Individualized Adjuvant Chemotherapy for Surgically Resected Lung Cancer and the Roles of Biomarkers

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Review

Individualized Adjuvant Chemotherapy for Surgically Resected Lung Cancer and the Roles of Biomarkers

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Several prospective randomized trials for patients with completely resected stages II and IIIA nonsmall cell lung cancer have confirmed a survival benefit with cisplatin-based adjuvant chemotherapy. The Lung Adjuvant Cisplatin Evaluation, which is based on pooled analyses of five randomized trials, has demonstrated a 4.2% absolute survival benefit at 5 years. The stage is the benchmark standard used to decide the indication for adjuvant chemotherapy; however, it is important to identify and select the patients who would benefit from adjuvant chemotherapy and to choose the optimal regimen for each case. The translational research was performed using specimens obtained in the above adjuvant trials also to obtain information concerning biomarkers and subsets of patients who would benefit from adjuvant chemotherapy. The extent to which individualized treatment of lung cancer can be provided, especially adjuvant chemotherapy, is discussed in this manuscript. (Ann Thorac Cardiovasc Surg 2009; 15: 144–149)

Key words: lung cancer, adjuvant chemotherapy, individualized treatment, biomarker

Introduction

Surgery is considered to be the standard treatment for early-stage nonsmall cell lung cancer (NSCLC). However, distant metastasis occurred in nearly 60% of patients with stages I to IIIA NSCLC after complete resection. Micrometastasis of the tumor is generally regarded as the cause of recurrence; therefore systemic chemotherapy after surgery is a rational strategy to reduce the risk of recurrence and metastasis.

Recent large-scale randomized trials have confirmed a survival benefit of adjuvant cisplatin-based chemotherapy following complete surgical resection of NSCLC (Table 1). The Lung Adjuvant Cisplatin Evaluation (LACE) study was based on a pooled meta-analysis of individual patient data from 5 trials (Adjuvant Lung Project Italy [ALPI];[1] Adjuvant Navelbine International Trialist Association [ANITA];[2] Big Lung Trial [BLT];[3] International Adjuvant Lung Cancer Trial [IALT];[4] and JBR.10[5]). The overall hazard ratio (HR) of death was 0.89 (95% confidence interval [CI]; 0.82–0.96; p <0.005), which corresponds to a 5-year survival benefit of 4.2% with chemotherapy.[6] The survival benefit varied with stage, and the results showed that the cisplatin-based adjuvant chemotherapy improved survival in patients with completely resected stage II and stage III NSCLC (Table 1). Japanese adjuvant trials showed that a survival benefit was obtained with adjuvant chemotherapy using uracil-tegafur (UFT) in stage I adenocarcinoma.[7] Meta-analysis revealed that the benefit was limited to those with a tumor size of 2 cm or more.[8] This suggests that the indications of adjuvant chemotherapy might extend from pathological stage I to stage III, which means that most operated NSCLC cases should be recommended to receive postoperative chemotherapy after surgery.

However, it is a sad scenario when many patients receive toxic agents with few benefits; therefore the

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Table 1. Results of representative adjuvant trials

	ALPI	IALT	JBR.10	ANITA01	BLT
Publication year	2003	2004	2005	2006	2004
Stage	I–IIIA	I–IIIA	IB, II	IB–IIIA	I–IIIA
No. of cases	1,209	1,867	482	840	381
Regimen	CDDP + VDS + MMC	CDDP + ETP CDDP + VLB/ VDS/VNR	CDDP + VNR	CDDP + VNR	CDDP + MMC + IFO/VLB CDDP + VDS/ VNR
HR (95% CI)	0.96 (0.81–1.13)	0.86 (0.76–0.98)	0.69 (0.52–0.91)	0.80 (0.66–0.96)	1.02 (0.77–1.35)
P value	0.589	<0.03	0.009	0.017	0.90
Survival benefit at 5 years (%)	3	4.1	15	8.6	–
TRD (%)	0.5	0.8	0.8	1.7	3.1

ALPI, Adjuvant Lung Project Italy; IALT, International Adjuvant Lung Cancer Trial; ANITA, Adjuvant Navelbine International Trialist Association; BLT, Big Lung Trial; HR, hazard ratio; 95% CI, 95% confidence interval; TRD, total radiation dose; CDDP, cisplatin; VDS, vindesin; MMC, mitomycin C; ETP, etoposide; VLB, vinblastine; VNR, vinorelbine; IFO, ifosfamide.

pursuit to identify patients who would really benefit from specific regimens is important. To classify them and to apply the optimal therapy to each subgroup would provide a breakthrough in lung cancer management. The ability to identify responder patients with particular drugs or regimens is a challenge that requires the application of translational research to clinical practice.[9,10] The strong relationship between epidermal growth factor receptor (EGFR) mutation and high response to gefitinib[11,12] is a typical example of the individualized treatment of lung cancer.

A resection of NSCLC usually yields large amounts of tissue for molecular analysis. Rapid advances in technology have led to advanced assays to measure changes in deoxyribonucleic acid (DNA), ribonucleic acid (RNA), and proteins, which help to identify potential molecular biomarkers of clinical outcome. Translational research performed using specimens obtained in some of the adjuvant trials mentioned above has provided some information about biomarkers and identification of the subsets of patients who would benefit from adjuvant chemotherapy. Many biomarkers have been evaluated in the context of the largest positive adjuvant NSCLC trials, such as gene expression signatures, p53 expression, and K-*ras* mutations;[13] DNA-repaired genes;[14] and class III β-tubulin (βTubIII) expression status.[15] The individualized treatment for the determination of adjuvant chemotherapy is extensively discussed in this manuscript.

Excision Repair Cross-Complementation Group 1[14]

The excision repair cross-complementation group 1 (ERCC1) enzyme plays a role in the nucleotide excision repair pathway that recognizes and removes cisplatin-induced DNA adducts.[16] IALT demonstrated a 5% survival benefit in overall 5-year survival among 1,867 NSCLC patients who received adjuvant cisplatin-based chemotherapy after curative surgery.[4]

ERCC1 expression was evaluated by immunohistochemistry in a total of 761 consecutive tumor samples from IALT. It was found to be positive in 335 (44%) and negative in 426 (56%). Cisplatin-based adjuvant chemotherapy significantly prolonged the survival in ERCC1 negative cases (HR: 0.65; 95% CI: 0.50–0.86), but not in ERCC1 positive cases (HR: 1.14; 95% CI: 0.84–1.55). Among patients who received no adjuvant chemotherapy, those with ERCC1 – positive tumors survived longer than those with ERCC1 – negative tumors (HR: 0.66; 95% CI: 0.49–0.90). The result showed that completely resected ERCC1 – negative NSCLC cases could benefit from cisplatin-based adjuvant chemotherapy (Table 2). The evaluation of ERCC1 expression in NSCLC before chemotherapy predicts the effect of cisplatin-based adjuvant chemotherapy, and this is therefore our promising biomarker for individualized treatment.

Table 2. Results of IALT and ERCC1

Group	All patients	Chemotherapy group	Control group	Hazard ratio for death (95% CI)	P value
Patients with ERCC1 – negative tumors				0.65 (0.50–0.86)	0.002
Deaths – no./total no. of patients	218/426	105/224	113/202		
Rate of survival at 5 yr – % (95% CI)	44 (38–49)	47 (40–55)	39 (32–47)		
Median survival – months	48	56	42		
Patients with ERCC1 – positive tumors				1.14 (0.84–1.55)	0.40
Deaths – no./total no. of patients	172/335	92/165	80/170		
Rate of survival at 5 yr – % (95% CI)	43 (37–49)	40 (32–49)	46 (37–55)		
Median survival – months	52	50	55		

ERCC1, excision repair cross-complementation group 1; 95% CI, 95% confidence interval; yr, year.

Table 3. Results of JBR.10 and β-tubulin III expression

	RFS	OS
Low expression (132 cases)		
Observation (60)	1	1
Chemotherapy (72)	0.78 95% CI: 0.44–1.37 (p = 0.4)	1.00 95% CI: 0.57–1.75 (p = 0.99)
High expression (133 cases)		
Observation (65)	1	1
Chemotherapy (68)	0.45 95% CI: 0.27–0.75 (p = 0.002)	0.64 95% CI: 0.39–1.04 (p = 0.007)

RFS, relapse-free survival; OS, overall survival; 95% CI, 95% confidence interval.

Class III β-Tubulin[15]

Tubulins constitute a family of globular proteins that make up microtubules in cells; they are vital for cell structure, movement, mitosis, and metabolism (vesicular transport). High expression βTubIII in advanced NSCLC is known to correlate with both reduced response rates and inferior survival following treatment with antimicrotubule agents.

Winton et al. published the results of a randomized trial of adjuvant vinorelbine and cisplatin compared with observation in completely resected stage IB and stage II NSCLC (National Cancer Institute of Canada Clinical Trials Group [NCIC] JBR.10).[5] A total of 482 patients were randomly assigned to either an adjuvant group (cisplatin/vinorelbine) or an observation group. The adjuvant group had a statistically significant longer survival than the observation group (69% vs. 54% at 5 years; p = 0.002).[5] Tumor tissues of resected specimens were collected from 265 out of 482 patients. Immunohistochemical staining was performed to evaluate the expression of βTubIII. High βTubIII expression is a sign of poor relapse-free survival (RFS) (HR: 1.52; 95% CI: 1.05–2.22; p = 0.03), and a similar trend was observed in overall survival (OS) (HR: 1.39; 95% CI: 0.96–2.01; p = 0.08). However, the high βTubIII expression group (n = 133) cases in the adjuvant group had more significantly favorable RFS (HR: 0.45; 95% CI: 0.27–0.75; p = 0.002) than in the observation group, and similar results were observed in OS (HR: 0.64; 95% CI: 0.39–1.04; p = 0.007). These results showed that adjuvant chemotherapy might prolong the RFS and OS in the high-tubulin expression patients, but the effect was unclear for the low-tubulin expression cases (Table 3).

KRAS and p53[13]

Protein expression of p53 and gene mutation of p53 and RAS were retrospectively evaluated using NSCLC samples obtained in the NCIC JBR.10 study. A total of 132 out of 253 cases showed p53 protein overexpression. And

Table 4. Results of JBR.10 and p53, RAS expression

Marker	No. of patients	Overall survival			
		Median	Hazard ratio	95% CI	P
p53 wild type					
Observation	136	6.2	1		0.04
Chemotherapy	137	7.8	0.67	0.46 to 0.98	
p53 mutant					
Observation	64	5.4	1		0.35
Chemotherapy	60	NR	0.78	0.46 to 1.32	
RAS wild type					
Observation	169	6.2	1		0.03
Chemotherapy	164	NR	0.69	0.49 to 0.97	
RAS mutant					
Observation	42	6.5	1		0.70
Chemotherapy	46	6.2	0.91	0.47 to 1.78	

95% CI, 95% confidence interval; NR, not reported.

though patients with p53-positive tumors had an overall significantly shorter survival than those with p53-negative tumors (HR: 1.89; 95% CI: 1.07–3.34; p = 0.03), p53-positive tumors did show significant benefits from adjuvant chemotherapy (HR: 0.54; 95% CI: 0.32–0.92; p = 0.02). Patients with p53-negative tumors, however, had no survival benefit from adjuvant chemotherapy (HR: 1.40; 95% CI: 0.78–2.52; p = 0.26).

In 333 patients with wild-type RAS, survival was significantly prolonged by adjuvant chemotherapy, compared with observation-only cases (HR: 0.69; 95% CI: 0.49–0.97; p = 0.03). But no survival benefit for adjuvant chemotherapy was recognized in patients with RAS mutant tumor (HR: 0.91; 95% CI: 0.47–1.78; p = 0.70) (Table 4).

Comments

Evidence-based medicine has been increasingly emphasized in medical practice in recent years, and standardized treatment methods have been developed mainly by multicenter randomized control trials. However, since the biological nature of tumors varies with each patient and their physical constitution, individualized treatment that takes both of these aspects into consideration could provide ideal optimal care for each cancer patient. Scientists have made enormous efforts to discover powerful biomarkers to help evaluate the biological behavior of cancer, and this strategy should be beneficial in selecting the most suitable therapy for each patient. Several biomarkers were evaluated using samples obtained in large adjuvant chemotherapy trials of lung cancer (Table 5), and some hold promise. The increased interest in identifying biomarkers with implications for personalized treatment is reflected in a recent decision by the Food and Drug Administration (FDA) to allow, under certain conditions, retrospective analyses of biomarkers from completed trials.[17] The relationship between the histological type of lung cancer and the sensitivity of pemetrexed has been reported in inoperable lung cancer cases.[18] Pemetrexed is currently approved in the United States in combination with cisplatin for the treatment of malignant mesothelioma and for second-line treatment of advanced NSCLC. A recent phase III trial compared cisplatin and gemcitabine with cisplatin and pemetrexed for the treatment of advanced NSCLC. This noninferiority phase III randomized study compared OS between two groups. The OS for cisplatin/pemetrexed was noninferior to cisplatin/gemcitabine. Statistically, OS was significantly superior for cisplatin/pemetrexed than cisplatin/gemcitabine was in adenocarcinoma patients and large cell carcinoma patients (12.6 vs 10.9 months, 10.4 vs 6.7 months, respectively).[18] Because pemetrexed is an antifolate that inhibits multiple enzymes involved in purine and pyrimidine synthesis, thymidylate synthase (TS) is its main target. Preclinical data indicate that overexpression of TS correlates with lower sensitivity to pemetrexed. The baseline expression of TS gene and TS protein was significantly higher in patients with squamous cell carcinoma than in those with adenocarcinoma. This might be

Table 5. Drug sensitivity, prognosis, and biomarkers

Biomarker		ERCC1		RRM1		BRCA1		Class III β-tubulin		KRAS	
Expression		High	Low	High	Low	High	Low	High	Low	Wild	Mutant
Prognosis		Good		Good		Poor		Poor			
Sensitivity	CDDP		○				○			○	
	Taxane					○		○		○	
	VNR							○		○	
	GEM				○					○	

ERCC1, excision repair cross-complementation group 1; RRM1, ribonucleotide reductase subunit M1; BRCA1, breast cancer 1, early onset; CDDP, cisplatin; VNR, vinorelbine; GEM, gemcitabine.

part of the explanation for the higher response of adenocarcinoma to pemetrexed. Further analysis of the relationship between the chemotherapy regimen and TS will be performed in a prospective manner; patients with stage II and stage III completely resected NSCLC are being treated with standard adjuvant chemotherapy or an individualized regimen determined by TS and ERCC1 expression (International Tailored Chemotherapy Adjuvant [ITACA] trial).[19]

Here is another approach to determine a suitable biomarker using proteomics. Maeda et al. performed a comprehensive protein analysis using surgically resected specimens of stage I adenocarcinoma by liquid chromatography tandem mass spectrometry, followed by bioinformatical investigations to identify protein molecules.[16] Two kinds of molecules (myosin IIA and vimentin) were identified as being related to prognosis and also to the responsiveness to adjuvant chemotherapy. Patients lacking expression of both myosin IIA and vimentin showed a significantly better outcome, regardless of postoperative adjuvant chemotherapy using UFT.

The nonrelapse survival of these patients at 5 years was 100%, which is better than that of patients positive for both myosin IIA and vimentin. Also, cases lacking expressions of both the two proteins had a good prognosis, irrespective of whether the patients had undergone adjuvant chemotherapy. In cases showing a positive expression of both myosin IIA and vimentin, the 5-year survival benefit was approximately 19% by adjuvant chemotherapy using UFT. Therefore these two proteins appear to be potentially useful biomarkers for the selection of adjuvant chemotherapy.[16]

The current retrospective data are by no means sufficient to support the routine use of molecular markers to guide adjuvant therapy for NSCLC outside of a clinical trial.

Before a molecular test can be adopted for routine practice, valid and standardized laboratory techniques must be established. The establishment of the feasibility of molecularly tailored adjuvant therapy for patients with resected NSCLC requires a prospective phase II trial. It is also important not only to select a suitable regimen, but also to develop innovative treatments, such as gene and molecular-targeted therapy.

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The Prognostic Impact of Main Bronchial Lymph Node Involvement in Non-Small Cell Lung Carcinoma: Suggestions for a Modification of the Staging System

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Background. The therapeutic strategies for non-small cell lung carcinoma (NSCLC) with N1 and N2 disease differ remarkably. Debate exists about the definition of the borderline between N1 and N2 stations. This study evaluated the prognostic effect of N1 disease, especially focused on the significance of the main bronchial node (No. 10) vs N2 disease.

Methods. The records of 1601 patients who underwent complete pulmonary resection for NSCLC were reviewed to examine the clinical features of lymph nodal involvement.

Results. There were 1086 patients (67.8%) with pN0 disease, 202 (12.6%) with pN1, and 274 (17.1%) with pN2 disease; overall 5-year survival rates were 74.7%, 56.1% and 28.9%, respectively ($p < 0.001$). Overall 5-year survival rates were 60.2% in hilar N1 and 49.6% in intralobar N1. Overall 5-year survival rates were 58.6% in N1 without node 10 and 35.1% in N1 with node 10. A significant difference was observed between N0 and N1 without node 10 ($p < 0.001$), and N1 without node 10 and N1 with node 10 ($p = 0.033$); however, the difference between N1 with node 10 and N2 was not significant. The status of node 10 involvement was an independent prognostic factor of pN1 patients, as well as age and gender.

Conclusions. Patients with node 10-positive N1 disease have an unfavorable prognosis, and the disease behaves like N2 disease. The definition of clear borderline between N1 and N2 is mandatory to achieve a uniform classification map. This study offers further information for clinical and therapeutic purposes.

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Staging of lung cancer based on the T N M classification is the method internationally accepted for the clinical setting of the disease to evaluate the prognosis, decide appropriate management, and analyze the results of treatment. The current T N M classification was initially proposed by Mountain in 1986 [1] and revised in 1997 [2].

Although this staging classification has been accepted, the anatomic definition of lymph node location—especially the boundary between N1 and N2 stations—has not been completely accorded. Currently, some variations of the lymph node map can be found, and thus considerable discordance exists regarding the designation of sites among investigators in the United States, Europe, and Japan [3]. The American Joint Committee on Cancer (AJCC) [4], Naruke and colleagues [5], and The American Thoracic Society (ATS) [6] introduced the concept of lymph node maps in 1973, 1978, and 1983, respectively.

The Mountain and Dresler modification of the ATS (MD-ATS) map was promulgated in 1997 [2]. Mountain and coworkers defined the boundary between N1 and N2 at the pleural reflection. The Naruke-Japanese map did not mention pleural reflection, however; they defined the lymph node station in relation to the bronchial tree and mediastinal structures [5, 7]. The main discrepancy between these two maps is that the Narke-Japanese map considers lymph nodes around the main bronchus and in the subcarinal space among the inferior border of the main bronchus to be station 10 (N1), whereas most of those are labeled as station 4 or 7 (N2) in the MD-ATS map [8].

A rational approach to the management of lung cancer requires accurate staging to plan the most appropriate treatment and to estimate the prognosis. Patients with pathologically proven N2 are no longer indicated for initial resection. Chemotherapy, chemoradiotherapy, or induction therapy, followed by resection, is the standard treatment of choice [9, 10]. Because the therapeutic strategies for patients with N1 and N2 disease differ greatly, the boundary between N1 and N2 stations where metastasis is confirmed pathologically is most essential. More-

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over, these discordant classifications may lead to a nonunivocal staging, rendering the comparison of different clinical studies difficult. Therefore, we need to define the boundary of lymph node location more precisely and reach consensus on the basis of the most recent evidence.

We reviewed the records of patients with completely resected non-small cell lung cancer (NSCLC) to examine the clinical features of lymph nodal involvement. The purpose of our study was to evaluate the prognostic impact of N1 disease, with a special focus on the significance of involvement of the main bronchial node (No. 10) compared with N2 disease.

Patients and Methods

Of 1601 patients who underwent complete pulmonary resection for NSCLC from 1990 to 2004 at Tokyo Medical University, 202 pN1 patients (12.6 %) without distant metastasis were the focus. Data collection and analyses were approved, and the need for obtaining informed consent from each patient was waived by the Institutional Review Board.

All of those patients underwent lobectomy or pneumonectomy with systemic lymph nodal dissection of the hilum and mediastinum. The histologic tumor type was determined according to the World Health Organization classification. Staging was determined according to the international T N M staging system [2]. All dissected lymph nodes were pathologically examined and classified according to anatomic location by the numbering system of Naruke and colleagues [5].

The station of N1 lymph nodes were classified main bronchial lymph node as No. 10, interlobar as No. 11, lobar bronchial as No. 12, segment bronchial as No. 13, and subsegmental as No. 14. N1 lymph nodes were generally classified into two groups as follows, hilar lymph nodes as No. 10 and 11, and intralobar lymph nodes as No. 12, 13, and 14. We further classified N1 lymph nodes involvement into two groups: pN1 disease who were No. 10-positive as the No. 10+ N1 group, and pN1 disease who were No. 10-negative as the No. 10− N1 group. Single-station metastasis was defined as involvement of only one station, whereas multiple-station metastasis was defined as involvement of more than one station.

For staging, all patients underwent a physical examination, chest roentgenogram, computed tomography (CT) imaging of the thorax, brain, and upper abdomen; bone scintigraphy, and bronchoscopy. The tumor marker, carcinoembryonic antigen (CEA) was also examined preoperatively. Serum CEA levels were measured using Latex photometric immunoassay (Mitsubishi Chemical Medience, Tokyo, Japan), and the upper limit of normal serum CEA levels was 3.0 ng/mL according to the manufacturers.

After resection, the patients were examined at 3-month intervals for 3 years, at 6-month intervals for the next 2 years, and thereafter at 1-year intervals in general. The evaluations included physical examination, chest roentgenogram, CT of the chest, and tumor marker measurement. Abdominal and brain CT as well as bone scintigraphy were done each year.

Table 1. Clinicopathologic Profiles of Patients With pN1 and pN2 Non-Small Cell Lung Cancer, 1990 to 2004

Variable	pN1 (n = 202)	pN2 (n = 274)
Age, median (range), y	64 (31–82)	65 (25–87)
Gender, No.		
Male	158	193
Female	44	81
pT status, No.		
T1	72	63
T2	99	153
T3	19	31
T4	12	27
Location, No.		
Right	117	176
Left	85	98
Histology, No.		
Adenocarcinoma	94	178
Squamous	84	68
Large	14	20
Adenosquamous	3	3
Other	7	5
Operation, No.		
Pneumonectomy	25	36
Lobectomy	177	238
Serum CEA, median (range) ng/mL	3.0 (1.0–213.5)	2.0 (1.0–140.0)

CEA = carcinoembryonic antigen.

Patients with cancer recurrences were carefully divided into two groups according to the site of initial relapse: locoregional or distant. Locoregional recurrence was defined as any recurrent site within the ipsilateral hemithorax, mediastinum, or supraclavicular lymph nodes. All other sites of recurrence were considered distant metastases.

Survival was calculated by the Kaplan-Meier method, and differences in survival were determined by log-rank analysis in which the initial day of treatment was the day of operation. The cause of death was recorded as cancer-related, due to other diseases, or unknown. Deaths that were not because of cancer were censored. Multivariate analysis of clinicopathologic factors was performed using the Cox proportional hazard regression model. A value of $p < 0.05$ was considered statistically significant. Hazard ratios (HR) and 95% confidence intervals (CI) are presented.

Results

Demographics

Of 1601 patients who underwent complete pulmonary resection for NSCLC from 1990 to 2004 at Tokyo Medical University, lymph node involvement was recognized in 1086 (67.8 %) as pN0, 202 (12.6 %) as pN1,

Table 2. Survival at 5 Years in Patients with pN1 Non-Small Cell Lung Cancer According to Prognostic Factors, 1994 to 2002

Variables	Patients, No.	5-Year Survival, %	*p* Value
Age, years			
<65	105	63.4	0.006
≥65	97	48.5	
Gender			
Male	158	50.7	0.007
Female	44	78.0	
pT status			
T1	72	58.7	0.319
T2/T3/T4	130	54.8	
Location			
Right	117	56.3	0.858
Left	85	56.0	
Histology			
Adenocarcinoma	94	55.7	0.830
Not adenocarcinoma	108	56.4	
Operation			
Pneumonectomy	25	46.4	0.303
Lobectomy	177	57.5	
Serum CEA, ng/mL			
<3.0	115	54.7	0.662
≥3.0	45	56.7	
pN1 status			
Intralobar[a]	124	60.2	0.146
Hilar[b]	78	49.6	
No. 10−	181	58.6	0.033
No. 10+	21	35.1	

[a] No. 12, 13, 14 lymph node metastasis. [b] No. 10, 11 lymph node metastasis.

CEA = carcinoembryonic antigen.

and 274 (17.1 %) as pN2 disease. The 202 pN1 patients (158 men, 44 women) were a median age of 64 years (range, 31 to 82 years). Operative procedures included 177 lobectomies and 25 pneumonectomies. The histologic classification was adenocarcinoma in 94 patients, squamous cell carcinoma in 84, large cell carcinoma in 14, adenosquamous in 3, and others in 7. The median value of preoperative serum CEA was 3.0 ng/mL (range, 1.0 to 213.5 ng/mL) (Table 1).

The distribution of pathologic T status was 72 pT1, 99 pT2, 19 pT3, and 12 pT4. The mean value of preoperative serum CEA was 3.0 ng/mL (range, 1.0 to 213.5 ng/mL). Among 202 patients with p-N1 disease, 124 had metastasis of intralobar nodes but not hilar nodes. Patients with hilar N1 nodes metastases were further categorized as 21 with No. 10+ N1 disease and 181 with No. 10− N1 disease (Table 2).

Prognosis

The median follow-up for survivors was 55 months (range, 1 to 200 months). The survival curves for the 1086 pN0, 202 pN1, and 274 pN2 patients are shown in Figure

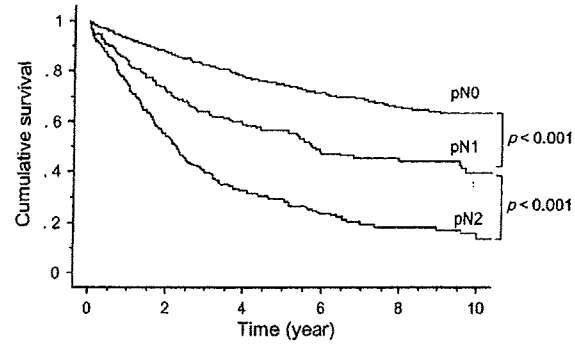

Fig 1. Kaplan-Meier curves show cumulative survival of patients undergoing complete resection for non-small cell lung carcinoma according to pathologic nodal status.

1. The overall 5-year survival rates were 74.7%, 56.1%, and 28.9%, respectively, and this difference was statistically significant ($p < 0.001$).

The association of various prognostic factors was examined by univariate analysis in 202 pN1 patients. Age and gender had a significant effect on survival ($p = 0.006$ and $p = 0.007$, respectively). The 21 No. 10+ N1 patients had significantly worse outcome than the 181 No.10− patients ($p = 0.033$). The overall 5-year survival of No. 10+ N1 patients was 35.1%, which was similar to that of pN2 patients (28.9%; Fig 2; Table 2). However, there was no significant difference in survival when pN1 patients were divided into hilar N1 (No. 10 and No. 11; n = 78) and intralobar N1 (No. 12, 13, and 14; n = 124; $p = 0.146$; Fig 3). There were also no significant differences on survival between the 156 patients with pN1 disease who had single-station metastasis and the 46 with multiple-station metastasis ($p = 0.742$; data not shown). This result implied that lymph node involvement of No. 10 is a poor

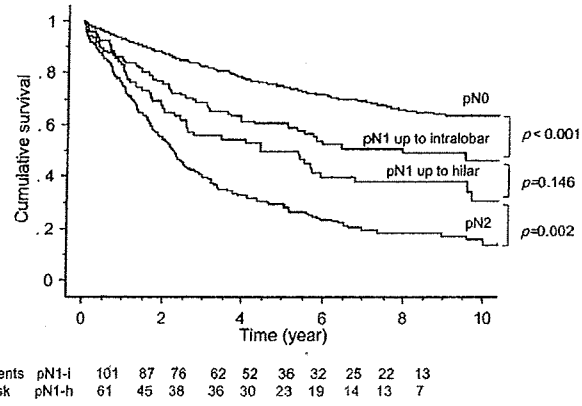

Fig 2. Kaplan-Meier curves show cumulative survival of patients undergoing complete resection for pN1 non-small cell lung carcinoma according to pathologic nodal status. Disease was classified as pN0, intralobar pN1, hilar pN1, and pN2. (Intralobar = No. 12, 13, 14 regional lymph nodes; hilar = No. 11, 10 regional lymph nodes.)

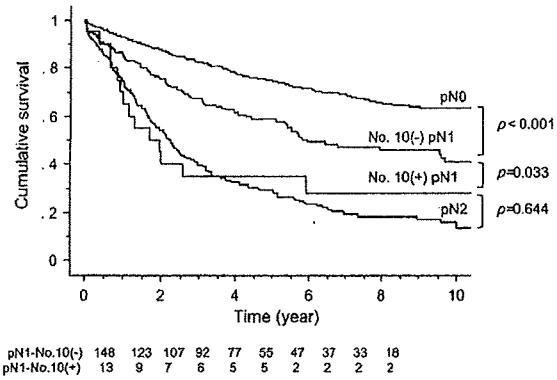

Fig 3. Kaplan-Meier curves show cumulative survival of patients undergoing complete resection for pN1 non-small cell lung carcinoma according to pathologic nodal status. Disease was classified as pN0, pN1 without No. 10 lymph nodes metastasis, pN1 with No. 10 lymph nodes metastasis, and pN.

prognostic marker in pN1 patients compared with No. 10 and No. 11 hilar lymph nodes. In multivariate analysis, the status of No. 10 lymph node involvement was an independent prognostic factor of pN1 patients as well as age and gender (HR, 1.933; 95% CI, 1.089 to 3.430; p = 0.024; Table 3).

We evaluated postoperative cancer recurrence in 100 pN1 patients for limited period, from 1996 to 2002, and 43 (43.0%) had cancer recurrence. Detailed data on cancer recurrence were not available for 6 patients. The initial relapse site was analyzed in the remaining 37 patients. The recurrences were locoregional in 10 (27.0%) and distant in 27 (73.0%). No statistical difference was observed in the distribution of the site of recurrence, locoregional or distant, between No. 10+ N1 and No. 10− N1 patients. Locoregional relapse occurred in 2 No. 10+ N1 patients (33.0%) and distant metastasis occurred 4 patients (67.0%). For those without No. 10 lymph node involvement, locoregional relapse occurred in 8 patients (25.8%) and distant metastasis in 23 (74.2%). The lung was the most common site for distant metastasis in both groups (data not shown).

Comment

During the past 30 years, different types of lymph node maps have been proposed. The distinction of lymph node stations is a most crucial topic that is still not entirely resolved by many lung cancer surgeons. One of the most significant problems concerning lymph node involvement under debate among thoracic oncologists is the definition of the borderline between N1 and N2 stations, which must be clarified, because this discordance could distort therapeutic strategies and stages reported in different studies.

In Naruke's map, lymph nodes in the subcarinal space along the inferior border of the mainstem bronchus are station No. 10 [5], whereas in MD-ATS map, these are labeled as level 7, hence N2 nodes [8]. The borderline between the N1 and N2 station is not clearly defined in Naruke's map. The No. 10 station is defined simply as "nodes around the main bronchus," and adjacent No.4 and No.7 were defined as "nodes at the tracheobronchial angle" and "nodes below tracheal carina," respectively [7].

In the MD-ATS map, the pleural reflection was set as a clear borderline for N1-N2 stations [8], with N1 nodes as those located distal to the mediastinal pleural reflection and within visceral pleura. This definition involves the proximal part of the hilar lymph nodes being classified within the N2 category because the proximal part of the mainstem bronchus lies within the mediastinal pleural envelope. However, concerning the borderline between N1 and N2 station, Asamura and colleagues [11] reported that the pleural reflection is recognized as a plane rather than as a line, and the reflecting line can be easily moved by retracting the lung anteriorly or posteriorly.

Some patients considered to have T1 2N1 M0 stage II disease in Japan would be considered to have T1 2N2 M0 stage IIIA disease in all other countries. This difference in nodal diagnosis might be a cause of staging migration.

In this study, we used Naruke's lymph node map to review the records of 1601 consecutive patients who had undergone complete resection for NSCLC. We also examined the spread pattern of lymph node metastases and investigated the outcome according to the level of the involved nodes.

Some investigators reported hilar lymph node metastasis is a significant unfavorable prognostic factor in p-N1 disease [12–18]. First, we divided N1 lymph nodes into two stations as follows, hilar lymph nodes (No. 10 and 11) and intralober lymph nodes (No. 12, 13, and 14). However, the difference in survival between hilar N1 and intralobar N1 was not significant.

Second, we further categorized patients with hilar N1 node metastases as those with and those without main bronchus node (No. 10) involvement. Among the 202 p-N1 patients, the 21(10.4%) identified with No. 10-positive N1 disease had a significantly worse prognosis than those with No. 10− N1 disease (p = 0.031). Moreover, the overall 5-year survival of patients with No. 10+

Table 3. Factors Influencing Survival in Patients With pN1 Non-Small Cell Lung Cancer by Multivariate Analysis

Variables	Favorable	Unfavorable	OR (95% CI)	p Value
Gender	Female	Male	2.109 (1.152–3.862)	0.016
Age, y	<65	≥65	1.771 (1.188–2.639)	0.005
No. 10 LN involved	Negative	Positive	1.933 (1.089–3.430)	0.024

CI = confidence interval, LN = lymph node; OR = odds ratio.

N1 disease was 35.1%, which was similar to the 28.9% survival in N2 disease. Multivariate analysis demonstrated that No. 10 lymph node involvement was one of the independent prognostic factors of pN1 patients as well as age and gender. Although the number of patients who were No. 10+ in this study is relatively small, we found that pN1 with No. 10+ disease behaves like a more advanced stage. Matsuoka and colleagues [19] reported the same results, including multivariate analysis, as ours concerning the survival benefit for the N1 disease with or without No. 10 involvement.

Several authors reported that the mode of metastasis in interlobar N1 tended to resemble that of N0, whereas that of hilar N1 behaved like N2 disease [12, 13, 15, 17, 18]; however, the modality of recurrence in our study for the limited period was not affected by the level of pN1 involvement. Our result that distant metastasis was predominant in the recurrent pattern over locoregional recurrence in p-N1 patients implies that nodal involvement might be a surrogate marker for distant metastasis, even if the site of metastasis is the interlobar lymph nodes.

Previous studies suggest that multiple levels of N1 stations are associated with a worse outcome than single-level disease [14, 20–23]. We were unable to identify the differences. Concerning the prognostic effect of the number of involved N1 stations, which may be one of the strong predictable factors for poor survival, it is possible that these analysis did not include enough patients to lead to a valid conclusion.

When taken together, the discrepancy between the Naruke map and the MD-ATS map might contribute to borderline cases between N1 and N2, such as multiple-station N1 disease or hilar N1 disease. The staging committee of the International Association for the Study of Lung Cancer (IASLC) is proposing a new international lymph node map that provides very precise definitions of the anatomic boundaries of each lymph node station and reconciles the differences between the Naruke map and the MD-ATS map [22].

The nodes around the junction of the hilum and mediastinum are key points at issue. Indeed, one of the most important problems is to decide whether the main bronchus nodes belong to the N1 or N2 station in relation to prognosis as well as anatomy. In this study, we found a difference in survival among patients with nodal metastasis up to either station 11 or station 10, whereas survival did not differ among patients with nodal metastases up to either station 10 or N2 station. This result suggested that nodes could be designated as intermediate between N1 and N2 and that there might be a borderline between N1 and N2 nodes around the main bronchus in accordance with the Naruke map. Moreover, our study demonstrated that the involvement of main bronchial nodes has a prognostic significance similar to that of single-station N2 and could be considered as an early N2 disease.

We fervently hope to have a single, accurate map of lymph node stations that can be used universally. Otherwise, it will be difficult to make progress in therapeutic strategies for lung cancer.

In conclusion, survival in patients with pN1 disease differs according to the type of lymph node involvement. Patients with No. 10 involvement have an unfavorable prognosis, and the disease behaves like N2 disease. The definition of a clear borderline between N1 and N2 is mandatory to achieve a uniform classification map. Further clinical studies may give more accurate information about the real prognostic value of No. 10 involvement to improve the clinical assessment and therapeutic strategies.

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Expression of podoplanin, CD44, and p63 in squamous cell carcinoma of the lung

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Recent molecular biological studies have identified podoplanin as a candidate cancer stem cell (CSC) marker in squamous cell carcinoma (SqCC). The purpose of this study was to examine the expression pattern of podoplanin, and the other stem cell markers CD44 and p63, and their relationship to clinico-pathological features including survival in pulmonary SqCC. We examined histologically the expression of podoplanin, CD44, and p63 in 162 consecutive SqCC by immunostaining. Podoplanin expression was observed in 107 (66%) tumors, CD44 in 145 (89.5%), and p63 in 151 (93.2%), respectively. In 95.3% of the podoplanin-positive tumors, tumor cells showing strong expression were localized in the periphery of the tumor nests. However, this peripheral localization was observed in only 55.9% of the CD44-positive and 43% of p63-positive tumors. In 88.8% of the podoplanin-positive tumors, positive cells were localized more peripherally in the tumor nests than CD44- or p63-positive cells and when CD44 and p63 expressions were compared in these podoplanin-positive tumors, p63-positive layers in the periphery of the tumor nests were broader compared to CD44-positive layers. These findings suggest tumor cells are aligned in the "hierarchical distribution pattern" according to the expression of these three markers. Patients who had podoplanin-positive tumors with the "hierarchical pattern" resulted in significantly better overall survival than those who had podoplanin-negative tumors ($P = 0.043$). These results suggest that podoplanin expression would reflect the most immature status in the differentiation process of SqCC, and SqCC with hierarchical expression would be a well-organized tumor group with lower biological aggressiveness based on the CSC concept. (*Cancer Sci* 2009; 100: 2054–2059)

Lung cancer is the leading cause of cancer mortality worldwide, and two main types of non-small-cell lung carcinoma (NSCLC), adenocarcinoma and squamous cell carcinoma (SqCC), account for over half the cases of lung cancer. There have been recent advances in molecularly targeted agents for the treatment of pulmonary adenocarcinoma, but not much progress has been made in the treatment of SqCC,[1–3] and the molecular mechanisms of SqCC are not completely understood.

Considering that the components of SqCC are heterogeneous and that its histology and marker expression are similar to those of normal epithelium, it suggests a "developmental hierarchy". Based on the concept that stem cells sit at the top of the developmental hierarchy, the cells at the basal (peripheral) region of SqCC nests may possess stem-cell-like properties. The notion that within established tumor, the great majority of the cancer cells cannot sustain the lesion and only a few cells, cancer stem cells (CSCs), are tumorigenic and possess the metastatic phenotype is CSC hypothesis. CSCs, a very small population of specialized cells, have self-renewal and extensively proliferative characteristics to sustain tumor formation.[4,5] Recent molecular biological studies have identified podoplanin, CD44, and p63 as candidate stem cell markers in normal squamous epithelium and SqCC.[6–8]

Podoplanin is a mucin-like transmembrane glycoprotein that is highly and specifically expressed in lymphatic endothelial cells.[9] Podoplanin on cancer cells has been shown to act as a platelet-aggregation factor and cell–cell adhesion promoter,[10] and induction of expression of PA2.26, a homologue of human podoplanin, in mouse epidermal cells and tumor cells has been shown to be related to increased cell migration and malignant transformation.[11] In addition, we have previously reported that podoplanin is a novel marker to enrich tumor-initiating cells with stem-cell-like properties in SqCC *in vivo* and *in vitro*. Using the human SqCC cell line A431, sorted podoplanin-positive cells have higher colony formation and tumorigenicity than podoplanin-negative cells, and xenografted tumors derived from podoplanin-positive cells are similar to those in human oral SqCC tissue and normal epithelium.[6]

The cell surface glycoprotein CD44 is involved in cell migration and cell adhesion. CD44 has been found to support anchorage-independent growth *in vitro* and tumor growth and metastasis in experimental models of solid cancer.[12] $CD44^+$ cells in breast and lung carcinoma, and head and neck SqCC, have been shown to possess the CSC properties of self-renewal and differentiation.[8,13–15]

p63, a homologue of the tumor suppressor p53, plays a crucial role in initiating epithelial stratification during development and in maintaining epidermal structures, including in the oral mucosa, skin, teeth, and other sites, and p63 has been shown to be a specific marker of human corneal and squamous epithelial stem cells.[7,16–18] The human *p63* gene codes for at least six protein isoforms as a result of initiation of transcription at two different promoter sites that contain (TA) or lack (ΔN) a transactivation domain. The isoforms have different functions and form complicated networks in different systems.[7,15,19]

The purpose of this study was to examine the expression pattern of podoplanin, CD44, and p63, and their implication for clinico-pathological features in pulmonary SqCC.

Materials and Methods

Patients. During the period from January 1998 to December 2003, a total of 1279 patients underwent surgical resection for primary lung cancer at the National Cancer Center Hospital East, Chiba, Japan, and we reviewed the cases of the 167 consecutive patients in whom complete resection of pulmonary SqCC had been possible. All patients signed the Institutional Review Board–approved informed consent form. Staging was performed according to the International Union Against Cancer's tumor-node-metastasis (TNM) classification. The tumors were histologically subtyped and graded according to the third

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edition of the World Health Organization (WHO) guidelines. Five of the 167 patients were not included in this study; three because they received preoperative chemotherapy and two because of the poor quality of the specimens obtained. Thus, 162 patients were ultimately eligible for inclusion in this study, and their median follow-up period was 5.0 years.

Clinical characteristics were retrieved from the clinical records available. The following clinico-pathological factors were assessed retrospectively in relation to immunohistochemical analysis: age (<70 years *vs* ≥70 years), tumor size (≤3 cm *vs* >3 cm), pathological nodal involvement (positive *vs* negative), grade of differentiation (well or moderately differentiated *vs* poorly differentiated), location of the tumor (central *vs* peripheral), vascular invasion (absent *vs* present), lymphatic invasion (absent *vs* present), and pleural invasion (absent *vs* present). Central location of a tumor was defined as a tumor location limited to the trachea, bronchi, or segmental bronchi, and peripheral location as location more peripheral than the subsegmental bronchi. Cumulative smoking was presented by a smoking index, defined as a product of the numbers of cigarette per day and the duration (years).

Pathological studies and tissue microarray (TMA) construction. After fixing the specimens with cold methanol and embedding in paraffin, serial 4-μm sections were stained with hematoxylin–eosin (H&E) and by the alcian blue-periodic acid-Schiff method to visualize cytoplasmic mucin and by the Verhoeff-van-Gieson (VvG) method to visualize elastic fibers. Sections stained by the VvG method were examined for the presence of vascular invasion and pleural invasion. The sections were reviewed by two pathologists (Y.S. and G.I.) and the histological diagnoses were based on the revised WHO histological classification.

For microarray construction, the above two pathologists marked morphologically representative tumor areas, avoiding necrotic areas and the area in which cancer cells and stromal cells are intermingled, and definitely containing interfaces between the tumor nests and stroma, on an H&E-stained slide of donor tissue. The TMAs were constructed with a manual tissue-arraying instrument (Azumaya, Tokyo, Japan). The microarray instrument is used to remove a tissue core from the donor block with a thin-walled needle having an inner diameter of approximately 2.0 mm. Core samples were precisely placed in an empty paraffin block (the recipient block) at a specifically assigned location. Two core samples of each tumor were routinely corrected from two different areas. Normal lung tissue from the some patient's specimen was used as a positive control for each staining. Specimens from the 162 cases were punched, and core samples were mounted in the same recipient blocks.

Immunohistochemical analysis. TMA recipient blocks were cut into 4-μm sections and mounted on silane-coated slides. After deparaffinizing the sections in xylene and dehydrating them in a graded ethanol series, the slides were washed three times in phosphate-buffered saline (PBS) and immersed in a 0.3% hydrogen peroxide solution in methanol for 15 min. to inhibit endogenous peroxidase activity. The slides were then washed three times in PBS, and nonspecific binding was blocked by preincubation with 2% normal swine serum in PBS (blocking buffer) for 30 min at room temperature. Individual slides were then incubated overnight at 4°C with anti-podoplanin (clone D2-40; Signet, Dedham, MA, USA) at a 1:50 dilution, and anti-CD44 (clone DF1485; Novocastra, Newcastle, UK) at a 1:40 dilution, and anti-p63 (clone 4A4; Dako Cytomation, Carpinteria, CA, USA) at a 1:200 dilution. Finally, the slides were washed three times with PBS and incubated with the EnVision+ System HRP (Dako, Glostrup, Denmark), and the reaction products were stained with diaminobenzidine and counterstained with hematoxylin.

When more than 10% of the tumor cells showed an unequivocally strong reaction with an antibody, the tumor was classified as positive. Cytoplasm and/or membrane immunoreactivity was considered to indicate podoplanin and CD44 expression. p63 expression was considered positive if distinct nuclear staining was present. Moreover, we discriminated between "peripheral expression pattern" and "diffuse expression pattern" in positive immunoreactivity by the tumor nests with these antibodies. Peripheral expression pattern was defined as cells showing strong expression localized to the periphery of the tumor nests with no or weak expression in the central area. Diffuse expression pattern was defined as a less clear staining intensity between the peripheral and central areas of the tumor nests.

Statistical analysis. The associations between immunohistochemical expression status and clinico-pathological parameters were analyzed by using the χ^2-test or Fisher's exact test. 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. All tests were two-sided, and P-values <0.05 were considered statistically significant. The Stat-view 5.0 software package was used to perform the statistical analysis (SAS Institute, Cary, NC, USA).

Results

Characteristics of the patients. The clinico-pathological characteristics of the 162 patients are summarized in Table 1. Male predominance and a high smoking index were outstanding characteristics, and there were only three never smokers. The

Table 1. Clinico-pathological features of squamous cell carcinoma cases (n = 162)

Gender	
Male	147
Female	15
Age	
Median (range)	67 (31–84)
Smoking index	
Median (range)	960 (0–2760)
Tumor size (cm)	
Median (range)	3.8 (1.0–9.4)
N Stage	
pN0	117
pN1/pN2	45
Tumor location	
Central	37
Peripheral	125
Differentiation	
Well/moderate	3/104
Poor	55
Vascular invasion	
Absent	113
Present	49
Lymphatic invasion	
Absent	105
Present	57
Pleural invasion	
Absent	116
Present	46
Pathological stage	
IA	50
IB	62
IIA	13
IIB	34
IIIA	3

5-year overall survival rate of the 162 patients as a whole was 67.7%.

Expression of podoplanin, CD44, and p63 by cancer cells and characteristic immunostaining. We examined the expression of podoplanin, CD44, and p63 by immunohistochemical staining in a series of 162 specimens of SqCC of the lung. Podoplanin and CD44 were expressed mainly in the cytoplasm and membrane of the tumor cells, and p63 was expressed in the nuclei. The results of the immunohistochemical analysis are shown in Figure 1. In normal lung tissue, podoplanin expression was consistently detected in the endothelium of the lymphatic vessels and basal cells in the bronchial epithelium (Fig. 1a). CD44 expression was observed in the basal layer of the bronchial epithelium and peribronchial mesenchymal cells (Fig. 1c), and p63 was expressed in the nuclei of basal cells in the bronchial epithelium (Fig. 1e). The tumor cells in 107 (66%) of the 162 specimens were positive for podoplanin. The tumor cells in 145 (89.5%) were positive for CD44, and the tumor cells in 151 (93.2%) were positive for p63 (Table 2). As discussed, there were two patterns of expression in the cases that immunohistochemically stained podoplanin positive. A total of 102 (95.3%) of the 107 podoplanin-positive cases showed peripheral expression patterns, (Fig. 1b) whereas only five cases (4.7%) showed diffuse expression patterns in the tumor nests. Figure 1(d,f) shows the results for CD44 and p63 staining of the same specimen as in Figure 1b. The CD44 staining pattern was similar to that of

Table 2. Expression of podoplanin, CD44, and p63 by cancer cells

Molecular marker	Negative (%)	Positive (%)
Podplanin	55 (34.0)	107 (66.0)
CD44	17 (10.5)	145 (89.5)
p63	11 (6.8)	151 (93.2)

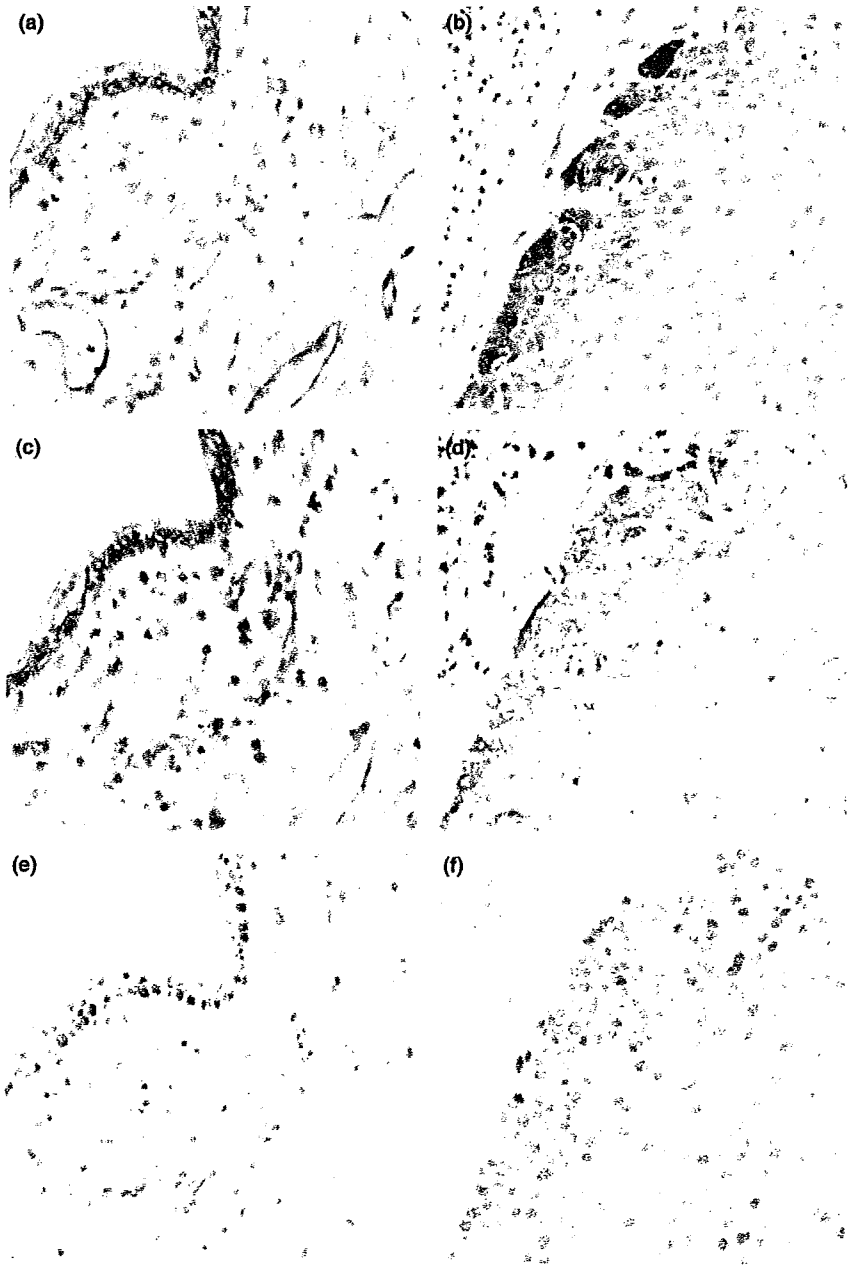

Fig. 1. Immunohistochemical analysis of podoplanin, CD44, and p63 expression in squamous cell carcinomas of the lung and a normal part of the specimen from one patient. (a) Podoplanin expression was detected in the endothelium of lymphatic vessels and in bronchial basal cells. (b) Podoplanin expression is mainly localized at the periphery of invading tumor nests. (c) CD44 expression was expressed in the bronchial basal cells. (d) CD44 expression was predominantly found in the peripheral areas of the tumor nests, but its distribution was broader than that of podoplanin. (e) p63 expression was observed in the nuclei of the bronchial basal cells. (f) Difference in p63 expression between the peripheral area and central area was less clear.

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podoplanin, but the area of intense CD44 staining was broader than that of podoplanin. Eighty-one (55.9%) of the CD44-positive cases showed peripheral expression patterns (Fig. 1d), whereas 64 cases (44.1%) showed diffuse expression patterns. In 65 (43%) of the p63-positive cases there were peripheral expression patterns, but the difference in the staining intensity of p63 between the peripheral and central areas was less clear compared with podoplanin and CD44 staining (Fig. 1f).

As shown in Figure 1(b,d,f), the distribution of podoplanin-positive cells appeared to be localized more peripherally within the tumor nests than the distribution of CD44- and p63-positive cells. When CD44 and p63 expressions were compared, p63-positive cell layers were broader compared to CD44-positive cell layers in the tumor nest periphery. We named this expression the "hierarchical distribution pattern" (Fig. 2).

The hierarchical distribution pattern was observed in 95 (88.8%) of the 107 podoplanin-positive cases (Table 3). Of the 12 remaining cases, there were diffuse extensive expression cases of all three markers in five. Further, there were CD44-negative/p63 peripheral staining patterns in two, negative staining cases for both CD44 and p63 in two, negative staining for CD44/diffuse staining pattern for p63 in one, p63-positive cells more restricted to the periphery of the tumor nests than in the CD44-positive cases in one, and CD44-positive cells expressed in only the central area in one.

Correlation between clinico-pathological features and the hierarchical distribution-positive cases. Correlations between the hierarchical distribution-positive cases and the clinico-pathological features of the patients are shown in Table 4. The hierarchical distribution-positive cases was significantly associated with the absence of lymphatic invasion ($P = 0.035$). No other clinico-pathological factors were correlated with them.

The hierarchical distribution cases showed better overall survival. The 5-year overall survival rate of patients with the podoplanin-positive cases and the podoplanin-negative cases was 74.4% and 54.8%, respectively. Patients with the podoplanin-positive cases had a longer overall survival time than those with the podoplanin-negative cases ($P = 0.018$; Fig. 3a), whereas staining with CD44 and p63 had no prognostic significance ($P = 0.941$ and 0.640, respectively; data not shown). Moreover, we examined the prognostic value of the hierarchical distribution–positive cases and podoplanin-negative cases. The 5-year overall survival rate of the hierarchical distribution–positive cases was 71.7% and the hierarchical distribution-positive cases had a more favorable outcome than podoplanin-negative cases ($P = 0.043$; Fig. 3b).

Discussion

In this study, we analyzed immunohistochemically the expression of podoplanin, CD44, and p63 in 162 pulmonary SqCCs.

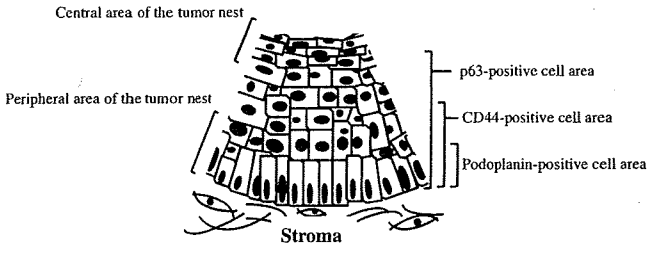

Fig. 2. Schema of the hierarchical distribution pattern of podoplanin, CD44, and p63 within tumor nests. The distribution of podoplanin-positive cells appeared to be more localized to the peripheral area of the tumor nests than the distribution of CD44- and p63-positive cells. The distribution of p63-positive cells was broader than that of the CD44-positive cells.

Table 3. Characteristic distribution of podoplanin-, CD44-, and p63-positive cells in podoplanin-positive squamous cell carcinoma

Podoplanin positive cases (n = 107)		
Hierarchical distribution pattern	Diffuse expression pattern	Others
95 (88.8%)	5 (4.7%)	7 (6.5%)

Table 4. Correlation between clinico-pathological features and the hierarchical distribution–positive cases

Variables	Hierarchical distribution cases (n = 95)	Podoplanin-negative cases (n = 55)	P-values
Gender			
Male	85	50	0.778
Female	10	5	
Age			
<70	48	33	0.262
≥70	47	22	
Tumor size			
≧3cm	42	21	0.471
>3 cm	53	34	
N Stage			
pN0	74	36	0.097
pN1 or pN2	21	19	
Differentiation			
Well or moderate	64	34	0.491
Poor	31	21	
Location			
Central	16	15	0.128
Peripheral	79	40	
Vascular invasion			
Absent	67	38	0.853
Present	28	17	
Lymphatic invasion			
Absent	68	30	0.035
Present	27	25	
Pleural invasion			
Absent	69	38	0.644
Present	26	17	

Focusing on the positive expression patterns, in 102 (95.3%) of the 107 podoplanin-positive tumors, tumor cells showing strong expression were localized in the periphery of the tumor nests. A similar pattern of podoplanin expression has been demonstrated immunohistochemically in head and neck, skin, and uterine cervix.[20–23] Meanwhile, this peripheral localization pattern was observed in only 55.9% (81/145) of the CD44-positive tumors and 43% (65/151) of p63-positive tumors. As shown in Figure 1(b,d), both the podoplanin-positive cells and CD44-positive cells resided at the periphery of tumor nests; however, the podoplanin-positive cells were more specifically restricted to the peripheral layers than the CD44-positive cells. Furthermore, when CD44 and p63 expressions were compared, p63-positive cell layers in the periphery of the tumor nests were broader compared to CD44-positive cell layers in the majority of cases (141/145; 97.2%; data not shown). The hierarchical distribution pattern was observed in almost 90% of the podoplanin-positive cases (Table 3). In non-cancerous squamous epithelium also, this hierarchical distribution pattern could be found (data not shown). Furthermore, we have previously reported that in human squamous SqCC cell line A431, almost all cultured

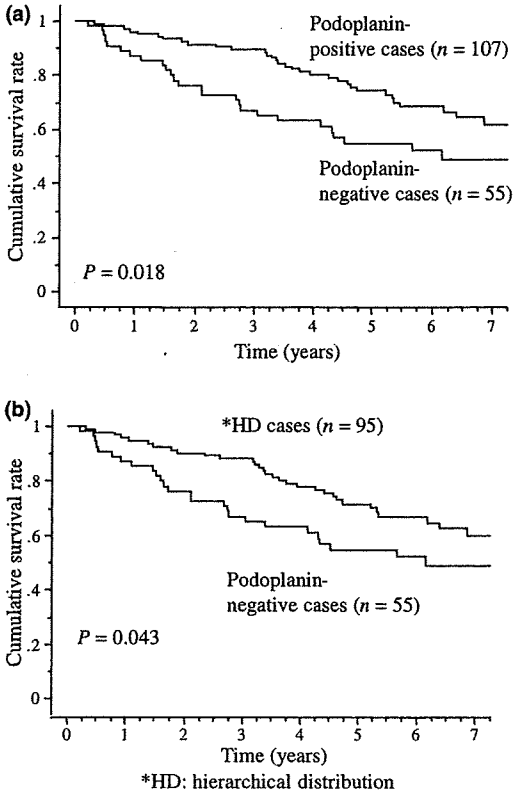

Fig. 3. Kaplan–Meier curves for overall survival. Overall survival curves of patients according to whether their tumor was podoplanin-positive or podoplanin-negative. The 5-year overall survival rates of the former and the latter were 74.4% and 54.8%, respectively. Overall survival curves of patients stratified according to whether their tumor was podoplanin-positive with the hierarchical distribution pattern or podoplanin-negative. The 5-year overall survival rates of the former and the latter were 71.7% and 54.8%, respectively.

A431 cells were positive for CD44 whereas the frequency of podoplanin-positive cells was approximately 35% in flow cytometric analysis.[6] This finding implies that podoplanin-positive A431 cells are a limited subpopulation of CD44-positive cells under *in vitro* condition, and is compatible with our immunohistochemical results. Considering a morphological representative for the developmental hierarchy based on the CSC hypothesis, podoplanin expression would reflect the most immature CSC status in its differentiation process, and podoplanin may be a marker of cells with a capacity for further maturation of SqCC. On the other hand, CD44 expression reflects even more differentiated cells of SqCC, and p63 expression may broadly reflect the CSC differentiation process ranging from immature CSC status to mature cells.

In the current study, podoplanin immunoreactivity had a prognostic significance, and this result is compatible with our previous study limited to pathological stage IB SqCC.[20] Furthermore it is notable that patients who had podoplanin-positive tumors with the hierarchical distribution pattern had significantly better overall survival than those who had podoplanin-negative tumors. Additionally these tumors showed a significant correlation of the absence of lymphatic invasion and had a certain tendency to no lymph node metastasis. These results suggest that SqCC with the hierarchical distribution pattern may indicate lower biological aggressiveness. It seems possible that SqCC showing the hierarchical distribution pattern is a well-organized tumor group based on the CSC concept, whereas SqCC with an unclear hierarchy is a disordered tumor group in terms of the developmental hierarchy.

In this study, 55 cases (34%) of SqCC were podoplanin-negative. This type of SqCC may fall into the following categories. First, CSCs of podoplanin-negative SqCC are enriched in the subpopulation expressing molecular markers other than podoplanin. Second, such cases may be a kind of tumor with no hierarchical structure based on the CSC concept. Some cancers contain small subpopulations of cancer-initiating cells, whereas others contain common tumorigenic cells with little evidence of hierarchical organization.[24] Podoplanin-negative SqCC might be the latter type of carcinoma. It will be important to analyze the biological features of podoplanin-negative pulmonary SqCCs in order to understand SqCC biology.

We examined the human SqCC cell lines, TE3, TE4, and TE10 other than A431, and the frequency of podoplanin expressing cells was 100%, 100%, and 0.5%, respectively (Atsumi *et al*, unpublished data). Given the expression frequencies in these SqCC cell lines, whether the podoplanin-positive cells can always represent a CSC subpopulation in a variety of SqCCs will remain a matter of debate.

CD44 is transmembrane hyaluronan receptor, and its cytoplasmic region, comprising 72 amino acid residues, has been shown to associate with actin filaments in various cells, a process mediated by ERM (ezrin/radixin/moesin) proteins.[25] Villar *et al.* demonstrated that the cytoplasmic domain of podoplanin also binds ERM proteins to promote epithelial–mesenchymal transition.[25] Considering the similar localization of podoplanin- and CD44-positive cells within the tumor nests and their common signaling via ERM proteins, it might be possible to think that signaling via Podoplanin and CD44 collectively mediate to express the biological properties of CSC .

Targeting CSCs has been proposed as an effective approach to cancer treatment, because CSCs are thought to be insensitive to conventional treatments and to be responsible for relapses. From this standpoint, the realization of CSCs due to a specific molecular marker, podoplanin, may lead to a new treatment strategy for SqCC.

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Disclosure Statement

No potential conflicts of interest are disclosed.

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Pulmonary Metastasectomy for Pulmonary Metastases of Head and Neck Squamous Cell Carcinomas

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Background. The lung is the major organ for distant metastasis from head and neck cancers, and pulmonary metastasectomy is indicated for selected cases. The efficacy of surgical treatment for pulmonary metastatic lesions from head and neck cancers has not been thoroughly examined.

Methods. The database developed by the Metastatic Lung Tumor Study Group of Japan was retrospectively reviewed. Between November 1980 and September 2006, 237 patients underwent resection of pulmonary metastases from primary head and neck cancers. After excluding nonsquamous cell carcinomas, 114 cases were analyzed, and the survival and prognostic factors for pulmonary metastasectomy for metastases from head and neck cancers were determined.

Results. The overall 5-year survival rate after pulmonary metastasectomy was 26.5%, and the median survival time was 26 months. As determined by univariate analysis, poor prognostic factors were oral cavity cancers, lymph node metastasis, a disease-free interval of 24 months or less, and incomplete resection. Multivariate analysis revealed that poor prognostic factors were being male, having oral cavity cancers, lymph node metastasis, and incomplete resection. When patients were divided into males with oral cavity cancers (n = 17) and all others (n = 97), the 5-year survival rates were 0% and 31.6%, respectively. Survival of male patients with oral cavity cancer that metastasized was significantly reduced ($p < 0.001$).

Conclusions. Male sex, oral cavity cancers, lymph node metastasis, and incomplete resection were poor prognostic factors for pulmonary metastases, but there is the potential for a good surgical outcome in carefully selected patients.

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The lung is the major organ of distant metastasis from head and neck cancers [1, 2]. Although surgical resection is an important treatment for pulmonary metastasis [3–5], pulmonary metastasis is commonly considered to reflect systemic disease, and the prognosis for pulmonary metastasis from head and neck cancers remains poor. The incidence of pulmonary metastasis from head and neck cancers is reported to range from 6.0% to 9.1% [6, 7]. With regard to pulmonary metastasectomy, Wedman and colleagues [6] reported a 5-year survival rate of 59% in a pulmonary metastasectomy group, but only 4% in a nonmetastasectomy group. Although Yamagata and associates [7] reported that docetaxel-based chemotherapy had a better response rate for pulmonary metastases than non–docetaxel-based chemotherapy, the role of chemotherapy seems to be limited.

The survival rate after surgery for pulmonary metastases from head and neck cancers is reported to range from 29.0% to 59.4% [6, 8–10]. However, owing to the small number of previous studies, prognosis after surgery for pulmonary metastases from head and neck cancers has not been thoroughly examined. The 2006 annual report by the Japanese Association for Thoracic Surgery documents 4,912 patients who underwent pulmonary metastasectomy, among whom were 260 patients (5.3%) with head and neck cancers [11]. The number of cases of pulmonary metastasectomy for head and neck cancers in a single institution is limited; therefore, we retrospectively reviewed cases registered in the database of the Metastatic Lung Tumor Study Group of Japan of patients who underwent surgical treatment for pulmonary metastases from head and neck squamous cell carcinomas. From this, we identified prognostic

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