancer patients using cDNA microarray analysis. A prospective study is required to confirm whether expression levels of these genes can be used for prognosis.

Introduction

Lung cancer is a leading cause of cancer death and most patients with this disease are candidates for chemotherapy. To improve the prognosis of lung cancer patients, attempts have been made to develop treatment of lung cancer and thereby decrease the mortality from this disease. To develop new therapeutic strategies for lung cancer we require a better understanding of the cell biology of this disease. Although a number of clinicopathological characteristics may affect the prognosis of lung cancer, these characteristics have not yet been defined. Several molecular markers have been evaluated in association with established histological and clinical prognostic parameters of non-small cell lung cancer (NSCLC) (1-5), although the intrinsic nature of gene dysregulation that leads small tumors to metastasize remains unclear. It is suspected that tumor invasion and metastasis involve complex alterations of gene expression that may be selective for specific cancer types.

We identified that survivin and cyclin D1 are indicators of poor prognosis in small adenocarcinoma of the lung (6,7). Moreover, other factors have also been reported to be prognostic factors in resected NSCLC, including cyclin E (1), FHIT (2), VEGF (3), cadherin (4) and RAR-ß (5). These factors have different functions in tumors, such as tumor suppression, angiogenesis, apoptosis, adhesion and cell differentiation. Clarification of the many genetic abnormalities that influence tumor progression in NSCLC is clearly required when considering new therapeutic strategies for resectable NSCLC.

The cDNA microarray method is now widely used to analyze the expression of thousands of genes simultaneously in cancer tissues, and its development has facilitated the analysis of genome-wide expression profiles that can generate a large body of information concerning genetic networks related to pathological conditions. Large-scale gene expression microarray studies of lung cancer have shown that expression patterns of various genes is associated with pathological characteristics (8,9). In other studies, different sets of genes were identified which may act as predictive markers for chemosensitivity to drugs in human cancer cell lines or tumor tissues using cDNA microarray (10-12).

In the present study, we used cDNA microarray screening to examine the expression levels of specific genes in tumor tissue obtained by transbroncheal biopsy, in order to determine any correlations with survival after chemotherapy.

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Key words: microarray, lung cancer

Patients and methods

Patients. This study was approved by the Institutional Review Boards of Kanagawa Cancer Center. The patients with histologically proven lung cancer treated with chemotherapy were entered into the present study. All were eligible for treatment. They had an expected survival of at least six weeks; measurable lesions; Eastern Cooperative Oncology Group performance status (PS) score ≤3; white blood count ≥4000/μl; hemoglobin ≥10 g/dl; platelet count ≥100000/μl; total serum bilirubin <2 mg/dl; aspartate aminotransferase and alanine aminotransferase less than twice the upper limit of the normal range; serum creatinine ≤1.5 mg/dl; and creatinine clearance >50 ml/min. None of the patients had received prior chemotherapy for the primary lesion. Written informed consent for chemotherapy and a genetic analysis of tumor tissue was obtained in every case. All patients with non-progressive cancer were treated with two or more courses of chemotherapy.

Tumor samples. Transbroncheal biopsy specimens of tumors were obtained before chemotherapy. One half of the specimens were fixed in formalin for pathological diagnosis and the other half were immediately frozen for storage at -80°C until genetic analysis.

Extraction and purification of RNA and preparation of probes. The total RNA of each sample was isolated and treated with DNaseI to avoid contamination of genomic DNA by silica membrane affinity chromatography using Macherey-Nagel's total RNA isolation kit (Macherey-Nagel GmbH and Co., KG, Germany). Total RNA (100 nanograms) for each sample was reverse transcribed into cDNA and amplified by SMART polymerase chain reaction (PCR) technology (18) using the Super SMART™ PCR cDNA Synthesis kit (BD Biosciences Clontech, CA, USA) according to the manufacturer's instructions. To represent the expression profile of the initial total RNA material, the optimal conditions for PCR cycling were determined for each sample by testing the amplified cDNA with gel electrophoresis. All samples were amplified for 19 to 23 cycles. Each cDNA sample was subjected to microarray expression profiling using the BD Atlas™ Human Cancer 1.2 Array (Clontech) based on the manufacturer's protocol. The following is a brief overview of the procedures used. A radioactively labeled probe mixture for hybridization with array membranes was synthesized from each cDNA sample using the CDS Primer Mix specific for the Atlas™ Human Cancer 1.2 Array and [α-^{32}P]-dATP.

cDNA microarray. Each labeled probe was hybridized into a separate Atlas Array. After appropriate washing, array membranes were exposed to a phosphor screen and the signal intensity for each spot, which corresponds to each gene examined, was determined using a STORM image analyzer (Amersham Bioscience, Picataway, NJ). The hybridization pattern and signal intensity were analyzed to determine changes in gene expression levels using AtlasImage™ 2.01 software (Clontech, Laboratory, Inc., Japan).

Table I. Patient characteristics.

	No. of patients
Total	47
Gender	
Male	36
Female	11
Smoker	38
PS (ECOG)	
0	5
1	30
2	9
3	3
Pathology	
SCLC	
Stage	
LD	2
ED	16
NSCLC	
Stage	
IIB/IIIA	4
IIIB	8
IV	17

PS, performance status; ECOG, Eastern Cooperative Oncology Group; SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; LD, limited disease; ED, extensive disease.

Statistical methods. To determine whether gene-expression profiles were associated with variety in cases of survival, Kaplan-Meier survival plots and log-rank tests were used. $p<0.01$ was considered statistically significant.

Results

Between September 2000 and December 2001, 47 patients were registered in the study. Patient characteristics are summarized in Table I. Thirty-six patients were male and eleven were female, with a median age of 66 years (range 35-81 years). Thirty-eight patients were smokers. The PS was 0 for five patients; 1 for 30 patients; 2 for nine and 3 for three patients. Eighteen patients had small cell lung cancer (SCLC), and the remaining had NSCLC. Of the patients with SCLC, two had limited disease and the other 16 had extensive SCLC. Of the patients with NSCLC, four had stage IIB/IIIA, eight had stage IIIB, and 17 had stage IV. None of the patients had received prior chemotherapy.

All patients except three who had been subscribed paclitaxel and irinotecan were given platinum-based chemotherapy. Three patients with SCLC and seven patients with NSCLC received thoracic radiotherapy concurrently or sequentially with chemotherapy (Table II). Sixteen of the 18 patients with SCLC (89%) and 12 of the 29 patients with NSCLC (41%) responded to chemotherapy, respectively. Eight out of the total 47 patients were alive at analysis.

Table II. Therapeutic regimens.

	No. of patients
SCLC	
Cisplatin + etoposide	6
Cisplatin + etoposide + TRT	2
Cisplatin + irinotecan	4
Cisplatin + irinotecan + etoposide	2
Carboplatin + etoposide	3
Cisplatin + TRT	1
NSCLC	
Cisplatin + gemcitabine	7
Cisplatin + vinorelbine	3
Cisplatin + vinorelbine + TRT	2
Cisplatin + vindesine + TRT	3
Cisplatin + irinotecan	1
Cisplatin +TRT	2
Carboplatin + etoposide	1
Carboplatin + paclitaxel	1
Nedaplatin + irinotecan	6
Paclitaxel + irinotecan	3

SCLC, small cell lung cancer; NSCLC, non-small cell lung cancer; TRT, thoracic radiation therapy.

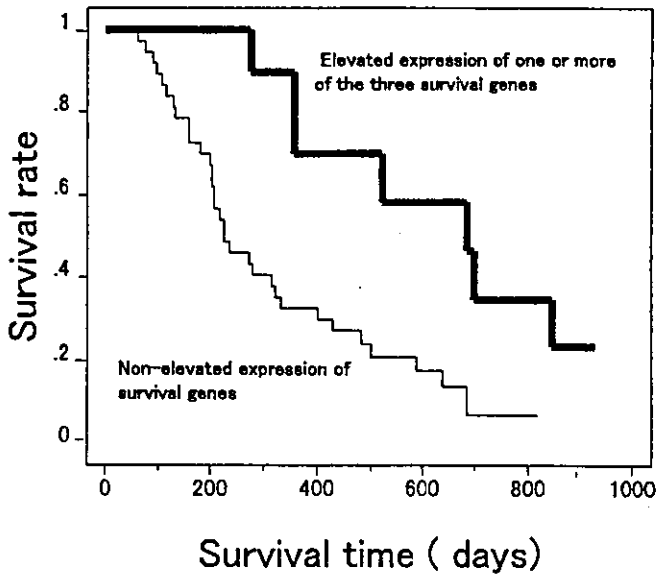

Figure 1. Survival curves constructed using the Kaplan-Meier method. Ten of 47 patients who showed an elevated expression of one or more of the three survival genes compared to the mean expression level of control genes had a significantly better chance of survival (log-rank, p=0.0056).

The expression levels of 1176 genes in the tumor specimens were analyzed using cDNA microarray screening. Four housekeeping genes which were expressed in all 47 tumor samples in the present study were used as controls for gene expression: ubiquitin, liver glyceraldehydes 3-phosphate dehydrogenase, 23-kDa highly basic protein, 60S ribosomal protein L13A and 40S ribosomal protein S9.

When we analyzed the relationship between gene expression level and survival, three genes, G1/S-specific cyclin, type II cGMP-dependent protein kinase and hepatocyte growth factor-like protein, were significantly correlated (Table III, log-rank test, $p<0.01$). Ten of 47 patients who showed an elevated expression of one or more of the three survival genes compared to the mean expression level of control genes had a significantly better chance of survival (Fig. 1, log-rank; p=0.0056).

Discussion

We examined cancer-related gene expressions in lung cancer samples obtained before chemotherapy using cDNA microarray screening, and analyzed the relationship between gene expression levels and survival after chemotherapy. We identified three genes whose expression could be used to predict the survival outcomes of patients in the present study. These genes were involved in cell cycling, adhesion and invasion. The families of G1-cyclins such as cyclins D and E, and their dependent kinases, control the transition through the restriction point of the middle and late G1 cells during cell cycles. A previous examination of gastric cancers revealed that positivity of cyclin D2 and negativity for p27 in the tumor tissue were independent of prognostic factors (13).

For cancer to metastasize, tumor cells present in the circulation must first adhere to the endothelium. An

Table III. Genes closely associated with patient survival.

Description	Symbol	p-value
G1/S-specific cyclin D2 (CCND2) + KIAK0002	M90813 + D13639	0.0055
Type II cGMP-dependent protein kinase	X94612	0.0016
Hepatocyte growth factor-like protein (HGF activator-like protein); hyaluronan-binding protein (PHBP)	D49742, S83182	0.0075

investigation of the mechanism of adhesion and trans-endothelial migration of cancer cells showed that stimulation of cancer cells by CD44 cross-linking or fragmented hyaluronan markedly induces the expression of lymphocyte function-associated antigen (LFA)-1; that stimulation of CD44 also induces expression of the hepatocyte growth factor (HGF) receptor c-Met on cancer cells; and that HGF further amplifies the LFA-1-mediated adhesion of cells (14). Another study demonstrated that HGF/SF-Met binding up-regulated the expression of CD44v6 in murine melanoma cells (15). These data support the hypothesis that HGF influences the outcome of patient survival.

Tumor hypoxia is associated with a poor prognosis for patients with various cancers, often resulting in an increased metastasis. A study demonstrated that culturing tumor cells under hypoxic conditions results in lower cyclic GMP levels. The study revealed that an important mechanism by which hypoxia increases tumor cell invasiveness requires inhibition of the nitric oxide signaling pathway involving protein kinase G activation (16). Moreover, in another study, a potent inhibitor of cyclic GMP-dependent protein kinase displayed cytostatic activity against *Toxoplasma gondii in vitro* (17). These data may support the hypothesis that the three survival genes identified in this study do influence the outcome of patient survival.

In this report we have discussed the mechanisms related to tumor cell survival with regard to three genes implicated in patient survival outcomes. We need to undertake prospective evaluations to determine whether the selected genes in this study are truly important and potentially useful for predicting patient survival. It is also necessary to determine whether administration of drugs will result in changes to the expression levels of the survival genes we identified, and if any such changes are related to survival. If the expression level of a gene changes with treatment, that gene will be the new target of cancer chemotherapy. In this study we measured the expression levels of genes in patients treated with platinum-based chemotherapy. Recently, patients with NSCLC have been treated with non-platinum chemotherapy. It is thus also necessary that the expression levels of our survival genes can be used to predict clinical outcome with non-platinum chemo-therapy. Accumulation of these data could eventually lead to the prescription of 'personalized chemotherapy' with effective anticancer drugs.

Acknowledgements

This work was supported in part by Kanagawa Health Foundation.

References

1. Fukuse T, Hirata T, Naiki H, *et al*: Prognostic significance of cyclin E overexpression in resected non-small cell lung cancer. Cancer Res 60: 242-244, 2000.
2. Tomizawa Y, Nakajima T, Kohno T, *et al*: Clinicopathological significance of Fhit protein expression in stage I non-small cell lung carcinoma. Cancer Res 58: 5478-5483, 1998.
3. Ohta Y, Tomita Y, Oda M, *et al*: Tumor angiogenesis and recurrence in stage I non-small cell lung cancer. Ann Thorac Surg 68: 1034-1038, 1999.
4. Herbst RS, Yano S, Kuniyasu H, *et al*: Differential expression of E-cadherin and type IV collagenase genes predicts outcome in patients with stage I non-small cell lung carcinoma. Clin Cancer Res 6: 790-797, 2000.
5. Khuri FR, Lotan R, Kemp BL, *et al*: Retinoic acid receptor-ß as a prognostic indicator in stage I non-small-cell lung cancer. J Clin Oncol 18: 2798-2804, 2000.
6. Ikehara M, Oshita F, Kameda Y, *et al*: Expression of survivin corelated with vessel invasion is a poor prognostic factor in small adenocarcinoma of the lung. Oncol Rep 9: 835-838, 2002.
7. Ikehara M, Oshita F, Ito H, *et al*: Expression of cyclin D1 but not of cyclin E is indicator of poor prognosis in small adeno-carcinoma of the lung. Oncol Rep 10: 137-139, 2003.
8. Bhattacharjee A, Richards WG, Staunton J, *et al*: Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. Proc Natl Acad Sci USA 98: 13790-13795, 2001.
9. Garber ME, Troyanskaya OG, Schluens K, *et al*: Diversity of gene expression in adenocarcinoma of the lung. Proc Natl Acad Sci USA 98: 13784-13789, 2001.
10. Dan S, Tsunoda T, Kitahara O, *et al*: An integrated database of chemosensitivity to 55 anticancer drugs and gene expression profiles of 39 human cancer cell lines. Cancer Res 62: 1139-1147, 2002.
11. Zembutsu H, Ohnishi Y, Tsunoda T, *et al*: Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. Cancer Res 62: 518-527, 2002.
12. Kikuchi T, Daigo Y, Katagiri T, *et al*: Expression profiles of non-small cell lung cancers on cDNA microarrays: identification of genes for prediction of lymph-node metastasis and sensitivity to anti-cancer drugs. Oncogene 22: 2192-2205, 2003.
13. Takano Y, Kato Y, van Diest PJ, *et al*: Cyclin D2 overexpression and lack of p27 correlate positively and cyclin E inversely with a poor prognosis in gastric cancer cases. Am J Pathol 156: 585-594, 2000.
14. Fujisaki T, Tanaka Y, Fujii K, *et al*: CD44 stimulation induces integrin-mediated adhesion of colon cancer cell lines to endothelial cells by up-regulation of integrins and c-Met and activation of integrins. Cancer Res 59: 4427-4434, 1999.
15. Recio JA and Merlino G: Hepatocyte growth factor/scatter factor induces feedback up-regulation of CD44v6 in melanoma cells through Egr-1. Cancer Res 63: 1576-1582, 2003.
16. Postovit LM, Adams MA, Lash GE, *et al*: Oxygen-mediated regulation of tumor cell invasiveness. Involvement of a nitric oxide signaling pathway. J Biol Chem 277: 35730-35737, 2002.
17. Nare B, Allocco JJ, Liberator PA, *et al*: Evaluation of a cyclic GMP-dependent protein kinase inhibitor in treatment of murine toxoplasmosis: gamma interferon is required for efficacy. Antimicrob Agents Chemother 46: 300-307, 2002.
18. Chenchik A, Zhu YY, Diatchenko L, Li R, Hill J, Siebert PD: Generation and use of high-quality cDNA from small amounts of total RNA by SMART PCR. In: Gene cloning and analysis by RT-PCR. Siebert PD and Larrick JW (eds). Bio Techniques Book, MA, USA, pp305-319, 1998.

Journal of Experimental Therapeutics and Oncology, Vol. 4, pp. 79-83
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SHORT COMMUNICATION

Phase II study of OK-432 intrapleural administration followed by systemic cisplatin and gemcitabine for non-small cell lung cancer with pleuritis carcinomatosa*

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(Received November 17, 2003; accepted December 28, 2003)

We conducted a phase II study of OK-432 intrapleural administration followed by systemic chemotherapy using cisplatin with gemcitabine to determine their combined effects on non-small cell lung cancer (NSCLC) with pleuritis carcinomatosa. Between December 1999 and October 2001, 15 patients were registered in the study. Fourteen patients had an Eastern Cooperative Oncology Group performance status (PS) of 1, and one patient had a PS of 2. Ten patients had adenocarcinoma, one had squamous cell carcinoma, and four had malignant mesothelioma. Patients underwent thoracocentesis and received an OK-432 intrapleural injection. They were then treated every three weeks with chemotherapy consisting of 80 mg/m2 cisplatin on day 1 and 1000 mg/m2 gemcitabine on days 1 and 8. Thirteen patients received two or more courses of chemotherapy. Grade 3 or 4 neutropenia, anemia and thrombocytopenia occurred in five, two and three patients, respectively. Non-hematological toxicities were mild, except for one patient who experienced a grade 3 elevation of transaminase and two patients who experienced grade 3 nausea. Of the 15 patients, one achieved partial response (PR), 13 a stable disease (SD) rating, and one a progressive disease (PD) rating, and the overall response rate was 6.7%. The median survival time was 13.5 months and the one-year survival rate was 60.0%. In conclusion, OK-432 intrapleural administration followed by cisplatin and gemcitabine systemic chemotherapy did not reduce patients' tumors but did prolong their survival time. A large-scale phase II study of the efficacy of this combination therapy is required.

* This work was supported in part by Kanagawa Health Foundation.

INTRODUCTION

Non-small cell lung cancer (NSCLC) is the leading cause of cancer death in Japan. To improve the prognosis of lung cancer patients, attempts have been made to develop tests that will facilitate the early diagnosis and treatment of lung cancer and thereby decrease the mortality from this disease. Pleuritis carcinomatosa is one type of advanced stage NSCLC, and shows poor prognosis due to micrometastatic lesions and respiratory failure by massive pleural effusion. Standard therapy for NSCLC with pleuritis carcinomatosa consists of drainage of pleural effusion followed by intrapleural administration of sclerosing agents. Until recently, there has been controversy regarding which agent was most effective for treatment of sclerosing pleural lesions. A randomized phase II study has been conducted to compare three regimens for intrapleural treatment in patients with pleuritis

carcinomatosa of NSCLC (1). The study suggested that intrapleural OK-432 administration was more effective for the management of malignant effusion compared to intrapleural administration of bleomycin or cisplatin plus etoposide.

Systemic chemotherapy is usually performed after sclerosing modality treatment for patients. In the past decade, a number of new anti-cancer agents have been approved for the treatment of advanced NSCLC, including vinorelbine, gemcitabine, docetaxel and paclitaxel. Regimens based on the combination of these drugs with platinum compounds have presented interesting new possibilities for treatment of patients with NSCLC. Randomized studies comparing these platinum-based combinations with single-agent treatment have demonstrated a small but significant survival benefit with the combination treatments (2, 3). The treatment for NSCLC with pleuritis carcinomatosa, usually performed in accordance with the chemotherapy regime for metastatic NSCLC, is controversial. A phase II study of cisplatin and gemcitabine combination chemotherapy, one of the standard therapies for metastatic NSCLC, has been conducted to determine its effects on malignant mesothelioma (4). The study reported 10 responders out of the 21 patients treated, and a median survival time of 41 weeks, suggesting an efficacy of cisplatin and gemcitabine for treating malignant pleural lesions of NSCLC.

With reference to these data, we conducted a phase II study to determine the efficacy of intrapleural administration of OK-432 followed by cisplatin and gemcitabine systemic chemotherapy for the treatment of NSCLC with pleuritis carcinomatosa. For this study, we used a gemcitabine and cisplatin regimen with a 21-day schedule. In previous phase II studies, based on a 28-day cycle, gemcitabine was given at a dose of 1000 mg/m^2 on days 1, 8 and 15 (5, 6). However, the number of omissions and reductions of the day-15 gemcitabine dose was quite high. As a previous study has shown that cisplatin and gemcitabine treatment on a 21-day cycle has a high-dose intensity with high activity (7), we chose a 21-day cycle of this combination chemotherapy for the present study. This study allowed the entry of patients with malignant mesothelioma.

PATIENTS AND METHODS

Patients

Patients with histologically or cytologically diagnosed NSCLC with pleuritis carcinomatosa or malignant mesothelioma were registered for intrapleural therapy using OK-432 followed by cisplatin and gemcitabine systemic chemotherapy. The eligibility criteria were: expected survival time ≥6 weeks, age ≦ 75 years, Eastern Cooperative Oncology Group performance status (PS) score ≤1, leukocyte count ≥4,000/μl, hemoglobin count ≥10 g/dl, platelet count ≥100,000/μl, total serum bilirubin ≤1.5 mg/dl, aspartate aminotransferase and alanine aminotransferase ≤90 IU/L, serum creatinine ≤1.5 mg/dL, and creatinine clearance ≥60 ml/min. Patients who had already received radiotherapy to their metastatic sites were not eligible for the present study. Written informed consent was obtained from every patient.

Treatment

Patients underwent thoracocentesis, and a 19-Fr chest drainage tube was kept in place until the drained volume of pleural effusion was less than 100 ml/day. Then, a 5–10 Klinische Einheit unit of OK-432 diluted by 100 ml saline was administered into the pleural cavity. The chest tube was clamped for 1–3 hours and then released for drainage. When the drained effusion volume was less than 100 ml/day, the chest tube was removed. Following intrapleural therapy, patients were treated every three weeks with two or more courses of systemic chemotherapy consisting of 80 mg/m^2 cisplatin on day 1 and 1000 mg/m^2 gemcitabine on days 1 and 8. Subsequent courses of chemotherapy were started when the leukocyte count was ≧ 4000 /μL, with a platelet count ≧ 100,000 /μL. The dose of gemcitabine was reduced to 800 mg/m^2 for the subsequent course if the patient experienced grade 4 thrombocytopenia, or grade 4 neutropenia lasting four days. Physical examination, complete blood cell counts, biochemical tests, and chest roentgenograms were obtained weekly. Tumor responses were evaluated according to the Response Evaluation Criteria for Solid Tumors (8). Toxicities were evaluated according to the National Cancer Institute Common Toxicity Criteria (NCI-CTC Version 2.0).

Fifteen patients were treated in the first stage. We decided to stop the study if less than three of the 15 patients responded at this stage. If four or more patients responded, a total of 26 patients would be required. This regimen was defined as active if the number of responders was ≧ 10 and inactive if the number of responders was ≦ 9 (Simon minimax two stage; α <0.05 and β< 0.10) (9, 10). This plan allowed early termination of the study as soon as possible should it become evident that the true rate of response was less than 25% or greater than 45%. Overall survival time was estimated using the method devised by Kaplan and Meier. The Review Board of the Kanagawa Cancer Center reviewed and approved the protocol prior to commencement of the trial.

Table 1. Patient characteristic.

			No. of patients
Total			15
Age, years	Median		62
	Range		29 - 74
Gender	Male		10
	Female		5
Performance status (ECOG)		1	14
		2	1
Histology	Adenocarcinoma		10
	Squamous cell carcinoma		1
	Mesothelioma		4
No. ofmetastatic sites		0	10
		1	5

RESULTS

Between December 1999 and October 2001, 15 patients were registered in the study. Patient characteristics are summarized in Table 1. Ten patients were male and five were female, with a median age of 62 years (ranging from 29 to 74). Fourteen patients had a PS of 1 and one patient had a PS of 2. Ten patients had adenocarcinoma, one had squamous cell carcinoma, and four had malignant mesothelioma. No patients had received prior treatment, including any radiotherapy for metastatic lesions. All fifteen patients were assessed for their response and for toxicities. Thirteen patients received two or more courses of chemotherapy. Two patients were not given a second course of chemotherapy, one because of PD, and another because of no improvement from a depressed PS 3.

Patients' hematologic and non-hematologic toxicities are summarized in Table 2. Grade 3 or 4 neutropenia, anemia and thrombocytopenia occurred in five (33%,), two (13%) and three (20%) patients, respectively. Non-hematological toxicities were mild, except in one patient, who experienced a grade 3 elevation of transaminase, and in two patients who experienced grade 3 nausea.

The outcome of chemotherapy in 15 patients with measurable lesions is shown in Table 3. One patient achieved a PR, 13 an SD, and one a PD, and the overall response rate was 6.7%. As only one patient responded, no further patients were registered for the first stage. The overall survival curve is shown in Figure 1. The median potential follow-up time was 18.5 months (range, 10.1–34.4), and the median time to progression (MTP) was 3.7 months (range, 1.9–11.2). Four patients were still alive and the other 11 patients died during the follow-up period. The median survival time (MST) was 13.5 months and the one-year survival rate was 60.0%.

Table 2. Toxicities

	No. of patients with Toxicity NCI-CTC ver.2 grade				
Toxicity	0	1	2	3	4
Hemoblobin	0	8	5	2	0
Leukocytes	2	2	8	3	0
Neutrophils	2	2	6	3	2
Platelets	4	5	3	3	0
Bilirubin	14	0	1	0	0
AST	8	5	1	1	0
ALT	7	5	2	1	0
Creatinine	14	1	0	0	0
Nausea	4	5	4	2	0
Vomiting	9	0	5	1	0
Phlebitis	14	0	1	0	0
Headache	14	1	0	0	0
Weight loss	12	3	0	0	0
Stomototis	14	1	0	0	0

Table 3. Chemotherapeutic response.

Responce	Number of Patients
PR	1
NC	13
PD	1

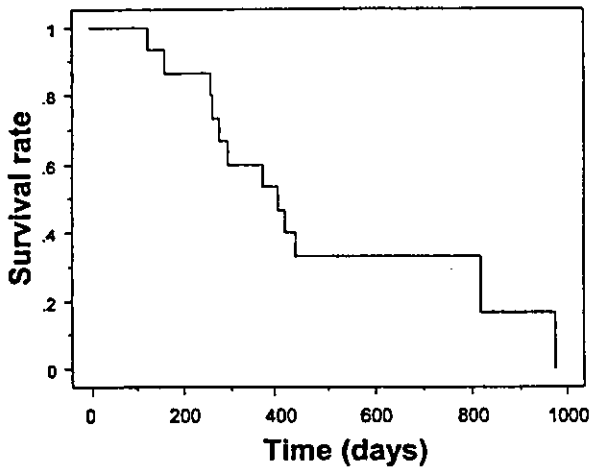

Figure 1. Kaplan-Meier estimation of overall survival of 15 patients with NSCLC with pleuritis carcinomatosa treated with cisplatin plus gemcitabine.

DISCUSSION

An effective treatment for NSCLC with pleuritis carcinomatosa has not been established. Sufferers usually experience massive pleural effusion and require pleurodesis before systemic chemotherapy. A randomized study conducted by the JCOG demonstrated the efficiency of intrapleural injection of OK-432 compared to bleomycin or cisplatin plus etoposide (1). Thirty-four patients in the study who received intrapleural treatment of OK-432 showed 28 weeks of median pleural progression-free survival and 48 weeks of MST. As these survival data were promising compared to those obtained with other treatments, we selected OK-432 administration for treating sclerosing pleural lesions in our study. Use of systemic chemotherapy after pleurodesis is also controversial, and the patients with pleuritis carcinomatosa are usually treated in accordance with the chemotherapy regimen for metastatic NSCLC. A large-scale, phase III study demonstrated an equal efficiency of cisplatin plus gemcitabine compared to cisplatin plus docetaxel, cisplatin plus paclitaxel, or carboplatin plus paclitaxel (11). We selected cisplatin plus gemcitabine for the treatment of pleuritis carcinomatosa after pleurodesis because the regimen was effective for malignant mesothelioma (4). Both pleuritis carcinomatosa and mesothelioma are pleural lesions, and the effective treatment for malignant mesothelioma is considered to also be effective for pleuritis carcinomatosa. It is also expected that cisplatin and gemcitabine shift to the thoracic cavity.

Unfortunately, only one patient responded to the combination of cisplatin and gemcitabine, so we terminated our study in the first stage. However, it should be noted that nine of the fifteen patients (60%) who entered our study survived over one year. While a combination of cisplatin and gemcitabine is one of the standard chemotherapies for advanced NSCLC, previous researchers have reported an MST of less than one year (5, 6, 11). Whether a measurable response is a good substitute for an increased survival time in the treatment of advanced cancer is still a matter of controversy (12). The survival time data in our study could not be confirmed as an outcome of treatment for pleuritis carcinomatosa because of the small number of patients analyzed, however it may suggest this combined therapy has potential for treatment of pleuritis carcinomatosa. Cisplatin and gemcitabine treatment induced a response rate similar to that of other standard chemotherapies in a randomized study against advanced NSCLC (11). The data showed that cisplatin and gemcitabine had a cytotoxic but not a cytostatic effect. The MTP was 3.7 months in our study, which is similar to other active regimens (11) and is considered to be long in spite of the poor response rate. The MTP is a measure of the quality of response, taking into account both objective response and stable disease qualifications. The reason why a good survival time was obtained in our study could not be explained; a tumor-stabilizing effect was certainly achieved with the treatment.

The JCOG study demonstrated that intrapleural sclerosing modality treatment using OK-432 is promising compared to intrapleural injection of anti-cancer agents such as bleomycin or cisplatin plus etoposide (1). OK-432 is not a cytotoxic agent and is used to achieve asclerosing effect for pleuritis carcinomatosa in Japan. The non-shrinking agent is more effective than cytotoxic agents for prolonging the survival of patients with lung cancer and pleuritis carcinomatosa, suggesting that stabilization of pleural lesions is most important for treatment of pleuritis carcinomatosa. OK-432 intrapleural administration followed by cisplatin and gemcitabine systemic chemotherapy did not reduce the tumor size in this study, but only one patient experienced tumor progression during the treatment. Chemotherapy regimens with a poor response rate usually have a 20–30% progression response and, therefore, the treatment used in this study may have the potential to stabilize pleural lesions and prolong survival.

We terminated this study in the first stage because of the poor response rate. In order to confirm the efficacy of OK-432 intrapleural administration followed by systemic chemotherapy with cisplatin and gemcitabine against pleuritis carcinomatosa, a large trial with survival time as the primary end-point is required.

REFERENCES

1. Yoshida K, Sugiura T, Takifuji S, Kawahara M, Matsui K, Kudoh S, Fukuoka M, Takada M, Ariyoshi Y, Ishizuka N, Saijo N. Randomized phase II trial of three intrapleural therapy regimens in non-small cell lung cancer (NSCLC) with malignant pleural effusion (MPE): Japan Clinical Oncology Group Study (JCOG9515). *Proc Am Soc Clin Oncol* 20:339a, 2001.

2. Sandler AB, Nemunaitis J, Denham C, von Pawel J, Cormier Y, Gatzemeier U, Mattson K, Manegold C, Palmer MC, Gregor A, Nguyen B, Niyikiza C, Einhorn LH. Phase III Trial of Gemcitabine Plus Cisplatin Versus Cisplatin Alone in Patients With Locally Advanced or Metastatic Non-Small-Cell Lung Cancer. *J Clin Oncol* 18 :122-130, 2000.

3. Wozniak AJ, Crowley JJ, Balcerzak SP, Weiss GR, Spiridonidis CH, Baker LH, Albain KS, Kelly K, Taylor SA, Gandara DR, Livingston RB. 122 Randomized trial comparing cisplatin with cisplatin plus vinorelbine in the treatment of advanced non-small-cell lung cancer: A Southwest Oncology Group study. *J Clin Oncol* 16:2459-2465, 1998.

4. Byrne MJ, Davidson JA, Musk AW, Dewar J, van Hazel G, Buck M, de Klerk NH, Robinson BW. Cisplatin and Gemcitabine

Treatment for Malignant Methothelioma : A Phase II Study. *J Clin Oncol* 17: 25-30, 1999.

5. Abratt RP, Bezwoda WR, Goedhals L, Hacking DJ. Weekly gemcitabine with monthly cisplatin: Effective chemotherapy for advanced non-small cell lung cancer. *J Clin Oncol* 15:744-749, 1997.

6. Crino L, Scagliotti G, Marangolo M, Figoli F, Clerici M, De Marinis F, Salvati F, Cruciani G, Dogliotti L, Pucci F, Paccagnella A, Adamo V, Altavilla G, Incoronato P, Trippetti M, Mosconi AM, Santucci A, Sorbolini S, Oliva C, Tonato M. Cisplatin-gemcitabine combination in advanced non-small cell lung cancer: A phase II study. J Clin Oncol 15:297-303, 1997.

7. Kunikane H, Kurita Y, Watanabe K, Yokoyama A, Noda K, Fujita Y, Yoneda S, Nakai Y, Niitani H. A study of the comboination of gemcitabine hydrochloride (LY188011) and cisplatin in non-small-cell lung cancer: 3-week schedule. *Int J Clin Oncol* 6:284-290, 2001.

8. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther. New guidelines to evaluate the response to treatment in solid tumors. *J Natl Cancer Inst* 92:205-215, 2000.

9. Simon R. Optimal two-stage designs for phase II clinical trial. *Control Clin Trial*10:1-10, 1989.

10. Lee YJ, Staquet M, Simon R, Catane R, Muggia F. Two stage plans for patient accrual in phase II cancer clinical trials. *Cancer Treat Rep* 63:1721-1726, 1979.

11. Schiller JH, Harrington D, Belani CP, Langer C, Sandler A, Krook J, Zhu J, Johnson DH; Eastern Cooperative Oncology Group. Comparison of four chemotherapy regimens for advanced non-small cell lung cancer. *New Eng J Med* 346: 92-98, 2002.

12 Buyse M and Piedbois P. On the relationship between response to treatment and survival time. *Stat Med* 15:2797-2812, 1996.

Cancer Chemother Pharmacol (2004) 54: 505–513
DOI 10.1007/s00280-004-0839-5

ORIGINAL ARTICLE

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Schedule-dependent synergism and antagonism between pemetrexed and paclitaxel in human carcinoma cell lines in vitro

Received: 4 August 2003 / Accepted: 24 March 2004 / Published online: 31 August 2004

Abstract Pemetrexed is a novel multitargeted antifolate with significant clinical activity against a variety of tumors. We studied the schedule-dependent cytotoxic effects of pemetrexed in combination with paclitaxel in vitro to improve our understanding of how this combination might be used clinically. Human lung cancer A549 cells, breast cancer MCF7, ovarian cancer PA1, and colon cancer WiDr cells were exposed to both pemetrexed and paclitaxel in vitro. Cell growth inhibition after 5 days was determined and the effects of drug combinations were analyzed by the isobologram method (Steel and Peckham). Simultaneous exposure to pemetrexed and paclitaxel for 24 h produced antagonistic effects in A549 and PA1 cells, additive/antagonistic effects in MCF7 cells, and additive effects in WiDr cells. Pemetrexed for 24 h followed by paclitaxel for 24 h produced synergistic effects in A549 and MCF7 cells and additive effects in PA1 and WiDr cells, while the reverse sequence produced additive effects in all four cell lines. Cell cycle analysis supported these observations. Our findings suggest that the simultaneous administration of pemetrexed and paclitaxel is suboptimal. The optimal schedule of pemetrexed in combination with paclitaxel is the sequential administration of pemetrexed followed by paclitaxel, and this schedule should be assessed in clinical trials for the treatment of solid tumors.

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Keywords Pemetrexed · Paclitaxel · Isobologram · Synergism · Antagonism

Introduction

The development of several new antifolates with distinctive chemical features and target enzymes has provided new opportunities to expand the role of antifolates in cancer chemotherapy. Multitargeted antifolate (MTA, pemetrexed) is a pyrrole-pyrimidine analogue of folate [33] currently in broad clinical evaluation. Pemetrexed is transported into cells mainly through the reduced folate carrier system and metabolized to polyglutamated forms [7] which inhibit thymidylate synthase, dihydrofolate reductase, and glycinamide ribonucleotide formyl transferase [30, 31], and has antithymidylate and antipurine effects [5]. Preclinical studies of pemetrexed have demonstrated its antitumor activity against a variety of human cancer cells [2, 29].

Phase I studies have shown that the dose-limiting toxicity includes neutropenia and thrombocytopenia, and other toxicities which are manageable, such as mucositis, skin rashes and transient elevations of transaminases [18, 23–25]. Daily and weekly schedules are associated with severe toxicity and 500 mg/m^2 of pemetrexed every 3 weeks was selected as the optimal schedule and dose for the further development of pemetrexed. Patients with a folate-deficient state showed severe toxicity. In preclinical models, folate supplementation reduced toxicity while maintaining antitumor activity. Based on these observations, folate and cobalamin administration before pemetrexed has been routine in recent clinical trials of pemetrexed [9, 26]. Pharmacokinetic studies have shown that pemetrexed undergoes biphasic plasma clearance with a terminal half-life of 1.1–3.1 h, depending on the schedule of administration [23]. The findings from the phase II trial results are encouraging: clear responses were observed in colorectal cancer, pancreatic cancer, lung cancer, breast cancer, mesothelioma, etc. [3, 4, 8, 10, 19–21, 26, 37]. A recent

phase III study has shown that treatment with pemetrexed and cisplatin results in survival times superior to those achieved with cisplatin alone in patients with malignant pleural mesothelioma [39].

Paclitaxel is an established anticancer agent with activity against a variety of solid tumors [1, 6]. Paclitaxel is a mitotic inhibitor that promotes the polymerization and stabilization of tubulin to microtubules [27]. Clinical studies have indicated that neutropenia is the dose-limiting toxicity of paclitaxel [1, 6]. Other toxicities include hypersensitivity reactions, neurotoxicity, mucositis, mild nausea and vomiting, and cardiac injury.

The combination of pemetrexed and paclitaxel may have a major role in the treatment of a variety of solid tumors. The wide range of antitumor activity of pemetrexed and paclitaxel, their different cytotoxic mechanisms and toxic profiles, and the absence of cross-resistance, provide the rationale for using combinations of these agents. Since pemetrexed and paclitaxel are cell cycle-specific agents [17, 38], the disturbances of the cell cycle produced by these agents may influence the cytotoxic effects of each agent, and the drug schedule may play a significant role in the outcome. Therefore, the design of a protocol using them in combination requires careful consideration. As expected, experimental studies for the combination of pemetrexed [22, 30, 36] or paclitaxel [13–15] with other agents have shown schedule-dependent interactions.

The aim of the present study was to elucidate the cytotoxic effects of combinations of pemetrexed and paclitaxel in various schedules on four human carcinoma cell lines. The data obtained were analyzed using the isobologram method of Steel and Peckham [32]. The combination showed schedule-dependent synergism and antagonism.

Materials and methods

Cell lines

Experiments were conducted with the human lung cancer A549, breast cancer MCF7, ovarian cancer PA1, and colon cancer WiDr cell lines. These cells were obtained from the American Type Culture Collection (Rockville, Md.) and maintained in 75-cm^2 plastic tissue culture flasks containing RPMI-1640 medium (Sigma Chemical Co., St Louis, Mo.) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Grand Island Biological Co.) and antibiotics. The cells used were devoid of mycoplasma infection. The doubling times of A549, MCF7, PA1, and WiDr cells under our experimental conditions were in the range 20–24 h.

Drugs

Pemetrexed was kindly provided by Eli Lilly and Company (Indianapolis, Ind.). Paclitaxel was purchased from Bristol-Myers Squibb Japan Co. (Tokyo). The drugs, at a concentration of 1 m*M*, were stored at −20°C and diluted with RPMI-1640 plus 10% FBS prior to use.

Cell growth inhibition using combined anticancer agents

On day 0, cells growing in the exponential phase were harvested with 0.05% trypsin and 0.02% EDTA and resuspended to a final concentration of 5.0×10^3 cells/ml in fresh medium containing 10% FBS and antibiotics. Cell suspensions (100 µl) were dispensed into the individual wells of a 96-well tissue culture plate (Falcon, Oxnard, Calif.). Each plate had one eight-well control column containing medium alone and one eight-well control column containing cells without drug. Eight plates were prepared for each drug combination. The cells were preincubated overnight to allow attachment.

Simultaneous exposure to pemetrexed and paclitaxel

After the overnight incubation for cell attachment, solutions of pemetrexed and paclitaxel (50 µl) at different concentrations were added to the individual wells. The plates were also incubated under the same conditions for 24 h. The cells were then washed twice with culture medium containing 1% FBS, and then fresh medium containing 10% FBS (200 µl) and antibiotics was added. The cells were incubated again for 4 days.

Sequential exposure to pemetrexed followed by paclitaxel or the reverse sequence

After overnight incubation, medium containing 10% FBS (50 µl) and solutions (50 µl) of pemetrexed (or paclitaxel) at different concentrations was added to individual wells. The plates were then incubated under the same conditions for 24 h. The cells were washed twice with culture medium containing 1% FBS; then fresh medium containing 10% FBS (150 µl) and antibiotics was added, followed by the addition of solutions (50 µl) of paclitaxel (or pemetrexed) at different concentrations. The plates were incubated again under the same conditions for 24 h. The cells were then washed twice with culture medium, and fresh medium containing 10% FBS (200 µl) and antibiotics was added. The cells were then incubated again for 3 days.

MTT assay

Viable cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously [12]. For all four cell lines examined, we were able to establish a linear relationship between the MTT assay value and the cell number within the range shown.