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CD90 Is a Diagnostic Marker to Differentiate Between Malignant Pleural Mesothelioma and Lung Carcinoma With Immunohistochemistry

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ABSTRACT

Objectives: *To pathologically distinguish mesothelioma from lung carcinoma, particularly adenocarcinoma.*

Methods: *We conducted immunohistochemical analyses on clinical specimens, including 26 cases of mesothelioma, 28 cases of lung adenocarcinoma, and 33 cases of lung squamous cell carcinoma.*

Results: *We found that CD90 expression was useful in making a differential diagnosis between epithelioid mesothelioma and lung adenocarcinoma, whereas sarcomatoid mesothelioma and lung carcinoma specimens, irrespective of the histologic types, were negative in general. The sensitivity and specificity of CD90 expression in epithelioid mesothelioma and lung adenocarcinoma were comparable to those of well-established markers used for the differential diagnosis.*

Conclusions: *These data collectively indicate that CD90 is a novel diagnostic marker that contributes to a diagnosis of epithelioid mesothelioma.*

Malignant mesothelioma is often associated with asbestos exposure and remains intractable despite recent treatment modalities.[1] No procedure is currently available to prevent mesothelioma development after asbestos exposure, and the patient numbers will increase in industrialized and newly developing countries in the next decades.[2] Mesothelioma is histologically classified into 3 categories—epithelioid, sarcomatoid, and biphasic types—and the epithelioid type is the major type among them. A differential diagnosis between mesothelioma, especially the epithelioid type, and lung carcinoma, particularly adenocarcinoma that invades into pleura, is often difficult in terms of surgical pathology but is quite important from the standpoint of therapeutic procedures. Previous studies demonstrated that immunohistochemical staining with a panel of various kinds of antibody (Ab) was valuable for diagnostics,[3-7] which used calretinin, D2-40, and Wilms tumor product 1 (WT-1) molecules as a positive marker. Recently, Amatya et al[8] showed that caveolin 1 could be a marker to differentiate epithelioid mesothelioma and lung adenocarcinoma, but contradictory data were also reported.[9] It is thereby important to identify other candidate molecules to increase diagnostic accuracy. In this study, we found that CD90, expressed mainly in immunological and nervous systems, was positive for human mesothelioma cell lines but negative for lung carcinoma. We then demonstrated that immunohistochemical staining of CD90 was useful to differentiate between mesothelioma, particularly the epithelioid type, and lung carcinoma.

Materials and Methods

Cells

Human mesothelioma cells (NCI-H2452, NCI-H2052, NCI-H226, NCI-H28, and MSTO-211H) and mesothelium-derived Met-5A cells that were immortalized by the SV40 T antigen[10] were obtained from American Type Culture Collection (Manassas, VA). Human lung squamous cell carcinoma cells (PC1, PC10, and QG56), human lung adenocarcinoma cells (PC9 and PC14), and human small cell lung carcinoma cells (QG90) were from Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan.

Cell Surface Expression of CD90

Cells were stained with anti–CD90 Ab (Calbiochem, Darmstadt, Germany) followed by fluorescein isothiocyanate (FITC)–conjugated goat anti–mouse immunoglobulin G (IgG) Ab (SouthernBiotech, Birmingham, AL) or with FITC-conjugated second Ab alone and were analyzed with FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA).

Immunohistochemical Staining

Twenty-six mesothelioma samples were collected from clinical specimens between 2000 and 2008 at Chiba University Hospital, Chiba Rosai Hospital, or Chiba East Hospital, Japan. The diagnosis was based on a combination of clinical findings, imaging analyses, and gross observations at surgery and on pathologic examinations. The histopathologic diagnosis was confirmed by several immunohistochemical staining results with Ab against calretinin, WT-1, D2-40, cytokeratin 5/6, cytokeratin CAM5.2, cytokeratin AE1/AE3, carcinoembryonic antigen, thyroid transcription factor 1, desmin, smooth muscle actin, and S100.[2,3,6,8] We also collected 28 lung adenocarcinoma and 33 lung squamous cell carcinoma specimens that were subjected to surgical resection at Chiba University Hospital between 2002 and 2004. Formalin-fixed, paraffin-embedded clinical specimens sliced at 4 μm thick were incubated with anti–CD90 Ab (AbD Serotec, Düsseldorf, Germany), followed by peroxidase-conjugated goat anti–mouse IgG Ab (Nichirei Biosciences, Tokyo, Japan) and developed with 3,3′-diaminobenzidine according to the manufacturer's instructions (Nichirei Biosciences). We judged the specimens as positive when more than 10% of the tumor cells were stained with the anti–CD90 Ab.[4]

Results

Expression of CD90 on Mesothelioma and Lung Carcinoma Cells

We examined 5 mesothelioma and 6 lung carcinoma cells for CD90 expression with flow cytometry **Figure 1**. All mesothelioma cells were positive for CD90, but all lung carcinoma cells tested were negative. MSTO-211H cells showed a biphasic staining pattern with a CD90-positive population as the majority. Interestingly, immortalized Met-5A cells of mesothelium origin were negative for CD90 expression.

CD90 Immunohistochemical Staining

We examined clinical specimens of mesothelioma and lung carcinoma for CD90 expression **Image 1**. We tested 26 cases of mesothelioma, including 15 epithelioid, 7 sarcomatoid, and 4 biphasic specimens, and 61 cases of lung carcinoma, including 28 adenocarcinoma and 33 squamous cell carcinoma specimens **Table 1**. CD90 expression was detected in cytoplasmic portions with a granular manner. Most mesothelioma specimens were positive for CD90, whereas lung carcinoma samples, irrespective of histologic type, were CD90 negative ($P < .01$). There was no statistical difference in CD90 expression between adenocarcinoma and squamous cell lung carcinoma ($P = .26$). We also classified mesothelioma according to histology and found that almost all epithelioid mesothelioma specimens were positive for CD90, whereas sarcomatoid types were in general CD90 negative **Table 2**. All biphasic types were CD90 positive in both epithelioid and sarcomatoid components, but the sample numbers were limited. We also compared the CD90-positive rate between epithelioid mesothelioma and lung adenocarcinoma or lung squamous cell carcinoma and found that the positivity was significantly greater in epithelioid mesothelioma than in lung carcinoma irrespective of the histologic types (Table 1). Furthermore, the sensitivity and specificity for CD90 expression were 93% and 82%, respectively, between epithelioid mesothelioma and lung adenocarcinoma and were 93% and 70% between epithelioid mesothelioma and lung squamous cell carcinoma. These data suggest that CD90 is a valuable marker for immunohistochemistry to differentiate epithelioid mesothelioma from lung carcinoma.

Discussion

We demonstrated in this study that CD90 was expressed in mesothelioma but not in lung carcinoma cell lines and that CD90 is a valuable marker to differentiate epithelioid mesothelioma from lung carcinoma. The differential pathologic diagnosis between epithelioid mesothelioma and lung carcinoma is often difficult, particularly in the case of adenocarcinoma that has developed adjacent to the pleura. Expression of CD90 in mesothelioma has not been well described, although a number of immunohistochemical analyses have been performed with clinical specimens.[4-8] A genome-wide gene expression analysis did not identify CD90 as a potential marker for mesothelioma,[11] but characterization

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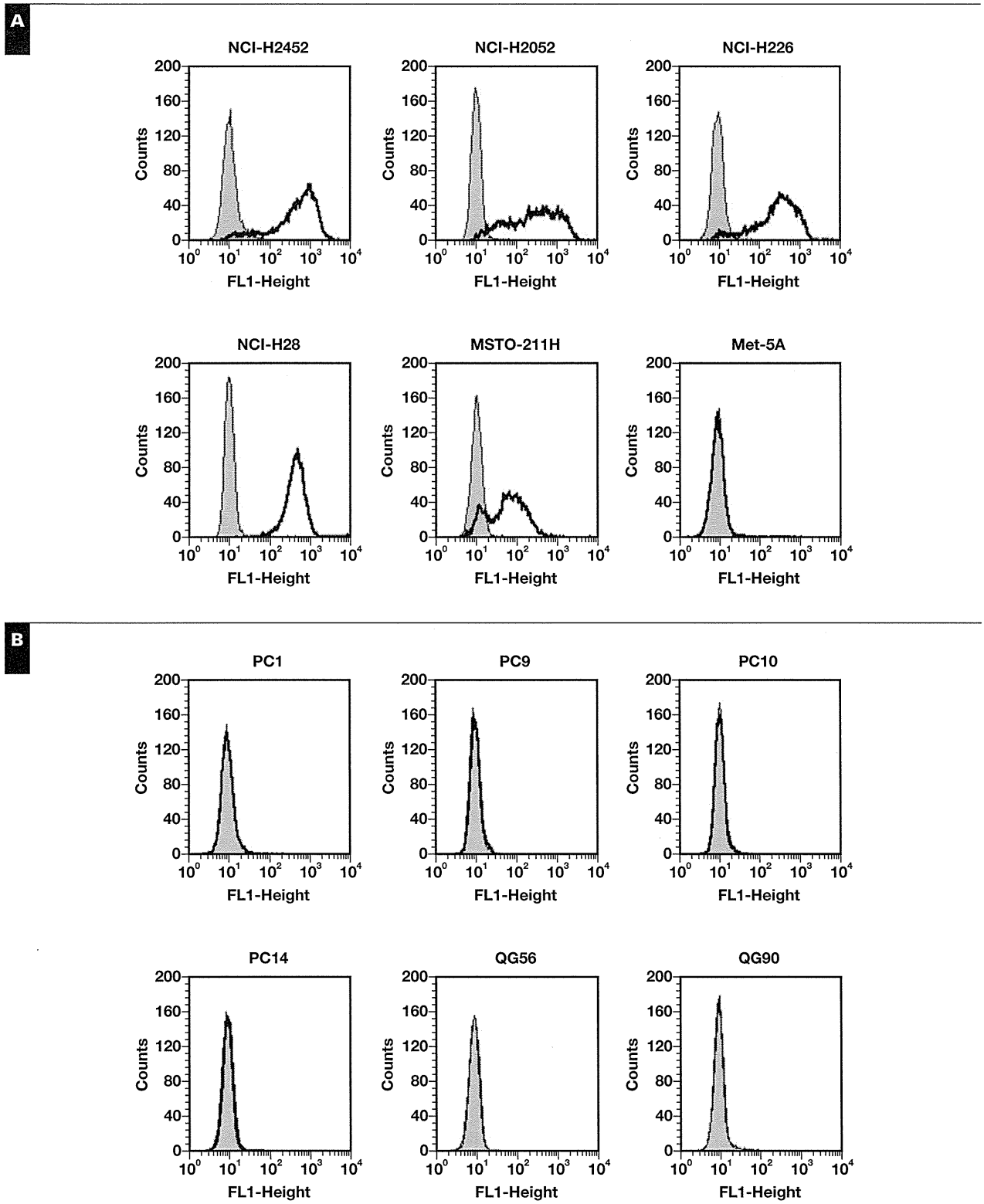

Figure 1 Cell surface staining profiles of CD90 analyzed with flow cytometry on human mesothelioma and Met-5A cells (**A**) and human lung carcinoma cells (**B**). Bold lines and shaded areas show staining with anti-CD90 antibody (Ab) and second Ab alone, respectively.

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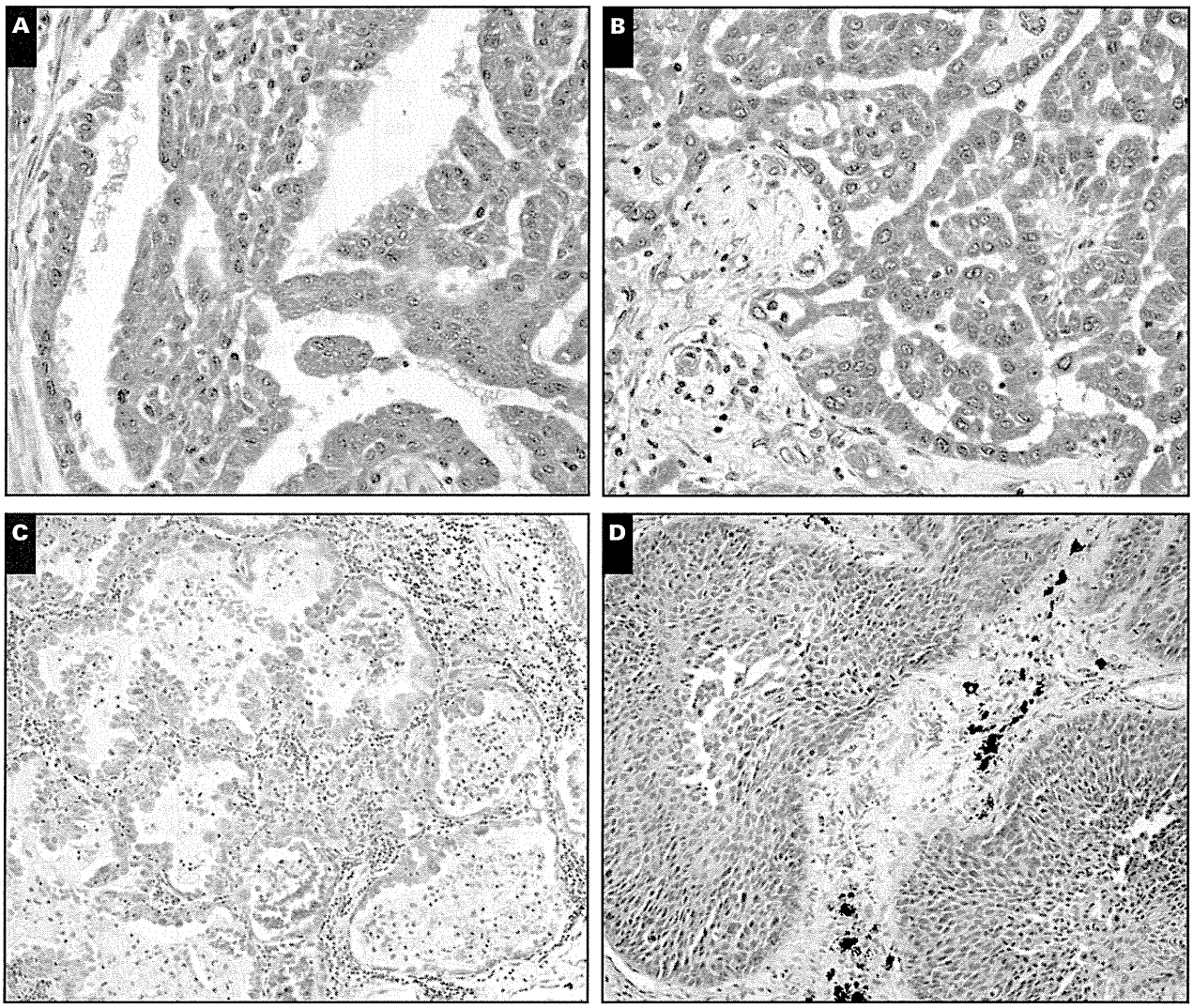

Image 1 Representative immunohistochemical staining of clinical specimens. Two different samples of epithelioid mesothelioma (**A**, ×20; **B**, ×20), lung adenocarcinoma (**C**, ×10), and lung squamous cell carcinoma (**D**, ×10) were stained with anti-CD90 antibody.

Table 1
CD90 Expression in Mesothelioma and Lung Carcinoma Specimens[a]

	Immunohistochemical Staining With Anti-CD90 Antibody		
Tumor Type (Subtype)	**Positive**	**Negative**	**Total**
Mesothelioma	19	7	26
(Epithelioid mesothelioma)	(14)	(1)	(15)
Lung adenocarcinoma	5	23	28
Lung squamous cell carcinoma	10	23	33
Total[b]	34	53	87

[a] χ^2 Test: mesothelioma vs lung adenocarcinoma, $P < .01$; mesothelioma vs lung squamous cell carcinoma, $P < .01$; lung adenocarcinoma vs lung squamous cell carcinoma, $P = .26$. χ^2 Test: epithelioid mesothelioma vs lung adenocarcinoma, $P < .01$; epithelioid mesothelioma vs lung squamous cell carcinoma, $P < .01$. Epithelioid mesothelioma vs lung adenocarcinoma: sensitivity, 93%; specificity, 82%. Epithelioid mesothelioma vs lung squamous cell carcinoma: sensitivity, 93%; specificity, 70%.
[b] Mesothelioma plus lung carcinoma.

Table 2
CD90 Expression in Mesothelioma Based on Histologic Types[a]

	Immunohistochemical Staining With Anti-CD90 Antibody		
Histologic Type (Component)	**Positive**	**Negative**	**Total**
Epithelioid	14	1	15
Sarcomatoid	1	6	7
Biphasic	4	0	4
(Epithelioid)	(4)	(0)	
(Sarcomatoid)	(4)	(0)	
Total	19	7	26

[a] χ^2 Test: epithelioid vs sarcomatoid, $P < .01$; epithelioid vs biphasic, $P > .99$; sarcomatoid vs biphasic, $P = .30$.

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of established mesothelioma cell lines showed that CD90 was one of the molecules expressed in mesothelioma.[12] CD90 is also known as one of the candidates for cancer stem cell markers,[13] and the present study showed that immortalized Met-5A cells of mesothelium origin were negative for CD90 expression. Our study, however, also showed that human immortalized fibroblasts, HFF[14] and OUMS-24,[15] were positive for CD90, and 3 of 5 reactive mesothelial hyperplasia specimens expressed CD90 with the same staining pattern as found in mesothelioma (data not shown). In contrast, 6 cases of normal mesothelium were negative for CD90 expression (data not shown). These data collectively imply that CD90 is not a representative marker for so-called stemness in mesothelioma, although a side population of mesothelioma seems to express CD90.[16] Recently, Ziegler et al[17] reported that CD90 could be a candidate protein marker to discriminate between pleural mesothelioma and lung adenocarcinoma based on their survey of proteomics for N-linked glycoprotein. They, however, examined CD90 expression just at the transcriptional level as their test for validity and showed only 2 respective clinical specimens with immunohistochemistry. In contrast, to our knowledge, our study is the first to reveal CD90 expression frequency with clinical specimens of mesothelioma and lung carcinoma.

A diagnostic value of CD90 needs to be compared with other markers. Four major studies[4,5,6,8] dealing with molecules that could differentiate epithelioid mesothelioma from lung adenocarcinoma examined the sensitivity and specificity of respective molecules **Table 3**. King et al[5] investigated 88 published studies, Yaziji et al[4] 65 epithelioid mesothelioma and 22 lung adenocarcinoma cases, Kushitani et al[6] 90 epithelioid mesothelioma and 51 lung adenocarcinoma cases, and Amatya et al[8] 80 cases of both kinds of tumors, including 20 mesothelioma of nonpleural origin. The number of specimens in the present study was not as large as in previous studies, but we showed that immunohistochemical staining of CD90 attained a similar level for the diagnostic values in terms of sensitivity and specificity, as demonstrated with calretinin, D2-40, and WT-1. These data suggest that CD90 could be integrated as one of the Ab marker panels for mesothelioma diagnostics. We examined 14 samples among CD90-tested mesothelioma cases for the expression of calretinin, D2-40, and WT-1. The respective antigen-positive rates were not different from each other (calretinin, 71%; D2-40, 77%; WT-1, 71%; and CD90, 71%). We, however, found that 4 cases were CD90 positive among 7 cases that were negative for calretinin, WT-1, or D2-40; furthermore, 2 cases were CD90 positive among 3 cases that were positive for only 1 of the 3 conventional markers. In contrast, only 1 case was CD90 negative among 7 cases that were positive for all markers. The results suggest that distribution of CD90-expressed mesothelioma is different from that of mesothelioma expressing the conventional markers and that CD90, as a positive marker, would be helpful to detect the marker-negative mesothelioma.

Table 3
Sensitivity and Specificity of Positive Immunohistochemical Markers That Distinguished Epithelioid Mesothelioma From Lung Adenocarcinoma in Previous Reports

Marker	Reference	Sensitivity, %	Specificity, %
Calretinin	4	95	87
	5	82	85
	6	95.5	66.7
	8	98.8	82.5
Cytokeratin 5/6	4	76	89
	5	83	85
	6	70	58.8
D2-40	8	92.5	83.7
HBME-1	4	84	48
	5	85	43
Mesothelin	4	75	71
	8	90.7	80
N-cadherin	5	78	84
Thrombomodulin	4	68	92
	5	61	80
	6	70.6	80.4
	8	80.9	90
Vimentin	4	69	84
	5	62	75
	6	91.0	52.9
WT-1	4	78	62
	5	77	96
	6	98.8	84.3
	8	60.5	92.5

HBME-1, Hector Battifora mesothelial cell 1; WT-1, Wilms tumor product 1.

Immunohistochemical differentiation between epithelioid mesothelioma and lung squamous cell carcinoma has not been well investigated, but Ordóñez[18] demonstrated that calretinin, mesothelin, and WT-1 are reliable positive markers based on his analysis with 30 respective cases. The sensitivity of calretinin, mesothelin, and WT-1 in his study was calculated as 100%, 100%, and 93%, respectively, and the specificity was 60%, 73%, and 100%, respectively. CD90 is thereby comparable to those molecules as a positive marker in immunohistochemistry that can differentiate epithelioid mesothelioma from lung squamous cell carcinoma. Interestingly, CD90 expression is almost negative in sarcomatoid mesothelioma, although sarcomatoid components of biphasic mesothelioma are CD90 positive. This could be partly due to putative etiologic differences between sarcomatoid mesothelioma and biphasic mesothelioma, but further investigations are required to confirm the CD90 reactivity with more clinical specimens.

The biological significance of CD90 expression in mesothelioma remains uncharacterized. CD90 molecules are primarily expressed in immunological and neurological systems with the glycosylphosphatidylinositol anchor, with knockout mice and other experimental systems showing that CD90 molecules have multiple roles in cell adhesion, apoptosis, and

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migration depending on experimental systems, as well as in neurite outgrowth and immunological activities.[19] Moreover, decreased expression of CD90 levels in lung fibroblasts has increased fibrogenesis probably through transforming growth factor β signaling, demonstrating a possible crosstalk of CD90 with other signaling systems.[20] CD90 is a soluble protein, and inflammatory responses can increase the secretion.[20] Detecting CD90 molecules in pleural effusion thereby can be a diagnostic method for epithelioid mesothelioma, and CD90 can also be a target of mesothelioma treatments, such as in the case of immunotherapy. In conclusion, CD90 is a novel marker for mesothelioma diagnosis and has a comparable ability to other conventional markers to differentiate epithelioid mesothelioma from lung carcinoma, particularly adenocarcinoma.

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Transbronchial Biopsy Needle Rinse Solution Used for Comprehensive Biomarker Testing in Patients with Lung Cancer

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Introduction: Although genetic information is essential for molecular targeted therapy for personalized medicine, tissue sampling for genetic analysis remains challenging. We investigated the utility of bronchoscopic sampling in non–small-cell lung cancer (NSCLC) patients compared with conventional histological materials for multiple genetic analyses.

Methods: Patients with NSCLC proven by onsite cytological evaluation during bronchoscopic survey were eligible for this study. After conventional needle aspiration biopsy by flexible bronchofiberscopy of primary lesions or convex-probe endobronchial ultrasound of lymph nodes, the used needle was rinsed with saline, and the ultra-microsample (uMS) was used for cytological diagnosis and genetic analysis. Gene mutations and fusion genes were examined by high-resolution melting analysis and direct sequencing. The results from the uMS and those from conventional histological samples were compared.

Results: A total of 134 lesions (48 primary and 86 metastatic) were analyzed. Adenocarcinoma (n = 80), squamous-cell carcinoma (n = 43), and NSCLC (n = 11) samples were pathologically confirmed in histological cores; however, malignancies were detected in only 45 (34%) of the corresponding uMS. In 62 samples, genetic disorders, including epidermal growth factor receptor (n = 21), *K-ras* (n = 11), and *BRAF* mutations (n = 1); anaplastic lymphoma kinase (n = 5), receptor tyrosine kinase (n = 1), and *RET* fusion genes (n = 1); and silent mutations (n = 22), were identified. In total, 1474 molecular tests were performed, and 1464 tests (99.3%) were identical for both histological samples and uMS.

Conclusion: Bronchoscopic uMS (biopsy needle rinsed fluids) are useful for multiple genetic examinations in NSCLC.

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The rise of molecular targeted chemotherapy has changed the fundamental concept of treatment in non–small-cell lung cancer (NSCLC). A relationship between epidermal growth factor receptor (EGFR) and EGFR–tyrosine kinase inhibitors was verified by large-scale clinical trials.[1,2] Additional relationships between companion diagnoses are under investigation, and treatment with new agents are under development, including crizotinib for anaplastic lymphoma kinase (ALK), c-ros oncogene 1, or receptor tyrosine kinase (ROS1) fusion gene–positive lung cancer[3,4]; sunitinib for ret proto-oncogene (RET) fusion gene–positive lung cancer[5,6]; and vemurafenib for BRAF mutation (V600E)–positive lung cancer.[7]

Therefore, the existence of gene mutations or fusion genes becomes a key factor for treatment decisions in NSCLC. However, the methodology used to detect such gene mutations is still under development. Although operational specimens are suitable for genetic analysis with respect to sample assurance and containing massive tumor cells, genetic analysis before the operation is preferable for acquisition of genetic information for initial treatment planning, an issue in the application of companion biomarkers. Bronchoscopic samples are general samples that may provide definitive diagnoses and that can be obtained during preoperative stages of clinical evaluation. To overcome this issue, molecular analyses of microhistological samples (e.g., bronchoscopic histological samples) have been established, and we have reported the utility of endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) sampling, which can be challenging, for molecular analysis.[8,9] Thus, establishing a methodology for the molecular analysis of cytological specimens is the next step to developing personalized medicine by using genetic information and molecular targeted therapy.[10]

The aim of this study was to determine the utility of bronchoscopic ultra-microsamples (uMSs), which were obtained from rinsed fluid of the used needle as well as endoscopic histological samples (histological cores) for multiple molecular profiling analyses in NSCLC.

PATIENTS AND METHODS

Patients

The patient eligibility criteria were as follows: (1) patients who had undergone conventional bronchoscopy for the diagnosis of primary lesions or EBUS-TBNA for the diagnosis of metastatic lymph nodes; (2) patients from whom both histological and cytological specimens could be obtained; (3) patients with malignant cells proven by rapid onsite cytology in both cytological and histological specimens; and (4) patients providing informed consent for the study. A well-trained operator carried out all sampling procedures under local anesthesia and conscious sedation, without intubation. In general, all procedures were performed in the outpatient clinics of our hospitals.

Bronchoscopic Sampling from the Primary Tumor

For the diagnosis of primary lung lesions, including both peripheral and central lesions, transbronchial forceps biopsy (TBFB) was performed with a flexible bronchoscope (BFS-type260; Olympus, Tokyo, Japan). to obtain histological specimens after TBNA to obtain cytological specimens, and these procedure were repeated until malignant cells were confirmed by rapid onsite cytology with a touch smear (for TBFB) or smear (for TBNA) slide stained with Diff-Quik (Sysmex Corporation, Kobe, Japan). In eligible cases, a portion of the TBFB sample was preserved on ice immediately and stored at −80°C with 1 ml lysis buffer (MagNA Pure Compact RNA Isolation Kit; Roche Diagnostics, Mannheim, Germany); this sample was the control sample for molecular analysis (endoscopic histological core; core), whereas the remaining portion of the TBFB sample was used for pathological diagnosis. Used TBNA needles were rinsed in a 20-ml saline bottle and washed after every TBNA procedure. The remnant cells in the bottle were referred as considered the uMS in this study; these cells were well mixed and divided into three bottles: 5 ml for bacterial culture, 5 ml for the diagnosis, and 10 ml for molecular analysis. The uMS for molecular analysis was equally divided and immediately centrifuged for 2 minutes at 2000 rpm. The obtained cell pellet was stored at −80°C. This uMS preparation procedure was performed within 20 minutes of sample collection. Pathologists confirmed the final cytological diagnosis of the uMS, and the results, including those from molecular profiling, were compared with those of histological core samples as a control. Malignant diagnosis in cytological specimen was defined by findings of strongly suggestive of malignancy (class IV) or conclusive for malignancy (class V) according to Papanicolaou's classification. This sampling sequence is described in the left (TBNA) and center (TBFB) columns of Figure 1.

EBUS-TBNA from Metastatic Nodes

For the diagnosis of metastatic lymph nodes, including both mediastinal and hilar nodes, a convex-probe EBUS (BF-UC260F-OL8, Olympus), dedicated ultrasound scanner (EU-C2000/EU-C60; Olympus), and dedicated 22-gauge needle equipped with an internal stylet (NA-201SX-4022; Olympus) were used to obtain histological and cytological specimens. Malignant cells in TBNA droplets from every puncture were confirmed by stained smears in onsite screening. Histological cores were preserved on ice immediately and stored at −80°C; these cores were used for control samples in molecular analyses. Used needles were rinsed in a 20-ml saline bottle for every puncture, and the uMSs were obtained and divided into three bottles for analysis using the same methods as those used for bronchoscopic samples. This sampling sequence is described in the right column of Figure 1.

DNA and RNA Extraction

The uMS pellet and tissue from the core were used to obtain DNA or RNA. These samples were homogenized with

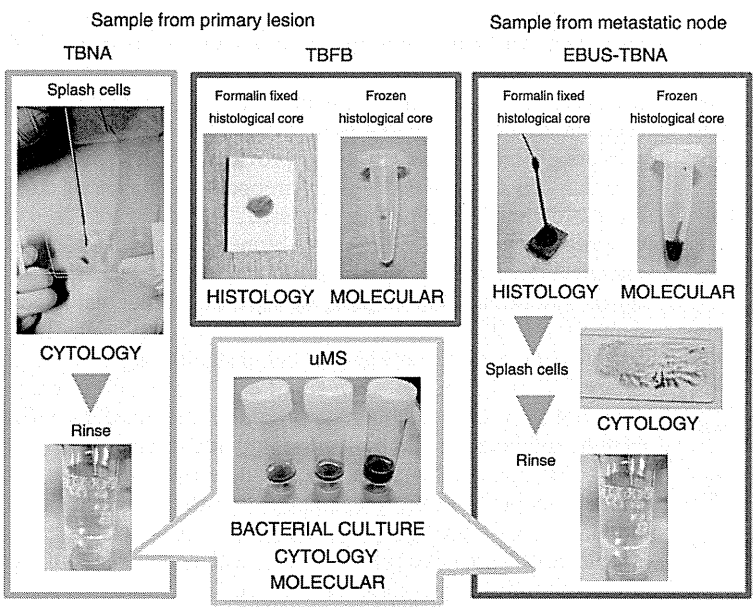

FIGURE 1. Sampling sequence for primary lesions and metastatic nodes. For primary lesions, TBNA with needle and TBFB was performed by using conventional bronchofiberscopy. For metastatic nodes, EBUS-TBNA was performed. Used needles were washed in saline and divided into three bottles, and these uMSs were used for bacterial culture, cytological analysis, and molecular analysis. All samples in this study were confirmed as malignant cells in each modality by rapid onsite cytological evaluation. TBNA, transbronchial needle aspiration; TBFB, transbronchial forceps biopsy; EBUS, endobronchial ultrasound-guided; uMS, ultra-microsamples.

a MagNA Lyser for 60 seconds at 6500 rpm and placed on a cooling block for 1 minute. Supernatants (100 μl) were used for DNA extraction with a MagNA Pure Compact Nucleic Acid Isolation Kit. Supernatants (350 μl) from homogenates were centrifuged and RNA was extracted using a MagNA Pure Compact RNA Isolation Kit. cDNA cloning was performed with a First Strand cDNA Synthesis Kit (Roche). Extracted DNA and cDNA were stored at 4°C.

Detection and Sequencing of Mutations

For the screening of mutations or fusion genes, we applied high-resolution melting (HRM) analysis[11] for both DNA and RNA analysis. We used a protocol and primer sequences that have been described previously to detect *EGFR* (4 primer sets),[12] *KRAS* (2 primer sets),[11,12] and *BRAF* (2 primer sets)[13] mutations in DNA extracts in one run. For reverse-transcriptase polymerase chain reaction (RT-PCR) analysis, optimized primer sets based on previously reported primer sets were used in two runs (*ALK* and *ROS1*, *RET*). For detection of the *ALK* fusion gene, a new forward primer (EML4-ALK v3b1; 5′-CAAGCATAAAGATGTCATCATCAAC-3′) was added to the eight previously reported primers,[14] and multiplex RT-PCR was performed with these nine primer sets in one test. For *ROS1* fusion gene detection, two optimized forward primers (TPM3-ROS1, 5′-GCTGAGTTTGCTGAGAGATCGGTAG-3′ and LRIG3-ROS1, 5′-CCAACACAGATGAGACCAACTTGC-3′) and four reported primers (SDC4-ROS1, SLC34A2-ROS1, CD74-ROS1, and EZR-ROS1)[15] with an optimized reverse primer (5′-CGCAGCTCAGCCAACTCTTTGTC-3′) to avoid the amplification of nonspecific products. These six primer sets were used in multiplex RT-PCR in one test. The *RET* fusion gene was tested by multiplex RT-PCR with three reported primer sets in one test.[15] After PCR amplification, purified products were then sequenced with a capillary sequencer (3130 Genetic Analyzer; Life Technologies, Carlsbad, CA), and the actual mutation sequence or fusion locus was defined. All mutation/fusion gene detections were repeated thrice to validate the results. In total, three runs, including 11 molecular tests, were needed for each sample.

Ethics Committee Approval

The bioethics committee of Chiba University Graduate School of Medicine approved this research (No. 275). Written consent was obtained from patients, and all samples were coded and managed independently.

RESULTS

Patients and Pathological Diagnosis

From November 2010 to October 2012, 146 patients (149 samples) were enrolled in this study; samples consisted of 52 primary tumors and 97 metastatic lymph nodes. By the revealed final pathological diagnosis, 15 samples were omitted and 134 samples were eligible for this study (107 from men and 27 from women). The reasons for the omitted cases included no malignant findings obtained in the histological core of the primary tumor (n = 4), metastasis of renal cell carcinoma (n = 1), gastric carcinoma (n = 1), small-cell lung cancer (n = 2), sarcoma (n = 1), malignant lymphoma (n = 1), and inflammation with severe atypia (n = 5). The latter 11 samples were diagnosed from core samples obtained by EBUS-TBNA. Final histological diagnoses in eligible samples of primary tumors and metastatic nodes consisted of adenocarcinomas in 30 and 50 (63% and 58%), squamous cell carcinoma in 16 and 27 (33% and 31%), and other cancers in 2 and 9 (4% and 10%) samples, respectively. Target lesion and size of primary tumor and metastatic node are listed in Supplementary Table 1 (Supplemental Digital Content 1, http://links.lww.com/JTO/A490). TBNA or EBUS-TBNA was attempted an average (±SD) of 2.37 ± 1.15 or 6.45 ± 3.26 times, and malignant cells were confirmed 1.71 times (81%) or 3.92 times (72%), respectively. No pathogens were cultured from uMSs in this study. Sample enrollment and pathological results are illustrated in Figure 2.

DNA and RNA Extraction

DNA was successfully extracted from both uMSs and histological cores for every sample. DNA was eluted in a final volume of 200 μl, and extracted DNA was obtained at concentrations of 9.3 ± 8.8 ng/μl from core samples and 3.7 ± 3.4 ng/μl from uMSs in bronchoscopic samples of primary tumors. In EBUS-TBNA samples of metastatic nodes, the extracted DNA concentrations were 24.8 ± 27.3 ng/μl from cores and 5.2 ± 4.1 ng/μl from uMSs. RNA was also successfully extracted from both uMSs and histological cores for every sample. RNA was eluted in a final volume of 50 μl. Extracted RNA was obtained at concentrations of 64.0 ± 45.0 ng/μl from cores and 12.8 ± 9.9 ng/μl from uMSs by bronchoscopy or 129.8 ± 140.3 ng/μl from cores and 18.1 ± 14.5 ng/μl from uMSs by EBUS-TBNA. These results are summarized in Supplementary Table 2 (Supplemental Digital Content 1, http://links.lww.com/JTO/A490).

Mutation/Fusion Gene Detection

We confirmed that our primer sets work properly by using following artificial sequences as the positive control; EGFR Exons 18–21 Genomic DNA Reference Standards, K-Ras Codons 12 & 13 Genomic DNA Reference Standards, B-Raf Codon 600 Genomic DNA Reference Standards (Horizon Diagnostics, Cambridge, United Kingdom), *EML4-ALK* (variant 1, 2, 3a), *KIF5B-ALK*, *KIF5B-RET*, *CCDC6-RET*, *TPM3-ROS1*, *SDC4-ROS1*, *SLC34A2-ROS1*, *CD74-ROS1*, *EZR-ROS1*, *LRIG3-ROS1* (GenScript, Piscataway, NJ). HRM curve of these control sequences are illustrated in Supplementary Figure 1 (Supplemental Digital Content 3, http://links.lww.com/JTO/A492). In total, 73 genetic disorders were identified, including *EGFR* mutations (n = 21), *KRAS* mutations (n = 11), *BRAF* mutations (n = 1), *ALK* fusion genes (n = 5), *ROS1* fusion genes (n = 1), *RET* fusion genes (n = 1), and silent mutations (n = 22). Double mutations (including silent mutation) were detected in 12 samples (Table 1), and HRM-PCR curves (melting peaks) of each mutation and fusion, along with the identified sequences, are shown in Figure 3.

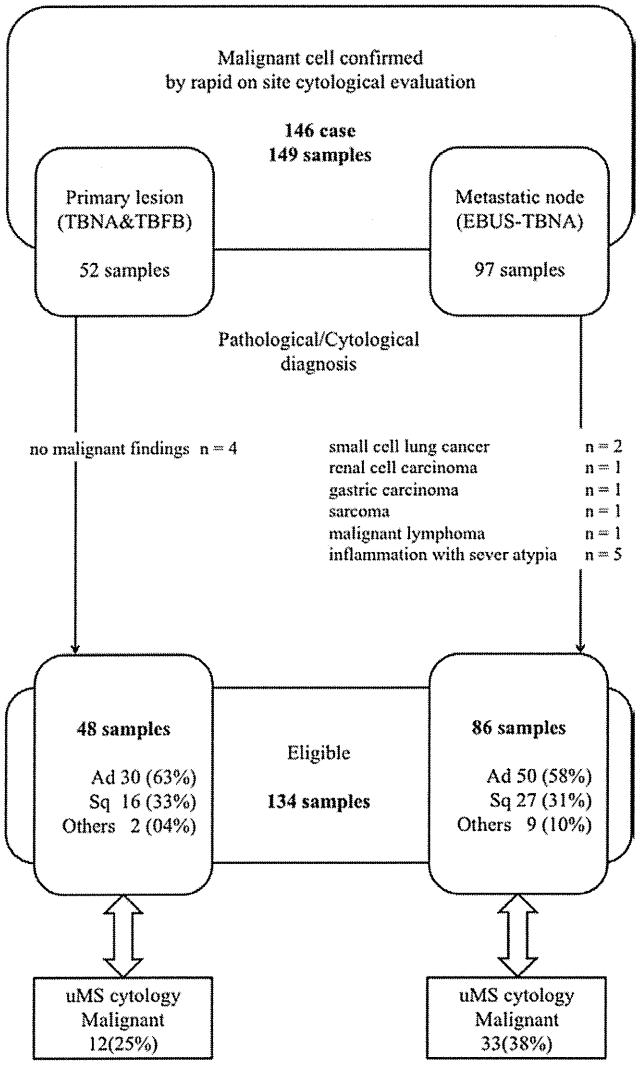

FIGURE 2. Case enrollment. From 149 samples, 15 samples were omitted because of the pathological/cytological final evaluation. Only non–small-cell lung cancer samples were enrolled in this study. Ad, adenocarcinoma; Sq, squamous-cell carcinoma; TBNA, transbronchial needle aspiration; TBFB, transbronchial forceps biopsy; EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; uMS, ultra-microsample.

Comparison of the Result from Histological Cores and uMSs

The results of comparisons between cores and uMSs are summarized in Table 2. Final cytological evaluations of uMSs revealed only 34% of malignant cells, despite that all eligible samples showed malignant findings in the final cytological and histological evaluation of cores. In uMSs from primary tumors, 25% (12 of 48) of malignant cells were confirmed, whereas 38% (33 of 86) of malignant cells were confirmed in uMSs from metastatic lymph nodes. In total, 1474 genetic tests were conducted, and complete concordance was confirmed in 1464 tests (99.3%). Major discrepancies occurred in six tests (0.4%); mutations were detected only in the histological core in four tests or only in uMSs in two tests. All major discrepancies occurred in samples from primary tumors. Minor discrepancies occurred in four tests (0.3%), and although these mutations were identified by HRM-PCR, their sequences were not identified by direct sequencing. This discrepancy occurred in uMSs, and the sequences were successfully identified in histological cores.

DISCUSSION

Through this study, we showed the utility of uMSs and bronchoscopic histological cores with respect to multiple molecular profiling analyses in NSCLC. Our data suggested that molecular analysis using uMSs provides accurate, easy-to-obtain data that can be used to replace conventional sampling methods in the development of molecular targeted therapies for patients with NSCLC.

The methodology was designed such that the uMS contained malignant cells, both through the sampling procedure itself and through onsite cytological evaluation. Generally, the primary objective of rinsing the biopsy needle is to clean the needle for the next biopsy procedure; usually this rinse liquid is considered waste. Our method allowed us to conduct molecular analysis using this waste without any additional biopsies or without repeated biopsies using other modalities. A comparison of the final pathological diagnosis and rapid onsite cytological evaluation revealed that only 34% (45 of 134) of samples were confirmed as malignant, an issue that has been previously reported;[16] however, our study allowed us to perform molecular testing by a logically well-designed sampling procedure known as the "recycled method," which enabled us to obtain malignant cells. uMS analysis may to increase the sensitivity to detect cancer through the molecular analysis in cases where we do not get enough tissue even for standard cytology.

In this study, 0.7% (10) of tests showed molecular profile discrepancies between histological cores and uMSs. Three minor discrepancies were caused by problems with sensitivity, which will be discussed later. We also observed about six major discrepancies between samples, and all these discrepancies occurred during sampling from primary lesions. For sampling from primary lesions, it consisted of two steps: TBFB and TBNA. In contrast, EBUS-TBNA was consisted of one step in which both histological and cytological specimens were obtained and no major discrepancies occurred in uMSs versus EBUS-TBNA samples. Thus, we assumed that the separate biopsy procedure was one cause of the discrepancies between bronchoscopic biopsy samples and uMSs. In addition, we retrospectively investigated the six major discrepancy cases; TBNA was attempted an average of 2 ± 1.09 times, and malignant cells were detected 1.33 times. These numbers are lower than those for EBUS-TBNA (6.45 ± 3.26 and 3.92 times, respectively). Multiple appropriate punctures may increase the total cell amount in uMS collected, and the collection of more cells may prevent the occurrence of major discrepancies. Notably, four of the major discrepancies were mutations detectable only in uMSs. Clinically, biopsy cores contain some normal lung tissue, and the rate of tumor occurrence is variable. Even when touch smear cytology shows

TABLE 1. Results of Mutation and Fusion Gene Analyses

	Bronchoscopic Samples (n = 48)		EBUS-TBNA Samples (n = 86)	
	Concordant (Minor Concordant)[a]	Discrepant Core/uMS	Concordant (Minor Concordant)[a]	Discrepant Core/uMS
Mutation analysis				
EGFR	7 (15%)	1/1 (2/2%)	12 (14%)	0/0 (0/0%)
L858R	2	0/1	4	0
E746_A750 del	2	1/0	2	0
E746_A750 delinsIP	0	0	1	0
L747_P753 delinsS	1	0	0	0
L858R+A871G	0	0	1	0
L858R+c.2361 G>A	2	0	3	0
L747_P753 delinsS + c.2361 G>A	0	0	1	0
Silent mutations	5 (10%)	1/2 (2/4%)	14 (16%)	0/0 (0/0%)
c.2316 C>T	0	0/1	0	0
c.2361 G>A	5 (1)	1/1	14	0
KRAS	5 (10%)	0/0 (0/0%)	6 (7%)	0/0 (0/0%)
G12V	1	0	3 (1)	0
G12C	2 (1)	0	1	0
G12D	2 (1)	0	1	0
G13C	0	0	1	0
BRAF	0 (0%)	0/1 (0/2%)	0 (0%)	0/0 (0/0%)
V600E	0	0/1	0	0
Fusion gene analysis				
ALK	1 (2%)	0/0 (0/0%)	4 (5%)	0/0 (0/0%)
Variant 1 + c.2361 G>A	1	0	1	0
Variant 2	0	0	1	0
Variant 3a/b	0	0	1	0
New variant + c.2361 G>A	0	0	1	0
RET	0 (0%)	0/0 (0/0%)	1 (1%)	0/0 (0/0%)
CCDC6-RET + c.2361 G>A	0	0	1	0
ROS1	0 (0%)	0/0 (0/0%)	1 (1%)	0/0 (0/0%)
SCL34A21-ROS1 + c.2361 G>A	0	0	1	0
Wild-type	24 (50%)	0/0 (0/0%)	48 (56%)	0/0 (0/0%)
Total	42 (88%)	2/4 (4/8%)	86 (100%)	0/0 (0/0%)

Concordant: same results were obtained between uMSs and histological samples. Discrepant: mutation was detected only in histological core or uMS.
[a]Minor concordant: mutations were detected by HRM-PCR analysis, but not identified in sequencing of uMSs.
EBUS-TBNA, endobronchial ultrasound-guided transbronchial needle aspiration; *EGFR*, epidermal growth factor receptor; HRM-PCR, high-resolution melting-polymerase chain reaction; uMSs, ultra-microsamples.

malignant cells, whole core samples sometimes contain very small amounts of tumor cells, and this may be one cause for the faults observed in molecular analysis. In this regard, EBUS-TBNA is a preferable sampling procedure, avoiding quality discrepancies between core samples and uMSs.

The relationship between molecular biomarkers and molecular targeting agents has been well recognized, and the importance of molecular testing has increased. Therefore, a universal and accessible method through which to analyze samples is needed. Because of this, molecular testing costs have become an issue. The goal of molecular testing is to accomplish individualization and optimization of treatment using molecular targeting agents, which can be expensive. Obviously, less-expensive testing is favorable; in the United States, *EGFR* mutation gene tests cost approximately $700. Our PCR-based gene profiling method used a universal DNA/RNA extraction kit and conventional PCR methods and consisted of automated DNA/RNA extraction and three runs (1 for DNA and 2 for RNA) for all 11 molecular tests in each case. Thus, the HRM-PCR method can reduce the running cost because PCR primers are the only additional consumable required when a new target is discovered. The quality of the test result was assured by the combination of the biopsy procedure itself (containing malignant cells) and the high sensitivity of the method to detect mutations/fusion genes. Thus, the total cost for the present analyses was approximately U.S. $350 per patient in this study, suggesting that this method must be cost-effective. The actual molecular screening cost is

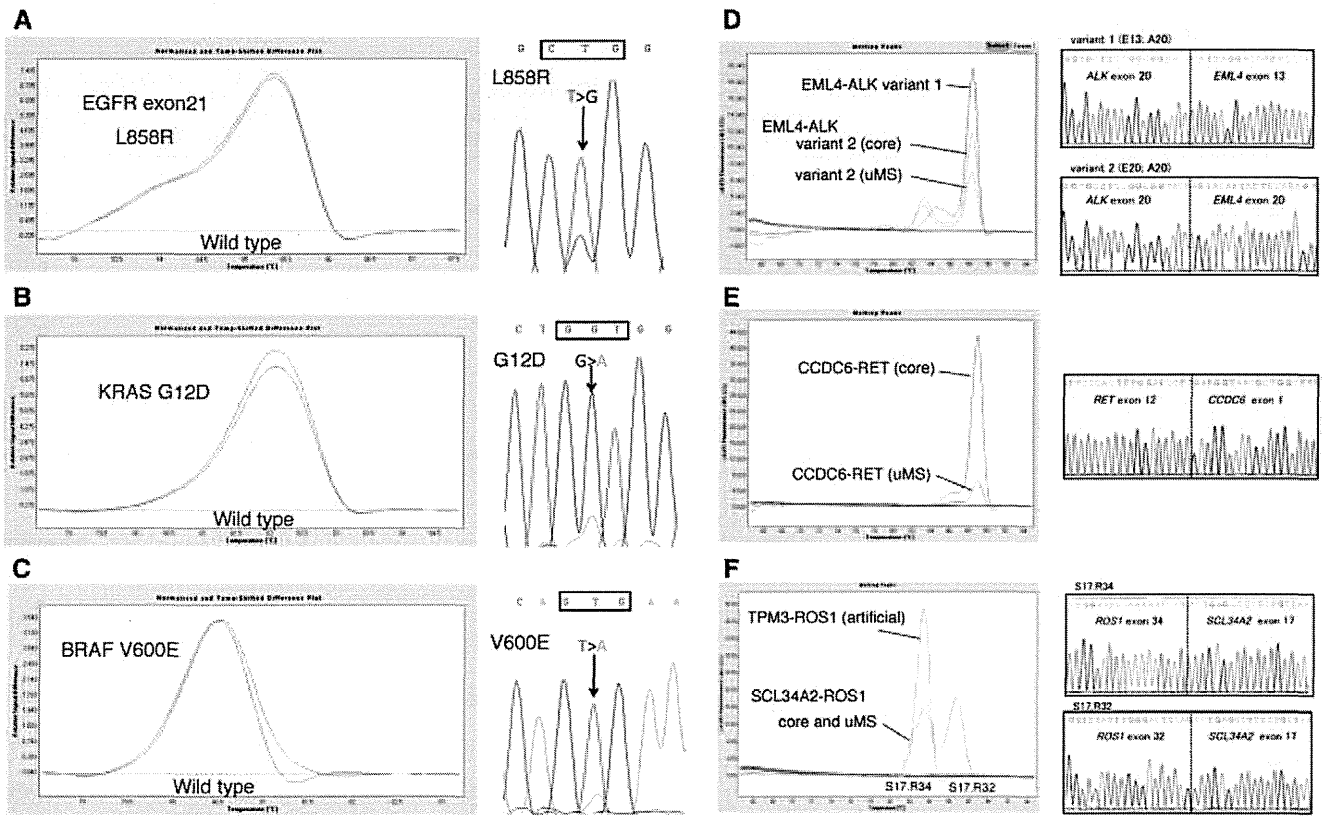

FIGURE 3. DNA and cDNA analyses for EGFR, KRAS, and BRAF mutation and *ALK*, *RET*, and *ROS1* fusion genes. HRM analysis and direct sequencing of *EGFR*-, *KRAS*-, and *BRAF*-positive samples are shown (A–C). The blue baseline shows the HRM curve of the wild-type sample, and the red curves show the existence of each mutation (A: *EGFR* exon21, L858R; B: *KRAS* exon12, G12D; C: *BRAF* V600E) by HRM analysis. Loci were identified by direct sequencing by using the PCR products from HRM analysis. HRM analysis and direct sequencing of EML4-ALK–, CCDC6-RET– and SCL34A2-ROS1–positive samples are shown (D–F). D, HRM curves for EML4-ALK variant 1 (uMS) and variant 2 (histological core and uMS) and the results of direct sequencing. Each variant showed a different curve. E, HRM curve and sequencing for CCDC6-RET fusion gene–positive samples. F, HRM curve for the TPM3-ROS1 fusion sequence (artificial sequence for the positive control) and HRM curve for the detected SCL34A2-ROS1 fusion gene. The sequence of the SCL34A2-ROS1 fusion gene was identified. EGFR, epidermal growth factor receptor; ALK, anaplastic lymphoma kinase; ROS1, receptor tyrosine kinase; uMS, ultra-microsample; HRM, high-resolution melting; PCR, polymerase chain reaction; KRAS, K-rat sarcoma; BRAF, B-rapidly accelerated fibrosarcoma.

listed and compared in Supplementary Table 3 (Supplemental Digital Content 1, http://links.lww.com/JTO/A490), and our institution absorbed all costs for molecular testing in this study because this survey was conducted for research purposes.

In the near future, molecular targeted therapy will become the mainstream treatment for anticancer chemotherapy, and new agents and corresponding new biomarkers will be found. Our screening method is able to accommodate newly discovered biomarkers with only the design of appropriate primer sets and may be easy to apply with high sensitivity for known mutations/fusion genes. In addition, using uMS screening methods allows for the preservation of small biopsy samples. If needed, preserved cores (frozen tissues) or paraffin-fixed cores may be used for secondary molecular marker investigations.

There were some limitations to this study. The first was the sensitivity of the PCR-based method. Our focus here was to show the quality of concordance between biopsy core samples and uMSs, and, as emphasized earlier in this article, these molecular tests need to be conducted conveniently and inexpensively. HRM analysis can achieve these goals by allowing us to perform multiple molecular analyses in one assay. For RNA testing, PCR-based methods have been reported to have sufficient sensitivity,[17] whereas for DNA testing, previously reported methods (e.g., peptide nucleic acid–locked nucleic acid PCR-clamp methods,[18] Scorpion amplification refractory mutation system methods,[19] etc.) showed higher sensitivity, allowing detection of mutations with 1% of the tumor amount; HRM analysis requires greater than 5% of the tumor amount to achieve sufficient molecular analysis.[20] For the verification of molecular analysis, we also surveyed the tumor ratio of core samples and uMSs in 20 randomly selected samples in our series, which could be evaluated both pathologically and cytologically. All these core samples and uMSs showed greater than 5% tumor content. Furthermore, we also analyzed EGFR mutations by the PCR-clamp method for verification in 15 cases, and the results were completely concordant with the results of HRM analysis. Therefore, we propose that this study limitation is acceptable, and we now need to explore

TABLE 2. Differences between Core Samples and uMSs

Pathological evaluation	uMS	
	Malignant cell (+)	Malignant cell (−)
Histologic core		
Malignant cell (+)	89 cases	45 cases
Malignant cell (−)	0	0
Molecular test	**uMS**	
	Mutation/fusion detected	Wild-type
Histologic core		
Mutation detected	64+4[a] tests	2 tests
Wild-type	4 tests	1400 tests

Molecular analysis required 11 tests for every 134 samples, with 1474 tests in total. Mutation/fusion gene detection was based on HRM-PCR analysis. Test concordance rate was 99.4% (1465 of 1474) in the genetic analyses.

[a]Mutations were detected by HRM-PCR analysis, but not identified in sequencing of uMSs.

HRM-PCR, high-resolution melting-polymerase chain reaction; uMSs, ultra-microsamples.

the respective high-sensitivity methods for each genetic disorder to improve sensitivity. Further investigations are required to compare the sensitivity between mass samples and biopsy samples.

The second limitation of this study was the heterogeneity of the tumors. Bronchoscopic or EBUS-TBNA samples represented only partial sections and may not have been representative of the whole tumor in heterogeneous lung cancer. This issue is common in the analysis of microsamples and is difficult to overcome because this problem arises from the nature of the tumor itself. Metastatic lesions sometimes show different genetic profiles from the primary resected tumor,[21] so this limitation may not even be overcome by using large operational specimens. Repeated biopsy of the same target lesion is one way to reduce this limitation, and from this point of view, analysis by uMS may lessen this limitation by representing accumulated malignant cells from multiple biopsy procedures.

The third limitation was that a single gene-analysis modality was applied for all uMSs. Especially for fusion gene detection, we have previously reported that multimodal analyses are desirable.[9] This screening method using uMSs was specialized for molecular analysis, and, because only one third of uMSs showed malignant cells, pathological evaluation was limited by using uMS. For multimodal analysis, histological cores can be used for fluorescent in situ hybridization or immunohistochemistry if needed and may allow for improved sensitivity by additional molecular surveys.

In conclusion, appropriately prepared uMSs, in addition to histological core samples, are useful for multiple molecular profiling with respect to accuracy, cost, and convenience in NSCLC.

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A Feasibility Study of Carboplatin Plus Irinotecan Treatment for Elderly Patients with Extensive Disease Small-cell Lung Cancer

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Objective: The role of platinum agents plus irinotecan has been unclear for elderly patients with extensive disease small-cell lung cancer. We conducted a feasibility study to evaluate the safety and efficacy of carboplatin plus irinotecan in preparation for a planned Phase III study.
Methods: Based on another Phase I study, carboplatin area under the curve of four Day 1 plus irinotecan 50 mg/m^2 Days 1 and 8 every 3 weeks for four courses was administered. Patients aged ≥70 years with a performance status of 0–2 were eligible. The primary endpoint was feasibility, defined as the percentage of patients who have received three or more courses of chemotherapy. If the feasibility was ≥60% in the first 10 patients, this endpoint would be considered to be met.
Results: Eleven patients were registered. The median age was 77 years, and nine patients had a performance status of 1. Ten patients completed four courses of treatment, and neither dose omission nor modification was required. The feasibility was 91% (10/11) and the relative dose intensity was 76.9%. Because neutropenia was frequently prolonged, the next course was delayed in 53% of all courses. Other toxicities were generally mild, and the only Grade 4 toxicity was hyponatremia. The overall response rate was 90% (9/10), and the progression-free survival and the overall survival were 5.1 and 10.9 months, respectively.
Conclusions: This regimen appears to be feasible and effective. Based on these results, a Phase II/III trial comparing carboplatin plus etoposide with carboplatin plus irinotecan for elderly patients with extensive disease small-cell lung cancer is being planned by the Japan Clinical Oncology Group.

Key words: chemo-respiratory tract – chemo-Phase I–III – clinical trials – lung medicine

INTRODUCTION

Approximately 30–40% of patients with small-cell lung cancer (SCLC) are ≥70 years old, and the proportion of elderly SCLC patients is continuously increasing in Japan (1–3). However, as elderly patients have been frequently excluded from clinical trials, no standard chemotherapeutic regimen has been established for this patient population. Moreover, standard chemotherapeutic regimens for non-elderly SCLC patients are not always suitable for older patients due to their vulnerable organ function and/or co-morbidities. Therefore, the establishment of a chemotherapeutic regimen that is well balanced between safety and efficacy for this population should be pursued.

The Japan Clinical Oncology Group (JCOG) 9702 study compared carboplatin plus etoposide (CE) versus split-dose cisplatin plus etoposide (SPE) in elderly and poor-risk patients with extensive disease (ED)-SCLC (4). Based on the results of this study, the JCOG concluded that the SPE regimen should remain as the standard treatment for elderly and poor-risk patients with ED-SCLC, the CE regimen being an alternative. However, because the CE regimen does not require hydration and can be administered in an outpatient setting, elderly patients with ED-SCLC in Japan more commonly receive this regimen.

In contrast, the Phase III JCOG 9511 study has shown that irinotecan plus cisplatin (IP) is more effective than etoposide plus cisplatin (EP) for treating non-elderly patients with ED-SCLC (5). However, elderly patients (age $\geq$71 years) were excluded from this trial. When considering the treatment plan for elderly patients with ED-SCLC, the 1-day bolus administration of this cisplatin-based regimen would be difficult because hydration is required. Until now, the carboplatin plus irinotecan (CI) regimen has been repeatedly reported. Although several studies included patients 70 years of age or older, few studies were especially designed for the elderly. Therefore, it would be meaningful to consider a CI regimen for the elderly. Two randomized trials have compared CI with CE for ED-SCLC patients. Although Schmittel et al. (6) did not show a significant survival benefit in the CI arm, survival was marginally better and fewer hematological toxicities were observed. In contrast, Hermes et al. (7) reported a significant survival advantage of CI over CE. Although these trials were not specifically designed for elderly patients and the doses used differed from Japanese standard doses, we believed it was worthwhile to investigate the efficacy of CI in elderly patients with ED-SCLC. Furthermore, a recent meta-analysis of camptothecins compared with etoposide in combination with platinum in ED-SCLC showed a survival benefit associated with camptothecins plus platinum (excluding nogitecan) over etoposide plus platinum in a subgroup analysis (8). Thus, a Phase III trial comparing CE with CI in elderly patients with ED-SCLC is being warranted in the JCOG Lung Cancer Study Group (LCSG).

In our previous study (9), we reported the 4-weekly schedule of CI regimen using prophylactic granulocyte colony-stimulating factor (G-CSF) support in elderly patients with SCLC. However, this study was not a Phase I study and had a heterogeneous patient population. In addition, because not only chemotherapy-naïve but also pretreated patients were included and the treatment drug dose was changed according to the patient's characteristics, the recommended dose could not be decided in the study. Recently, prophylactic use of G-CSF has not been preferred in clinical practice in Japan because more expensive cost and prolonged hospital stays are required. For the reason given above, we cannot apply the previous data to plan the Phase III study and we think that optimal schedule and dose of CI for elderly patients with SCLC have not been established. On the other hand, Thoracic Oncology Research Group (TORG) decided a recommended dose of 3-weekly schedule of CI regimen for elderly patients with limited disease (LD)-SCLC in a Phase I study (unpublished data). Because thoracic radiotherapy was sequentially administered after four courses of chemotherapy in this Phase I study, it might be justified that the recommended dose of CI for LD-SCLC could be used in elderly patients with ED-SCLC based on these data. Furthermore, because members of JCOG and TORG were much different, JCOG-LCSG recommended a further feasibility study by only JCOG members for elderly patients with ED-SCLC. Therefore, we conducted a feasibility study to evaluate the safety and efficacy of CI in elderly patients with ED-SCLC in preparation for a future JCOG Phase III study designed to compare CE with CI in this patient population. This study is registered with the UMIN Clinical Trials Registry as trial 000003208.

PATIENTS AND METHODS

Patient Selection

Patients with the following inclusion criteria were enrolled: age $\geq$70 years; cytologically or histologically confirmed SCLC; ED stage (defined as at least one of the following: distant metastasis, contralateral hilar-node metastasis, malignant pleural effusion and pericardial effusion); no prior chest radiotherapy or chemotherapy; an Eastern Cooperative Oncology Group performance status (PS) of 0–2; no other co-existing malignancy and adequate hematologic, hepatic and renal organ function (leukocyte count $\geq$4000/mm^3, absolute neutrophil count [ANC] $\geq$2000/mm^3, platelet count $\geq$100 000/mm^3, hemoglobin level $\geq$9.0 g/dl, aspartate aminotransferase [AST]/alanine aminotransferase [ALT] levels $\leq 2\times$ upper limit of normal range, total bilirubin $\leq$1.5 mg/dl, creatinine $\leq$1.5 mg/dl, creatinine clearance $\geq$50 ml/min and $PaO_2 \geq$ 60 mmHg). The additional criteria were: no symptomatic pericardial or pleural effusion requiring drainage, no active concomitant malignancy, no senile dementia, no diarrhea and provision of written informed consent. The exclusion criteria included brain metastases requiring radiotherapy, superior vena cava syndrome requiring radiotherapy and serious medical or psychiatric illness. Patients with interstitial pneumonitis detected by chest computed tomography (CT) scan were excluded. All the patients had chest X-ray, CT scan of the chest and abdomen, CT scan or magnetic resonance imaging of the brain and isotope bone scanning or positron emission tomography within 28 days before registration.

Treatment Plan

Based on our previous feasibility study using CI for elderly patients with SCLC (9), the TORG conducted a Phase I study of the CI regimen and sequential thoracic radiotherapy for elderly patients with LD-SCLC. In that study, the recommended dose was carboplatin area under the curve (AUC) of four Day 1 and irinotecan 50 mg/m^2 Days 1 and 8 every 3 weeks (unpublished data). Although the TORG study

included only elderly patients with LD-SCLC, we elected to use the recommended dose from this study in the current study of elderly patients with ED-SCLC. Thus, all the patients were assigned to carboplatin AUC 4 intravenously (IV) on Day 1 plus irinotecan 50 mg/m^2 IV on Days 1 and 8 every 21 days. Irinotecan on Day 8 was withdrawn if leukocyte counts were $<3000/mm^3$, platelet counts were $<100\,000/mm^3$ or if diarrhea Grade ≥ 1 occurred. Treatment was repeated for up to four cycles. Subsequent cycles were permitted only if the ANC was $\geq 1500/mm^3$, the leukocyte count was $\geq 3000/mm^3$, the platelet count was $\geq 100\,000/mm^3$, serum creatinine was ≤ 1.57 mg/dl, AST/ALT levels were $\leq 2.5\times$ upper limit of normal range, PS was 0–2, neither infection nor fever was present and treatment-related non-hematologic toxicities (excluding alopecia) had resolved to Grade ≤ 2 after Day 21. A treatment delay of ≤ 2 weeks was permitted. Use of G-CSFs was recommended in accordance with their package inserts or clinical recommendations. If G-CSF therapy was administered, the criteria for the next cycle had to be satisfied both after Day 21 and ≥ 2 days after discontinuation of G-CSF. Antiemetic prophylaxis with 5-HT_3 antagonists plus dexamethasone was routinely administered. Dose modifications were allowed only once if Grade 4 leukopenia or neutropenia lasting ≥ 4 days, Grade 4 thrombocytopenia or Grade 3 non-hematological toxicities, except for nausea/vomiting, constipation, hyponatremia and creatinine, occurred. When dose modification was needed, the next treatment course was started with carboplatin AUC 4 on Day 1 plus irinotecan 40 mg/m^2 on Days 1 and 8 every 21 days.

The protocol treatment was terminated if any of the following occurred: disease progression, a treatment delay ≥ 2 weeks, need for dose modification two times, Grade 2–4 pneumonitis and Grade 4 non-hematological toxicities. Because this was a feasibility study, post-protocol treatments were left to the discretion of the treating physicians.

Study Design

This trial was designed as a multicenter prospective feasibility study. The study protocol was approved by the institutional review board at each institution prior to study initiation. The primary objective was feasibility, defined as the percentage of patients who have received three or more courses of chemotherapy. Patients showing disease progression prior to receiving three courses of chemotherapy were excluded from the feasibility evaluation. In addition, even if irinotecan was not administered on Day 8 due to toxicity, the chemotherapy course was judged as being complete. In the JCOG9702 (4), the percentages of patients who have received three and four courses of CE regimen were 69 and 63%, respectively. In this study, we considered that the completion rate of three or more courses of chemotherapy was a more appropriate endpoint than that of four courses because CI regimen might be more toxic than the CE regimen. Therefore, we concluded that the study treatment was feasible when the completion rate of three or more courses of chemotherapy was $\geq 60\%$. Ten patients were initially registered into this study. If the feasibility (completion rate) was $>60\%$, the study would be considered to have yielded positive results and to be finished. If the completion rate was 30 to $<60\%$, we planned to enroll 10 more patients to confirm whether the low rate was due to the treatment regimen or to chance. If the feasibility remained at $<60\%$ in a total of 20 patients, the study would be considered to have yielded negative results. The secondary objectives were toxicity status, overall response rate (ORR), progression-free survival (PFS) and overall survival (OS). Tumor responses were evaluated according to the Response Evaluation Criteria in Solid Tumors criteria, version 1.0. Toxicity was evaluated using the National Cancer Institute Common Toxicity Criteria version 3.0.

If a patient was documented as having a complete response (CR) or a partial response (PR), a confirmatory evaluation was performed after an interval of at least 4 weeks. The patient was considered to have a stable disease (SD) if it was confirmed and sustained for 6 weeks or longer.

The relative dose intensity (RDI) of irinotecan was calculated by dividing the actual received dose of the agent among all chemotherapy courses (mg/m^2/week) by the total projected dose of the four treatment courses (mg/m^2/week). When chemotherapy was completed without any delays or skipping of agents, the RDI was 100%.

Table 1. Patient characteristics

Median age, years (range)	77.5 (70–82)
Gender	
Male/female	10/0
ECOG PS 0/1	1/9
TNM classification	
T 4/3/2/1	4/2/1/3
N 0/1/2/3	1/1/2/6
M 0/1	1/9
Brinkman index	
Median (range)	1110 (840–3000)

ECOG, Eastern Cooperative Oncology Group; PS, performance status.

RESULTS

Patient Characteristics

From March 2010 through March 2011, 11 patients were registered in three institutions. One patient withdrew consent after Day1 of the first course. Because this patient did not experience acute toxicities and the reason seemed to be related to other personal problems, we thought one more additional patient to the previously scheduled 10 patients were appropriate for this study. The median age was 77 (range, 70–82) years and nine patients had a PS of 1, all of whom were male (Table 1). The median Brinkman Index was 1110 (range,