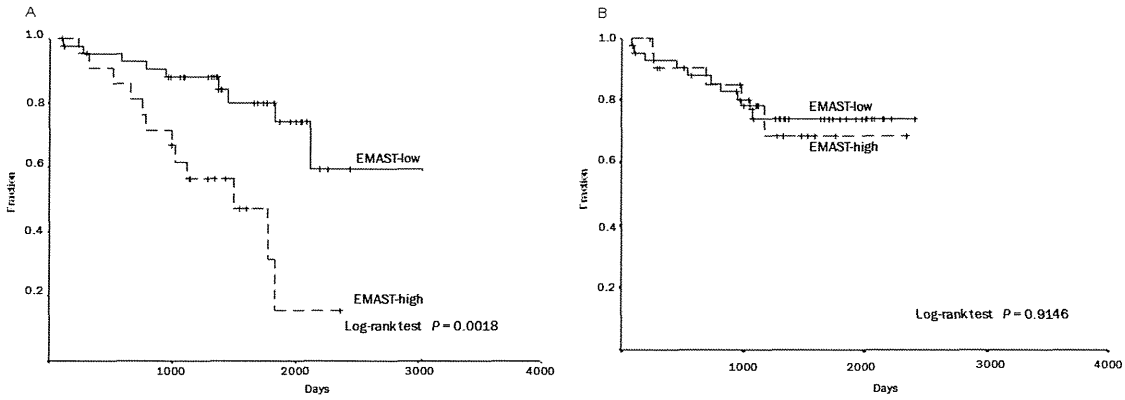

Figure 6. Association between EMAST and 5-year overall survival (A) and 5-year disease-free survival (B). Kaplan-Meier survival curves are shown. EMAST-high: tumors exhibiting EMAST in two or more of the tetra-nucleotide-repeating regions. EMAST-low: tumors exhibiting the EMAST in one or none of the regions. Five-year overall survival rates were 79.9% and 31.4% in EMAST-low and –high groups, respectively (Log-rank test, P = 0.0018) (A). Five-year disease-free survival rates were 73.9% and 68.5% in EMAST-low and –high groups, respectively (Log-rank test, P = 0.9146) (B).

or di-nucleotide-repeating regions of the Bethesda panel (**Figure 2B**). Traditional MSI was found at three markers, BAT 26 (4/65, 6.2%), D2S8123 (2/65, 3.1%) and D17S250 (1/65, 1.5%) (**Figure 3B**).

EMAST and traditional MSI appear to occur independently, as no significant association in their incidence was found (Fisher's exact test, *P* = 0.146).

LOH in selected tetra-nucleotide-repeats and Bethesda panel

All the tumors examined were heterozygous in at least two markers among the ten tetra-nucleotide-repeating regions, and 58 (89.2%) were heterozygous in at least one marker of the Bethesda panel. LOH at the tetra-nucleotide-repeated regions was found in 41 of 65 (63.1%) tumors (25/39 [64.1%] ADCs, 11 of 19 [57.9%] SQCs, 4 of 6 [66.7%] LCCs and one adenosquamous cell carcinoma) (**Figure 4A**). LOH tended to preferentially occur at D8S348 (7/18, 38.9%), D21S1436 (10/28, 35.7%), MYCL1 (11/31, 35.5%), D9S304 (11/44, 25.0%), D9S303 (11/45, 24.4%), D20S82 (6/27, 22.2%), and D8S321 (7/32, 21.9%), than at D2S443 (5/11, 15.2%), D9S747 (5/59, 8.5%), and UT5037 (0/41, 0.0%) (**Figure 5A**).

LOH at the mono- or di-nucleotide regions of the Bethesda panel was found in 11 of 58 (19.0%) tumors (3/19 [15.8%] squamous cell carcinomas, 5 of 39 [12.8%] adenocarcinomas and 3 of 6 [50%] large cell carcinomas) (**Figure 4B**). LOH tended to preferentially occur at BAT25 (2/17, 11.8%), BAT 26 (2/4, 50.0%), and D2S123 (4/38, 10.5%), than at D5S346 (2/27, 7.4%), and D17S250 (3/43, 7.0%) (**Figure 5B**).

Association between EMAST/MSI and clinicopathologic parameters

Tumors exhibiting EMAST in two or more of the tetra-nucleotide-repeating regions were defined as EMAST-high (22/65, 33.8%), and all other tumors were defined as EMAST-low (43/65, 66.2%), according to the previous studies [15, 28]. The level of EMAST showed significant association with medical history of an overlap with other malignant neoplasms (**Table 3**). There were no significant correlations between the level of EMAST and other clinicopathologic parameters (i.e., sex, age, smoking history, family history of malignancies, histological subtype, pathological T factor (pT), vascular and lymphatic invasion, proliferative activity [Ki-67 index], LOH of p53 locus, and immunohistochemical expression of p53 protein) (**Table 3**).

Tumors exhibiting traditional MSI in two or more regions of the Bethesda panel were defined as MSI-high (0/8, 0%), and all other tumors were defined as MSI-low (8/8, 100%). There were no significant correlations between the level of

Table 5. Essential clincopathologic information and EMAST among cases with multiple malignant neoplasms

No	Sex	Age	Histology	EMAST level	alternation in the ten selected tetra-nucleotide-repeating regions										Overlapped neoplasms	Outcome	Cause of death
					D8S321	D20S82	UT5037	D8S348	D2S443	D21S1436	D9S747	D9S303	D9S304	MYCL1			
1	M	66	SQC	H	-	Ins	-	-	-	-	-	Ins	-	-	GC+RCC	Dead	RCC
2	M	68	SQC	H	-	-	-	-	Ins	-	-	Ins	-	-	LC+RC	Dead	unknown
3	M	79	SQC	H	-	-	-	-	Ins	Ins	-	-	LOH	-	GC	Dead	AMI
4	M	74	ADC	H	-	Ins	-	-	Ins	-	-	-	-	-	PC	Dead	pneumonia
5	M	60	SQC	H	-	-	-	Ins	-	-	Ins	LOH	-	LOH	RC	Dead	RC
6	M	58	SQC	H	Ins	-	-	LOH	-	Ins	-	-	LOH	-	BC	Dead	BC
7	F	77	ADC	H	Ins	Ins	-	-	LOH	-	-	Ins	-	LOH	MSC	Alive	-
8	M	53	ADC	H	Ins	-	-	-	Ins	-	-	-	-	LOH	GC	Alive	-
9	M	64	ADC	H	Ins	Ins	-	-	-	-	-	-	-	LOH	GC	Alive	-
10	M	82	SQC	L	-	-	-	-	-	LOH	Ins	-	-	-	GC	Dead	NSCLC
11	F	76	ADC	L	-	-	-	-	-	-	-	-	-	-	UC	Alive	-
12	M	55	ADC	L	-	LOH	-	NA	-	Ins	-	-	-	LOH	ML	Dead	ML
13	M	75	ADC	L	-	-	-	-	-	-	-	-	LOH	-	SS	Alive	-
14	M	78	ADC	L	-	-	-	-	-	-	-	-	-	-	PC	Alive	-
15	M	77	SQC	L	LOH	-	-	NA	Ins	-	-	-	NA	-	SCLC	Dead	SCLC
16	M	73	SQC	L	-	-	-	-	-	-	-	-	-	-	GC	Alive	-

EMAST, elevated alterations of selected tetra-nucleotide; SQC, squamous cell carcinoma; ADC, adenocarcinoma; M, male; F, female; H, high; L, low; Ins, instable; LOH, loss of heterozygosity; NA, Not available; -, no alteration; NSCLC, Non-small cell lung carcioma; SCLC, small cell lung carcinoma; GC, gastric cancer; RCC, renal cell cancer; LC, laryngeal cancer; RC, rectal cancer; UC, uteirne cancer; ML, malignant lymphoma; PC, prostate cancer; SS, synovial sarcoma; BC, bladder cancer; MSC, maxillary sinus cancer; AMI, acute myocardial infarction.

MSI and the clinicopathologic parameters examined (data not shown).

Association between LOH and clinicopathologic parameters

Tumors exhibiting LOH in two or more tetra-nucleotide-repeating regions were defined as LOH/selected tetra-nucleotide (ST)-high (20/65, 30.8%), and all other tumors were defined as LOH/ST-low (45/65, 69.2%). There were no significant correlations between the level of LOH/ST and the clinicopathologic parameters (**Table 4**).

Similarly, tumors exhibiting LOH in two or more regions of the Bethesda panel were defined as LOH/Bethesda panel (BP)-high (2/58, 3.4%), and all other tumors were defined as LOH/BP-low (56/58, 96.6%). There were no significant correlations between the level of LOH/BP and the clinicopathologic parameters (data not shown).

Association between EMAST and clinical outcome

The EMAST-high group showed a poorer postoperative overall survival than the EMAST-low group (mean survival time was 1394 days for the EMAST-high group and 2396 days for the EMAST-low group; log-rank test, $P = 0.0018$) (**Figure 6A**). Of the 22 patients with EMAST-high tumors, 12 died; 3 died of NSCLCs (the primary cause), 3 other malignant neoplasms (i.e., renal cell cancer, rectal cancer, bladder cancer), and 6 non-neoplastic diseases (i.e., acute myocardial infarction, cardiac failure, and pneumonia). Of the 43 patients with EMAST-low tumors, 10 died; 6 died of NSCLCs (the primary cause), 2 other malignant neoplasms (i.e., small cell lung cancer and malignant lymphoma), and 2 non-neoplastic diseases (i.e., cardiac failure and decrepitude).

There was no significant difference in the disease-free survival (mean survival time was 1844 days for the EMAST-high group and 1947 days for the EMAST-low group; log-rank test, $P = 0.9146$) (**Figure 6B**), and no association between the level of EMAST and disease recurrence (recurrent rate, 5/22 in the EMAST-high group versus 11/43 in the EMAST-low group, Chi-square test, $P = 0.962$). Moreover, no significant associations were found between the level of LOH/ST, LOH/BP, or MSI and any of the clinicopathologic parameters examined (data not shown).

Discussion

The present study demonstrated that a considerable fraction of NSCLCs was unstable in the ten tetra-nucleotide-repeating regions and that the incidence of EMAST is unequivocally higher than that of traditional MSI. These findings are comparable to those of previous studies which showed an incidence of EMAST in NSCLC of 35-51% [21-23]. The incidence of EMAST differs among the types of malignant neoplasms, 5% in prostate cancer [6], 13% in ovarian cancer [29], 75% in skin cancer [27], and 43.9-45% in bladder cancer [27, 28]. These findings suggest a potential molecular basis for the unique properties in different types of malignant neoplasms.

The most interesting finding of the present study is that patients with EMAST-high NSCLC were affected by additional malignant neoplasms including gastric cancer and renal cell cancer at a significantly higher incidence (42.9% [9/21] in the EMAST-high group versus 16.3% [7/43] in the EMAST-low group). For the 16 patients who were affected by multiple neoplasms, essential information of their clinicopathologic characteristics and alterations in the tetra-nucleotide-repeating markers are described in **Table 5**. Similarly, patients with HNPCC (Lynch syndrome) are also often affected by additional neoplasms, such as endometrial and gastric cancer [14, 15, 31, 32]. HNPCC is an autosomal dominant disease with germ line mutations in the mismatch repair genes (i.e., *h*MSH2, *h*MLH1, and *h*MSH6) [10, 11, 14, 15, 32]. Defects in DNA mismatch repair due to mutations cause traditional MSI and manifest as frame-shift mutations in mono- or di-nucleotide-repeating regions [10, 11, 14, 15, 31, 32]. Traditional MSI has been found in 85-95% of HNPCC (and in 10-15% of sporadic colorectal cancers, in which the mismatch repair genes are silenced by the acquired epigenetic modification) and is well accepted to be an important molecular basis for promoting carcinogenesis of certain types of malignant neoplasms [7-12]. On the other hand, EMAST, distinct from traditional MSI, is not associated with defects in mismatch repair [23, 28]. Although the actual

molecular mechanism of EMAST remains unclear, previous studies of some AAAG-type tetra-nucleotide repeating regions suggest that p53 alterations could be involved [22]. One recent study demonstrated an association between the heterogeneous nuclear expression of hMSH3 and EMAST in colorectal cancer cells, suggesting that an acquired hMSH3 alteration could be its molecular mechanism [36]. The present study investigated the involvement of p53 (LOH of p53 gene and its potential mutations evaluated by immunohistochemistry) in EMAST, but failed to obtain a result that supports previous findings [16, 21-24, 27]. The difference in the tetra-nucleotide repeating regions examined might be responsible for this discrepancy. Thus, establishment of universal markers to evaluate EMAST, like the Bethesda panel, is necessary to verify its clinicopathologic significance. Moreover, a comprehensive search for potential alterations of DNA replication/repair molecules like hMSH3 may lead to elucidation of the molecular mechanism of EMAST.

As for the clinical outcome, a pronounced difference in the overall survival was found between the EMAST high- and low-groups. However, no significant difference was found in the disease-free survival and the recurrent rate, or in histological grade and proliferating activity of neoplastic cells. Notably, 3 of 22 (13.6%) patients with EMAST-high tumor died of other malignant neoplasms, while 2 of 43 (4.7%) patients with EMAST-low tumor died of other neoplasms. These findings suggest that the poorer overall survival in the EMAST-high group might be due to a high susceptibility to malignant neoplasm, and EMAST itself does not promote the progression process of their carcinogenesis (that is, it does not promote the acquisition of highly malignant activity of neoplastic cells).

In conclusion, impairment of molecular machinery that maintains stable replication of the tetra-nucleotide-repeated regions may elevate susceptibility to NSCLCs and certain neoplastic diseases. Elucidation of the potential molecular mechanism of EMAST could lead to discovery of a novel genetic background determining susceptibility to NSCLCs and establishment of a novel disease susceptible for multiple neoplasms including NSCLCs.

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Declaration of Conflicts of interest

None declared.

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Case Report

Three-dimensional simulation, surgical navigation and thoracoscopic lung resection

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This report describes a 3-dimensional (3-D) video-assisted thoracoscopic lung resection guided by a 3-D video navigation system having a patient-specific 3-D reconstructed pulmonary model obtained by preoperative simulation. A 78-year-old man was found to have a small solitary pulmonary nodule in the left upper lobe in chest computed tomography. By a virtual 3-D pulmonary model the tumor was found to be involved in two subsegments (S1 + 2c and S3a). Complete video-assisted thoracoscopic surgery bi-subsegmentectomy was selected in simulation and was performed with lymph node dissection. A 3-D digital vision system was used for 3-D thoracoscopic performance. Wearing 3-D glasses, the patient's actual reconstructed 3-D model on 3-D liquid-crystal displays was observed, and the 3-D intraoperative field and the picture of 3-D reconstructed pulmonary model were compared.

INTRODUCTION

Although conventional open thoracotomy has been replaced with video-assisted thoracoscopic surgery (VATS) for many pleuro-pulmonary diseases, video-assisted thoracoscopic lung resection such as segmentectomy and subsegmentectomy requires surgeons to have much experience and skill for performing the procedure safely. Recently, 3-dimensional (3-D) images, which help surgeons, have been used in preoperative simulation for VATS lung resection [1].

Previously, our department has reported a patient-specific virtual 3-D pulmonary model for thoracoscopic lung resections [2, 3]. This report describes 3-D video-assisted thoracoscopic lung resection guided by a 3-D video navigation system having a patient-specific 3-D reconstructed pulmonary model obtained by preoperative 3-D simulation.

CASE REPORT

A 78-year-old man with a history of pulmonary tuberculosis and gastric cancer was found to have a small solitary pulmonary nodule in the left upper lobe 4 months ago in chest computed tomography (CT) done for postoperative follow-up for gastric cancer (Fig. 1A). Routine laboratory study results, including the tumor markers carcinoembryonic antigen, sialyl Lewis X-1, and squamous cell carcinoma antigen, were within normal limits. He was preoperatively staged as N0 by positron emission tomography, high-resolution CT (HRCT) of the chest and abdomen, and the magnetic resonance imaging of brain. He was also examined by thin-sliced chest CT scan. Based on thin-sliced plain chest CT scan, based on the data of which a virtual 3-D pulmonary model was designed for him on a personal computer (PC) before operation. Patient-specific 3-D pulmonary model was created by the following methods. Homemade software, called CTTRY (Tokyo Women's Medical University, Tokyo, Japan), was used [2]. By using 120 of 1-mm thin-sliced CT scan images of the tumor and the hilum in digital imaging and communications in medicine format, CTTRY allowed us to mark pulmonary arteries, veins, bronchi and tumor in the thin-sliced CT scan images. CTTRY attempted to reconstruct an anatomical model with the help of anatomically correct

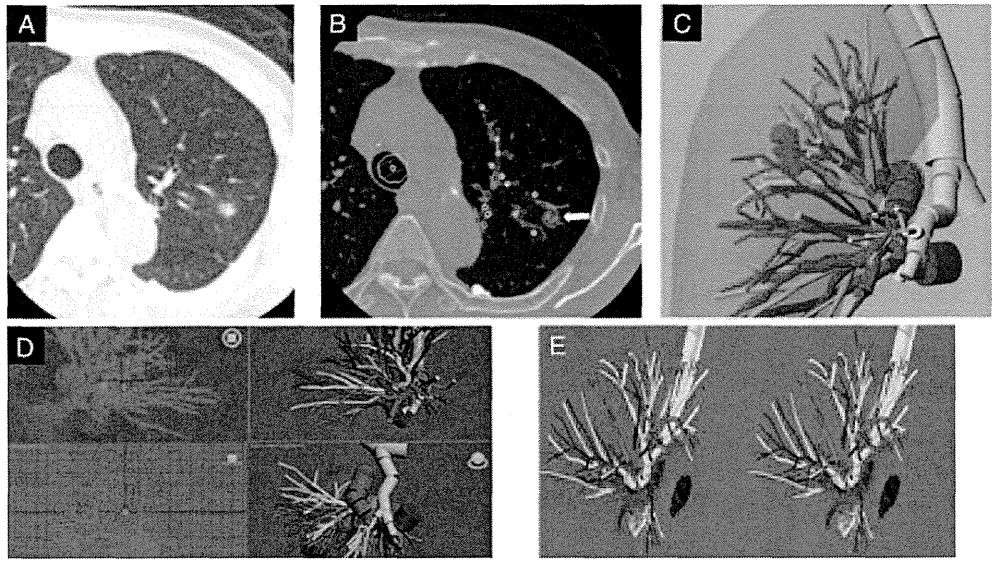

Figure 1: (**A**) Chest CT image shows a primary lesion as ground glass opacity in 2 segments (S1 + 2 and S3) of the left upper division. (**B**) Locations and thicknesses of tumor. Yellow dots indicate the bronchi; red dots, the pulmonary arteries; blue dots, the pulmonary veins; white, tumor, which were rendered as different-sized cylinders by the home-made software program (CTTRY). The striped rectangles of each color were a trace of the dots of each color. (**C**) Virtual 3-D image was reconstructed using shareware (Metasequoia). Yellow indicates the trachea and bronchi; red, pulmonry arteries; blue, pulmonary veins; the veiled area, the lung. (**D**) Data of the reconstructed 3-D images were converted with Autodesk® 3ds Max® 2012. (**E**) REMO Exporter®, which reproduces Autodesk 3ds Max data with real-time CG, can indicate with a stereoscopic vision display by 3-DCG data. On a PC, reconstructed 3-D pulmonary images were provided side-by-side and were output to a monitor. Dark yellow indicates the stapled bronchi; dark blue, resected pulmonary veins.

images (Fig. 1B). After feeding the reconstructed model images into a PC, the pulmonary arteries, veins and bronchi of patient were traced and marked by an experienced thoracic surgeon for the anatomic structure on the images. The location and thickness of the bronchi and pulmonary vascular were rendered as various-sized cylinders. Then, based on the resulting numerical data, a 3-D image, which was converted by the surface-rendering technique, was reconstructed using software (Metasequoia, http://www.metaseq.net/) (Fig. 1C). When his virtual 3-D pulmonary model was reconstructed by CTTRY, the tumor was found to be involved in two subsegments [horizontal subsegment of apicoposterior segment (S1 + 2c) and posterior subsegment of anterior segment (S3a)] (Fig. 1E). Further, the data of the reconstructed 3-D images by Metasequoia were converted with Autodesk® 3ds Max® 2012 (Autodesk, San Rafael, CA, USA) (Fig. 1D). Finally, image software, REMO Exporter® (3D Incorporated, Kanagawa, Japan), which reproduced Autodesk data with real-time computer graphics (CG), was able to indicate with a stereoscopic vision display by 3-DCG data. On a PC, reconstructed 3-D pulmonary images were provided side by side and were output to a monitor. Surgeons were able to watch the 3-DCG of the reconstructed 3-D pulmonary model with a 3-D liquid-crystal (LC) monitor by REMO Viewer® (3D Incorporated). The reconstructed 3-D images on a 3-D LC monitor were able to be manipulated by virtual surgical procedures such as reshaping, cutting and moving (Fig. 1E). The simulation result was able to allow the subsegmentectomy of S1 + 2c and S3a to be performed with a sufficient surgical margin.

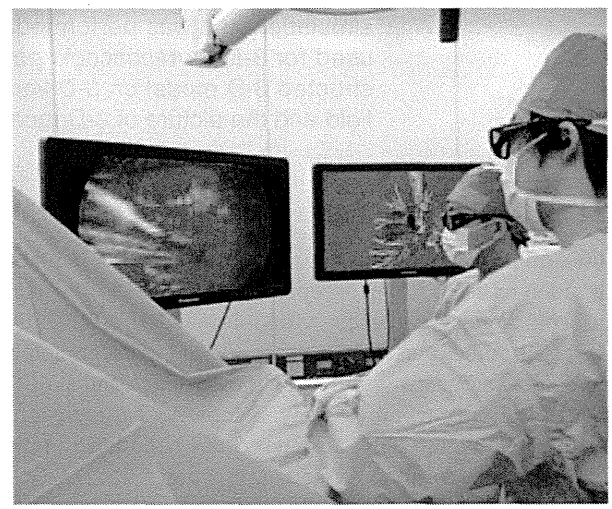

Figure 2: In 3-D thoracoscopic surgery with 3-D navigation, wearing glasses, comparing 3-D intraoperative field during VATS and the picture of 3-D reconstructed pulmonary model, with 3-D LC displays, the surgeons performed thoracoscopic procedures.

A 3-D digital vision system (Shinko Optical, Tokyo, Japan) was used for 3-D thoracoscopic performance. This system included a 11-mm, 30° stereo digital scope and a 3-D data-processing unit. Wearing glasses, thoracoscopic procedures were performed by the surgeons and by means of intraoperative navigation, the patient's actual reconstructed 3-D pulmonary model was able to be watched with 3-D LC displays (Fig. 2). Complete VATS bi-subsegmentectomy was selected

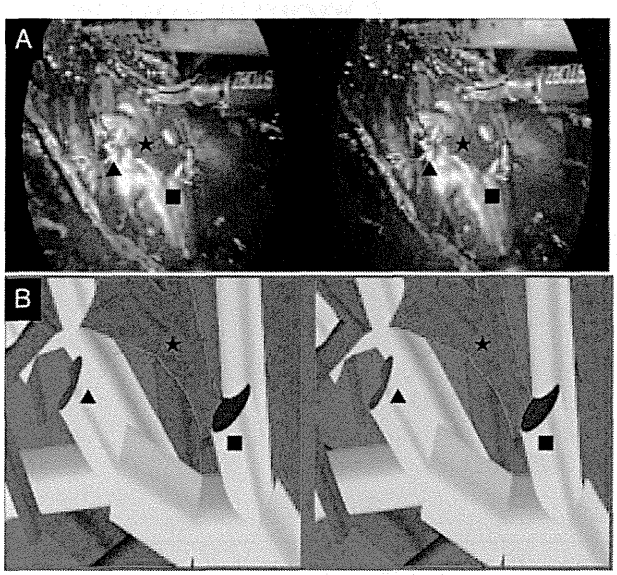

Figure 3: Comparison between the 3-D navigation of patient-specific 3-D reconstructed pulmonary model and the operative view. (**A**) The operative view of the patient was extracted from digital video data taken during the operation and was provided on a PC after operation. (**B**) In patient-specific 3-D image, the target bronchus and vascular branching pattern were similar to intraoperative findings. B1 + 2c (Filled Square) and B3a (Filled Triangle) were dissected and stapled separately. A1 + 2c (Filled Star) was detected.

in preoperative simulation and was performed with intraoperative assessment of lymph nodes; hilar node dissection with mediastinal lymph node sampling was done using conventional thoracoscopic instruments (Fig. 3A and B). Final histologic examination revealed T1aN0M0 pulmonary adenocarcinoma. The patient's postoperative course was uneventful.

DISCUSSION

Recently, smaller size lung cancers in a peripheral location are detected by HRCT. For these lung cancers, lung segmentectomy/subsegmentectomy has become a good option and has been reported to be able to yield promising outcomes comparable with those of lobectomy. On the other hand, video-assisted thoracoscopic lung resection such as segmentectomy and subsegmentectomy requires surgeons to have much experience and skill for performing the procedure safely.

Advances in HRCT scans have enabled reconstitution of fine virtual 3-D pulmonary models. Virtual reconstructed 3-D models can show easily the positional relationship among the target nodule, pulmonary vessels and bronchi, and allow surgeons to access more peripheral vascular branches and target segmental/subsegmenatal bronchi with an accurate detection during a thoracoscopic procedure [1]. As a result, reconstructed 3D pulmonary models guide surgeons in avoiding vascular injury in complete VATS lung resections. Further, simulation using a patient-specific 3-D pulmonary model helps surgeons to select the most suitable type of VATS lung resection [2–4].

In 3-D thoracoscopic surgery with 3-D navigation onto a 3-D LC display, depth perception is improved, compared with a conventional thoracoscopic surgery, and 3-D surgery is similar to an operative field vision of open thoracotomy [5]. Although 3-D vision was hampered by technical and financial limitations previously, a 3-D thoracoscopic system has been improved to real-time transmission. There is no study that performs 3-D endoscopic surgery using both presurgical 3-D simulation and actual navigation with a reconstructed patient pulmonary model. In our 3-D thoracoscopic surgery under 3-D navigation, comparing 3-D intraoperative field and the picture of 3-D reconstructed pulmonary model, surgeons simply wore glasses and were able to perform thoracoscopic surgery, such as segmentectomy and subsegementectomy, more easily and precisely. This technique can be applied to tumor <2 cm. We ensured that the safety surgical margin was >2 cm from the tumor.

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CHEST

Original Research

LUNG CANCER

Prognostic Factors and the Significance of Treatment After Recurrence in Completely Resected Stage I Non-small Cell Lung Cancer

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Objective: **The objective of this study was to identify the clinicopathologic factors influencing postrecurrence survival (PRS) in and the effect of postrecurrence therapy (PRT) on patients with completely resected stage I non-small cell lung cancer (NSCLC).**

Methods: **We reviewed the data of 919 patients in whom complete resection of stage I NSCLC had been performed.**

Results: **Of the 919 patients, 170 (18.5%) had recurrent disease. Initial PRT was performed in 118 patients (69.1%) (surgery in eight, chemotherapy in 79, radiotherapy in 10, and chemoradiotherapy in 21). On multivariate analyses, PRT (hazard ratio [HR], 0.542; 95% CI, 0.344-0.853; $P = .008$), female sex (HR, 0.487; 95% CI, 0.297-0.801; $P = .005$), and differentiation (HR, 1.810; 95% CI, 1.194-2.743; $P = .005$) demonstrated a statistically significant association with favorable PRS. Bone metastasis (HR, 3.288; 95% CI, 1.783-6.062; $P < .001$), liver metastasis (HR, 4.518; 95% CI, 1.793-11.379; $P = .001$), chemotherapy (HR, 0.478; 95% CI, 0.236-0.975; $P = .040$), epidermal growth factor receptor-tyrosine kinase inhibitors treatment (EGFR-TKIs) (HR, 0.460; 95% CI, 0.245-0.862; $P = .015$), and nonadenocarcinoma (HR, 2.136; 95% CI, 1.273-3.585; $P = .004$) were independently and significantly associated with PRS in the 118 patients who underwent any PRT. Subgroup analysis with a combination of these five PRS factors in the patients who underwent any PRT revealed median PRS times of 42.4 months for 20 patients lacking all five risk factors and 18.8 months for 98 patients with at least one of these risk factors ($P = .001$).**

Conclusions: **PRT, sex, and differentiation were independently associated with PRS. In the patients who underwent any PRT, PRS was related to EGFR-TKIs, chemotherapy, histology, and initial recurrence sites. One challenge for the future will be to create systematic treatment strategies for recurrent NSCLC according to the risk factor status of individual patients.**

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Abbreviations: EGFR-TKI = epidermal growth factor receptor-tyrosine kinase inhibitor; HR = hazard ratio; NSCLC = non-small cell lung cancer; PRS = postrecurrence survival; PRT = postrecurrence therapy; PS = performance status; RFP = recurrence-free proportion

Surgical resection with a curative intent is considered the standard of care for early stage non-small cell lung cancer (NSCLC), but > 20% of patients had recurrence, even in pathologic stage I cases.[1-6] Recurrence after complete resection for stages I to III of NSCLC ranges from 30% to 75%, and has been reported to depend on pathologic staging and follow-up period.[1,6-8] The majority of recurrences occur within the first 2 years,[1,6] although there are several studies showing late recurrences ≥ 5 years after resection.[9-11] Long-term, continuous follow-up is required to establish accurate recurrence rates and patterns.

Although several studies focusing on postrecurrence survival (PRS) of patients in stage I or stage I-III NSCLC have been reported,[2-4,8,12-14] no standard treatment strategy for recurrent disease based on prospective studies has been established. However, a standard treatment strategy is necessary because much longer

follow-up periods and robust protocols are required to evaluate PRS objectively. It is difficult to generalize about multifactorial patient backgrounds, which depend on disease, treatment, and performance status (PS) at recurrence. The prognostic factors predicting PRS or the appropriate treatment are still controversial.

Encouraging new treatments (including epidermal growth factor receptor-tyrosine kinase inhibitors [EGFR-TKIs], anaplastic lymphoma kinase inhibitors, pemetrexed, and bevacizumab) have afforded benefits to certain patients with advanced or recurrent NSCLC.[15-21] Advances in postrecurrence therapy (PRT) may provide improvement in overall survival among the patients who undergo surgery. The objective of the present study was to identify the clinicopathologic factors influencing PRS and their effect of PRT on stage I NSCLC.

Materials and Methods

From January 1990 through December 2007, 1,214 patients underwent complete resection for pathologic stage I NSCLC at our hospital. Complete resection was defined as demonstrating cancer-free surgical margins, both grossly and histologically. All patients underwent radical, anatomic, lobar resection and systematic, mediastinal lymph node dissection. The following exclusion criteria were applied: preoperative chemotherapy, radiation therapy, or both (n = 38); low-grade malignant tumors, including carcinoids, mucoepidermoid carcinomas, or adenoid cystic carcinomas (n = 20); and death within 30 days of operation (n = 9). Of the remaining 1,147 patients, complete follow-up was available for 919 patients, who composed the subjects of this study.

Preoperative evaluation included physical examination, chest radiography, CT scan of the chest and abdomen, bone scintigraphy, blood examination, and, since the early 2000s, PET scan (recently integrated PET-CT scan). Histologic subtypes of lung cancer were determined according to the World Health Organization classification,[22] and disease stage was determined in accordance with the *TNM Classification for Lung and Pleural Tumours*, 7th ed.[23]

The follow-up schedule consisted of a clinic visit every 3 months in the first year after resection, every 6 months from the second to the fifth year, and annually thereafter on an outpatient basis, and aimed at continuing follow-up for 10 years after resection. Follow-up procedures included physical examination, chest radiography, and blood examination (including serum tumor markers). CT scans of the chest and abdomen was performed every 6 months in the first 2 years, and annually from the third to the fifth year. Whenever any symptoms or signs of recurrence were detected, MRI of the brain and bone scintigraphy were performed.

Recurrences were diagnosed by physical examination and diagnostic imaging. Histologic or cytologic confirmation of the recurrence was made when clinically feasible. Local recurrence was defined as disease recurrence at the surgical margin, ipsilateral hemithorax, or mediastinum. Radiographic lymph node recurrence was defined as enlarged lymph nodes measuring > 1 cm on the short axis by CT scan and/or hypermetabolic lymph nodes on PET-CT scans. Pathologic confirmation of recurrence was made by endobronchial ultrasound-guided transbronchial needle aspiration of enlarged lymph nodes during follow-up. Distant metastasis was defined as disease recurrence in the contralateral lung or outside the hemithorax and mediastinum. A second primary tumor was recorded when a patient presented with a new histologic type, and with clinical features consistent with a new primary tumor. Data collected from our department database of patients, telephone interviews, and correspondence from outside sources during the follow-up periods were included.

Clinical characteristics were retrieved from available clinical records. The following clinicopathologic factors were assessed in the PRS analysis: age, sex, smoking status, primary tumor status (T1 vs T2), tumor size (0-30 mm vs > 30 mm), tumor differentiation (well/moderate vs poor), pathologic vascular invasion, pleural invasion, histology (adenocarcinoma vs others), and extent of resection (single lobe lobectomy vs more extensive resection, namely bilobectomy/pneumonectomy).

Length of the recurrence-free period was calculated in months from date of resection to date of initial recurrence or last follow-up showing no recurrence. To calculate the recurrence-free proportion (RFP), patients who died without recognized recurrence or who were known to have no recurrence at the date of last contact were censored. Length of PRS was measured from date of initial recurrence to date of death from any cause or date on which the patient was last known to be alive. PRS and RFP curves were plotted using the Kaplan-Meier method, and differences in variables were determined using the log-rank test or the Breslow tests. Categorical comparison was performed using the χ^2 test for discrete data and Student t test for continuous data. Multivariate analyses were performed using the Cox proportional hazards regression model. A backward stepwise selection procedure was implemented. All tests were two-sided, and P values < 0.05 were considered to indicate a statistically significant difference. Statview 5.0 software (SAS Institute Inc) was used for statistical analyses.

Data collection and analyses were approved, and the need to obtain written informed consent from each patient was waived, by the institutional review board at Tokyo Medical University (No. 2133).

Results

Median follow-up time for survivors was 62.0 months (range: 1.4-247.6 months). The RFP was 82.2% at 5 years after operation. Of the 919 patients, 170 (18.5%) had recurrent disease, with a median age of 66 years at the time of initial recurrence. Median PRS time for these patients was 17.6 months (range: 0.4-103.0 months). The 1- and 2-year PRS proportions were 73.5% and 51.4%, respectively (Fig 1).

Table 1 shows 5-year RFPs and univariate/multivariate analyses of recurrence according to clinicopathologic characteristics of patients with stage I NSCLC. Univariate analysis identified five significant risk factors:

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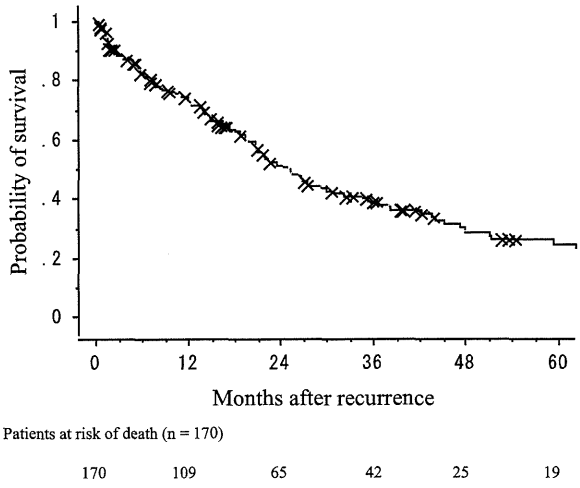

FIGURE 1. Postrecurrence survival curve of patients with non-small cell lung cancer recurrence.

male sex, pathologic vascular invasion, pleural invasion, poorly differentiated carcinoma, and nonadenocarcinoma. Multivariate analysis demonstrated that pathologic vascular invasion (hazard ratio [HR], 2.306; 95% CI, 1.621-3.280; $P < .001$), pleural invasion (HR, 1.489; 95% CI, 1.048-2.115; $P = .026$), and poorly differentiated carcinoma (HR, 1.842; 95% CI, 1.328-2.555; $P < .001$) were statistically significant predictors of recurrence.

Initial recurrence sites and PRT are shown in Table 2. Type of recurrence included only local recurrence in 43 patients (25.3%), distant in 113 (66.5%), and both in 14 (8.2%). Most commonly involved organs were the lung (the site of recurrence in 66 patients: ipsilateral in 23, contralateral/bilateral in 43), followed by regional lymph nodes in 37, brain in 30, bone in 21, and liver in 16. Initial PRT was performed in 118 patients (69.4%), and included surgery for 8, chemotherapy for 79, radiotherapy for 10, and chemoradiotherapy for 21. Surgical resections (n = 8) were performed in three patients with solitary pulmonary metastasis, three with solitary brain metastasis, one with adrenal gland metastasis, and one with chest wall and axillary lymph node involvement. Forty-one patients (24.1%) had no treatment for recurrent disease. Of the 118 patients who underwent any PRT, 66 (55.9%) underwent second-line or subsequent therapy, including

Table 1—*Patient Characteristics and Univariate and Multivariate Analyses of Recurrence*

	Univariate Analysis			Multivariate Analysis		
Factors	Patients, No.	5-y RFP, %	*P* Value	HR	95% CI	*P* Value
Age,[a] y						
< 65	439	84.1	...	...	...	...
≥ 65	480	80.4	.129	...	...	...
Sex						
Male	542	78.0	...	...	...	...
Female	377	87.8	< .001	...	...	...
Smoking status						
Never smoker	347	85.2	.134	...	...	...
Ever smoker	572	80.2	...	...	...	...
T category						
T1	512	84.7	...	...	...	...
T2	407	78.9	.100	...	...	...
Tumor size						
0-30 mm	663	84.0	...	...	...	...
> 30 mm	256	81.5	.112	...	...	...
Pathologic vascular invasion						
Absent	481	91.0	...	1	...	...
Present	421	72.1	< .001	2.306	1.621-3.280	< .001
Pleural invasion						
Absent	719	84.9	...	1	...	...
Present	191	71.8	< .001	1.489	1.048-2.115	.026
Histology						
Adenocarcinoma	706	83.8	...	...	...	...
Nonadenocarcinoma	213	76.3	.039	...	...	...
Differentiation						
Well or moderate	656	86.7	...	1	...	...
Poor	216	67.7	< .001	1.842	1.328-2.555	< .001
Type of surgery						
Single lobectomy	873	81.9	...	...	...	...
Bilobectomy or pneumonectomy	46	87.2	.942	...	...	...

HR = hazard ratio; RFP = recurrence-free proportion.
[a]Median age = 65 y.

Table 2—*Initial Recurrence Site and Postrecurrence Therapy*

Data of Recurrence Sites and Postrecurrence Therapies	Patients, No.
Overall	170
Type of recurrence	
Distant	113
Local	43
Both	14
Initial recurrence site	
Ipsilateral lung	23
Contralateral/bilateral lung	43
Regional lymph nodes	37
Malignant effusion/dissemination	13
Stump	9
Brain	30
Bone	21
Liver	16
Adrenal gland	10
Others	14
Postrecurrence therapy	
Initial therapy	
Surgery	8 (lung, 3; brain, 3; adrenal gland, 1; lymph nodes, 1)
Surgery alone	6
Surgery + chemotherapy	3
Chemotherapy	79
Radiation therapy	10
Chemoradiotherapy	21
None	41
Unknown	11
Second-line or the subsequent therapy	66
Chemotherapy	58
EGFR-TKIs	27 (gefitinib, 22; erlotinib, 3; both, 2)
EGFR mutation status/histology	Positive 12 (Ad, 11; Sq, 1) Wild 4 (Ad, 3; LCC, 1) Unknown 11 (Ad, 10; LCC, 1)
Others	7

Ad = adenocarcinoma; EGFR-TKI = epidermal growth factor receptor-tyrosine kinase inhibitor; LCC = large cell carcinoma; Sq = squamous cell carcinoma.

chemotherapy for 58, and EGFR-TKIs for 27 (gefitinib, 22 patients; erlotinib, three patients; and both, two patients). Among the latter 27 patients, *EGFR* mutations were detected in 12; four had wild-type *EGFR*.

Table 3 shows univariate/multivariate analyses of PRS. Univariate analysis identified six significant risk factors for PRS: male sex, smoking, poorly differentiated carcinoma, nonadenocarcinoma, no PRT, and shorter recurrence-free interval (≤ 24 months; median recurrence-free period was 24 months). Multivariate analysis demonstrated that PRT (HR, 0.542; 95% CI, 0.344-0.853; $P = .008$), female sex (HR, 0.487; 95% CI, 0.297-0.801; $P = .005$), and differentiation (HR, 1.810; 95% CI 1.194-2.743; $P = .005$) had a statistically significant association with favorable PRS.

The results of multivariate analysis of PRS determined that PRT had a strong impact on PRS. Therefore, we further examined PRS in the 118 patients who underwent any PRT (Table 4). Univariate analysis identified nine significant risk factors for PRS: male sex, smoking, poorly differentiated carcinoma, bone metastasis, liver metastasis, no chemotherapy or EGFR-TKI, no second-line therapy, and multiple organ metastases. Multivariate analysis demonstrated that bone metastasis (HR, 3.288; 95% CI, 1.783-6.062; $P < .001$), liver metastasis (HR, 4.518; 95% CI, 1.793-11.379; $P = .001$), chemotherapy (HR, 0.478; 95% CI, 0.236-0.975; $P = .040$), EGFR-TKI therapy (HR, 0.460; 95% CI, 0.245-0.862; $P = .015$), and nonadenocarcinoma (HR, 2.136; 95% CI, 1.273-3.585; $P = .004$) had a statistically significant association with PRS.

Subgroup analysis with a combination of these five PRS factors (no EGFR-TKI and chemotherapy, presence of liver or bone metastasis, nonadenocarcinoma) in patients with recurrence who underwent any PRT revealed median PRS times of 42.4 months for 20 patients lacking all five unfavorable factors and 18.8 months for 98 patients with one of these risk factors, respectively (Fig 2). The difference in PRS was statistically significant between the two groups ($P = .001$).

Discussion

We set out to identify clinicopathologic factors influencing PRS of patients with stage I NSCLC. Although curative surgical resection is the most effective therapy for stage I NSCLC, a considerable number of patients will develop recurrence. In the current study, overall incidence of recurrence was 18.5%, and median PRS time was 17.6 months. Initial location of recurrence was at a distant site in 74.7%, and the proportions of recurrences within 2 or 3 years after surgery were 48.2% and 66.5%, respectively (unpublished data). Previous studies have reported that the incidence of recurrence in patients with stage I NSCLC was 14% to 36%, with the 1-year survival rate ranging from 30% to 68% (Table 5).[1-6,8,24]

We examined risk factors for recurrence in stage I NSCLC, and identified three: pathologic vascular invasion, pleural invasion, and poorly differentiated carcinoma. These standard pathologic factors have also been reported to be good predictors of overall survival for patients with stage I NSCLC.[25-36] In our study, univariate analysis for PRS identified six significant risk factors (male sex, smoking, poorly differentiated carcinoma, nonadenocarcinoma, no PRT, and shorter recurrence-free interval [≤ 24 months]), while multivariate analysis revealed that sex, PRT, and differentiation were independent prognostic factors. Only differentiation was a significant predictor of recurrence and poor PRS, and pathologic vascular invasion and pleural invasion had no significant impact on PRS. PRS may be associated

Table 3—*PRS Analyses*

Factors	Univariate Analysis			Multivariate Analysis		
	Patients, No.	Median PRS, mo	*P* Value	HR	95% CI	*P* Value
Age at recurrence,[a] y						
<66	76	18.9	...	...	...	...
≥66	94	15.8	.242	...	...	...
Sex						
Male	118	15.5	...	1	...	...
Female	52	25.6	<.001	0.487	0.297-0.801	.005
Smoking status						
Never smoker	59	25.0	...	...	...	...
Ever smoker	111	14.1	.006	...	...	...
T category						
T1	87	15.8	...	...	...	...
T2	83	19.6	.476	...	...	...
Tumor size						
0-30 mm	132	16.9	...	...	...	...
>30 mm	38	20.9	.632	...	...	...
Pathologic vascular invasion						
Absent	53	15.8	...	...	...	...
Present	113	17.0	.088	...	...	...
Pleural invasion						
Absent	115	15.8	...	...	...	...
Present	53	18.8	.393	...	...	...
Histology						
Adenocarcinoma	124	20.9	...	...	...	...
Nonadenocarcinoma	46	12.4	<.001	...	...	...
Differentiation						
Well or moderate	97	20.8	...	1	...	...
Poor	65	14.1	.002	1.810	1.194-2.743	.005
Type of surgery						
Single lobectomy	162	17.3	.152	...	...	...
Bilobectomy or pneumonectomy	8	19.5	...	...	...	...
Adjuvant therapy						
Without	134	15.9	.547	...	...	...
With	36	21.0	...	...	...	...
Postrecurrence therapy						
Without	41	7.2	...	1	...	...
With	118	21.4	.021	0.542	0.344-0.853	.008
Recurrence free interval						
≤24 mo	82	16.2	...	...	...	...
>24 mo	88	18.4	.021	...	...	...
Type of recurrence						
Distant	127	15.8	...	...	...	...
Local only	43	18.8	.087	...	...	...
Number of recurrent sites						
Single	132	16.8	...	...	...	...
Multiple	38	18.6	.305	...	...	...

PRS = postrecurrence survival. See Table 1 legend for expansion of other abbreviations.
[a]Median age at recurrence = 66 y.

with recurrent disease characteristics, including the recurrence site, PRT, recurrence-free interval, or PS at time of recurrence, rather than with the biologically aggressive characteristics of lung cancer.

Previous studies have demonstrated the survival benefit of PRT in patients with stage I NSCLC. Nakagawa et al[4] and Hung et al[2,3] demonstrated that patients with stage I NSCLC treated either surgically or nonsurgically had a significantly better PRS than those with supportive care alone. In our study, PRT provided a more favorable PRS than that of no treatment, similarly to previous reports. However, the results of PRS in the patients who underwent any PRT showed that surgical resection was not related to a favorable outcome. This may have been because the number of patients who received surgery for recurrent disease was too small to provide any supportive data in terms of survival benefit. However, in cases of surgical resection for recurrent lung metastasis, objective evidence supporting the role of surgery is limited because it

Table 4—*PRS Analyses in 118 Patients Who Underwent Postrecurrence Therapy*

Factors	Univariate Analysis			Multivariate Analysis		
	Patients, No.	Median PRS, mo	*P* Value	HR	95% CI	*P* Value
Age at recurrence, y						
< 66	63	22.4	...	...	...	...
≥ 66	55	19.5	.151	...	...	...
Sex						
Male	79	20.0	...	...	...	...
Female	39	27.2	.002	...	...	...
Smoking status						
Never smoker	43	27.6	...	...	...	...
Ever smoker	75	17.6	.035	...	...	...
Histology						
Adenocarcinoma	84	24.4	...	1	...	...
Nonadenocarcinoma	34	13.9	< .001	2.136	1.273-3.585	.004
Differentiation						
Well or moderate	66	23.1	...	...	...	...
Poor	46	18.8	.019	...	...	...
Lung metastasis						
Absent	68	19.8	...	...	...	...
Present	49	21.4	.053	...	...	...
Brain metastasis						
Absent	96	19.6	...	...	...	...
Present	21	22.6	.584	...	...	...
Bone metastasis						
Absent	100	21.9	...	1	...	...
Present	17	15.8	.001	3.288	1.783-6.062	< .001
Liver metastasis						
Absent	110	21.9	...	1	...	...
Present	7	10.5	.001	4.518	1.793-11.379	.001
Chemotherapy						
Without	15	9.6	...	1	...	...
With	103	22.7	.009	0.478	0.236-0.975	.040
Surgical resection						
Without	110	20.8	...	...	...	...
With	8	33.7	.209	...	...	...
EGFR-TKI therapy						
Without	91	17.0	...	1	...	...
With	27	41.4	.002	0.460	0.245-0.862	.015
Second line therapy						
Without	52	14.0	...	...	...	...
With	66	27.2	.004	...	...	...
Recurrence free interval						
≤ 24 mo	59	17.0	...	...	...	...
> 24 mo	59	22.4	.394	...	...	...
Type of recurrence						
Distant	85	20.8	...	...	...	...
Local only	33	21.8	.086	...	...	...
Number of recurrent sites						
Single	89	21.0	...	...	...	...
Multiple	29	20.8	.049	...	...	...

See Table 1 and 3 legends for expansion of abbreviations.

may be difficult to distinguish second primary tumors from recurrent pulmonary metastasis. Advances in genomic analysis, molecular biologic tools, or diagnostic imaging may enable more accurate diagnosis of a solitary pulmonary lesion.

Among the cohort of 118 patients with any PRT, we identified five independent favorable prognostic factors of PRS by multivariate analysis: the absence of bone or liver metastasis, chemotherapy, EGFR-TKI therapy, and nonadenocarcinoma. Moreover, the result of the study showed an important aspect of a prognostic-factor based risk stratification. Median PRS times were 42.4 months for the patients lacking all five factors and 18.8 months for the patients with one of these risk factors ($P = .001$).

Some authors have found that the site of initial recurrence was a prognostic factor for PRS, which agrees with the current study. Yoshino et al[8] demonstrated

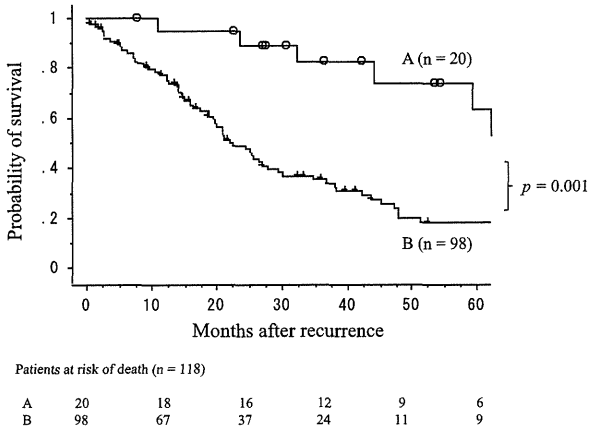

FIGURE 2. Postrecurrence survival curves of the patients lacking (A) all five unfavorable factors (not receiving epidermal growth factor receptor-tyrosine kinase inhibitor therapy and chemotherapy, liver or bone metastasis positive, nonadenocarcinoma), and (B) the patients with one of the five risk factors.

that bone metastasis was a marginally prognostic factor for PRS in patients with stage I-III NSCLC at the first resection. Assessment of bone metastatic type, osteoblastic or osteolytic, may be important as a part of postrecurrence therapeutic strategy because it has been noted that osteoblastic tumors lead to both a better prognosis and activating *EGFR* mutation presence.[37]

Major advances in NSCLC management have resulted from the understanding of molecular biology, development of molecule-targeting agents, and identification of biomarkers for targeted treatment. Since 2002, gefitinib has been used in Japan for the treatment of inoperable or recurrent NSCLC, and we started to administer it around the same period. It is now felt that EGFR-TKIs can improve the survival of some previously treated and untreated patients with advanced NSCLC, with the overall benefit being driven primarily by the subgroup with *EGFR* mutations.[15-17,38,39] EGFR-TKIs have also improved endurance and health-related quality of life compared with platinum-based doublet chemotherapy.[15-17] EGFR-TKIs are, therefore, good candidates for first-line PRT in patients who have had resected adenocarcinoma with distant metastases, but only in those with *EGFR* mutations.

There are several limitations in the present study. This study is retrospective, and bias may exist. First, patient-selection bias regarding PRT was unavoidable. Curative intent therapy or systematic treatment is difficult to perform in patients with poor PS. In the current study, PS or comorbidities at the time of recurrence were not accurately evaluated. Second, distinguishing second primary tumors from recurrent pulmonary metastasis was difficult. Even if a pathologic specimen was obtained, definitive diagnosis could be difficult under the current morphology-based diagnostic criteria. Third, complete follow-up was not available for all eligible patients.

There are presently no clinical guidelines for PRT regarding resected NSCLC based on large-scale prospective studies. Molecularly targeted therapy, chemotherapeutic regimens, and surgical strategies have evolved substantially over the decades. A challenge for the future will be to create systematic treatment strategies for recurrent NSCLC according to the individual patient's recurrent-disease characteristics, including the initial recurrence site, age, sex, PS, or recurrence-free interval, and original tumor characteristics.

CONCLUSION

This study showed that male sex, the absence of PRT, and poorly differentiated carcinoma were independent unfavorable prognostic factors of PRS in patients

Table 5—*PRS of Patients With Stage I Non-small Cell Lung Cancer in Previous Series*

Series/Year	Patients, No.	Incidence of Recurrence, No. (%)	PRS, % (y)	Type of Recurrence	Independent Favorable Factors of PRS
Martini et al[6]/1995	598	159 (26.6)	NR	L/D	NR
al-Kattan et al[1]/1997	123	36 (29.3)	NR	L/D	NR
Nakagawa et al[4]/2008	397	87 (21.9)	67.7 (1)	L/D	Symptom at recurrence, negative
			34.4 (3)		Cervicomediastinum metastases, negative
					Liver metastases, negative
					PRT (surgery/nonsurgery)
Hung et al[2]/2009	933	74 (7.9)	48.7 (1)	L	PRT (surgery, chemotherapy, and/or radiotherapy)
			17.6 (2)		
Hung et al[3]/2010	933	166 (17.8)	30.2 (1)	D	Disease-free interval > 16 mo
			15.1 (2)		PRT
Current series/2013	919	170 (18.5)	73.5 (1)	L/D	PRT
			51.4 (2)		Female sex

D = distant recurrence; L = local recurrence, NR = not reported; PRT = postrecurrence therapy. See Table 3 legend for expansion of other abbreviation.

with resected stage I NSCLC. Moreover, in patients who underwent any PRT, who were receiving EGFR-TKIs and chemotherapy, and with absence of liver or bone metastasis, and with nonadenocarcinoma had a statistically significant association with favorable PRS. Further clinical studies may give more accurate information about the benefits of PRT for survival and lead to the improvement of clinical assessment and therapeutic strategies in recurrent NSCLC.

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Dr Shimada: contributed to the design and coordination of the study, prepared the manuscript, read and approved the final manuscript, and served as principal author.
Dr Saji: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Yoshida: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Kakihana: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Honda: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Nomura: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Usuda: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Kajiwara: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Ohira: contributed to preparing the manuscript and read and approved the final manuscript.
Dr Ikeda: contributed to the design and coordination of the study, revised the article for important intellectual content, and read and approved the final manuscript.
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ORIGINAL RESEARCH

Cancer stem cell-related marker expression in lung adenocarcinoma and relevance of histologic subtypes based on IASLC/ATS/ERS classification

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Background: The cancer stem cell (CSC) theory has been proposed to explain tumor heterogeneity and the carcinogenesis of solid tumors. The aim of this study was to clarify the clinical role of CSC-related markers in patients with lung adenocarcinoma and to determine whether each CSC-related marker expression correlates with the histologic subtyping proposed by the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS), and the European Respiratory Society (ERS) classifications.

Methods: We reviewed data for all 103 patients in whom complete resection of adenocarcinoma had been performed. Expression of CSC-related markers, ie, aldehyde dehydrogenase 1A1 (ALDH1A1), aldo-keto reductase 1C family member 1 (AK1C1), and 1C family member 3 (AK1C3), was examined using immunostaining on whole-mount tissue slides, and the tumors were reclassified according to the IASLC/ATS/ERS classification.

Results: ALDH1A1 expression was observed in 66.0% of tumors, AK1C1 in 62.7%, and AK1C3 in 86.1%. Immunoreactivities with the frequency of mean expression of ALDH1A1 in papillary predominant adenocarcinoma were significantly higher than those of solid predominant adenocarcinoma ($P<0.05$). Papillary predominant adenocarcinoma had significantly lower expression of AK1C1 when compared with noninvasive or solid predominant adenocarcinomas ($P<0.05$). On multivariate analysis, larger tumor size (hazards ratio 1.899, $P=0.044$), lymph node metastasis (hazards ratio 2.702, $P=0.005$), and low expression of ALDH1A1 (hazards ratio 3.218, $P<0.001$) were shown to be independently associated with an unfavorable prognosis.

Conclusion: Immunohistochemistry of ALDH1A1 expression is strongly associated with prognosis. Expression of each CSC-related marker varies according to subtype, suggesting that a comprehensive histologic subtyping approach in the IASLC/ATS/ERS classification provides new molecular biology insights into the genesis of lung adenocarcinoma according to CSC theory.

Keywords: cancer stem cell marker, adenocarcinoma, ALDH1A1, AK1C1, AK1C3, prognosis

Introduction

Lung cancer is the most lethal of all cancers, and adenocarcinoma (ADC) is the most common histopathologic type of lung cancer worldwide.[1] Major advances in ADC management have resulted from the understanding of molecular biology, development of molecular targeting agents, and identification of biomarkers for targeted treatment. However, there exists a widely divergent clinical, radiologic, molecular and pathologic spectrum in lung ADC. In this context, the International Association for the Study of Lung Cancer (IASLC), the American Thoracic Society (ATS), and the European Respiratory Society (ERS) have proposed a new subclassification of lung ADCs that relies on the predominant structural morphology.[2] Histologic subtyping according to

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the IASLC/ATS/ERS classification has been reported to have a strong relationship with prognosis in several studies.[3–7]

Cancer stem cell (CSC) theory has been proposed to explain tumor heterogeneity and the carcinogenesis of solid tumors, including lung cancer.[8] CSCs, a very small population of specialized cells, have potential for self-renewal and extensively proliferative characteristics that sustain tumor formation.[8,9] Various molecules are being investigated as putative markers of CSCs. In this study, we focused on aldehyde dehydrogenase 1A1 (ALDH1A1), aldo-keto reductase 1C family member 1 (AK1C1), and 1C family member 3 (AK1C3), which have been previously identified as labeling CSCs in breast, colon, prostate, and lung cancer.[10–15] In particular, ALDH1 has already been evaluated as an effective prognostic marker in lung cancer.[12,15,16]

In the current study, we attempted to determine whether expression of each CSC-related marker is correlated with the IASLC/ATS/ERS classification, and whether expression of CSC-related markers has any bearing on overall survival.

Materials and methods

Patients

The subjects recruited for this study consisted of 103 patients with lung ADC who underwent complete surgical resection at Tokyo Medical University Hospital between December 1999 and January 2002. All of these patients underwent complete lobar resection and systematic mediastinal lymph node dissection. We excluded patients who had undergone preoperative chemotherapy or radiotherapy. Diagnoses were made according to the criteria of the current World Health Organization classification for lung cancer and the IASLC/ATS/ERS international multidisciplinary classification of lung ADC.[2] The 7th edition International Union Against Cancer/American Joint Committee on Cancer TNM classification was applied to all ADCs.[17] Data collection and analyses were approved, and the need to obtain written informed consent from each patient was waived by the Tokyo Medical University institutional review board.

Clinical characteristics were retrieved from the clinical records available. The following clinicopathologic factors were assessed retrospectively in relation to immunohistochemical analysis: age, gender, smoking history, pathologic staging, tumor size, pathologic nodal involvement, grade of differentiation, vascular invasion, and pleural invasion.

Histopathology

After the specimens were fixed with formalin and embedded in paraffin, serial 4 μm sections were stained with hematoxylin and eosin and by the Alcian Blue-periodic acid-Schiff method to visualize cytoplasmic mucin and by the Elastica van Gieson method to visualize elastic fibers. All slides were evaluated by three of the authors (YS, MN, JM) together using a multiheaded microscope and discussed until consensus was achieved.

All tumor areas were evaluated on the slides. If several tumor foci were present, all foci were included in the analysis. Evaluation was done according to the criteria of the IASLC/ATS/ERS classification, recording the percentage of each histologic component in 5% increments: adenocarcinoma in situ (Figure 1A), minimally invasive adenocarcinoma (Figure 1B), lepidic and acinar (Figure 1C), solid (Figure 1D), papillary, micropapillary (Figure 1E), and mucinous predominant (Figure 1F). The predominant pattern was defined as the pattern with the largest area percentage.

Immunohistochemistry

Expression of three CSC-related proteins was tested with the following commercially available antibodies according to the respective manufacturer's protocols: monoclonal rabbit anti-ALDH1A1 antibody (Abcom Japan, Tokyo, Japan), polyclonal anti-AK1C1 antibody (GeneTex, Irvine, CA, USA), and monoclonal anti AK1C3 antibody (Sigma Japan, Tokyo, Japan). Sections were briefly incubated with xylene, rehydrated with graded ethanol solutions, and incubated with methyl alcohol containing 3% hydrogen peroxide to remove endogenous peroxidase activity. After washing thoroughly with phosphate-buffered saline, sections were incubated with adequately diluted primary antibodies and then with Histofine® simple stain (Nichirei Bioscience, Tokyo, Japan), and finally visualized with products of the peroxidase and diaminobenzidine reaction.

Antibody binding was microscopically recognizable as brown cytoplasmic staining. We categorized immunoreactivity by the percentage of the immunopositive area. In tumor cells of interest, when more than 5% of the staining extensiveness showed an unequivocally strong reaction with an antibody, the tumor was classified as positive.

Statistical analysis

Overall survival was measured from the date of surgery to the date of death from any cause or the date on which the patient was last known to be alive. Survival curves were plotted according to the Kaplan–Meier method and compared using the log-rank test. Categoric comparisons were performed using the Pearson chi-squared test. Multivariate analysis was performed using the Cox proportional hazards model. A