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## Clinical Experience with Autofluorescence Imaging System in Patients with Lung Cancers and Precancerous Lesions

Kiyonobu Ueno<sup>a</sup> Yoko Kusunoki<sup>a</sup> Fumio Imamura<sup>a</sup> Mana Yoshimura<sup>a</sup>  
Suguru Yamamoto<sup>a</sup> Junji Uchida<sup>a</sup> Yoshitane Tsukamoto<sup>b</sup>

Departments of <sup>a</sup>Pulmonary Oncology, and <sup>b</sup>Pathology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

### Key Words

Lung cancer · Autofluorescence endoscopy ·  
Autofluorescence imaging system

### Abstract

**Background:** It is important to detect preinvasive bronchial lesions before they become invasive cancer, because detection of early cancer is expected to lead to a cure. Autofluorescence bronchoscopy is a useful device in the detection of preinvasive and cancerous lesions. Recently, a new autofluorescence bronchoscopic system, autofluorescence imaging (AFI) system, has been developed. **Objectives:** We evaluated the efficacy of AFI in the diagnosis of precancerous and cancerous lesions. **Methods:** A total of 31 patients underwent both conventional white-light bronchoscopy (WLB) and AFI from January 2002 to September 2004. We evaluated autofluorescence findings using a four-point scale: AFI-I, II, III, and B. The findings in WLB were evaluated on a three-point scale: WLB-I, II, and III. Abnormal areas by WLB and AFI were biopsied for histopathological examinations. **Results:** A total of 64 lesions were evaluated. When the AFI-III finding was regarded as positive in AFI and WLB-III as positive in WLB, sensitivity for severe dysplasia or worse was 94.7% with AFI and 73.7% with

WLB, respectively. **Conclusions:** AFI is an effective system for the detection of precancerous and cancerous lesions.

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### Introduction

Both the incidence and mortality rate of lung cancer are increasing. In 1998, lung cancer deaths first surpassed deaths from gastric cancer, and became the leading cause of death from cancer in both men and women in Japan. Detection of cancer, especially carcinoma in situ (CIS) and microinvasive carcinoma, has been widely expected to lead to a cure. Endobronchial treatments such as photodynamic therapy and brachytherapy have improved the cure rate of the central type of early-stage lung cancer [1-3]. Before endobronchial treatments became clinically applicable, surgical resection was a standard treatment for this stage of lung cancer. Recently, these treatments are considered to be good alternatives for surgery because they can preserve lung volume. Since it is difficult even for experienced bronchoscopists to identify dysplastic and early-stage carcinomatous lesions including CIS by conventional white-light bronchoscopy (WLB) alone, fluorescence endoscopy has been applied to detect these le-

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Kiyonobu Ueno, MD, PhD  
Department of Pulmonary Oncology  
Osaka Medical Center for Cancer and Cardiovascular Diseases  
1-3-3 Nakamichi, Higashinari-ku, Osaka 537-8511 (Japan)  
Tel. +81 6 6972 1181, Fax +81 6 6971 7636, E-Mail ueno-ki@mc.pref.osaka.jp

sions. The laser-induced fluorescence endoscopy (LIFE) system (Xillix, Vancouver, Canada) is one of the autofluorescence bronchoscopic systems developed by Hung and Lam et al. [4–6]. LIFE has been developed to detect the lesions of dysplasia, CIS, and cancers. Recently, a new autofluorescence bronchoscopic system, autofluorescence imaging (AFI), has been developed by Olympus (Olympus Medical Systems Corp., Tokyo Japan). AFI utilizes a videoscope system, and second-generation AFI is capable of displaying both the images of conventional mode and AFI mode on the same monitor by use of a switch. In AFI mode, a combination image of autofluorescence signal and two independent signals from two restricted wavelengths is displayed. By adding autofluorescence information to high-resolution images by conventional mode, holistic diagnosis of endobronchial lesions is expected to become possible. In this paper, we evaluated the efficacy of AFI in the diagnosis of precancerous and cancerous lesions.

## Subjects and Methods

### AFI System

A charge-coupled device is equipped with filters that cut excitation light in the optical system of the AFI bronchovideoscope to construct images by a 460–690 nm wavelength. The outer diameter of the distal end of the AFI bronchovideoscope is 6.1 mm. From a light source equipped with a 300 W xenon lamp, three lights are irradiated sequentially in AFI mode – the excitation light (395–445 nm) for taking autofluorescence images, and G'-light (550 nm) and R'-light (610 nm) for taking reflected images (G'-image and R'-image, respectively) [7]. Both autofluorescence (460–690 nm) signals and the reflected signals of G'-light and R'-light are processed in the videoprocessor, and composite images are displayed on the monitor as AFI images. The wavelengths of G'-light and R'-light are determined by the hemoglobin's absorption features. Hemoglobin absorbs a large volume of G'-light wavelength and the areas containing more hemoglobin are displayed dark in the G'-image. Consequently, the blood volume (hemoglobin quantity) in the tracheobronchial mucosa is well reflected in the G'-image. On the other hand, hemoglobin absorbs a lesser quantity of R'-light wavelength, and R'-images can afford a basic intensity of signals from the mucosa independently of blood supply [7]. Allocating autofluorescence images to green, G'-images to red, and R'-images to blue, the combined images are constructed by standardizing the intensities of autofluorescence images and G'-images by that of R'-images. AFI displays a green image for normal epithelium. The abnormalities in AFI are basically classified into two patterns, the area expressing magenta color where red is relatively dominant and the area expressing blue color where blue is relatively dominant. In the first generation, AFI lacked the switch that changed two modes of observation images, conventional and AFI mode. In the second generation, a switch was added allowing for the alternating between two modes.

### Subjects

A total of 31 patients with risk factors for central-type early lung cancer, 30 men and 1 woman, underwent AFI in our hospital from January 2002 to September 2004. The reasons patients underwent AFI are classified as follows: 10 patients with positive sputum cytology, 8 patients with symptoms including bloody sputum and cough, 11 patients as monitoring after previously treated lung cancer (photodynamic therapy 6, radiotherapy 1, surgical resection 4), and 2 patients as an examination of thoracic malignancy. The mean age was 69 (range 57–80) years old.

### Methods

After informed consent was obtained, both WLB and AFI were performed under local anesthesia. We used bronchoscopes (Olympus BF200 or BF240) as WLB and a AFI device as autofluorescence bronchoscopy. Using the first generation of the AFI system, we performed WLB followed by AFI. In this series, 9 patients were enrolled from January to October in 2002. To avoid the bias by bronchoscopic procedure, we performed AFI followed by WLB when the second generation of the AFI device was used. In this series, 22 patients were enrolled from October 2003 to September 2004.

We evaluated the autofluorescence findings using a four-point scale. These evaluations for lesions were done by two or more bronchoscopists. AFI-I images had normal autofluorescence intensity and were green, AFI-III images had much less autofluorescence intensity than AFI-I and appeared magenta. AFI-II images fell in between AFI-I and AFI-III and appeared pinkish-brown. When increased blue color was observed in a lesion, AFI-B was allocated. However, if the lesion showed AFI-II or III in blue image, AFI-II or III was allocated.

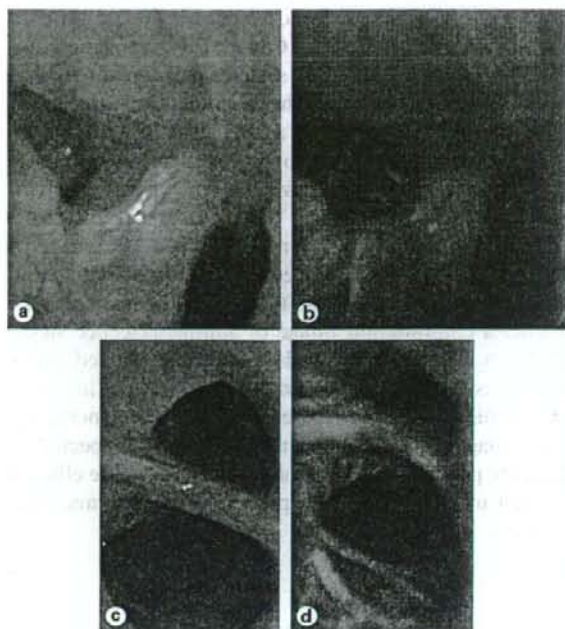
Similarly, the findings in WLB were evaluated on a three-point scale. WLB-I was defined as normal mucosa, WLB-II as slightly abnormal mucosa (changes suggesting mild/moderate dysplasia), and WLB-III as severely abnormal mucosa (changes suggesting severe dysplasia and cancer). All abnormal areas with a scale of II or III or B in AFI, or a scale of II or III in WLB underwent biopsies for histopathological examinations. Some areas showing WLB-I and AFI-I also underwent biopsies as negative control.

## Results

The mean cigarette index (packs of cigarettes per day × years at that consumption rate) for the 30 smokers was 56 packs (range 3.5–151 packs) and 1 patient was a non-smoker. The lesions receiving preceding biopsy, radiation therapy, or photodynamic therapy showed AFI-II or AFI-III in spite of their almost normal histology. The same results were seen in LIFE in our previous study. For this reason, we excluded these lesions from the evaluation. Moreover, acute bronchitis showed as a blue image in a wide extent of bronchial mucosa, and the diagnosis of acute bronchitis was facilitated in WLB. These lesions were also excluded in our study. Therefore, 64 lesions were evaluated: 11 lesions were normal, 7 lesions were benign changes including inflammation, fibrosis and hy-

perplasia. Of 29 dysplastic lesions, 16 were of mild, 11 were moderate, and 2 were severe. Seventeen lesions were cancer, and 1 of these lesions was CIS. The relationship between the AFI scale and histopathological diagnosis is shown in table 1.

Sensitivity for severe dysplasia and cancer by WLB-III was 73.7% and that by AFI-III was 94.7%. Sensitivity for moderate dysplasia or worse by WLB-III was 53.5% and that by AFI-III was 76.6%. Sensitivity and specificity with AFI and WLB were summarized in table 2. A typical case is shown in figure 1a and b. It is noted that 3 squamous cell carcinomas showed AFI-III and WLB-II, and detected correctly by AFI (fig. 1c, d). Examination time for AFI and WLB was 15 min on average. There were no adverse events in adding AFI in bronchoscopic examination.



**Fig. 1.** Squamous cell carcinoma of left B6 (a WLB, b AFI). Squamous cell carcinoma of right B8+9B10 spur (c WLB, d AFI).

**Table 1.** Diagnosis by AFI system and WLB

Histopathology	Biopsy specimens (n = 64)	AFI				WLB		
		AFI-I (n = 19)	AFI-II (n = 11)	AFI-III (n = 31)	AFI-B (n = 3)	WLB-I (n = 17)	WLB-II (n = 17)	WLB-III (n = 18)
Normal + benign changes	18	15	3	0	0	11	7	0
Mild dysplasia	16	2	4	8	2	3	11	2
Moderate dysplasia	11	1	4	5	1	2	7	2
Severe dysplasia	2	1	0	1	0	0	22	0
Cancer	17	0	0	17	0	0	3	14

**Table 2.** Sensitivity and specificity of AFI and WLB to detect each histopathology

Histopathology	AFI-III		WLB-III	
	sensitivity	specificity	sensitivity	specificity
Moderate dysplasia or worse	76.6%	76.5%	53.3%	94.1%
Severe dysplasia or worse	94.7%	71.1%	73.7%	93.0%
Moderate dysplasia or worse	AFI-II & AFI-III		WLB-II & WLB-III	
	sensitivity	specificity	sensitivity	specificity
Moderate dysplasia or worse	90.0%	50.0%	93.0%	41.2%



**Fig. 2.** Photomicrograph in the case of AFI-B. Small capillary grows into the moderate dysplastic epithelium. Arrow indicates capillary.

## Discussion

Lung cancer is the major cause of cancer death in Japan and most developed countries, and tobacco smoking is one of the causes of this disease. Not only the cessation of smoking, but also early detection and treatment may improve the prognosis of this disease. It is well known that autofluorescence bronchoscopy can improve the detection rate of endobronchial lesions in high-risk groups [8], although Kurie et al. [9] reported that LIFE did not improve the detection of dysplasia or squamous metaplasia. The patient population in Kurie's study may have had a lower possibility of preinvasive cancer than in the other studies.

LIFE, D-light system (Storz, Tuttlingen, Germany), SAFE 1000 autofluorescence system (Pentax, Asahi Optical Tokyo, Japan), and DAFE system (Wolf, Knittlingen, Germany) are commercially available autofluorescence bronchoscopy systems [10–12]. We have been using the LIFE-lung system in clinical practice. LIFE-Lung uses a helium-cadmium laser for excitation at 442 nm, and the emission spectrum is captured by two charge-coupled device cameras and processed through a fluorescence collection sensor and optical multichannel analyzer. Real-time digitized images are acquired by the ratios of red to green (520–630 nm) fluorescence emissions. In comparison, AFI uses a xenon lamp, and lacks a laser generator. AFI afforded clearer images than LIFE because of the utilization of a video system. Moreover, the switch for independent viewing of the two modes of observation reduced the examination time for AFI. In practical use, this switch will be useful because of the need for repeated observations of suspicious areas.

We found the sensitivity of AFI in detection of severe dysplasia and cancer was 94.7%, and the sensitivity of

AFI was 21.0% higher than that of WLB. We previously reported that sensitivity of LIFE for detection of severe dysplasia or worse was 83.7% [13]. Sensitivity of LIFE for detection of moderate dysplasia or worse is reported in other literature to lie between 38.0 and 91.0% [14]. In this study, the sensitivity of AFI for detection of moderate or worse was 76.6%. In our study, 3 squamous cell carcinomas diagnosed as moderate dysplasia (WLB-II) in WLB and cancer (AFI-III) in AFI. Haussinger et al. [15] reported that detection of CIS is not increased by autofluorescence bronchoscopy. The reasons for this discrepancy are as follows: sample size of our study was small, and we used a four-point scale in AFI and a three-point scale in WLB, but Haussinger et al. used a two-point scale. Indeed, these lesions were abnormal in both AFI and WLB if we used a two-point scale.

If only WLB-III in WLB is judged as abnormal without considering the AFI finding, 4 lesions with severe dysplasia or worse are missed. These results suggested that AFI has comparable diagnostic ability for detecting precancerous lesion to the other autofluorescence bronchoscopy systems such as LIFE.

Recently, Chiyo et al. [7] compared prospectively AFI with WLB and LIFE. Sensitivity and specificity of AFI for mild, moderate, and severe dysplasia was 80 and 83.3%, respectively. They excluded 2 squamous cell carcinomas which could be detected by all 3 modalities from their analysis; AFI-II and AFI-III in our criteria were judged to be positive in their analysis. In the analysis of our data on the same condition as theirs, sensitivity and specificity were 76.7% (23/30) and 83.0% (15/18), respectively. These results were almost comparable with theirs.

Sensitivity and specificity of the first generation of AFI were not significantly different from those of the second (data not shown). This means that the diagnostic ability of AFI is not dependent upon the order of bronchoscopic procedure. Hirsch et al.'s [14] report also showed that there was no difference in the sensitivity and specificity, regardless of the order of the bronchoscopic procedure.

Tracheobronchial mucosa that contains a large amount of blood should be indicated by blue images with AFI. The extended vessels in mucosa and any bleeding area are actually shown in blue. Most of the lesions that exhibited blue in AFI were inflammation unless they were bleeding. However, three blue lesions in AFI were mild or moderate dysplasias (fig. 2). The reason for this is not clear at present. Angiogenic squamous dysplasia (ASD) is a newly recognized morphological entity. ASD consists of capillary blood vessels closely juxtaposed to and pro-

jected into metaplastic or dysplastic squamous epithelium [16]. Keith et al. [16] reported that ASD was observed in many high-risk smokers and patients with squamous cell cancer and may represent an important intermediate pathological biomarker preceding lung cancer development. Two of three blue lesions showed an increased number of microcapillaries and this might result in increasing blood supply in the mucosa; an increase in the blood supply results in an increase in the blue signal. LIFE detected more ASD lesions than WLB. Regarding hemoglobin content, AFI has more information than LIFE because of adding R'-image information. AFI may be useful for detecting ASD.

We conclude that AFI is a useful system to detect cancer and precancerous lesions, and is used for screening of the high-risk group. Since the number of severe dysplasias and CIS was small in our study, multicenter trials will be necessary to confirm this conclusion.

#### Acknowledgement

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## Letter to the Editor

**Pleural sarcomatoid malignant mesothelioma consisting of histiocytoid cells***To the Editor:*

Pleural sarcomatoid malignant mesothelioma (PSMM) consists of spindle cells arranged in fascicles or having a haphazard fashion. The pattern often resembles fibrosarcoma, but marked anaplasia and bizarre multinucleated tumor cells may result in a picture closely mimicking that of malignant fibrous histiocytoma.<sup>1</sup> We herein report an extremely rare case of PSMM consisting of histiocytoid cells without any inflammatory infiltrate. We discuss its differential diagnosis and the key to establishing an accurate diagnosis.

A 76-year-old Japanese woman, a non-smoker, consulted Osaka Prefectural Medical Center for Respiratory and Allergic Diseases complaining of left chest pain that had persisted for the previous 2 weeks. She had worked as a spinning-mill worker for more than 30 years and occupational exposure to asbestos was highly supported. She had a past history of pleuritis and pericarditis of unknown origin. Chest X-ray demonstrated two fist-sized subpleural nodules in the left mediastinal and parietal costal pleura without any pulmonary lesions. There was a large amount of bloody pleural effusion on the left side. Pleural effusion cytology did not demonstrate any neoplastic findings. The course of the disease was rapidly progressive. A tentative clinical diagnosis of pleuritis carcinomatosa was made and percutaneous needle biopsies were obtained from the subpleural lesions. Histologically, most tumor cells infiltrated into a myxoid stroma and grew as a diffuse pattern (Fig. 1a). Sheets and nests of cells were absent. Inflammatory infiltrate was slight. The tumor cells were mainly composed of histiocytoid cells (Fig. 1b). These histiocytoid cells were ovoid or polyhedral in shape, with pale eosinophilic or foamy cytoplasm. Their nuclei were medium to large, ovoid or angulated, contained fine chromatin, and inconspicuous nucleoli (Fig. 1c). Multinucleated giant tumor cells were rarely recognized. Mitoses were 2/10 high-power fields (HPF). Necrosis and hemorrhagic areas were absent. Reactive histiocytes were sometimes present among these neoplastic cells. Immunohistochemical studies showed that these tumor cells were positive for AE1/AE3 (Fig. 2a), D2-40 (Fig. 2b), HBME-1, vimentin and WT-1 (Fig. 2c), but negative for BerEP4, CA125, calretinin, CAM5.2, CD3, CD15, CD30, CD34, CD45RB, CD79 $\alpha$ , CEA, cytokeratin 5/6, cytokeratin 7, desmin, epithelial membrane antigen, napsin A, PE-10, PG-M1, S-100, Smooth-muscle actin, thrombomodulin and thyroid transcription factor-1. MIB-1 was positive in 20% of tumor cells. PAS reaction was positive in some

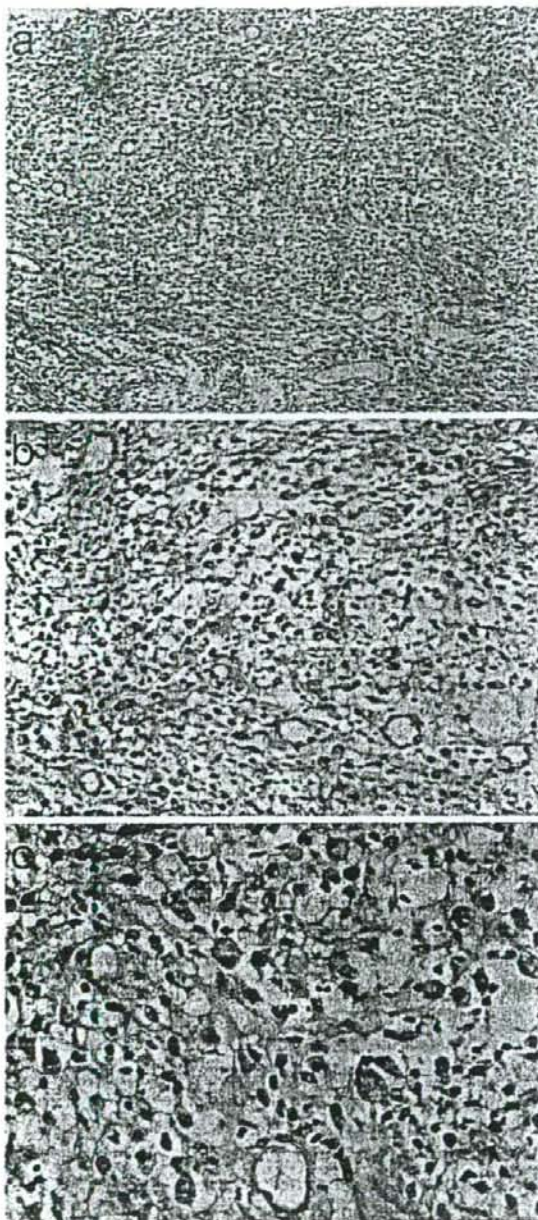
tumor cells and was partly digested with diastase. Alcian blue stain was positive in stroma digested with hyaluronidase and negative in the cytoplasm. Colloidal iron stain was positive in the stroma and digested with hyaluronidase. Taken together, we made a diagnosis of pleural sarcomatoid malignant mesothelioma consisting of histiocytoid cells.

Recognition of this rare variant of malignant mesothelioma is important because of the possibility of confusing it with lymphohistiocytic variant of anaplastic large cell lymphoma (LHALCL), sarcomatoid carcinoma (SC) of the lung or thymus, inflammatory malignant fibrous histiocytoma (IMFH), inflammatory myofibroblastic tumor (IMT) or lymphohistiocytoid mesothelioma (LHM). In LHALCL, large-sized atypical cells with immunohistochemical CD30 positivity are admixed with histiocytes and plasma cells.<sup>2</sup> However, in this tumor, immunohistochemical studies showed that the atypical cells were negative for CD30. As for SC, lesions of the lung and anterior mediastinum were absent, and spindle or giant atypical cells were not apparent histologically in the present case. IMFH is composed of xanthogranulomatous inflammation with scattered atypical large cells with prominent nucleoli. The present tumor lacked xanthogranulomatous inflammation, and histiocytoid cells were not so large and nucleoli were inconspicuous. IMT is composed of a variable mixture of collagen, inflammatory cells and cytologically bland spindle cells having myofibroblastic differentiation. However, in the present case, collagen and these bland spindle cells were absent. LHM is a variant of sarcomatoid malignant mesothelioma, characterized by diffuse proliferation of large, ovoid histiocyte-like and spindle cells, uniformly intermixed with a prominent lymphocytic or lymphoplasmacytic infiltrate.<sup>3,4</sup> But in the present case the lymphocytic or lymphoplasmacytic infiltrate was subtle.

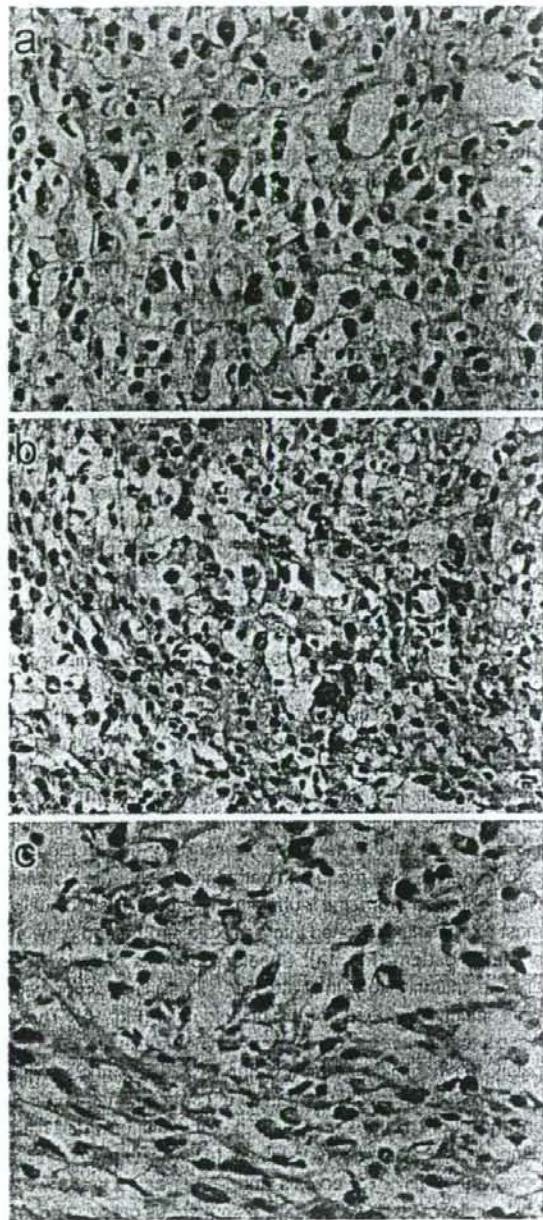
We made a diagnosis of PSMM consisting of histiocytoid cells based on percutaneous needle biopsy specimens. The keys to accurate diagnosis were as follows: (i) recognition of serosal tumor; (ii) immunohistochemical positivity for AE1/AE3, vimentin and mesothelial markers including D2-40,<sup>5</sup> WT1 and HBME-1; and (iii) being aware of the varied histopathological manifestations of PSMM.

Kunimitsu Kawahara,<sup>1</sup> Teruaki Nagano,<sup>1</sup> Kaoru Matsui,<sup>2</sup>  
Toshiaki Kawai,<sup>3</sup> and Teruo Iwasaki<sup>4</sup>

Departments of <sup>1</sup>Pathology, <sup>2</sup>Thoracic Malignancy and  
<sup>4</sup>Thoracic Surgery, Osaka Prefectural Medical Center for  
Respiratory and Allergic Diseases, Habikino and  
<sup>3</sup>Department of Pathology, National Defense Medical  
College, Tokorozawa, Japan



**Figure 1** Microscopic view of the tumor. (a) Tumor cells infiltrating diffusely in a myxoid stroma. (b) Tumor cells mainly composed of histiocytoid cells. (c) The tumor cells were ovoid or polyhedral in shape with pale eosinophilic or foamy cytoplasm. Stain: HE.



**Figure 2** Immunohistochemical staining of the tumor. The histiocytoid cells had positive staining for (a) AE1/AE3, (b) D2-40 and (c) WT1.

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# Proteomics-based identification of $\alpha$ -enolase as a tumor antigen in non-small cell lung cancer

Ping He,<sup>1</sup> Tetsuji Naka,<sup>1,2,4</sup> Satoshi Serada,<sup>3</sup> Minoru Fujimoto,<sup>1,2</sup> Toshio Tanaka,<sup>1</sup> Shoji Hashimoto,<sup>4</sup> Yoshihito Shima,<sup>1</sup> Tomoki Yamadori,<sup>1</sup> Hidekazu Suzuki,<sup>3</sup> Tomonori Hirashima,<sup>3</sup> Kaoru Matsui,<sup>3</sup> Hiroyuki Shiono,<sup>6</sup> Meinoshin Okumura,<sup>5</sup> Toshiro Nishida,<sup>7</sup> Isao Tachibana,<sup>1</sup> Naoko Norioka,<sup>3</sup> Shigemi Norioka<sup>3</sup> and Ichiro Kawase<sup>1</sup>

<sup>1</sup>Department of Molecular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871; <sup>2</sup>Laboratory for Immune Signal, National Institute of Biomedical Innovation, 7-6-8 Saito-asagi, Ibaragi, Osaka 567-0085; <sup>3</sup>Functional Proteomics Group, Osaka University Graduate School of Frontier Biosciences, 1-3 Yamadaoka, Suita, Osaka 565-0871; <sup>4</sup>Division of Infection Control and Prevention, Osaka University Hospital, 2-2 Yamadaoka, Suita, Osaka 565-0871; <sup>5</sup>Department of Thoracic Malignancy, Osaka Prefectural Medical Center for Respiratory and Allergic Diseases, 3-7-1 Habikino, Habikino, Osaka 583-8588; <sup>6</sup>Department of Surgery, Division of General Thoracic Surgery, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871; <sup>7</sup>Department of Surgery (E1), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan

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Autoantibodies against tumor antigens represent one type of biomarker that may be assayed in serum for detection of cancer and monitoring of disease progression. In the present study, we used a proteomics-based approach to identify novel tumor antigens in non-small cell lung cancer (NSCLC). By combining two-dimensional electrophoresis, western blotting, mass spectrometry and enzyme-linked immunosorbent assay technology, we detected autoantibodies against  $\alpha$ -enolase in a subset of NSCLC patients' sera. When 'Mean OD<sub>healthy control sera</sub> + 3 SD<sub>healthy control sera</sub>' was used as the cut-off point, the prevalence of this autoantibody was 27.7% in patients with NSCLC (26 of 94), 1.7% in healthy control subjects (1 of 60), and not detectable in sera from 15 patients with small cell lung cancer, 18 patients with gastrointestinal cancer and nine patients with *Mycobacterium avium* complex infection of lung. Immunohistochemical staining showed that expression of  $\alpha$ -enolase was increased in cancer tissues of NSCLC patients, and flow cytometric analysis confirmed the expression of  $\alpha$ -enolase at the surface of cancer cells. The combined detection of autoantibodies against  $\alpha$ -enolase, carcinoembryonic antigen and cytokeratin 19 fragment (CYFRA21-1) enhanced sensitivity for the diagnosis of NSCLC. Therefore, autoantibodies against  $\alpha$ -enolase may constitute a promising biomarker for NSCLC. (*Cancer Sci* 2007; 98: 1234–1240)

Lung cancer is the leading cause of cancer death,<sup>(1)</sup> and NSCLC accounts for nearly 80% of lung cancer cases. There is an urgent need for a better understanding of the biological mechanisms of NSCLC as well as the identification of reliable biomarkers for its diagnosis and prognosis. To date, a number of NSCLC markers have been evaluated, including CEA, CYFRA 21-1, SCC antigen, CA125 and NSE.<sup>(2–4)</sup> Autoantibodies against several tumor antigens such as L-myc and c-myc, p53 and antinuclear/antinuclear antigens have also been investigated.<sup>(5–12)</sup> Recently, autoantibodies against PGP9.5, peroxiredoxin-I, annexin-I and annexin-II were identified in the sera of lung cancer patients using a proteomic approach.<sup>(13–15)</sup> However, the sensitivity and specificity of these biomarkers are not yet satisfactory and there are currently no data to support any particular method for screening for lung cancer.<sup>(16)</sup>

Autoantibodies against tumor antigens represent one type of biomarker that may be assayed in serum for detection of cancer and monitoring of disease progression. In spite of the fact that the quantity of any tumor antigen in cancer cells or in the circulation is usually very small, especially in the early stages of cancer, the body's immune response to such antigens represents a remarkable phenomenon of biological amplification of these weak signals from tumor antigens.<sup>(17)</sup> The identification of panels of tumor antigens that elicit an immune response may thus be useful for

detecting potential specific biomarkers as well as for the initiation of immunotherapy against NSCLC. The aim of the present study was to identify novel candidate tumor antigens in NSCLC by means of a proteomics-based approach. One of these antigens was identified as  $\alpha$ -enolase, and its immunogenicity was confirmed by western blotting using recombinant protein. The results obtained with enzyme-linked immunosorbent assay (ELISA) demonstrated that when 'Mean OD<sub>healthy control sera</sub> + 3 SD<sub>healthy control sera</sub>' was used as the cut-off point, a humoral immune response directed against  $\alpha$ -enolase occurred in 27.7% of NSCLC patients, but in only 1.7% of healthy control subjects. Immunohistochemical staining showed that  $\alpha$ -enolase was overexpressed in cancer tissues of NSCLC patients. The combined detection of autoantibodies against  $\alpha$ -enolase, CEA and CYFRA 21-1 enhanced sensitivity for NSCLC diagnosis. Therefore, autoantibodies against  $\alpha$ -enolase may constitute a promising biomarker for NSCLC.

## Materials and Methods

**Subjects.** Sera and tumor tissue were obtained at the time of diagnosis after informed consent had been given by the subjects. The experimental protocol was approved by the ethics committee of Osaka University. Sera from 94 patients with NSCLC, 15 patients with SCLC, 18 patients with gastrointestinal cancer (10 patients with gastric cancer, 8 patients with colon cancer) and nine patients with MAC were analyzed. In terms of TNM stages, the NSCLC patients comprised 17 cases of stage I, 14 cases of stage II, 34 cases of stage III and 29 cases of stage IV. The histological distribution of NSCLC was 73 adenocarcinoma cases and 21 SCC cases. Clinical data for the serum tumor marker CEA and CYFRA 21-1 were also collected for investigation. Sera from 60 asymptomatic healthy subjects, whose average age and sex were comparable to those of the NSCLC patient group, were used as controls.

**1-DE and 2-DE.** Proteins were extracted from NSCLC tumor tissues using the Complete Mammalian Proteome Extraction Kit (Calbiochem, Darmstadt, Germany). For 1-DE, extracted proteins

\*To whom correspondence should be addressed. E-mail: tnaka@nibio.go.jp  
Abbreviations: 1-DE, one-dimensional electrophoresis; 2-DE, two-dimensional electrophoresis; a.a., amino acids; CA, cancer antigen; CBB, Coomassie brilliant blue; CEA, carcinoembryonic antigen; CYFRA21-1, cytokeratin 19 fragment; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; IEF, isoelectric focusing; IHC, immunohistochemical; LC, liquid chromatography; MAC, *Mycobacterium avium* complex; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MS, mass spectrometry; NSCLC, non-small cell lung cancer; NSE, neuron-specific enolase; OD, optical density; PCR, polymerase chain reaction; PMF, peptide mass fingerprinting; PVDF, polyvinylidene difluoride; SCC, squamous cell carcinoma; SCLC, small cell lung cancer; TNM, tumour-node-metastasis.

were resolved by using 10% Bis-Tris Criterion XT Precast gels (Bio-Rad Laboratories, Hercules, CA, USA), transferred to PVDF membranes or stained with CBB. For 2-DE, IEF was carried out using the PROTEAN IEF cell (Bio-Rad Laboratories) according to the manufacturer's instructions. Extracted proteins were reconstituted in a rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2 mM tributyl phosphine (TBP), 0.0002% bromophenol blue (BPB), 0.2% bio-lyte ampholyte 3-10) and applied to ReadyStrip IPG strips (11 cm, pH 3-10). IEF was run for 45 000 Vh, and 2-DE was carried out using 10% Bis-Tris Criterion XT Precast gels. The gels were then stained with the Silver Stain MS Kit (Wako Pure Chemical Industries, Osaka, Japan) or used for protein transfer to PVDF membranes.

**Western blotting.** After blocking with 5% skim milk, the PVDF membranes were incubated with serum at a 1:100 dilution or rabbit anti-enolase antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1:1500 dilution. The membranes were then incubated with sheep anti-human IgG or donkey anti-rabbit IgG (Amersham Biosciences UK, Buckinghamshire, UK). Membranes incubated with sheep anti-human IgG only were used as negative controls. Finally, the signals were visualized with an enhanced chemiluminescence reaction system (Perkin Elmer Life Sciences, Boston, MA, USA).

**Identification of protein bands or spots.** Protein bands on gels stained with CBB or protein spots on gels stained with silver, which corresponded to positive bands or spots on western blot membranes, were excised from the gel and digested with trypsin (Promega, Madison, WI, USA) according to published procedures.<sup>(18)</sup> For protein bands, the LC-MS/MS analysis was carried out using an LCQ ion trap mass spectrometer (ThermoElectron, San Jose, CA, USA) coupled on-line with Magic 2002 capillary high-performance liquid chromatography (Michrom BioResources, Auburn, CA, USA). For protein spots, all PMF spectra were obtained by using an ultraflex TOF/TOF MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). MS/MS or PMF data were then searched with Mascot software (Matrix Science, London, UK) against the NCBI nr or swiss-prot databases. Protein database searching was carried out with following parameters for PMF: *Homo sapiens*, maximum of one missed, cleavage by trypsin, monoisotopic mass value, charge state of 1+, allowing a mass tolerance of 100 p.p.m., and carbamidomethyl modification of cysteine.

**Preparation of recombinant protein.** To prepare recombinant proteins, the human full-length  $\alpha$ -enolase complementary DNA (1-434 a.a.) was amplified by PCR from the Hep3B cell line cDNA library using the primers: sense 5'-GTGGCTAGAAGTTCACCATG-3', antisense 5'-TTACTTGGCCAAAGGGGTTTC-3'. To map the autoepitope on  $\alpha$ -enolase, three cDNA fragments that encode C-terminal deletion mutant proteins ( $\alpha$ -Eno1,  $\alpha$ -Eno2,  $\alpha$ -Eno3) were similarly amplified. The nucleotide sequences of the primers for PCR were:  $\alpha$ -Eno1 (1-334 a.a.) sense 5'-TGTCTATTCTCAAGATCCATGCC-3', antisense 5'-TTACTCGTTCACGGCCCTGGC-3';  $\alpha$ -Eno2 (1-234 a.a.), sense 5'-TGTCTATTCTCAAGATCCATGCC-3', antisense 5'-TTAAGCTTTCCCAATAGCAGTC-3'; and  $\alpha$ -Eno3 (1-134 a.a.) sense 5'-TGTCTATTCTCAAGATCCATGCC-3', antisense 5'-TTAGATGTGGCGGTACAGGGG-3'. These cDNA fragments were then subcloned into the pET-28a(+) vector (Novagen, Madison, WI, USA), resulting in expression of  $\alpha$ -enolase or its fragments with a 6  $\times$  His tag. Recombinant proteins were produced in *Escherichia coli* BL21-CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, CA, USA) and purified by affinity chromatography using Ni-NTA resin (QIAGEN, Tokyo, Japan). Recombinant human full-length or C-terminal deletion mutant  $\alpha$ -enolase, rabbit  $\beta$ -enolase (Sigma, St Louis, MO, USA) and human  $\gamma$ -enolase (Calbiochem) were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis, using a 4-20% precast gel, then stained with CBB or transferred to PVDF

membrane and probed with anti-enolase antibody, anti-6  $\times$  His monoclonal antibody or sera as described above.

**Flow cytometry.** Human lung adenocarcinoma cell line A549 was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100  $\mu$ g/mL streptomycin. Cells ( $10^6$ ) were incubated with rabbit anti-enolase antibody at a 1:100 dilution and labeled with fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin (BD Biosciences, San Jose, CA, USA). Normal rabbit IgG was used as a control. Stained cells were analyzed using a FACS Canto cytometer (Becton-Dickinson, Mountain View, CA, USA) and the results were analyzed using FlowJo software (Tree Star, Stanford, CA, USA).

**ELISA.** To assess the potential of these autoantibodies as a diagnostic marker, their frequencies in the sera were determined by means of ELISA using recombinant human full-length  $\alpha$ -enolase protein. The ELISA was carried out as published elsewhere, with modifications.<sup>(19)</sup> Briefly, each well of a Microtiter plate (MaxiSorp; Nunc A/S, Roskilde, Denmark) was coated with 1  $\mu$ g of recombinant human full-length  $\alpha$ -enolase. After blocking with 1% bovine serum albumin, all wells were incubated with human serum at a 1:500 dilution at room temperature for 1 h. To reduce the background level originating from the non-specific reactivity of sera with bacterial proteins, the sera were diluted and incubated with 100  $\mu$ g/mL *E. coli* BL21-CodonPlus (DE3)-RIL cell lysate for 2 h at room temperature before incubation with coated recombinant human  $\alpha$ -enolase. The antigen-antibody complexes were detected with 1:5000-diluted HRP-conjugate sheep anti-human IgG with TMB (Dako, Carpinteria, CA, USA) as the substrate. OD was read at 450 nm. The antibody titer was expressed by using arbitrary binding units calculated according to the formula:

$$\text{binding units of sample} = \frac{\text{OD}_{\text{sample}}}{[\text{Mean OD}_{\text{healthy control sera}} + 3 \text{ SD}_{\text{healthy control sera}}]} \times 100.$$

Based on this formula, 100 binding units was used as the cut-off point.

**IHC staining.** After deparaffinization, tissue sections were treated with 100% cold methanol containing freshly prepared 0.3% hydrogen peroxide for 30 min, blocked in 10% normal goat serum for 20 min and incubated with rabbit anti-enolase antibody (Santa Cruz Biotechnology) at a 1:250 dilution overnight. Incubation of parallel sections omitting the first antibody was done to generate negative controls. Staining of sections was completed with a biotin-conjugated secondary antibody, HRP-conjugated streptavidin and diaminobenzidine.

**Statistical analysis.** Significant differences between groups were assessed with the  $\chi^2$ -test and Fisher's exact test.  $P < 0.05$  was considered significant.

## Results

**Detection of autoantigens associated with NSCLC by 1-DE western blotting and LC-MS/MS.** In order to screen for autoantibodies against cancer cells in patients with NSCLC, proteins extracted from a given patient's tumor tissue were subjected to 1-DE, transferred to membranes, and incubated with sera from the same patient or from healthy control subjects. Membranes incubated with only the secondary antibody were used as negative controls. An approximately 47-kDa band was recognized only by a subset of NSCLC patient sera, whereas no such reaction was observed with healthy control sera or negative controls (Fig. 1a). To identify this 47-kDa protein, the corresponding band on the gel stained with CBB was digested and analyzed using LC-MS/MS. The eight proteins, including  $\alpha$ -enolase and elongation factor 1- $\alpha$  1, which were identified by database searching through Mascot software, are listed in Table 1. Many of these proteins

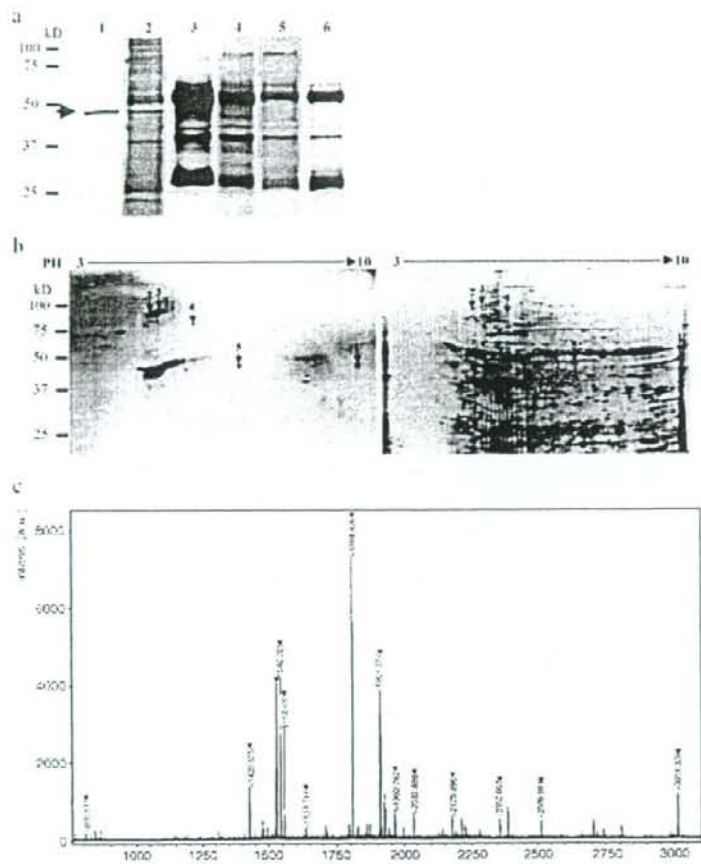


Fig. 1. (a) Screening by means of one-dimensional electrophoresis (1-DE) western blotting analysis for autoantigen associated with non-small cell lung cancer (NSCLC). Lane 1: ~47-kDa positive band (arrow), which was recognized by anti-enolase antibodies. Lanes 2, 3: 47-kDa positive band, which was recognized by NSCLC sera. Lane 4: no 47-kDa positive band was observed in a negative NSCLC case. Lane 5: no positive reaction was observed with healthy control sera. Lane 6: no positive reaction was observed in negative controls. (b) Detection by means of two-dimensional electrophoresis (2-DE) western blotting analysis of autoantigen associated with NSCLC. Left panel: Representative 2-DE western blotting analysis. Right panel: corresponding 2-DE silver-stained image. Protein spots recognized only by NSCLC sera are marked with arrows and numbers. (c) Peptide mass fingerprinting spectra of positive spot 5. For spot 5, 14 peptide masses were matched with human  $\alpha$ -enolase by executing an NCBI database search, yielding 52% protein sequence coverage. The matched mass peaks are marked with arrow heads.

Table 1. Mascot search results of the liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) data

Protein name	Swiss Prot Accession number	Molecular weight (Da)	pI	Score*	Peptide matched	Protein coverage (%)
Elongation factor 1- $\alpha$ 1	P68104	50 451	9.10	123	4	7
Cytokeratin 17	Q04695	48 230	4.97	271	13	25
$\alpha$ -Enolase	P06733	47 037	6.99	96	3	5
Elongation factor Tu	P49411	49 852	7.26	56	1	3
$\alpha$ -1-acid glycoprotein 1 precursor	P02763	23 725	4.93	160	4	18
Vimentin	P08670	53 545	5.06	115	5	9
Albumin precursor	P02768	71 317	5.92	106	4	6
Actin-like protein 3	P61158	47 797	5.61	46	2	7

\*Scores > 39 indicate identity or extensive homology ( $P < 0.05$ ). To identify the 47-kDa protein recognized only by non-small cell lung cancer patient sera, the corresponding band stained with Coomassie brilliant blue was digested and analyzed by LC-MS/MS. The eight proteins identified are listed.

are of similar molecular weight and one of them may be the autoantigen associated with NSCLC. We used western blotting with rabbit anti-enolase antibodies to confirm that the expression of  $\alpha$ -enolase occurred at the same position as that of the 47-kDa positive band (Fig. 1a).

Autoantibodies against  $\alpha$ -enolase present in NSCLC patient sera. To characterize autoantibodies in NSCLC sera, proteins

extracted from a given patient's tumor tissue were separated by 2-DE, transferred to membranes, and incubated with sera from the same patient or from healthy control subjects. Compared with the sera of healthy control subjects, 2-DE western blotting with NSCLC patient sera showed five positive protein spots (Fig. 1b, left panel), including one spot (spot 5) that also had a molecular weight of approximately 47 kDa and a pI value of

Table 2. Mascot search results of matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) data

Spot no.	Protein name	Sequence coverage (%)	Molecular weight (Da)	pI
1	Chain D, myeloperoxidase	22	53 806	9.43
2	Tumor rejection antigen-1 (gp96)	17	92 696	4.76
3	Not identified			
4	$\alpha$ glucosidase II $\alpha$ subunit	15	86 236	5.71
5	$\alpha$ -enolase	52	47 037	6.99

To identify the immunoreactive spots in two-dimensional electrophoresis western blotting analysis recognized by non-small cell lung cancer patient sera, corresponding silver-stained spots were digested and analyzed by MALDI-TOF/mass spectrometry. 'Spot no.' corresponds to spots marked in Fig. 1b.

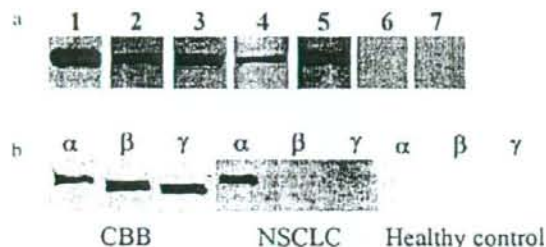


Fig. 2. (a) Western blotting analysis of recombinant human full-length  $\alpha$ -enolase protein. Recombinant human full-length  $\alpha$ -enolase protein was probed with rabbit anti-enolase antibodies (lane 1), with sera from non-small cell lung cancer (NSCLC) patients (lane 2-5), and with sera from healthy control subjects (lane 6-7). (b) Western blotting analysis of  $\alpha$ ,  $\beta$  and  $\gamma$ -enolase.  $\alpha$ ,  $\beta$  and  $\gamma$ -enolase protein were stained with Coomassie brilliant blue (left panel) or probed with sera from NSCLC patients (middle panel) and from healthy control subjects (right panel).

approximately 7.0. The corresponding spots on the silver-stained gel (Fig. 1b, right panel) were identified by MALDI-TOF/MS and database search. The identified proteins are summarized in Table 2. Spot 5 was recognized as  $\alpha$ -enolase, as in the previous LC-MS/MS analysis. Its PMF spectrum is shown in Fig. 1c and the database search produced 14 peptide masses that coincided with human  $\alpha$ -enolase, thus yielding 52% protein sequence coverage.

Next, western blotting with full-length recombinant human  $\alpha$ -enolase protein was used to confirm and analyze the immunogenicity of  $\alpha$ -enolase. Correct expression of the recombinant protein was verified by western blotting using rabbit anti-enolase antibodies (Fig. 2a). The recombinant proteins were then probed with sera from NSCLC patients or healthy control subjects, and positive bands were detected only in sera from the former, not from the latter (Fig. 2a). In addition, western blotting was used to determine the reactivity of NSCLC patient sera to enolase isoforms, which contain  $\alpha$ ,  $\beta$  and  $\gamma$ -enolase. The sera that were positive for autoantibodies against  $\alpha$ -enolase reacted with neither  $\beta$ -enolase nor  $\gamma$ -enolase (Fig. 2b), whereas healthy control sera did not react with any of the enolase isoforms. This indicates the specificity of autoantibodies against  $\alpha$ -enolase in NSCLC patient sera, and the overall results suggest that a subset of NSCLC patient sera contains autoantibodies against  $\alpha$ -enolase.

Frequencies of autoantibodies against  $\alpha$ -enolase in the sera were determined by means of ELISA using recombinant human full-length  $\alpha$ -enolase protein. We tested 94 sera from patients with NSCLC, 15 from patients with SCLC, 18 from patients with gastrointestinal cancer (10 patients with gastric cancer, 8 patients with colon cancer), nine from patients with MAC infection of lung and 60 from healthy control subjects. When

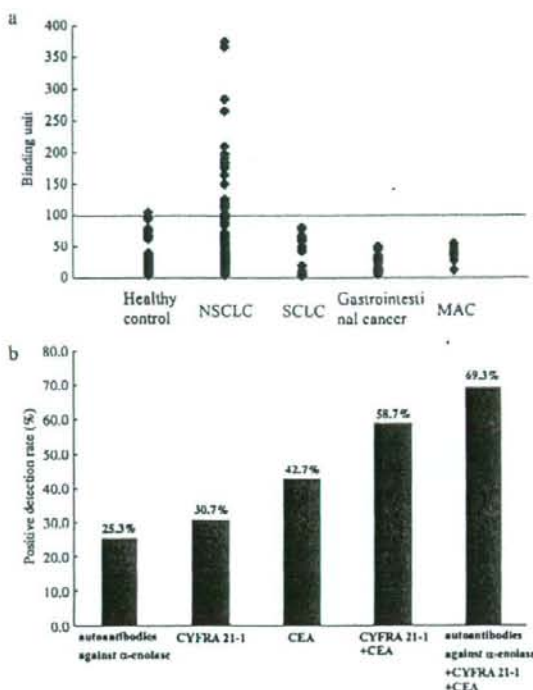


Fig. 3. (a) Prevalence of autoantibodies against  $\alpha$ -enolase determined by enzyme-linked immunosorbent assay (ELISA). The y-axis denotes binding units. The solid horizontal line represents the positive cut-off limit. The prevalence of autoantibodies against  $\alpha$ -enolase was 27.7% in patients with non-small cell lung cancer (NSCLC) (26 of 94), 1.7% in healthy control subjects (1 of 60), and not detectable in small cell lung cancer gastrointestinal cancer (10 patients with gastric cancer, 8 patients with colon cancer) and *Mycobacterium avium* complex infection of lung. (b) Positive detection rate of autoantibodies against  $\alpha$ -enolase, cytokeratin 19 fragment (CYFRA21-1) and carcinoembryonic antigen (CEA) in NSCLC patients. Detection of CYFRA 21-1 and CEA in combination increased the positive detection rate to 58.7%. Furthermore, combined detection of autoantibodies against  $\alpha$ -enolase, CEA and CYFRA 21-1 achieved a positive detection rate of up to 69.3%.

'Mean OD<sub>healthy control sera</sub> + 3 SD<sub>healthy control sera</sub>' was used as the cut-off point, the prevalence of autoantibodies against  $\alpha$ -enolase was 27.7% in patients with NSCLC (26 of 94), 1.7% in healthy control subjects (1 of 60), and not detectable in SCLC, gastrointestinal cancer or MAC infection of lung (Fig. 3a). These results

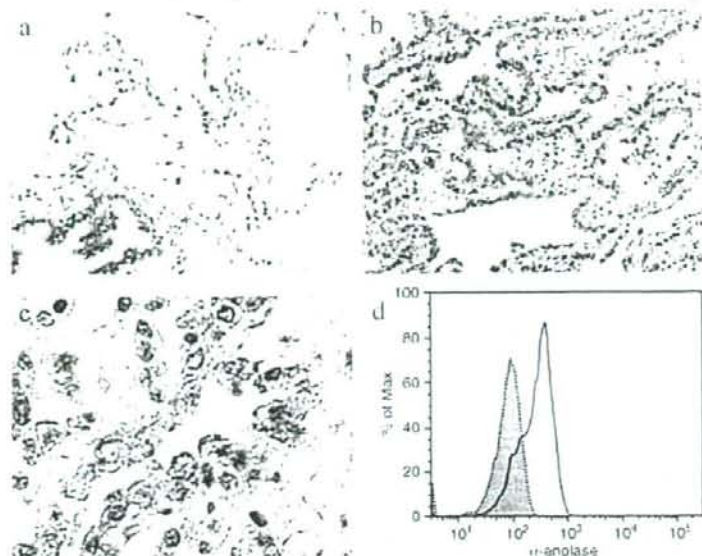


Fig. 4. Immunohistochemical and flow cytometric analysis of  $\alpha$ -enolase. (a) Normal lung tissue ( $\times 100$ ). (b) Lung adenocarcinoma ( $\times 100$ ). (c)  $\alpha$ -Enolase staining showing a mixture of cytoplasmic and membranous immunoreactivity ( $\times 400$ ). (d) A549 cells were stained with fluorescein-isothiocyanate-conjugated goat anti-rabbit immunoglobulin, and analyzed on a FACS Canto (open histogram). Shaded histogram indicates staining with control IgG.

showed that titers of autoantibodies against  $\alpha$ -enolase are increased in a subset of NSCLC patients. Next, we examined the correlation between the prevalence of autoantibodies against  $\alpha$ -enolase and clinicopathological features in NSCLC patients. Positive reactivity was detected in 21 of the 73 sera from adenocarcinoma patients (28.8%) and in five of the 21 sera from SCC patients (23.8%). There was no significant correlation between the occurrence of autoantibodies against  $\alpha$ -enolase and pathological types ( $P = 0.654$ ). In addition, there was a tendency for autoantibodies against  $\alpha$ -enolase to be more prevalent in patients with advanced NSCLC cases (stage III/IV, 33.3%, 21 of 63) than in stage I/II cases (16.1%, 5 of 31), although the results of the statistical analysis suggest that the prevalence has no significant correlation with disease stage ( $P = 0.08$ ). We also investigated the relationship between autoantibodies against  $\alpha$ -enolase and other tumor markers (CEA, CYFRA 21-1) that have been applied to clinical practice in NSCLC patients. In a total of 94 NSCLC patients, clinical data of both CEA and CYFRA 21-1 were available for 75 patients. In these patients, 25.3% (19/75) were positive for autoantibodies against  $\alpha$ -enolase, 42.7% (32/75) were positive for CEA, and 30.7% (23/75) were positive for CYFRA 21-1. The occurrence of autoantibodies against  $\alpha$ -enolase didn't show a significant correlation with CEA ( $P = 0.63$ ) or with CYFRA 21-1 ( $P = 0.92$ ). Detection of CYFRA 21-1 and CEA in combination increased the positive detection rate to 58.7% (44/75). Furthermore, positive detection rate was enhanced up to 69.3% (52/75) when combined detection of autoantibodies against  $\alpha$ -enolase, CEA and CYFRA 21-1 was used (Fig. 3b).

**IHC and flow cytometric analysis of  $\alpha$ -enolase.** We used IHC staining to compare  $\alpha$ -enolase expression in non-malignant and malignant lung tissues from 20 NSCLC patients, including 10 patients with autoantibodies against  $\alpha$ -enolase and 10 patients without autoantibodies against  $\alpha$ -enolase. The staining results showed that expression of  $\alpha$ -enolase was increased in malignant lung tissue of NSCLC patients (Fig. 4a-c). Additionally, IHC staining showed not only cytoplasmic but also membranous immunoreactivity in cancer cells (Fig. 4c). Flow cytometric analysis of human lung adenocarcinoma cell line A549 also confirmed the expression of  $\alpha$ -enolase at the surface of lung cancer cells (Fig. 4d).

**Epitopes located at the N-terminal region (1-134 a.a.) of  $\alpha$ -enolase that are recognized by autoantibodies.** To locate the serological epitopes of  $\alpha$ -enolase, full-length and C-terminal deletion mutant proteins ( $\alpha$ -Eno1,  $\alpha$ -Eno2,  $\alpha$ -Eno3) were prepared. The full-length  $\alpha$ -enolase (1-434 a.a.),  $\alpha$ -Eno1 (1-334 a.a.),  $\alpha$ -Eno2 (1-234 a.a.) and  $\alpha$ -Eno3 (1-134 a.a.) recombinant proteins were clearly shown by an anti-6  $\times$  His antibody or stained with CBB, which verified their expression (Fig. 5). A commercially available rabbit anti-enolase antibody reacted only with the full-length  $\alpha$ -enolase,  $\alpha$ -Eno1 and  $\alpha$ -Eno2 recombinant proteins (Fig. 5). However, sera from NSCLC patients who showed the presence of autoantibodies against  $\alpha$ -enolase reacted with the  $\alpha$ -Eno1,  $\alpha$ -Eno2, and  $\alpha$ -Eno3 recombinant proteins (Fig. 5), indicating that in NSCLC patients at least the N-terminal region of  $\alpha$ -enolase contains epitopes.

## Discussion

In the present study we used a proteomics-based screen test to identify proteins such as  $\alpha$ -enolase and gp96 that may elicit a humoral immune response in NSCLC patients. We then confirmed that some NSCLC patients' sera contained autoantibodies against  $\alpha$ -enolase by means of western blotting using recombinant protein. Furthermore, the results obtained with ELISA demonstrated that when 'Mean OD<sub>healthy control sera</sub> + 3 SD<sub>healthy control sera</sub>' was used as the cut-off point, the humoral immune response directed against  $\alpha$ -enolase occurred in 27.7% of NSCLC patients, but in only 1.7% of healthy control subjects.  $\alpha$ -enolase is an isoenzyme of enolase, a key protein that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate, which is the second of the two high-energy intermediates that generate ATP in glycolysis.<sup>(20)</sup> Three isoforms of enolase have been identified and are known as  $\alpha$ ,  $\beta$  and  $\gamma$ -enolase.  $\alpha$ -enolase is present in most tissues and is predominant in early embryonic tissue.  $\beta$ -enolase is expressed in muscle tissue, and  $\gamma$ -enolase, also known as NSE, is found only in neuronal tissues.

Autoantibody responses to tumors are generally thought to be elicited in three ways. These are overexpression of specific proteins, especially on the cell surface, gene mutation or post-translational modification of proteins, which shows new epitopes

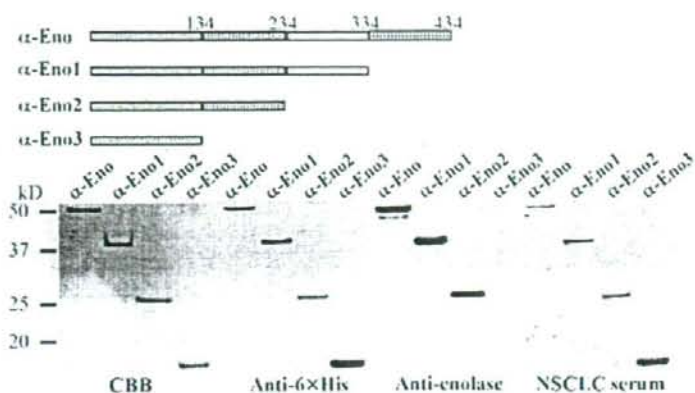


Fig. 5. Upper panel: preparation of recombinant human full-length  $\alpha$ -enolase protein:  $\alpha$ -Eno (1-434 a.a.), C-terminal deletion mutant  $\alpha$ -enolase proteins:  $\alpha$ -Eno1 (1-334 a.a.),  $\alpha$ -Eno2 (1-234 a.a.) and  $\alpha$ -Eno3 (1-134 a.a.). Lower panel: recombinant proteins were stained with Coomassie brilliant blue or probed with anti-6x His antibody, anti-enolase antibody or non-small cell lung cancer sera.

as immunogens, and other types of protein processing in tumor tissue.<sup>(21)</sup> In the present study we used normal recombinant proteins to confirm the immunogenicity of  $\alpha$ -enolase in NSCLC patients. In addition, although a past study reported that expression of  $\alpha$ -enolase was downregulated in NSCLC tissues,<sup>(22)</sup> IHC staining used in our study showed that  $\alpha$ -enolase expression is commonly increased in malignant lung tissue from NSCLC patients compared to the expression in non-malignant lung tissue, which is consistent with other previous reports.<sup>(23-27)</sup> Interestingly, IHC staining also showed not only cytoplasmic but also membranous immunoreactivity. Moreover, flow cytometric analysis demonstrated expression of  $\alpha$ -enolase on the surface of lung cancer cells. We think that enhanced expression of  $\alpha$ -enolase on cancer-cell surface might be one reason for autoantigenicity, and might be required for induction of autoantibody responses. However,  $\alpha$ -enolase expression alone is insufficient for autoantibody production as increased expression was also found in these patients without autoantibodies against  $\alpha$ -enolase. Further study is necessary to investigate the detailed mechanisms involved in this autoantibody response.

It is widely accepted that the propensity for glycolysis is enhanced in cancer cells because of increased cell proliferation. In fact,  $\alpha$ -enolase, a key enzyme in the glycolysis pathway, was found to be overexpressed in 18 cancers.<sup>(25)</sup> Furthermore, although the mechanism of its surface expression and orientation on the membrane are not yet clearly understood, it is known that the C-terminal a.a. of  $\alpha$ -enolase, lysine, is exposed at the cell surface and is involved in binding to plasminogen, which is then activated and converted to plasmin.<sup>(26)</sup> Once plasmin is stabilized at the cell surface, it in turn induces fibrinolysis.<sup>(26)</sup> In response to the upregulation of  $\alpha$ -enolase expression, progression of the fibrinolytic system is markedly accelerated, and the resultant increase in local fibrinolysis may contribute to cancer cell invasion and metastasis. This is consistent with our finding that there was a tendency for autoantibodies against  $\alpha$ -enolase to be more prevalent in patients with advanced NSCLC (stage III/IV) than in stage I/II cases.

Tumor markers for NSCLC are potentially useful for both diagnostic and therapeutic practice. To date, a variety of NSCLC

tumor markers have been identified and the most extensively investigated circulating protein markers include CEA, CYFRA 21-1, SCC antigen, NSE and CA125.<sup>(5)</sup> The percentages of NSCLC patients who have elevated serum protein levels of the above markers are 26%–42% for CEA, 51%–74% for CYFRA 21-1, 20%–32% for SCC, 28–32% for NSE, and 46–55% for CA125, with variations depending on histology and stage.<sup>(5-8)</sup> However, because elevated serum protein levels of these markers have been observed in tumors other than NSCLC, their sensitivity and specificity are not satisfactory and their clinical applicability is limited. Our study demonstrated that when 'Mean OD<sub>healthy control sera</sub> + 3 SD<sub>healthy control sera</sub>' was used as the cut-off point, autoantibodies against  $\alpha$ -enolase occurred in 27.7% of NSCLC patients but not in those with SCLC, gastrointestinal cancer or MAC infection of the lungs. Moreover, combined detection of autoantibodies against  $\alpha$ -enolase, CEA and CYFRA 21-1 enhanced sensitivity for the diagnosis of NSCLC. These results suggest that using autoantibodies against  $\alpha$ -enolase has potential as a clinical biomarker for serological screening of NSCLC. Further large-scale validation studies will be needed to determine the sensitivity, specificity and positive prognostic value of this marker in real-world screening scenarios.

Autoantibodies against  $\alpha$ -enolase have been detected in some autoimmune diseases,<sup>(20)</sup> and Fujii *et al.* report that in Hashimoto's encephalopathy one of these autoantibodies recognizes the N-terminal region of  $\alpha$ -enolase.<sup>(29)</sup> Because our results show this same recognition in NSCLC, further experiments are warranted to compare epitopes in  $\alpha$ -enolase as detected in NSCLC and in autoimmune diseases to determine its utility as a biomarker for NSCLC.

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## Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: Four-Arm Cooperative Study in Japan

Y. Ohe<sup>1\*</sup>, Y. Ohashi<sup>2</sup>, K. Kubota<sup>3</sup>, T. Tamura<sup>1</sup>, K. Nakagawa<sup>4</sup>, S. Negoro<sup>5</sup>, Y. Nishiwaki<sup>3</sup>, N. Saijo<sup>3</sup>, Y. Ariyoshi<sup>6</sup> & M. Fukuoka<sup>4</sup>  
For the FACS Cooperative Group

<sup>1</sup>Department of Internal Medicine, National Cancer Center Hospital, Tokyo; <sup>2</sup>Department of Biostatistics/Epidemiology and Preventive Health Sciences, School of Health Sciences and Nursing, The University of Tokyo, Tokyo; <sup>3</sup>Thoracic Oncology Division, National Cancer Center Hospital East, Kashiwa, Chiba; <sup>4</sup>Department of Medical Oncology, Kinki University School of Medicine, Osakasayama, Osaka; <sup>5</sup>Department of Thoracic Oncology, Hyogo Medical Center for Adults, Akashi, Hyogo; <sup>6</sup>Aichi Cancer Center Aichi Hospital, Okazaki, Aichi, Japan

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**Background:** To compare the efficacy and toxicity of three platinum-based combination regimens against cisplatin plus irinotecan (IP) in patients with untreated advanced non-small-cell lung cancer (NSCLC) by a non-inferiority design.

**Patients and methods:** A total of 602 patients were randomly assigned to one of four regimens: cisplatin 80 mg/m<sup>2</sup> on day 1 plus irinotecan 60 mg/m<sup>2</sup> on days 1, 8, 15 every 4 weeks (IP); carboplatin AUC 6.0 min × mg/mL (area under the concentration–time curve) on day 1 plus paclitaxel 200 mg/m<sup>2</sup> on day 1 every 3 weeks (TC); cisplatin 80 mg/m<sup>2</sup> on day 1 plus gemcitabine 1000 mg/m<sup>2</sup> on days 1, 8 every 3 weeks (GP); and cisplatin 80 mg/m<sup>2</sup> on day 1 plus vinorelbine 25 mg/m<sup>2</sup> on days 1, 8 every 3 weeks (NP).

**Results:** The response rate, median survival time, and 1-year survival rate were 31.0%, 13.9 months, 59.2%, respectively, in IP; 32.4%, 12.3 months, 51.0% in TC; 30.1%, 14.0 months, 59.6% in GP; and 33.1%, 11.4 months, 48.3% in NP. No statistically significant differences were found in response rate or overall survival, but the non-inferiority of none of the experimental regimens could be confirmed. All the four regimens were well tolerated.

**Conclusion:** The four regimens have similar efficacy and different toxicity profiles, and they can be used to treat advanced NSCLC patients.

**Key words:** carboplatin, cisplatin, gemcitabine, irinotecan, non-small-cell lung cancer, paclitaxel, randomized phase III study, vinorelbine

### Introduction

Nearly 60 000 patients in Japan died of lung cancer in 2004, and the mortality rate is still increasing [1]. Even old-generation cisplatin-based chemotherapy provides a survival benefit and symptom relief in patients with inoperable non-small-cell lung cancer (NSCLC) [2]. Several anticancer agents including irinotecan, paclitaxel, docetaxel, gemcitabine, and vinorelbine, were developed in the 1990s and most of them have mechanisms of action that differ from those of the old-generation agents [3–7]. The combinations of platinum and these new agents developed in the 1990s are more useful against advanced NSCLC than old-generation combination

chemotherapy, and doublets of platinum and new-generation anticancer agents are considered standard chemotherapy regimens for advanced NSCLC, although no consistent standard regimens have yet been established [8–17].

Two phase III studies comparing cisplatin plus irinotecan (IP) with cisplatin plus vindesine for advanced NSCLC have been conducted in Japan [18, 19]. Fukuoka et al. [20] reported the results of a combined analysis of the 358 eligible stage IV patients in these studies. They carried out a multivariate analysis using the Cox regression model with adjustment for well-known prognostic factors, and the Cox regression analysis demonstrated that treatment with IP was one of significant independent favorable factor. Based on their data, we selected IP for the reference arm in our study.

The Ministry of Health, Labour and Welfare of Japan approved the prescription of paclitaxel, gemcitabine, and

\*Correspondence to: Dr Y. Ohe, Department of Internal Medicine, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan. Tel: +81-3-3542-2511; Fax: +81-3-3542-7006; E-mail: yoh@ncc.go.jp

vinorelbine for NSCLC in 1999 and requested a phase III study to confirm the efficacy and safety of these agents. The Japanese investigators and the pharmaceutical companies decided to conduct a four-arm randomized phase III study for NSCLC, the so-called FACS, Four-Arm Cooperative Study. The purpose of the study was to compare the efficacy and toxicity of three platinum-based combination regimens, carboplatin plus paclitaxel (TC), cisplatin plus gemcitabine (GP), cisplatin plus vinorelbine (NP), with IP as the reference arm.

## patients and methods

### patient selection

Patients with histologically and/or cytologically documented NSCLC were eligible for participation in the study. Each patient had to meet the following criteria: clinical stage IV or IIIB (including only patients with no indications for curative radiotherapy, such as malignant pleural effusion, pleural dissemination, malignant pericardial effusion, or metastatic lesion in the same lobe), at least one target lesion >2 cm, no prior chemotherapy, no prior surgery and/or radiotherapy for the primary site, age 20–74 years, Eastern Cooperative Oncology Group performance status (PS) of 0 or 1, adequate hematological, hepatic and renal functions, partial pressure of arterial oxygen ( $\text{paO}_2$ )  $\geq 60$  torr, expected survival >3 months, able to undergo first course treatment in an inpatient setting, and written informed consent. The study was approved by the Institutional Review Board at each hospital. Written informed consent was obtained from every patient.

### treatment schedule

All patients were randomly assigned to one of the four treatment groups by the central registration office by means of the minimization method. Stage, PS, gender, lactate dehydrogenase (LDH) and albumin values, and institution were used as adjustment variables. The first group received the reference treatment, 80 mg/m<sup>2</sup> of cisplatin on day 1 and 60 mg/m<sup>2</sup> of irinotecan on days 1, 8, and 15, and the cycle was repeated every 4 weeks. The second group received 200 mg/m<sup>2</sup> of paclitaxel (Bristol-Myers K.K., Tokyo, Japan) over a 3-h period followed by carboplatin at a dose calculated to produce an area under the concentration–time curve of 6.0 min  $\times$  mg/mL on day 1 and the cycle was repeated every 3 weeks. The third group received 80 mg/m<sup>2</sup> of cisplatin on day 1 and 1000 mg/m<sup>2</sup> of gemcitabine (Eli Lilly Japan K.K., Kobe, Japan) on days 1, 8 and the cycle was repeated every 3 weeks. The fourth group received 80 mg/m<sup>2</sup> of cisplatin on day 1 and 25 mg/m<sup>2</sup> of vinorelbine (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) on days 1, 8 and the cycle was repeated every 3 weeks. Each treatment was repeated for three or more cycles unless the patient met the criteria for progressive disease or experienced unacceptable toxicity.

### response and toxicity evaluation

Response was evaluated according to the Response Evaluation Criteria in Solid Tumors, and tumor markers were excluded from the criteria [21]. Objective tumor response in all responding patients was evaluated by an external review committee with no information on the treatment group. Toxicity grading criteria in National Cancer Institute Common Toxicity Criteria Ver 2.0 were used to evaluate toxicity.

### quality of life assessment

Quality of life (QoL) was evaluated by means of the Functional Assessment of Cancer Therapy—Lung (FACT-L) Japanese version and the QoL Questionnaire for Cancer Patients Treated with Anticancer Drugs (QoL-ACD), before treatment, immediately before the second cycles of chemotherapy, and 3 and 6 months after the start of treatment [22–24].

### statistical analysis and monitoring

The primary end point of this study was overall survival (OS), and the secondary end points were response rate, response duration, time to progressive disease (TTP), time to treatment failure (TTTF), adverse event, and QoL. The 1-year survival rate of the control group in this study was estimated to be 43% based on the data in published papers, and the 1-year survival rate in the other treatment group was expected to be 50%. The lower equivalence limit for 1-year survival rate was set as  $^1-10\%$ . The criterion for the non-inferiority of each treatment was a lower limit of the two-sided 95% confidence interval (CI) of the 1-year survival rate of treatment minus that of control larger than the lower equivalence limit. Because the non-inferiority of each treatment versus the control was to be evaluated independently, a separate null hypothesis was stated for each treatment, and for that reason no multiple comparison adjustment was included in the study. Based on the above conditions and binomial distribution, 135 patients were needed per arm for a one-sided Type I error of 2.5% and 80.0% power. In view of the possibility of variance inflation due to censoring, the sample size was set at 600 (150 per arm).

Central registration with randomization, monitoring, data collection, and the statistical analyses were independently carried out by a contract research organization (EPS Co., Ltd, Tokyo, Japan).

## results

### patient characteristics

From October 2000 to June 2002, a total of 602 patients were registered by 44 hospitals in Japan. All patients had been followed up for >2 years, and 447 patients had died as of June 2004. Of the 602 patients registered, 151 were allocated to the reference treatment, IP, and 150, 151, and 150 patients were allocated to TC, GP, and NP, respectively. Since 10 patients did not receive chemotherapy and 11 patients were subsequently found to be ineligible, 592 patients were assessable for toxicity and 581 patients were assessable for efficacy. Four patients did not receive chemotherapy due to electrolytic disorder, fever, symptomatic brain metastases, and rapid tumor progression in IP, two patients due to refusal and pneumonia in TC, four patients due to lower WBC counts (two patients), rapid tumor progression, and nephritic syndrome in NP. Two patients were ineligible due to wrong stage in IP, two patients were wrong stage and one patient had double cancer in TC, two patients were wrong diagnosis, one patient had massive pleural effusion, one patient received prior chemotherapy in GP, one patient had no target lesions in NP. Age, gender, PS, stage, and LDH and albumin values were well balanced in each arm (Table 1). Fewer patients with adenocarcinoma and more patients with squamous cell carcinoma were, however, entered in three experimental arms than in IP.

### objective tumor response and response duration

Objective tumor response is shown in Table 2. Forty-five partial responses occurred in the 145 assessable patients in the reference arm, IP, for an objective response rate of 31.0% with a median response duration of 4.8 months. The response rate and median response duration were 32.4% and 4.0 months in TC, 30.1% and 3.5 months in GP, and 33.1% and 3.4 months in NP. The response rates in TC, GP, and NP were not statistically different from the rate in IP according to the results of the  $\chi^2$  test.

Table 1. Patient characteristics and treatment delivery

	Cisplatin + irinotecan	Carboplatin + paclitaxel	Cisplatin + gemcitabine	Cisplatin + vinorelbine
Assessable patients	145	145	146	145
Gender (male/female)	97/48	99/46	101/45	101/44
Age, median (range)	62 (30-74)	63 (33-74)	61 (34-74)	61 (28-74)
PS (0/1)	44/101	44/101	45/101	45/100
Histology				
Adenocarcinoma	121	104	108	109
Squamous cell carcinoma	16	31	29	29
Others	8	10	9	7
Stage (IIIB/IV)	31/114	28/117	30/116	26/119
No. of cycles				
Mean $\pm$ SD	3.0 $\pm$ 1.3	3.5 $\pm$ 1.5	3.2 $\pm$ 1.2	3.1 $\pm$ 1.3
Median	3	3	3	3
Range	1-7	1-10	1-7	1-8

PS, performance status; SD, standard deviation.

Table 2. Survival, TTP, TTTF, response rate, and response duration

	N	Median survival months	1-year survival (%)	Difference in 1-year survival from IP	2-year survival (%)	TTP (median), months	TTTF (median), months	Response rate (%)	Response duration (median), months
Cisplatin + irinotecan	145	13.9	59.2	-	26.5	4.7	3.3	31.0	4.8 (n = 45)
Carboplatin + paclitaxel	145	12.3	51.0	-8.2% (95% CI -19.6% to 3.3%)	25.5	4.5 (P = 0.355) <sup>a</sup>	3.2 (P = 0.282) <sup>a</sup>	32.4 (P = 0.801) <sup>b</sup>	4.0 (n = 47)
Cisplatin + gemcitabine	146	14.0	59.6	0.4% (95% CI -10.9% to 11.7%)	31.5	4.0 (P = 0.170) <sup>a</sup>	3.2 (P = 0.567) <sup>a</sup>	30.1 <sup>†</sup> (P = 0.868) <sup>b</sup>	3.5 (n = 44)
Cisplatin + vinorelbine	145	11.4	48.3	-10.9% (95% CI -22.3% to 0.5%)	21.4	4.1 (P = 0.133) <sup>a</sup>	3.0 (P = 0.091) <sup>a</sup>	33.1 (P = 0.706) <sup>b</sup>	3.4 (n = 48)

<sup>a</sup>Compared with IP by the generalized Wilcoxon test.

<sup>b</sup>Compared with IP by the  $\chi^2$  test.

CI, confidence interval; IP, cisplatin plus irinotecan; TTP, time to progressive disease; TTTF, time to treatment failure.

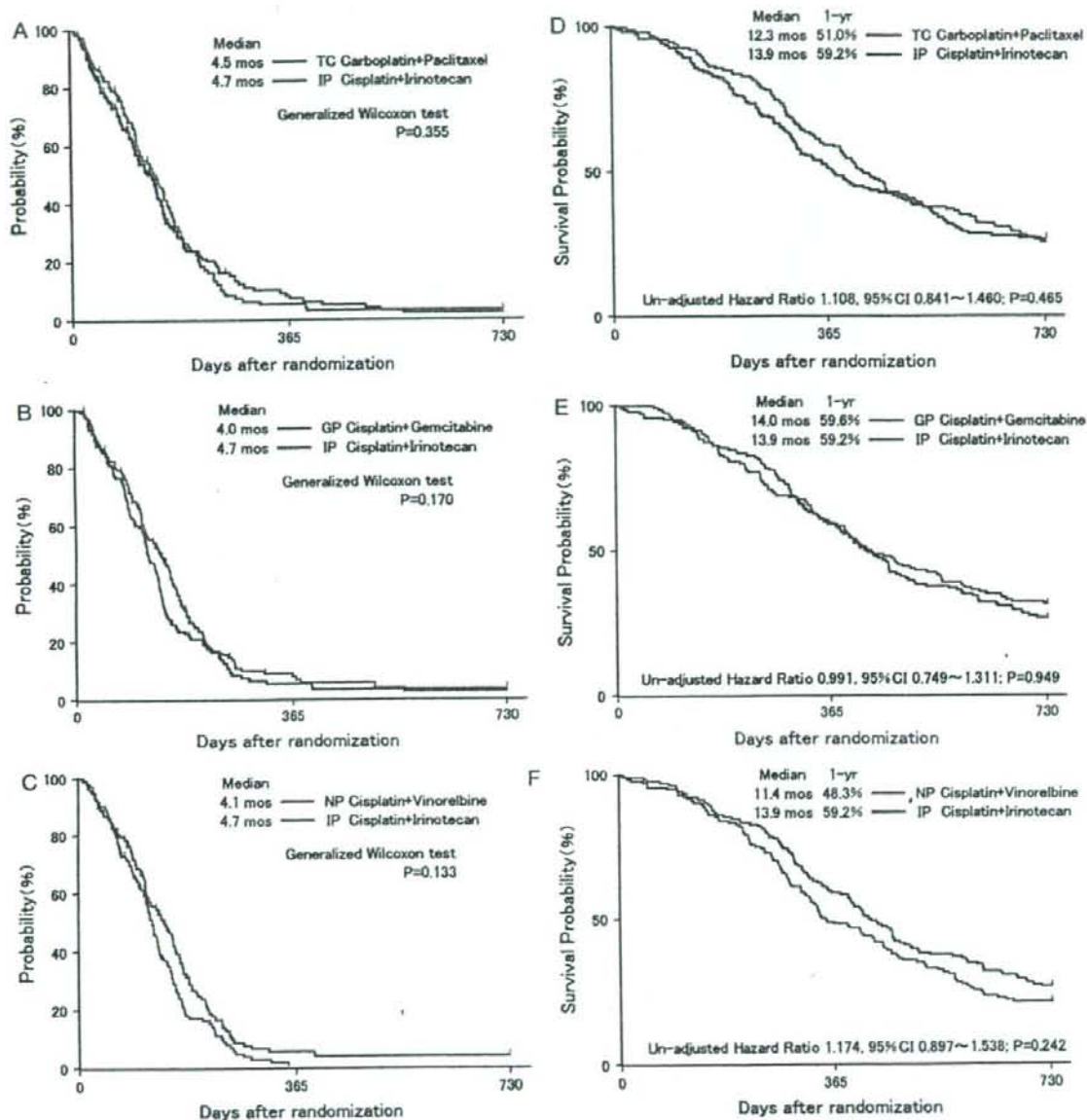
## OS, TTP disease, and TTTF

OS and TTP are shown in Figure 1. Median survival time (MST), the 1-year, and 2-year survival rate in IP were 13.9 months, 59.2%, and 26.5%, respectively. The MSTs, 1-year, and 2-year survival rates were, respectively, 12.3 months, 51.0%, and 25.5% in TC; 14.0 months, 59.6%, and 31.5% in GP; and 11.4 months, 48.3%, and 21.4% in NP. The lower limits of the 95% CI of the difference in 1-year survival rate between IP and TC (-19.6%), GP (-10.9%), and NP (-22.3%) were below -10%, which was considered the lower equivalence limit (Table 2). Thus, the results did not show non-inferiority in three experimental regimens compared with reference treatment. Median TTP and median TTTF were 4.7 and 3.3 months, respectively in IP. Median TTP and TTTF were, respectively, 4.5 and 3.2 months in TC, 4.0 and 3.2 months in GP, and 4.1 and 3.0 months in NP. There were no statistical differences in either TTP or TTTF in TC, GP, or NP, compared with IP according to the results of the generalized Wilcoxon test (Table 2).

## hematologic and non-hematologic toxicity

In IP, 47.6% and 83.7% of patients developed grade 3 or worse leukopenia and neutropenia, respectively (Table 3). The incidences of grade 3 or worse leukopenia (33.1%,  $P = 0.010$ ) and neutropenia (62.9%,  $P < 0.001$ ) were significantly lower in GP than in IP. The incidence of grade 3 or worse leukopenia (67.1%,  $P < 0.001$ ) was significantly higher in NP than in IP. Grade 3 or worse thrombocytopenia developed in 5.4% of the patients in IP, and the incidence was significantly higher in GP (35.1%,  $P < 0.001$ ). The incidence of febrile neutropenia in IP was 14.3%, and was significantly lower in GP (2.0%,  $P < 0.001$ ).

Grade 2 or worse nausea, vomiting, anorexia, and fatigue occurred in 60.5%, 51.0%, 65.3%, and 38.8%, respectively, of the patients in IP. The incidences of grade 2 or worse nausea (TC: 25.0%,  $P < 0.001$ , NP: 47.3%,  $P = 0.022$ ), vomiting (TC: 22.3%,  $P < 0.001$ , NP: 36.3%,  $P = 0.011$ ), and anorexia (TC: 32.4%,  $P < 0.001$ , NP: 49.3%,  $P = 0.005$ ) were significantly lower in TC and NP than in IP. Grade 2 or worse diarrhea was



**Figure 1.** Overall survival (OS) and time to progressive (TTP) disease. TTP and OS in the carboplatin plus paclitaxel (TC) (A, D), cisplatin plus gemcitabine (GP) (B, E), and cisplatin plus vinorelbine (NP) (C, F) were not statistically significantly different from the values in the cisplatin plus irinotecan.

significantly less frequent in TC (6.8%), GP (8.6%), and NP (11.6%) than in IP (48.3%,  $P < 0.001$ ). The incidences of grade 2 or worse sensory neuropathy (16.9%,  $P < 0.001$ ), arthralgia (21.6%,  $P < 0.001$ ), and myalgia (17.6%,  $P < 0.001$ ) were significantly higher in TC than in IP. Grade 2 alopecia occurred in 30.6% of the patients in IP, and its incidence was significantly higher in TC (44.6%,  $P = 0.013$ ) and significantly lower in GP (15.2%,  $P = 0.001$ ) and NP (8.9%,  $P < 0.001$ ). Grade 2 injection site reactions were more frequent in NP (26.7%) than in IP (4.8%,  $P < 0.001$ ).

A total of five patients died of treatment-related toxicity: three in IP (cerebral hemorrhage, interstitial pneumonia, acute circulatory failure/disseminated intravascular coagulation: 2.0%), one in TC (acute renal failure: 0.7%), and one in NP (pulmonary embolism: 0.7%).

#### second-line treatment

Data on second-line treatment, but not third-line or later treatment, was available in this study, and they showed that