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Figure 1. Histopathology of SQSTM1-ALK-positive large B-cell lymphoma.

(A) The pattern of tumor infiltration was diffuse. The lymphoma cells were large with abundant cytoplasm and had round, vesicular nuclei, each containing a centrally located large nucleolus. These features may be consistent with immunoblasts or plasmablasts, but the size of tumor cells was extremely large compared with these typical cell types (40× objective). (B) Some lymphoma cells expressed cytokeratin (AE1/AE3) (20× objective). (C) Syndecan1/CD138 was strongly expressed (20× objective). (D) In anti-ALK immunohistochemistry, a diffuse cytoplasmic staining pattern with ill-demarcated spots was clearly shown (20× objective).

Figure 2. Discovery of *SQSTM-ALK* fusion gene.

(A) A chromosome translocation, t(2;5)(p23.1;q35.3), generates a cDNA fusion in which exon 5 of *SQSTM* is joined to the *ALK* cDNA for the intracellular region of its encoded protein (containing the tyrosine kinase domain). Numbers indicate amino acid positions of each protein. PB1: Phox and Bem1p; Z: atypical zinc finger; U: ubiquitin-associated. (B) A section of the specimen for the present case was subjected to FISH with an *SQSTM1-ALK* fusion assay. Nuclei are stained blue with DAPI. (C) Murine 3T3 fibroblasts were infected with retroviruses expressing SQSTM1-ALK. The cells were photographed after culture for 14 days. (D) A nude mouse was injected subcutaneously with 3T3 cells infected as in (C), and tumor formation was examined after 20 days.

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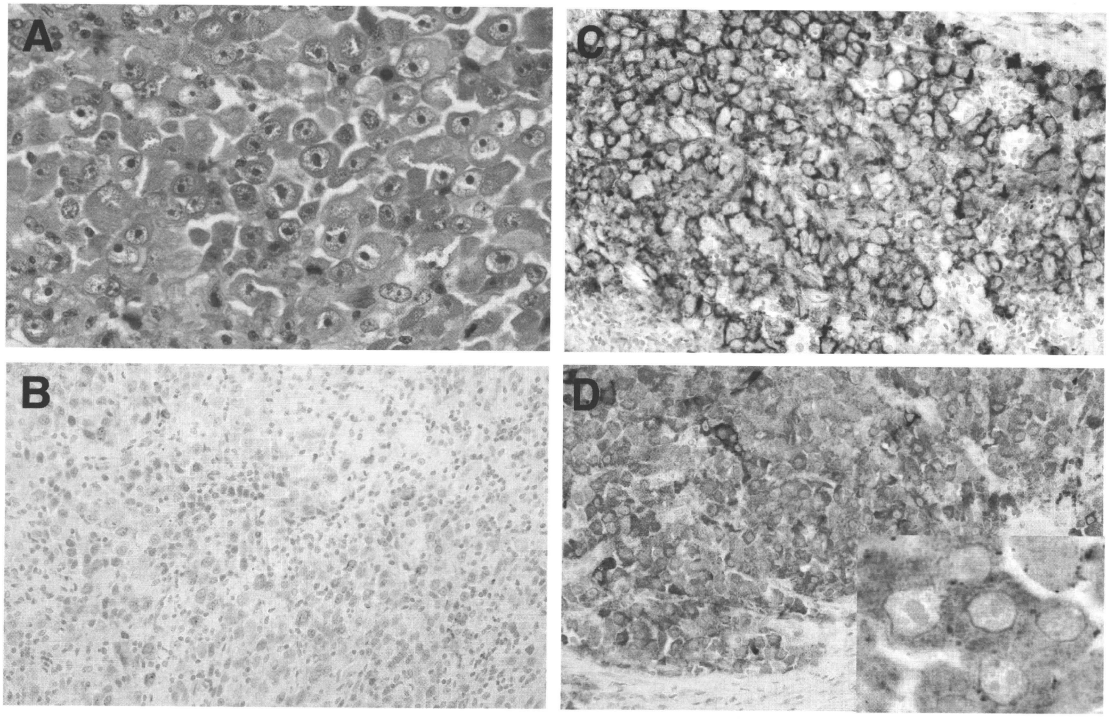

Figure 1

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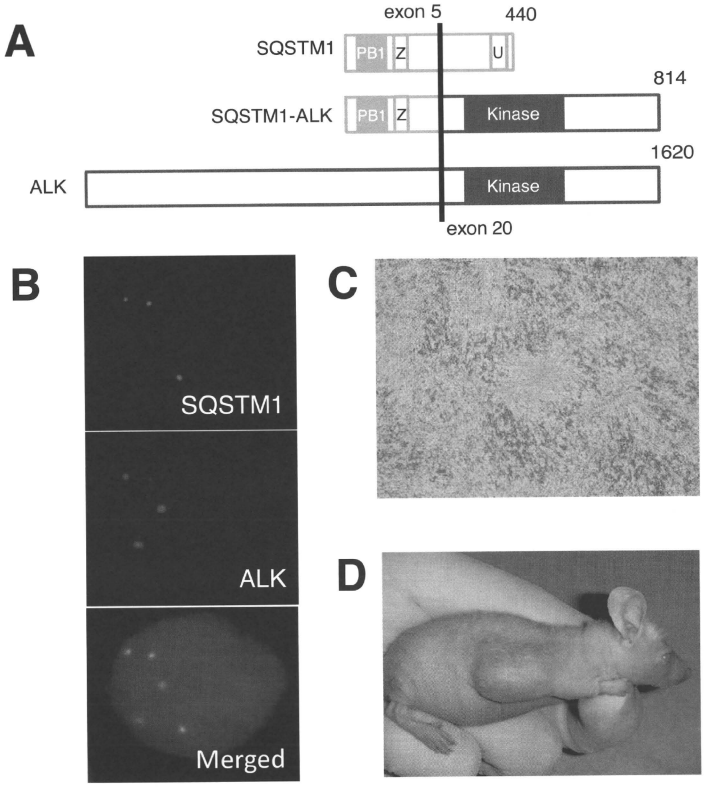

Figure 2

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SQSTM1-ALK fusion cDNA

2499 bp (*SQSTM1* 760bp ~exon 5: *ALK* 1739bp exon 20~)

ORF: 7–2448

ctcgctATGGCGTCGCTCACCGTGAAGGCCTACCTTCTGGGCAAGGAGGACGCGGCGCGCGA
GATTCGCCGCTTCAGCTTCTGCTGCAGCCCCGAGCCTGAGGCGGAAGCCGAGGCTGCGGCGG
GTCCGGGACCCTGCGAGCGGCTGCTGAGCCGGGTGGCCGCCCTGTTCCCCGCGCTGCGGCCT
GGCGGCTTCCAGGCGCACTACCGCGATGAGGACGGGGACTTGGTTGCCTTTTCCAGTGACGA
GGAATTGACAATGGCCATGTCCTACGTGAAGGATGACATCTTCCGAATCTACATTAAAGAGA
AAAAAGAGTGCCGGCGGGACCACCGCCCACCGTGTGCTCAGGAGGCGCCCCGCAACATGGTG
CACCCCAATGTGATCTGCGATGGCTGCAATGGGCCTGTGGTAGGAACCCGCTACAAGTGCAG
CGTCTGCCCAGACTACGACTTGTGTAGCGTCTGCGAGGGAAAGGGCTTGCACCGGGGGCACA
CCAAGCTCGCATTCCCCAGCCCCTTCGGGCACCTGTCTGAGGGCTTCTCGCACAGCCGCTGG
CTCCGGAAGGTGAAACACGGACACTTCGGGTGGCCAGGATGGGAAATGGGTCCACCAGGAAA
CTGGAGCCCACGTCCTCCTCGTGCAGGGGAGGCCCGCCCTGGCCCCACGGCAGAATCAGCTT
CTGGTCCATCGGAGGATCCGAGTGTGAATTTCCTGAAGAACGTTGGGGAGAGTGTGGCAGCT
GCCCTTAGCCCTCTGGTGTACCGCCGGAAGCACCAGGAGCTGCAAGCCATGCAGATGGAGCT
GCAGAGCCCTGAGTACAAGCTGAGCAAGCTCCGCACCTCGACCATCATGACCGACTACAACC
CCAACTACTGCTTTGCTGGCAAGACCTCCTCCATCAGTGACCTGAAGGAGGTGCCACGGAAA
AACATCACCCTCATTCGGGGTCTGGGCCATGGAGCCTTTGGGGAGGTGTATGAAGGCCAGGT
GTCCGGAATGCCCAACGACCCAAGCCCCCTGCAAGTGGCTGTGAAGACGCTGCCTGAAGTGT
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CAGAACATTGTTCGCTGCATTGGGGTGAGCCTGCAATCCCTGCCCCGGTTCATCCTGCTGGA
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TATTTGGAGGAAAACCACTTCATCCACCGAGACATTGCTGCCAGAAACTGCCTCTTGACCTG
TCCAGGCCCTGGAAGAGTGGCCAAGATTGGAGACTTCGGGATGGCCCGAGACATCTACAGGG
CGAGCTACTATAGAAAGGGAGGCTGTGCCATGCTGCCAGTTAAGTGGATGCCCCCAGAGGCC
TTCATGGAAGGAATATTCACTTCTAAAACAGACACATGGTCCTTTGGAGTGCTGCTATGGGA
AATCTTTTCTCTTGGATATATGCCATACCCCAGCAAAAGCAACCAGGAAGTTCTGGAGTTTG
TCACCAGTGGAGGCCGGATGGACCCACCCAAGAACTGCCCTGGGCCTGTATACCGGATAATG
ACTCAGTGCTGGCAACATCAGCCTGAAGACAGGCCCAACTTTGCCATCATTTTGGAGAGGAT
TGAATACTGCACCCAGGACCCGGATGTAATCAACACCGCTTTGCCGATAGAATATGGTCCAC
TTGTGGAAGAGGAAGAGAAAGTGCCTGTGAGGCCCAAGGACCCTGAGGGGGTTCCTCCTCTC
CTGGTCTCTCAACAGGCAAAACGGGAGGAGGAGCGCAGCCCAGCTGCCCCACCACCTCTGCC

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TACCACCTCCTCTGGCAAGGCTGCAAAGAAACCCACAGCTGCAGAGGTCTCTGTTCGAGTCC
CTAGAGGGCCGGCCGTGGAAGGGGGACACGTGAATATGGCATTCTCTCAGTCCAACCCTCCT
TCGGAGTTGCACAGGGTCCACGGATCCAGAAACAAGCCCACCAGCTTGTGGAACCCAACGTA
CGGCTCCTGGTTTACAGAGAAACCCACCAAAAAGAATAATCCTATAGCAAAGAAGGAGCCAC
ACGAGAGGGGTAACCTGGGGCTGGAGGGAAGCTGTACTGTCCCACCTAACGTTGCAACTGGG
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ACCTCTGTTCAGGCTACGTCACTTCCCTTGTGGGAATGTCAATTACGGCTACCAGCAACAGG
GCTTGCCCTTAGAAGCCGCTACTGCCCCTGGAGCTGGTCATTACGAGGATACCATTCTGAAA
AGCAAGAATAGCATGAACCAGCCTGGGCCCtgagctcggtcgcacactcacttctcttcctt
gggatccctaagaccgtgg

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SQSTM1-ALK fusion protein

814 aa (SQSTM1 251aa; ALK 563aa)

MASLTVKAYLLGKEDAAREIRRFSFCCSPEPEAEAEAAAGPGPCERLLSRVAALFPALRPGG
FQAHYRDEDGDLVAFSSDEELTMAMSYVKDDIFRIYIKEKKECRRDHRPPCAQEAPRNMVHP
NVICDGCNGPVVGTRYKCSVCPDYDLCSVCEGKGLHRGHTKLAFPSPFGHLSEGFSHSRWLR
KVKHGHFGWPGWEMGPPGNWSPRPPRAGEARPGPTAESASGPSEDPSVNFLKNVGESVAAAL
SPLVYRRKHQELQAMQMELQSPEYKLSKLRTSTIMTDYNPNYCFAGKTSSISDLKEVPRKNI
TLIRGLGHGAFGEVYEGQVSGMPNDPSPLQVAVKTLPEVCSEQDELDFLMEALIISKFNHQN
IVRCIGVSLQSLPRFILLELMAGGDLKSFLRETRPRPSQPSSLAMLDLLHVARDIACGCQYL
EENHFIHRDIAARNCLLTCPGPGRVAKIGDFGMARDIYRASYYRKGGCAMLPVKWMPPEAFM
EGIFTSKTDTWSFGVLLWEIFSLGYMPYPSKSNQEVLEFVTSGGRMDPPKNCPGPVYRIMTQ
CWQHQPEDRPNFAIILERIEYCTQDPDVINTALPIEYGPLVEEEEKVPVRPKDPEGVPPLLV
SQQAKREEERSPAAPPPLPTTSSGKAAKKPTAAEVSVRVPRGPAVEGGHVNMAFSQSNPPSE
LHRVHGSRNKPTSLWNPTYGSWFTEKPTKKNNPIAKKEPHERGNLGLEGSCTVPPNVATGRL
PGASLLLEPSSLTANMKEVPLFRLRHFPCGNVNYGYQQQGLPLEAATAPGAGHYEDTILKSK
NSMNQPGP

Treatment of Lung Cancer with an ALK Inhibitor After *EML4-ALK* Fusion Gene Detection Using Endobronchial Ultrasound-Guided Transbronchial Needle Aspiration

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A 40-year-old man who had complained of bloody sputum was referred to our hospital for workup. Chest computed tomography showed a significant mediastinal lymphadenopathy (Figure 1*A*). Bronchoscopic examination revealed a tumor compressing the right mainstem bronchus (Figure 2*A*). Massive bleeding from the tumor was caused by passage of the bronchoscope. Therefore, a diagnosis of pulmonary adenocarcinoma was made by sputum cytology. The patient first received conventional chemotherapy in the form of four courses of cisplatin plus vinorelbine (CDDP + VNR), two cycles of cisplatin plus gemcitabine (CDDP + GEM), and four cycles of carboplatin plus gemcitabine (CBDCA + GEM). However, both the size of the tumor and the serum carcinoembryonic antigen level continued to increase. Fluorodeoxyglucose positron emission tomography suggested systemic metastasis in hilar and mediastinal lymph nodes and bone (Figure 1*B*).

We performed endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) to avoid bleeding from the tumor. Metastatic adenocarcinoma was revealed in an upper paratracheal lymph node (#2R) (Figures 2*B*, *C*). Because the epidermal growth factor receptor gene was wild type, we examined the presence of ALK fusion genes. Immunohistochemistry by the intercalated antibody-enhanced polymer (iAEP) method[1] showed an expression of ALK protein in the samples obtained by EBUS-TBNA (Figure 2*D*). *EML4-ALK* fusion gene was also confirmed by both fluorescence in situ hybridization (Figure 2*E*) and reverse transcriptase-polymerase chain reaction (Figure 2*F*). Direct sequencing of the PCR product revealed the presence of *EML4-ALK* variant 1. Thus, we referred the patient for enrollment in a clinical trial with crizotinib (PF-02341066).[2] Six weeks after administration of the crizotinib (250 mg twice a day, oral administration), the bloody sputum disappeared, and the tumor size decreased on chest computed tomography (Figure 1*C*). The carcinoembryonic antigen level also normalized. Five months after administration, an abnormal accumula-

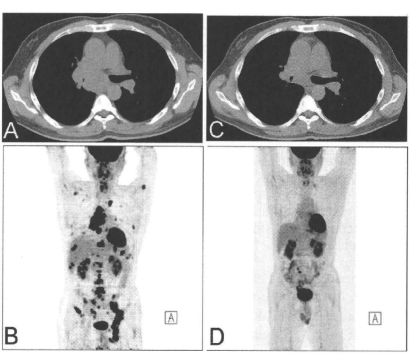

FIGURE 1. *A*, Chest computed tomography showed a narrowing of the right main bronchus due to massive lymphadenopathy. *B*, FDG-PET suggested multiple lymph node metastases and bone metastases. *C*, Six weeks after administration of the ALK inhibitor, the effect of the treatment was judged as partial response based on RECIST. *D*, Five months after administration of the ALK inhibitor, abnormal accumulation on FDG-PET had disappeared. FDG-PET, fluorodeoxyglucose positron emission tomography.

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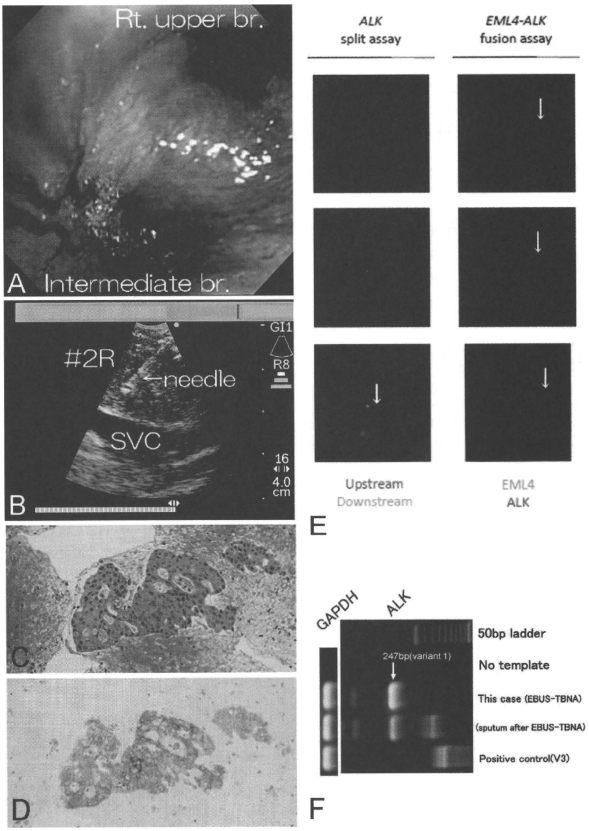

FIGURE 2. *A,* Bronchoscopic examination showed tumor compression of the right main bronchus, and the tumor had hyperplastic vessels on its surface. *B,* EBUS-TBNA was performed for a pretracheal lymph node (#2R). *C,* Histologic core revealed metastatic adenocarcinoma in #2R node. *D,* Immunohistochemistry was positive for ALK protein using the iAEP method. *E,* FISH revealed the EML4-ALK fusion gene. EML4-ALK split assay with labeled probes for the upstream (red) and downstream (green, arrow) region of the ALK locus. EML4-ALK fusion assay with labeled probes for EML4 (green, arrow) or ALK (red, arrow). Fusion gene showed EML4-ALK (arrow). *F,* RT-PCR using specific primer set for each variant also confirmed the presence of EML4-ALK variant 1 (274bp). The presence of variant 1 type fusion was also confirmed by direct sequence of the RT-PCR product (data not shown). RT-PCR, reverse transcriptase-polymerase chain reaction; FISH, fluorescence in situ hybridization.

tion almost disappeared on fluorodeoxyglucose positron emission tomography scan (Figure 1*D*). The observed side effects were only slight nausea during the early period of administration. The patient remains in good condition without tumor relapse for 10 months. The patient suddenly complained bilateral lower extremities paralysis, and the spinal cord metastasis was revealed. The patient was discontinued treatment during the trial in April 2010 because of disease progression.

DISCUSSION

Fusion of *ALK* with *EML4* gives rise to a highly potent oncogene in non-small cell lung cancer,[3] being detected in ~5% of all non-small cell lung cancer cases.[1,3,4] Presence of the ALK fusions can be detected by immunohistochemical screening[4] and can be also confirmed by fluorescence in situ hybridization and reverse transcriptase-polymerase chain reaction.[4] Recently, with progress in chemotherapeutic research, molecular targeted therapeutic agents have been developed, including ALK kinase inhibitors that are now being clinically tested.[2] Ideally, ALK fusion gene assessment should be performed using minimally invasive means to obtain biopsy samples sufficient for genetic analysis for subsequent targeted molecular therapy. Histologic as well as cytologic samples can be obtained by EBUS-TBNA, and we have previously reported that high-quality cores are adequate for molecular analyses for biomarkers.[5] The dramatic

effect of the ALK inhibitor in this patient demonstrates that adequate biomarker assessment contributes to the optimum selection of reagents in targeted molecular therapy and in individualized treatment.

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Original Article

Cancer of Unknown Primary Site: A Review of 28 Cases and the Efficacy of Cisplatin/Docetaxel Therapy at a Single Institute in Japan

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We evaluated the efficacy and toxicity of cisplatin/docetaxel (CDDP/TXT) chemotherapy and identified prognostic factors in Japanese patients with cancer of unknown primary site (CUP). Twenty-eight consecutive patients seen at a single institute were reviewed retrospectively. Sixteen patients were treated with TXT 80 mg/m^2, followed by CDDP 75 mg/m^2. The overall response rate to CDDP/TXT treatment was 62.5%, with a median survival time (MST) of 22.7 months. Common adverse reactions were myelosuppression and hyponatremia. The MST of all 28 patients with CUP was 8.3 months, and the 1-year overall survival rate was 45.6%. Univariate analysis identified 5 prognostic factors:performance status, liver involvement, bone involvement, pleural involvement, and lymph node involvement. In conclusion, CDDP/TXT chemotherapy is effective with tolerable toxicity in patients with CUP. Japanese patients with CUP might be chemosensitive and may survive longer.

Key words: cancer of unknown primary site (CUP), cisplatin, docetaxel, prognosis

Cancer of unknown primary site (CUP) is defined as the presence of metastatic cancer documented in the absence of an identifiable primary tumor site. These tumors are not rare; they represent 3-5% of all malignancies diagnosed in oncology practice [1, 2]. CUP occurs in a heterogeneous group of patients, and subgroups with treatment-responsive diseases exist that may achieve long-term, disease-free survival [1]. Generally, however, the prognosis of CUP is poor, with median survival times of 6-12 months, and the benefits of chemotherapy compared with best supportive care remain unclear [3].

Chemotherapy for patients with CUP is improving, but no chemotherapy regimen has been established as a standard first-line therapy for these patients [2]. Recent clinical reports have shown that cisplatin (CDDP)-containing regimens have good response rates of 32-55% in patients with CUP and are relatively well-tolerated [3, 4]. Docetaxel (TXT) has definite antitumor activity in various solid tumors and seems to

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be a good candidate for inclusion in a chemotherapy regimen for patients with CUP [5]. A CDDP plus TXT phase II study revealed a 26% response rate with 42% 1-year survival [6].

This paper presents a retrospective analysis of 28 consecutive Japanese patients with CUP to clarify the disease course and prognostic factors. We also report an excellent response rate and survival of Japanese patients with CUP who were treated using a combination regimen of CDDP/TXT.

Patients and Methods

Patients. Twenty-eight consecutive patients referred to the Division of Medical Oncology and Hematology at the Cancer Institute Hospital between April 1, 2000 and September 30, 2004 were reviewed retrospectively. Patients referred with a presumed diagnosis of CUP were identified and registered in the database at the time of their initial clinical evaluation. All patients diagnosed with CUP during this period were registered; however, 2 female patients with adenocarcinoma involving only the axillary lymph nodes were treated for occult breast cancer and were excluded from this analysis. The medical records of the patients were reviewed for the results of diagnostic studies and pathologic and cytologic diagnosis before referral, the results of subsequent radiographic evaluations, pathology review, involved disease sites, treatment, and survival.

Clinical evaluation. All patients with CUP underwent a basic evaluation consisting of a complete medical history, a physical examination (including careful palpation of the thyroid, breasts, lymph nodes, and prostate), general laboratory studies, chest radiography, and computed tomography from the neck to pelvis. If possible, gastrointestinal endoscopy, nose and pharyngeal endoscopy, and bronchoscopy were conducted. Positron emission tomography (PET) was performed in some patients when all other tests were inconclusive. In some cases, an extensive immunohistochemical study was carried out with the biopsied specimen to minimize the possibility of a misdiagnosis of other malignancies such as non-Hodgkin's lymphoma, extragonadal germ cell tumor, malignant melanoma, or undifferentiated sarcoma [2]. The most commonly used markers were the leukocyte common antigen, cytokeratins, neuron-specific enolase or chromogranin, S-100 protein, vimentin, thyroid transcription factor-1 (TTF-1), estrogen receptors, HMB45, and prostate-specific antigen (PSA). The blood concentrations of CA19-9, CA15-3, CA125, and carcinoembryonic antigen (CEA) were assessed in most cases.

Decision-making in the 'cancer board meeting'. Determining whether the primary site is unknown or whether it will be possible to detect with further evaluation is difficult. In the present study, members of the cancer board, including medical oncologists, hematologists, surgeons, pathologists, and radiation oncologists, evaluated the diagnosis and treatment strategies for the patients with CUP.

Treatment schedule for CDDP/TXT therapy. Eligible patients with CUP were treated with CDDP/TXT combination chemotherapy. All patients gave written informed consent. TXT 80 mg/m^2 in 300 mL of normal saline was administered over 2 h, followed by CDDP 75 mg/m^2, which was administered via a 120-min intravenous infusion. Premedication included intravenous administration of 4 mg of dexamethasone 24 h before treatment, 30 min before starting the docetaxel infusion, and 24 h after the infusion. A single 3-mg intravenous dose of granisetron was given to all patients as an antiemetic. Concurrent radiotherapy for symptom control in the absence of disease progression was allowed, but the drugs were held for at least 2 weeks after irradiation. Chemotherapy cycles were repeated every 3 weeks. Doses were modified for some patients mainly due to hematological toxicity.

Assessment of response and toxicity. Responses were defined according to the World Health Organization criteria [7]. Briefly, complete response (CR) was defined as the entire disappearance of all assessable lesions and signs of disease for at least 4 weeks. Partial response (PR) was defined as a reduction of 50% or more in the sum of the products of the perpendicular dimensions of measurable lesions and the appearance of no new lesion for at least 4 weeks. No change (NC) was defined as a decrease of less than 50% or an increase of less than 25% in the 2 greatest dimensions of measurable lesions and the appearance of no new lesions. Progressive disease (PD) was defined as any evidence of disease progression of 25% or more, or the appearance of a new lesion. Chemotherapy-related adverse events were recorded according to the National Cancer Institute's Common

Terminology Criteria, version 3.0 [8].

Statistical methods. Survival was calculated from the first day of pathologically or cytologically diagnosed malignancy. Survival following CDDP/TXT therapy was calculated from the first day of treatment. Survival curves were estimated using the Kaplan-Meier method [9] and compared using the Cox-Mantel log-rank test [10]. StatView 5.0 (SAS Institute, Cary, NC, USA) was used for the statistical analyses.

Results

General patient characteristics. The characteristics of our 28 patients (19 men and 9 women) are listed in Tables 1 and 2. One female patient was excluded from the survival analysis because she was postoperatively diagnosed with ovarian cancer. The median age at diagnosis was 58.5 years (range 32–76 years). Performance status according to the Eastern Cooperative Oncology Group (ECOG) [11] was 0–1 in 17 patients (60.7%). The sites of metastasis documented pathologically, cytologically, or radiographically are listed in Table 3. Lymph nodes were involved most frequently (64.3%), and visceral metastases including bone, lung, or liver were also common. The lymph node involvement was further subclassified by anatomic site. Of the 18 patients with nodal metastases, 11 had retroperitoneal, 8 had supraclavicular or cervical, 7 had mediastinal, 4 had axillary, and 4 had inguinal lymph nodes.

Table 1 Characteristics of 28 CUP patients

Characteristic	No. of Patients
Sex	
Female	9
Male	19
Age, years	
0–40	1
41–50	6
51–60	8
61–70	10
71–80	3
Median	58.5
Range	32–76
Performance status	
0–1	17
2–3	11

The pathological diagnoses of the patients are also listed in Table 2. Thirteen patients (46.4%) were diagnosed with adenocarcinoma, 9 (32.1%) with poorly differentiated carcinoma, 2 (7.1%) with squamous cell carcinoma, and 4 with unknown or other diagnoses. One patient was diagnosed based only on the cytology of ascites. No patients with neuroendocrine carcinoma were included in this study. No patients appeared to belong to subgroups with a favorable prognosis [1].

Twelve patients (42.9%) had a single metastatic organ site, 4 (14.3%) had 2, 7 (25.0%) had 3, and 5 (17.9%) had 4 or more. Serum tumor markers at baseline were assessed in all 16 patients who underwent CDDP/TXT therapy.

Twenty-five patients (89.3%) were treated with chemotherapy with or without concurrent radiotherapy for symptom control. One patient was treated with radiotherapy only. Two patients were treated with supportive care alone.

CDDP/TXT treatment. Sixteen patients who received the CDDP/TXT combination according to the protocol were assessable for response. The patient

Table 2 Sites of tumor involvement and histologic diagnoses in 28 patients with CUP

Site of involvement	No. of patients
Lymph nodes	18
Bone	10
Lung	7
Liver	6
Pleura/pleural space	4
Peritoneum	4
Skin	3
Adrenal	2
Others	9
Histologic Diagnosis	**No. of patients**
Adenocarcinoma	13
Poorly differentiated	4
Papillary	1
No descriptor/other	8
Poorly differentiated carcinoma	9
Squamous cell carcinoma	2
Unknown/other	4
No. of involved organ sites	**No. of patients**
1	12
2	4
3	7
4 or more	5

Table 3 Characteristics of 16 patients treated with the CDDP/TXT regimen

Characteristic	No. of Patients
Sex	
Female	6
Male	10
Age, years	
Median	62.5
Range	41–76
PS	
0–1	12
2–3	4
Histology	
Adonocarcinoma	10
Poorly differentiated	2
Papillary	1
No descriptor/other	7
Poorly differentiated Carcinoma	4
Squamous cell carcinoma	0
Unknown/other	2
Metastatic sites at presentation	
Lymph nodes	10
Bone	6
Lung	2
Liver	1
Pleura/pleural space	1
Peritoneum	2
Skin	1
Adrenal	1
Others	4
No. of involved organ sites	
Single site	9
Multiple (≥2) sites	7
No. of courses given	
Median	3
Range	1–6

characteristics were similar to those of all 28 patients (Table 3): 10 men and 6 women, median age 62.5 years (range 41–76 years). However, performance status (PS) and the number of metastatic sites were lower, with PS 0–1 in 12 patients and single-site involvement in 9. In 8 patients (50%), more than 2 tumor markers had increased at diagnosis. The median duration from the day of the pathological diagnosis of metastatic carcinoma to the first day of CDDP/TXT therapy was 43 days (range 0–154 days). A total of 44 cycles of therapy was given, and the patients underwent a median of 3 treatment cycles (range 1–6 cycles). Doses were modified mainly because of hematological toxicity; 4 patients had a 20% dose reduction.

The overall response rate was 62.5% (95% CI 8.6–81.5%), with CR in one patient and PR in nine patients. Six of 10 patients with adenocarcinoma responded, and all 4 patients with poorly differentiated carcinoma responded. Tumor markers decreased in most responding patients. Fig. 1 shows the survival curve for these patients. The median follow-up was 20.4 months (range 1.7–60.2 months), the median disease-free survival (DFS) was 8.7 months, the 1-year overall survival (OS) rate was 68.8% (95% CI 40.6–91.5%), and the median OS was 22.7 months. The median hospitalization stay of the 16 patients treated with CDDP/TXT therapy was 65.5 days (range 26–162 days).

Toxicity data are listed in Table 4. Grade 3–4 neutropenia was frequent (14 patients, 87.5%). One patient who had multiple lung, liver, and bone metastases died of bacterial pneumonia due to neutropenia. Hyponatremia occurred in 14 patients (87.5%) and was grade 3–4 in 3 patients; however, all patients were able to continue the treatment. The hyponatremia was caused by a loss of sodium due to renal tubule damage caused by cisplatin or the syndrome of inappropriate secretion of antidiuretic hormone (SIADH). Grade 3 allergic reactions due to docetaxel occurred in 2 patients during the first course. These 2 patients were given hydrocortisone and treated with cisplatin-only beginning with the next course. Other recorded toxicities were mild to moderate.

Prognostic factors for survival in the 28 patients with CUP. Fig. 2 shows the survival

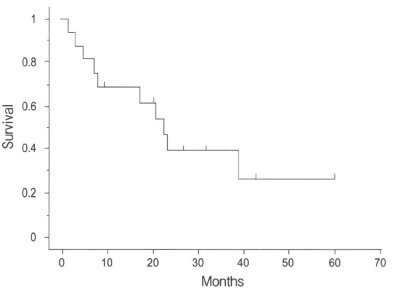

Fig. 1 Kaplan-Meier survival curve for the CUP patients treated with CDDP/TXT chemotherapy (n = 16).

curve for the 28 patients with CUP calculated from the day of diagnosis. The 1-year OS rate for all 28 patients was 45.6% (95% CI 26.8–64.4%), and the median survival was 8.3 months.

Table 5 lists the median survival of the CUP patient subgroups according to various factors. The univariate analysis revealed that 4 factors were deleterious: performance status 2–4, liver involvement, bone involvement, and pleural involvement. The advantageous clinical feature was lymph node involvement.

Discussion

In this study, we obtained an excellent response rate and survival with CDDP/TXT therapy for patients with CUP. An overall response rate of 62.5% was seen in patients with CUP who were given the CDDP/TXT combination once every 3 weeks. The median disease-free survival was 8.7 months. The 1-year OS was 68.8%, and the median OS was 22.7 months. The response rate and survival were superior to those obtained in the reported phase II trials of platinum plus taxane-based chemotherapy [2, 5, 6, 12]. Greco *et al.* reported a prospective phase II study of the CDDP/TXT regimen with a response rate of 22% and a 1-year survival of 40% [6]. The patient characteristics and dose intensity were similar to those in our study, although they had fewer patients with a single metastasis.

Yakushiji *et al.* have reported that 35 Japanese patients received a median of four cycles of CDDP and TXT, and had a response rate of 57.1%. The median survival time was 13.2 months [13]. These results in Japanese patients together with ours in the present study seem to be better than those reported from other countries. Although the prognostic factors in our reports are similar to those for other countries, Japanese patients with CUP might be chemosensitive and thus survive longer.

The treatment-related toxicity of the CDDP/TXT regimen mainly involved myelosuppression; in particular, grade 3–4 neutropenia was severe. Non-prophylactic G-CSF seemed to be the cause of this severity. One patient died of bacterial pneumonia due to neutropenia on day 12 of the first course. This patient was a 62-year-old man with PS 3. He had multiple metastases to lung, liver, and bone. He was therefore at high risk of pneumonia and had a very poor prognosis. Although hyponatremia occurred in 87.5% of our patients, it has not been reported in other studies that treated patients with CUP using platinum-containing regimens. Greco *et al.* have reported the toxicities of CDDP/TXT therapy to consist primarily of gastrointestinal events, with myelosuppression being moderate [6]. Based on urinalysis and the serum osmolarity, SIADH was the main cause of hyponatremia in the present study (data not shown). Since 1990, many Japanese researchers have reported SIADH following platinum administration for solid tumors [14, 15]. Collectively, Japanese patients seem to be more sensitive to platinum in terms of developing SIADH. Other recorded toxicities are mild to moderate. Overall, the regimen is generally tolerated in the majority of patients.

Table 4 Toxicity of CDDP/TXT therapy as worst grade per patient (n = 16)

Toxicity	No. of patients (%)			
	Grade 1	Grade 2	Grade 3	Grade 4
Anemia	6 (37.5)	6 (37.5)	1	0
Thrombocytopenia	4 (25.0)	1	0	1
Leukocytopenia	0	5 (31.3)	7 (43.8)	3 (18.8)
Granulocytopenia	0	1	3 (18.8)	11 (68.8)
Diarrhea	2 (12.5)	0	0	0
Nausea	3 (18.8)	5 (31.3)	4 (25.0)	0
Hyponatremia	11 (68.8)	0	3 (18.8)	0
High serum bilirubin	2 (12.5)	0	1	0
High serum creatinine	7 (43.8)	0	0	0
Allergic reaction	0	0	2 (12.5)	0

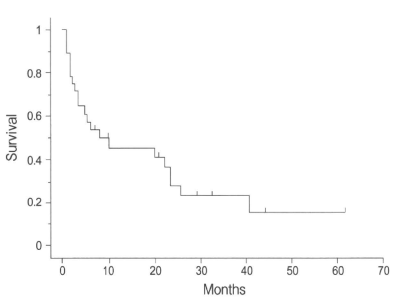

Fig. 2 Kaplan-Meier survival curve of all 28 CUP patients.

Table 5 Median survival duration of defined patient populations with CUP

Variable		No. of Patients	Median survival (days)	*p*-value
Sex				
Female		9	712	0.2599
Male		19	186	
No. of organ sites				
1		12	709	0.1180
2-6		16	108	
Performance status				
0-1		17	673	**0.0413**
2-3		11	79	
Involved organ sites				
Lymph nodes	+	18	673	**0.0310**
	−	10	148	
Liver	+	6	50	**0.0002**
	−	22	673	
Bone	+	10	108	**0.0380**
	−	18	673	
Lung	+	7	67	0.0642
	−	21	604	
Pleura/pleural space	+	4	31	**0.0040**
	−	24	604	
Brain	+	1	108	0.3704
	−	27	306	
Peritoneum	+	4	67	0.1458
	−	24	306	
Adrenal	+	2	33	0.6633
	−	26	249	
Skin	+	3	51	0.1820
	−	25	306	
Therapy				
CDDP/TXT		16	681	<0.0001
Other		12	73	
Histology				
Adenocarcinoma		13	673	
Poorly differentiated carcinoma		9	306	
Squamous cell carcinoma		2	33	
Unknown or others		4	67	

Clinical subsets of patients who are sensitive to platinum-containing treatment have been identified in the last 2 decades [2]. In this study, one patient almost fit into a favorable subset. She was a 73-year-old woman with peritoneal adenocarcinoma, but no papillary serous carcinoma, so she did not completely conform to a favorable subset. She was given three courses of CDDP/TXT, attained good PR, and was still alive at the 23.6-month follow-up. Therefore, although no patients completely matched the subgroups with a favorable prognosis, the CDDP/TXT regimen in this study was found to be very beneficial.

We also analyzed the course and prognostic factors of the patients with CUP. Univariate analysis identified 4 factors predicting a poor prognosis: performance status 2-4, liver involvement, bone involvement, and pleural involvement. The one favorable prognostic factor was lymph node involvement. Abbruzzese *et al.* examined prognostic factors in 657 consecutive patients and found that male sex, increased numbers of involved organ sites, adenocarcinoma histology, and hepatic involvement were negative prognostic factors in a multivariate analysis [16]. They also reported that lymph node involvement,

peritoneal involvement, and neuroendocrine histology are favorable prognostic factors [16]. Our results confirm the reported analyses; *i.e.*, visceral metastasis, multiple metastatic sites, and poor performance status may predict shorter survival.

Recently, a new combination of chemotherapy has been reported that includes gemcitabine, etoposide, or irinotecan combined with platinum plus taxane; these studies have shown similar response and survival rates [17, 18]. New agents such as vascular endothelial growth factor (VEGF) inhibitors and epidermal growth factor receptor (EGFR) inhibitors are being tested to improve the prognosis of CUP. Hainsworth *et al.* have reported that bevacizumab plus erlotinib therapy has a response rate of 10%, leading to stable disease in 61%, a median OS of 7.4 months, and a 1-year OS of 33% [19]. Therapeutic trials involving platinum and taxane plus VEGF or EGFR inhibitors should be conducted to improve the survival of patients with CUP.

In conclusion, although this was a retrospective study, we observed an excellent response rate and survival with CDDP/TXT chemotherapy in Japanese patients with CUP, despite their being in unfavorable subgroups. Our results show that bone, liver, and pleural metastasis, and poor performance status may predict a shorter survival.

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Original article

Combination of morphological feature analysis and immunohistochemistry is useful for screening of EML4-ALK-positive lung adenocarcinoma

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ABSTRACT

Background A subset of lung cancers harbours the fusion gene echinoderm microtubule-associated protein-like-4—anaplastic lymphoma kinase (EML4-ALK). Recently, immunohistochemistry for ALK has shown sensitivity for the detection of EML4-ALK-positive lung adenocarcinoma almost equal to that of the fluorescence in situ hybridisation (FISH) assay.

Aims To study the clinicopathological features of EML4-ALK-positive lung adenocarcinoma in a large number of surgically resected samples using immunohistochemistry, in order to establish a useful screening method for EML4-ALK-positive lung adenocarcinoma.

Methods Immunohistochemistry for ALK was used to screen for EML4-ALK-positive lung adenocarcinomas in 254 cases of surgically resected samples.

Results EML4-ALK-positive cases were detected in 3.1% of lung adenocarcinomas (8/254). EML4-ALK-positive lung adenocarcinomas showed significant associations with intra- and/or extra-cytoplasmic mucin ($p=0.0001$), and cribriform pattern with excessive extracytoplasmic mucin ($p<0.0001$). Signet-ring cell appearance alone lacked significance ($p=0.149$).

Conclusion EML4-ALK-positive lung adenocarcinoma has a tendency to express a characteristic morphological pattern. The combined use of morphological feature analysis and immunohistochemistry may be a useful and cost effective screening method for EML4-ALK lung adenocarcinoma.

INTRODUCTION

Lung cancer is the major cause of cancer-related deaths in the world. It is known that some gene mutations are involved in carcinogenesis of the lung. Epidermal growth factor receptor (EGFR) is an example of a gene mutation in lung cancer. Activation of the mutation in EGFR defines a group of patients with sensitivity to the chemical inhibitor for the kinase activity of EGFR, accounting for about 10% of primary lung cancers.[1 2] In 2007, Soda *et al* identified the fusion oncogene joining the echinoderm microtubule-associated protein-like-4 (EML4) and the anaplastic lymphoma kinase gene (ALK) in non-small-cell lung cancer (NSCLC).[3] The detection of EML4-ALK-positive lung cancer is needed to identify lung cancer patients for molecular target therapy. Some authors recommend molecular cascade screening for the detection of EML4-ALK-positive lung cancer.[4] The cascade is a stepwise approach to test for gene mutations in lung adenocarcinoma: first for KRAS, second for EGFR, and third for EML4-ALK translocation. However, it is an expensive and time-consuming method. Moreover, EML4-ALK-positive lung cancer accounts for only about 5% of all NSCLC patients according to previous reports.[3 5–8] To find a more efficient screening method for EML4-ALK-positive lung cancer, more precise clinical features and histopathological findings of EML4-ALK-positive lung cancer must be determined.

A previous report showed that EML4-ALK-positive lung cancers are characterised by smaller tumour size, and found in younger patients who are non-smokers or light smokers.[6] Another study reported that EML4-ALK-positive lung cancer showed acinar morphology[9] and signet-ring cell appearance.[5 10]

Recently, immunohistochemistry for ALK has shown sensitivity for the detection of EML4-ALK-positive lung adenocarcinoma almost equal to that of the fluorescence in situ hybridisation (FISH) assay.[11] In this study, the clinicopathological features of EML4-ALK-positive lung adenocarcinoma were studied in a large number of surgically resected samples using immunohistochemistry, in order to establish a useful screening method for EML4-ALK-positive lung adenocarcinoma.

MATERIALS AND METHODS

Patients and sample collection

This study included samples from 254 Japanese NSCLC patients with a diagnosis of lung adenocarcinoma who underwent surgery at Osaka Police Hospital (Osaka, Japan) between January 2000 and December 2009. Clinical data were collected from inpatient and outpatient medical records.

Histological analysis

All surgically resected lung tumour specimens were embedded in paraffin and serial 5 μm thick sections were prepared. The pathological examination was based on standard H&E stained slides from all blocks of tissues. Pathological stage and differentiation were evaluated according to the current international tumour node metastasis (TNM) staging system and the World Health Organization classification. All tumour slides (consisting in each case of 1–25 slides, with a mean of six slides) were reviewed, and classified according to WHO histological types. In this study, bronchioloalveolar carcinoma is a pure type of carcinoma without invasion. The mixed subtypes category contained varied lesions. Therefore, in this study, an

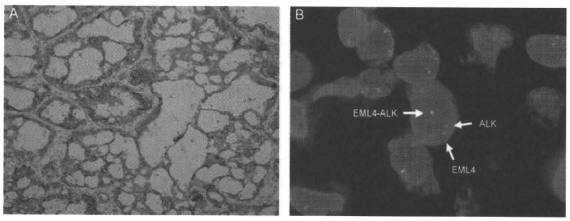

Figure 1 (A) Anaplastic lymphoma kinase (ALK) protein was expressed in the cytoplasm with no nuclear staining. (B) Fluorescence in situ hybridisation assay of echinoderm microtubule-associated protein-like-4—ALK.

additional predominance classification was used, as described in a previous report.[6] Regardless of percentage, the most dominant subtype was regarded as the predominant subtype. The remaining subtypes were regarded as minor components.

Three pathologists (RJ, TY, MT) reviewed all tumour slides of all cases. The decision on discrepant cases was made by consensus when two of the three pathologists agreed. All discrepant cases (10 cases) resulted in consensus.

Immunohistochemical analysis

Unstained paraffin-embedded sections were deparaffinised in xylene, hydrated through and rinsed in distilled water. Heat-induced epitope retrieval was performed with EnVision FLEX Target Retrieval Solution, High pH (Dako, Carpinteria, California, USA). Slides were then incubated at room temperature with antibody to ALK (clone 5A4, 1:100, Abcam) for 30 min. To increase the sensitivity of detection, the intercalated antibody-enhanced polymer (iAEP) method was used with minor modifications.[12] Slides were incubated at room temperature with EnVision FLEX+ Mouse Linker (Dako) for 15 min. The immune complexes were then detected with the dextran polymer reagent.

Table 1 Clinicopathological comparison between echinoderm microtubule-associated protein-like-4—anaplastic lymphoma kinase (EML4-ALK)-positive and -negative lung adenocarcinoma

	(n=254)	EML4-ALK (+) (n=8)	EML4-ALK (−) (n=246)	p Value
Age	Mean±SD	61.75±11.41	66.55±9.246	0.1524*
	Median	65.5	67.5	
	Range	44–77	42–87	
Sex	Male	4	126	1‡
	Female	4	120	
Smoking habit	Never smoker	2	49	0.114†
	Ever smoker	5	79	
	Unknown	1	118	
Tumour size (mm)	Mean±SD	25.88±10.3	23.58±11.65	0.581*
	Median	26.5	21	
	Range	10–45	5–75	
Differentiation	Well (G1)	0	132	0.0025‡
	Less (G2, G3)	8	114	(G1 vs G2, G3)
Tumour status	pT1	6	169	1‡ (pT1 vs T2-4)
	pT2–pT4	2	77	
Lymph node metastasis	pN0	4	193	0.0782‡ (pN0 vs pN1-3)
	pN1–3	4	53	
TNM stage	pStage I	4	175	0.2403‡ (pStage I vs pStage II–IV)
	pStage II–IV	4	71	
EGFR mutation (n=67)	(+)	0	33	
	(−)	8	26	

*Student's t test b.
†χ^2 test.
‡Fisher's exact test.
EGFR, epidermal growth factor receptor.

FISH assay

FISH was performed on formalin-fixed paraffin-embedded tumour tissues with use of fluorescently-labelled bacterial artificial chromosome clone probes specific to the ALK and the EML4 loci (EML4 RP11-996L7; ALK RP11-984I21, RP11-62B19) using a histology FISH accessory kit (Dako).

DNA extraction and mutation analysis of EGFR, KRAS

Because no fresh sample was available for this study, DNA was extracted from formalin-fixed paraffin-embedded tissues; mutations in the EGFR (exons 18–21) genes were analysed by the peptide nucleic acid locked nucleic acid PCR-clamp (PNA LNA PCR-clamp) technique[15] and in the KRAS (codons 12,13) genes by direct sequencing.[14] Mutation analysis was performed on 67 cases, for which informed consent was obtained from patients. The ethics committee of our institute approved the genetic analyses in the present study.

Statistical analysis

Statistical analysis for the tumour size and age was carried out using Student's t test. The values are shown as mean ± SD. The relationship between EML4-ALK expression and clinicopathological variables was analysed with the χ^2 test or Fisher's exact test. Statistical significance was defined as $p<0.05$.

RESULTS

Clinical presentation

Using immunohistochemistry for ALK, the expression of ALK was studied in 254 lung adenocarcinomas. Eight of 254 cases showed homogeneous ALK protein in the cytoplasm without nuclear staining. The percentage of positive tumour cells ranged from 80% to 100%. Varied intensity of ALK expression was observed. The FISH assay confirmed the presence of the EML4-ALK fusion gene in the eight cases that showed the ALK protein (figure 1).

Table 1 summarises details of the clinicopathological features of all cases. All patients were Japanese, ranging in age from 42 to 87 years, with a mean age of 66.4 years. The maximum diameter of their tumours ranged from 0.5 to 7.5 cm. EML4-ALK-positive

lung adenocarcinomas were significantly less differentiated than the EML4-ALK-negative lung adenocarcinomas (p=0.0025). EML4-ALK-positive and EML4-ALK-negative lung adenocarcinomas showed no difference in age, sex, tumour size, smoking habit and TNM stage. However, EML4-ALK-positive lung adenocarcinomas showed a tendency for association with younger patients, positive lymph nodes, adverse pathological stage and smoking habit. All of the EML4-ALK-positive cases had no mutation of the EGFR and KRAS genes.

Pathological findings and immunohistochemistry

Histologically, the 254 adenocarcinomas were comprised of 153 mixed subtypes, 34 acinar, 30 papillary, 2 others and 35 bronchioloalveolar carcinomas based on the WHO classification. The mixed subtypes category contained varied lesions. Therefore an additional predominance classification was used, as described in a previous report.[6] According to the predominance subtyping, the 254 adenocarcinomas were comprised of 85 acinar, 83 papillary, 74 bronchioloalveolar non-mucinous carcinomas, 7 bronchioloalveolar mucinous carcinomas and 5 others (table 2).

Table 2 also summarises the histological features of all cases. According to the predominance subtypes, six of eight EML4-ALK-positive lung adenocarcinomas (75%) were subclassified as acinar-predominant adenocarcinomas and the other two cases were papillary-predominant adenocarcinomas. When compared with EML4-ALK-negative lung adenocarcinoma, the association of EML4-ALK-positive lung adenocarcinoma and acinar morphology was statistically significant (p=0.0184, Fisher's exact test). In other words, six of 85 (7.0%) acinar-predominant adenocarcinomas were positive for EML4-ALK fusion.

Lung adenocarcinoma cells have intracytoplasmic mucin in varying degrees and sometimes have extracytoplasmic mucin. Some lung adenocarcinoma cells show signet-ring cell like appearance because of excessive intracytoplasmic mucin (figure 2). Also, other lung adenocarcinomas show a cribriform pattern with excessive extracytoplasmic mucin (figure 3). In the 254 cases examined, 36 had intracytoplasmic or extracytoplasmic mucin detected by Alcian blue stain.

Table 2 Subtypes by predominance classification

	Total	EML4-ALK (+)	EML4-ALK (−)
	254	8 (3.1%)	246
Papillary	83	2	81
Acinar	85	6	79*
Bronchioloalveolar carcinoma, non-mucinous	74	0	74
Bronchioloalveolar carcinoma, mucinous	7	0	7
Others	5	0	5

*Fisher's exact test, p=0.0184 (acinar predominant adenocarcinoma vs the other adenocarcinoma).
EML4-ALK, echinoderm microtubule-associated protein-like-4—anaplastic lymphoma kinase.

EML4-ALK-positive lung adenocarcinomas showed significant associations with intra- and/or extra-cytoplasmic mucin (p=0.0001), and cribriform pattern with excessive extracytoplasmic mucin (p<0.0001). Signet-ring cell appearance alone lacked significance (p=0.149) (table 3).

Two of eight EML4-ALK-positive cases did not have a characteristic morphological feature, nor did they show any mucin production (tables 4 and 5).

DISCUSSION

A large-scale screening conducted for EML4-ALK-positive lung adenocarcinomas in our institute detected eight cases. In this study, the association of EML4-ALK-positive lung adenocarcinoma and acinar-predominant morphology, extra- and/or intracytoplasmic mucin production and cribriform pattern with excessive extracytoplasmic mucin was demonstrated. A previous report showed that EML4-ALK-positive lung cancers were characterised by smaller tumour size, younger patients and non-smokers or light smokers when compared against

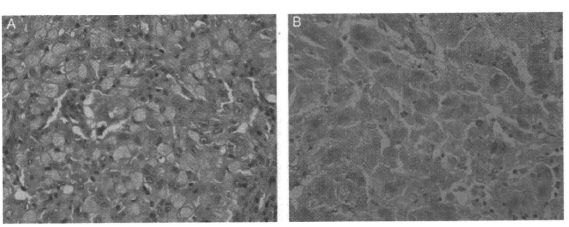

Figure 2 (A) Echinoderm microtubule-associated protein-like-4—anaplastic lymphoma kinase (EML4-ALK)-positive lung adenocarcinoma cells showing signet-ring cell like appearance because of excessive intracytoplasmic mucin. (B) Alcian blue staining showing excessive intracytoplasmic mucin.

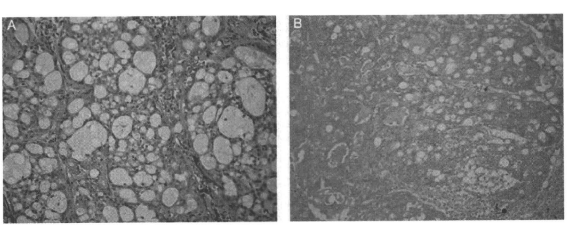

Figure 3 (A) Echinoderm microtubule-associated protein-like-4—anaplastic lymphoma kinase (EML4-ALK)-positive lung adenocarcinoma showing cribriform pattern with excessive extracytoplasmic mucin. (B) Alcian blue staining showing excessive extracytoplasmic mucin.

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