Table 5
Relationship between expression of BCRP and response to NPe6-PDT ($n = 24$).

NPe6-PDT	Lesion	BCRP		
		(−)	(1+)	(2+)
CR	22	0	4	18
[a]Rec, PR	2	0	2	0
CR rate	91.6%		66.7%	100%

[a] Rec, recurrence.

antitumor effect, regardless of the BCRP expression, and especially among lesions ≥10 mm in diameter (data not shown), the CR rate to NPe6-PDT was much higher than that to Photofrn-PDT.

4. Discussion

In this study, using the WST assay as the sensitivity test, we showed that BCRP-overexpressing cells were resistant to Photofrin-PDT as compared to the parental cells *in vitro* (Fig. 2A); we also showed, based on analysis of the treatment outcome of centrally located early lung cancers, that BCRP expression affected the efficacy of Photofrin-PDT (Table 2). We hypothesized that Photofrin may be a substrate of BCRP, which is a member of the ABC transporter protein family. *In* Fig. 1A and B, we examined the accumulation of the photosensitizers, Photofrin and NPe6, based on detection of the fluorescence intensity by fluorescence microscopy, and showed less pronounced accumulation of Photofrin in the A431/BCRP cells than in the parental A431 cells. Treatment with the specific inhibitor of BCRP, Fumitremorgin C, reversed the resistance against Photofrin-PDT in the A431/BCRP cells. We therefore hypothesized that Photofrin may be a substrate of BCRP. However, recently Liu et al. reported that Photofrin was minimally transported [38], and speculated that Photofrin is a mixture of multimeric photosensitizers that are considered to be clinically active fractions, together with monomers that are poorly retained in the cells and tissues and have little biological efficacy, and that only these inactive monomers are BCRP substrates [38]. It still remains under debate as to whether or not Photofrin is a substrate of BCRP. From our analysis of the BCRP expression in centrally located early lung cancers, we concluded that the expression of BCRP significantly affected the efficacy of Photofrin-PDT, and that BCRP could be a molecular determinant of the outcome of Photofrin-PDT.

On the other hand, as shown in Fig. 2B, in the case of NPe6-PDT, there was no significant difference in the sensitivity between A431 cells and A431/BCRP cells, and treatment with the specific inhibitor of BCRP, Fumutremorgin C, did not affect the sensitivity. Robey et al. reported that BCRP-overexpressing cells were not resistant to PDT with meso-tetra-3-hydroxyphenyl chlorine (m-THPC), which has a similar structure to the NPe6 chlorine annulus [24]. We concluded that NPe6 is not a substrate of BCRP and does not therefore affect the sensitivity of PDT.

In this study, all of the 81 centrally located lung lesions were found to express BCRP. Yoh et al. reported that 46% of all lung cancers were BCRP-positive in particular, 39% of squamous cell lung cancers [31]. Our data showed that all of the patients with centrally located early lung cancers were male, heavy smokers (>30 pack-years), and all of the tumors were BCRP-positive (100%). We hypothesized that smoking induces the expression of BCRP, and that some chemical products may act as substrates of BCRP. Recently, cigarette smoking was found to significantly lower both the exposure to irinotecan, which is a topoisomerase I inhibitor and a substrate of BCRP, and treatment-induced neutropenia, with a potential risk of treatment failure [39]. In addition, induction of ABC transporters by smoking can result in the elimination of irinotecan and its metabolites, suggesting that cigarette smoking may influence the pharmacokinetics of irinotecan via modulation of the ABC transporters [40,41]. Kolwankar et al. reported, based on immunohistochemical analysis of 94 patients with non-small cell lung carcinoma higher expression levels of ABCB1 (P-glycoprotein, MRP1) in smokers (58% vs. 9%; $P < 0.01$) [42]. In our study, we also examined the association between the efficacy of PDT and the expression of other ABC transporters, namely, MDR1/P-gp, MRP1/ABCC1. A431/MDR1 cells and KB3-1/MRP1 cells were slightly resistant to Photofrin-PDT as compared to the parental cells [30]. From analysis of the dose-modifying factor at the IC_{50} using these three cell lines, we suspect that BCRP may play the most important role among the three ABC transporters in influencing the outcome of clinical PDT. Although the data were not sufficient to arrive at any definitive conclusion in regard to the influence of smoking on the ABC transporters, we conclude that there may be some associations between the expression of the ABC transporters and smoking, and that BCRP, in particular, affected the efficacy of Photofrin-PDT. Additional investigation may be required to determine the mechanism underlying these results, and the expression of BCRP in centrally located early lung cancers may have important clinical implications.

For the treatment of centrally located early lung cancers, NPe6-PDT would appear to be superior to Photofrin-PDT, because the expression of BCRP did not seem to affect the antitumor effect of NPe6-PDT. Recently, it has been reported that the tyrosine kinase inhibitor imatinib mesylate (Gleevec) can block the functions of BCRP, to increase the intracellular accumulation of photosensitizers such as protoprphyrin IX (PpIX), in BCRP-overexpressing tumors [38]. Many advanced cancers have elevated expression levels of the BCRP protein and we hypothesize that PDT combined with administration of BCRP inhibitors such as imatinib can overcome any resistance conferred by elevated expression amounts of BCRP. In conclusion, for the treatment of centrally located early lung cancers with high expression levels of BCRP, NPe6-PDT would appear to be better than Photodrin-PDT, and individualized treatment based on the expression status of BCRP may improve the efficacy of PDT in patients with lung cancer.

Conflict of interest statement

None declared.

Acknowledgement

This study was supported in part by a Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) Fujita Memorial Fund for Medical Research (to J.U.).

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Management of Multiple Primary Lung Cancer in Patients with Centrally Located Early Cancer Lesions

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Background: Patients with centrally located early lung cancer (CLELC) are often heavy smokers with a considerably high risk of multiple primary lung cancer (MPLC) lesions; treatment strategies for such patients must preserve the cardiopulmonary function.

Methods: Between July 2004 and July 2008, patients with CLELC underwent photodynamic therapy (PDT) using NPe6, second-generation photosensitizer at Tokyo Medical University Hospital. Among these patients, we retrospectively analyzed MPLC, which was treated by surgery plus PDT or PDT alone and examined the effectiveness of PDT, and we propose a treatment strategy for patients with MPLC.

Results: A total of 64 patients with CLECL received NPe6-PDT, and MPLCs were found in 22 patients (34.4%) using sputum cytology and a bronchoscopical examination using autofluorescence bronchoscopy. Among these 22 patients, 10 patients underwent surgery for primary lung cancer and underwent NPe6-PDT for the treatment of secondary primary CLELC, one patient underwent PDT for CLELC as a primary lesion followed by an operation for peripheral-type lung cancer as a secondary primary lesion, and 11 patients underwent PDT alone for MPLC lesions (28 lesions) that were roentgenographically occult lung cancers. Among these 22 patients with MPLC including peripheral-type lung cancers, which were resected by surgery, all 39 CLELC lesions exhibited a complete response after PDT, and all patients were alive.

Conclusions: For patients with lung cancer with a long-term history of smoking, careful follow-up examinations after surgical resection are needed considering the incidence of metachronous primary lung cancers. PDT can play an important role for the treatment strategy for MPLC.

Key Words: Multiple primary lung cancer, Photodynamic therapy, Centrally located early lung cancer.

(*J Thorac Oncol.* 2010;5: 62–68)

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ISSN: 1556-0864/10/0501-0062

Photodynamic therapy (PDT) is widely used as a treatment option for solid cancers and also for some noncancerous diseases.[1–3] Early lung cancers can be divided into two categories, the peripheral type and the central type, according to the site of origin of the tumor.[4,5] In Japan, PDT is recommended as a treatment option for centrally located early lung cancers (CLELCs), which are roentgenographically occult squamous cell carcinomas not located distal to the segmental bronchi that are histologically determined to be carcinoma in situ or carcinoma showing only limited invasion, with no evidence of invasion beyond the bronchial cartilage, as defined in the therapeutic guidelines for lung cancer established by the Japanese Ministry of Health, Labor and Welfare based on the principles of evidence-based medicine.[4,5] The second-generation photosensitizer talaporfin sodium (NPe6 or Laserphyrin), which has a major absorption band at 664 nm, has been approved by the Japanese government for use in the diagnosis/treatment of CLELC.[4–7] A phase II clinical study using NPe6 and a diode laser for early-stage lung cancer demonstrated excellent antitumor effects and safety, including a significantly lower skin incidence of photosensitivity compared with that observed using Photofrin (Wyeth Japan K.K., Tokyo, Japan).[6,7] The Japanese government approved the use of NPe6 for PDT in 2003, and the product has been available in Japan since June 2004.[4–7]

Roentgenographically occult lung cancers, which are located in the central bronchus, can be detected in high-risk patients using either sputum cytology or bronchoscopic evaluation.[8–11] One to 4% of these patients have a synchronous lung cancer, and the risk of a second lung cancer ranges from 1 to 25% per year.[12–14] Some reports have revealed that 17% of newly diagnosed early lung cancer cases have a synchronous lesion.[15,16]

Multiple primary lung cancer (MPLC) is not an uncommon event, and Nakata et al.[17] reported that 7.9% of patients with surgically resected non-small cell lung cancers and 8.4% of patients with adenocarcinomas exhibited multiple lesions. In 1975, Martini and Melamed[18] outlined the criteria for differentiating between MPLC and recurrence, and they proposed that tumors were 'synchronous' when they were detected or resected simultaneously and 'metachronous' when the second tumor was found some time later. Recently, the

incidence of MPLC has been increasing as a result of the widespread use of early detection tools such as multislice spiral computed tomography (CT) and fluorescence endoscopy.[12–15] Nevertheless, no guidelines have detailed recommendations for the selection and treatment of patients with synchronous or metachronous MPLC.

In particular, patients with CLELC are often heavy smokers and have a considerably high risk of a second primary lung cancer; thus, these patients require treatment that will preserve their cardiopulmonary function. It has been reported that 70% of carcinoma in situ is not detected during white-light bronchoscopy, and that the prevalence of synchronous occult lesions after fluorescence bronchoscopy assessment might be higher than the previously reported values of 7 to 14%.[8,19,20] Moreover, after the successful treatment of a first occult cancer, as many as 5% of the metachronous tumors detected per year might actually result from the progressive evolution of synchronous, undetected early lung cancers. For patients treated using PDT, we routinely perform follow-up examinations using autofluorescence bronchoscopy (SAFE-3000) to avoid missing occult lung cancers.[4–7,21] In such patients with synchronous or metachronous MPLC, PDT is an effective treatment that also preserves lung function and is recommended in the evidence-based clinical practice guidelines of the American College of Chest Physicians.[4–7]

In this study, we retrospectively analyzed MPLC with CLELC lesions, which were treated by surgery plus PDT or PDT alone, and examined the effectiveness of PDT for the treatment of patients with CLELCs, and we propose a treatment strategy for patients with MPLC.

MATERIALS AND METHODS

Photosensitizer

NPe6 (Meiji Seika, Tokyo, Japan) is a second-generation, water-soluble photosensitizer with a molecular weight of 799.69 and a chlorine annulus; its highest absorption peak occurs at a wavelength of 407 nm, and a second peak occurs at a wavelength of 664 nm.[4–7] NPe6 exhibits superior in tumor affinity, compared with Photofrin, and is excited by visible red light with a longer wavelength of 664 nm, enabling deeper and superior penetration into living tissues.[4,22]

Laser Unit

A diode laser (Matsushita Electric Industrial Co., Osaka, Japan) emitting continuous-wave laser light at a wavelength of 664 nm was used as the light source for the excitation of NPe6.[4–7]

Criteria for the Diagnosis of CLELC

Lung cancers not located distal to the segmental bronchi, diagnosed histologically as squamous cell carcinoma, and determined to be carcinoma in situ or carcinoma showing only limited invasion with no evidence of invasion beyond the bronchial cartilage were defined as CLELCs.[4–7] We routinely determined the tumor depth using endobronchial ultrasonography and confirmed that the tumors had not invaded the bronchial wall beyond the level of the cartilage and were confined to the basal membrane of the mucosa, submucosa, or intracartilaginous layers of the bronchial wall.[4,5,7,23] In 2003, the Japan Photodynamic Association and Japanese Society of Laser Surgery and Medicine established the following therapeutic criteria for PDT in cases with CLELC: patients with (a) endoscopically assessable early lung cancer, (b) a normal chest x-ray and CT, and (c) no metastasis to lymph nodes or distant metastasis as revealed using routine clinical diagnostic methods, including fluorodeoxyglucose-position emission tomography for staging.[4,5]

PDT Procedures and Follow-Up

Local anesthesia was performed using 4% lidocaine spray. Additional sedation was necessary. Laser irradiation (664 nm) for NPe6-PDT was transmitted by means of quartz fibers inserted through the biopsy channel of the endoscope, 4 to 6 hours after the administration of the photosensitizer, NPe6 (40 mg/m^2). The total energy of the laser irradiation was 100 J/cm^2 (150 mW/cm^2).[4–7] The Japanese government approved the use of NPe6 for PDT against CLELCs in 2003, and the product became available in Japan in June 2004.[4–7] Ever since, we have used NPe6 for PDT. A fiber-optic bronchoscopy with cytologic and histologic examinations was performed at 1, 2, and 3 months after the PDT and at 3-month intervals during the first year and 6-month intervals during the second year thereafter. The antitumor effect of the initial treatment was rated based on the endoscopic measurement of the tumor size using forceps, the morphologic appearance, and the pathologic findings of the biopsy specimens, in accordance with the general rules of the Japan Lung Cancer Society and the Japan Society of Clinical Oncology.[4–7] The antitumor effect was evaluated again at 3 months after the PDT. The tumors were then classified as showing a complete response (CR) (no microscopically demonstrable tumor in the brushings and/or biopsy specimens over a period of 4 weeks).[4–7,21] We used fluorescence bronchoscopy (SAFE-3000) as part of the follow-up examination after NPe6-PDT.[5,7]

Patient Selection

From 2004 to 2008 at the Tokyo Medical University Hospital, we found 64 patients with CLELC by bronchoscopical examination using autofluorescence bronchoscopy (SAFE-3000) because of abnormal sputum production and/or sputum cytologic abnormalities in mass survey or follow-up after surgical resection or PDT. All 64 patients with CLELC received NPe6-PDT. PDT was undertaken in patients who met the criteria for PDT after obtaining their informed consent in accordance with institutional guidelines.[4–7] The clinicopathological characteristics of the patients are listed in Table 1. Their median age at diagnosis was 74 years (range, 67–84). All the patients were men and heavy smokers with a smoking history of more than 30 pack-years.

Efficacy Evaluation

The antitumor effect was rated, based on endoscopic measurement of tumor size using forceps, morphologic observation, and histopathological examination by biopsy. The antitumor effect was rated at 2 months after PDT. Antitumor effect was rated as CR (no demonstrable tumor for 4 weeks),

TABLE 1. Clinicopathological Characteristics of the Patients Who Underwent NPe6-PDT (July 2004–July 2008)

Characteristics	No. of Lesions
Patients	64
Age	67–83
Gender	
Male	64
Female	0
Smoking history	
Positive	64 (>30 pack-years)
Patients with multiple lesions	22 patients
Synchronous	10 patients
Metachronous	12 patients

PDT, photodynamic therapy.

TABLE 2. Treatment for Multiple Lung Cancer Lesions (July 2004–July 2008)

Treatment	No. of Patients (Metachronous)
Surgery ⟶ PDT	10 (8)
PDT ⟶ surgery	1 (1)
PDT alone	11 (3)
Total	22 (11)

PDT, photodynamic therapy.

partial response (50% or greater reduction in tumor size), no change (less than 50% reduction or less than 25% increase in tumor size), and progressive disease (more than 25% increase in tumor size).

RESULTS

Clinicopathological Characteristics of the Patients Who Underwent NPe6-PDT

The clinicopathological characteristics of the patients with roentgenographically occult lung cancer, who underwent PDT with NPe6, are listed in Table 1. Their median age at the time of the diagnosis of CLELC was 74 years (range, 67–83 years). All the patients were men and were heavy smokers with a smoking history of more than 30 pack-years. We performed skin photosensitivity test 2 weeks after PDT, and all patients had no photosensitivity.

MPLCs were noted in 22 patients (34.4%, 22 of 64). Synchronous lesions were noted in 10 patients (15.6%), and metachronous lesions were noted in 12 patients (18.8%).

Treatment for MPLC Lesions

The characteristics of the patients with MPLC lesions are summarized in Table 2. Among these 22 patients, 10 patients underwent surgery for peripheral-type lung cancer as their first primary lesion followed by PDT for CLELC (Tables 3 and 4), one patient underwent PDT for CELC as a primary lesion followed by an operation for peripheral-type lung cancer as a secondary primary lesion (Table 5), and 11 patients underwent PDT alone for MPLC lesions that were all centrally located and roentgenographically occult lung cancers (Table 6).

TABLE 3. Surgery Followed by PDT (July 2004–July 2008)

Treatment	No. of Patients (Metachronous)
Surgery ⟶ PDT	10 (8)
Lobectomy	
Adenocarcinoma	1 (1)
Squamous cell carcinoma	7 (6)
Small cell carcinoma	1 (1)
Pneumonectomy	
Squamous cell carcinoma	1 (1)

PDT, photodynamic therapy.

Surgery for Peripheral-Type Lung Cancer as Their First Primary Lesion Followed by PDT for CLELC

Ten patients underwent surgery (nine lobectomies and one pneumonectomy) for primary lung cancer at peripheral sites and then underwent NPe6-PDT for the treatment of secondary primary CLELCs (Table 3).

Information on 10 Patients Who Underwent Surgery Followed by PDT

Information on 10 patients, who underwent surgery (nine lobectomies and one pneumonectomy) for peripheral-type lung cancer as a first primary lesion and were then treated for CLELCs as secondary primary lung cancers, is presented in Table 4. The age distribution ranged from 63 to 70 years at the time of PDT for the treatment of secondary primary CLELCs. Two of these lesions (case 3 and case 6) were synchronous and eight were metachronous, and the interval between the diagnosis of the first and second cancers in this series ranged from 4 to 126 months (mean, 30 months). After operation, eight metachronous CLELCs were found by sputum cytology and bronchoscopical examination using autofluorescence bronchoscopy (SAFE-3000). Histologic examination of the surgically removed tumors before PDT revealed one adenocarcinoma, six squamous cell carcinomas, and one small cell carcinoma (Tables 3 and 4). In case 1, a left lower lobectomy was performed, and the pathologic diagnosis was adenocarcinoma (p-T1N0M0 stage IA); 72 months later, a CLELC was detected in the left main bronchus and PDT was performed. In case 2 and case 5, the pathologic stage was IIB (p-T2N1M0, squamous cell carcinoma); in case 10, the pathologic stage was IIIA (small cell carcinoma). In only one case (case 10), chemotherapy was performed after a right lower lobectomy. No evidence of metastasis or local recurrence was observed after the operation for the first primary lung cancer. In these 10 patients, the secondary tumors, all of which were roentgenographically occult, carcinoma in situ squamous cell carcinomas, were treated with NPe6-PDT. Three lesions were located in the trachea, and seven lesions were located in the segmental bronchus. In all 10 patients with CELCS, a CR was achieved after NPe6-PDT treatment (CR: 100%), and all the patients were alive at the time of writing.

TABLE 4. Surgery Followed by PDT (July 2004–July 2008)

Age (yr)	Operation	Pathology	Interval (mo)	PDT Lesion	PDT	Outcome
63	LL lobectomy	IA (adenocarcinoma)	72	Lt. main br. CR		Alive
63	LL lobectomy	IIB (sq.)	10	Rt. B1	CR	Alive
68	LL lobectomy	IA (sq.)	0	Rt. B3	CR	Alive
70	RL lobectomy	IA (sq.)	39	Rt. B6-basal CR		Alive
64	Lt. pneumonectomy	IIB (sq.)	17	Lt. main br. CR		Alive
66	RU lobectomy	IA (sq.)	0	Rt. B6	CR	Alive
67	RU lobectomy	IA (sq.)	49	Trachea	CR	Alive
69	RU lobectomy	IA (sq.)	4	Lt. B3	CR	Alive
70	RL lobectomy	IA (sq.)	43	Trachea	CR	Alive
65	RL lobectomy	IIIA (small)	126	Rt. B1	CR	Alive

PDT, photodynamic therapy; RU, right upper; LL, left lower; CR, complete response; Rt, right; Lt, left.

TABLE 5. PDT Followed by Surgery (July 2004–July 2008)

Age (yr)	Lesion	PDT	Interval (mo)	Operation	Pathology	Outcome
57	Lt. upper-lower	CR	25	RU lobectomy	IIIA (adenocarcinoma)	Alive

PDT, photodynamic therapy; CR, complete response; RU, right upper; Lt, left.

TABLE 6. PDT Alone for Multiple Primary Lung Cancers (July 2004–July 2008)

No. of Lesions	Patients (Metachronous)	Lesions	CR	Outcome
Two	7 (2)	14	14	Alive: 7
Three	3 (0)	9	9	Alive: 3
Five	1 (1)	5	5	Alive: 1
Total	11 (3)	28	28	Alive: 11

PDT, photodynamic therapy; CR, complete response.

Figure 1 shows case 4, a patient with metachronous double primary lung cancers. A 70-year-old man presented with coughing and hemoptysis. He had undergone a right upper lobectomy for squamous cell carcinoma 39 months previously. A chest CT scan revealed a clear lung field, and no mediastinal lymphadenopathy or abdominal lesions. A sputum cytologic examination revealed class V, squamous cell carcinoma. Conventional white-light bronchoscopy showed a 1.0 cm, thickened-type, roentgenographically occult lung cancer at the bifurcation between the right B6 and the basal bronchus that was identified as a secondary primary squamous cell carcinoma (Figure 1*B*). Four hours after the administration of NPe6 (40 mg/cm^2), we observed red fluorescence from the tumor using autofluorescence bronchoscopy (SAFE-3000) and were able to determine the tumor margin using a photodynamic diagnosis (Figure 1*C*); PDT was then performed using a diode laser (664 nm, 100J/cm^2, 150 mW) as previous reports.[4,5,7]

PDT for CELC as a Primary Lesion Followed by Surgery for Peripheral-Type Lung Cancer as a Secondary Primary Lesion

One patient underwent NPe6-PDT for a roentgenographically occult first primary lung cancer located at the bifurcation between the left upper and lower bronchus, and the metachronous lesion was resected by surgery (Table 5). This patient was followed using chest CT and sputum cytology, and 25 months later, a contralateral metachronous lesion (adenocarcinoma) was detected as a secondary primary lung cancer in the right S3 (Tables 2 and 5). The patient received a right upper lobectomy and then underwent chemotherapy because the pathologic stage was IIIA (T1N2M0) (Table 5).

PDT Alone for MPLC Lesions That Were All Centrally Located and Roentgenographically Occult Lung Cancers

In Table 6, the cases of multiple CLELCs that were treated with PDT alone are summarized. In 11 of the 22 patients, MPLCs (28 lesions in total) were observed. All lesions were roentgenographically occult lung cancers found by abnormal sputum production and were treated using NPe6-PDT alone (Tables 2 and 6). Seven patients had two roentgenographically occult lung cancer lesions, three patients had three lesions, and one patient had five lesions. Three of the 11 patients had metachronous occult lung cancer lesions and were followed using bronchoscopical examination with conventional and autofluorescence bronchoscopy after undergoing PDT (Tables 2 and 6). A CR was achieved in all 11 patients with a total of 28 roentgenographically occult lung cancer lesions (CR rate, 100%, 28 of 28); all the patients were alive at the time of writing.

Figure 2 shows a case with five CLELCs that were identified as metachronous MPLCs and treated using NPe6-PDT alone. This 65-year-old man with multiple CLELCs was diagnosed based on positive sputum cytologic findings performed during a mass screening. The performance status of this patient was 1 because of hemiparalysis caused by a cerebral infarction. All roentgenographic examinations were negative. The first tumor was a nodular-type early lung cancer located in the left B3, and NPe6-PDT was performed.

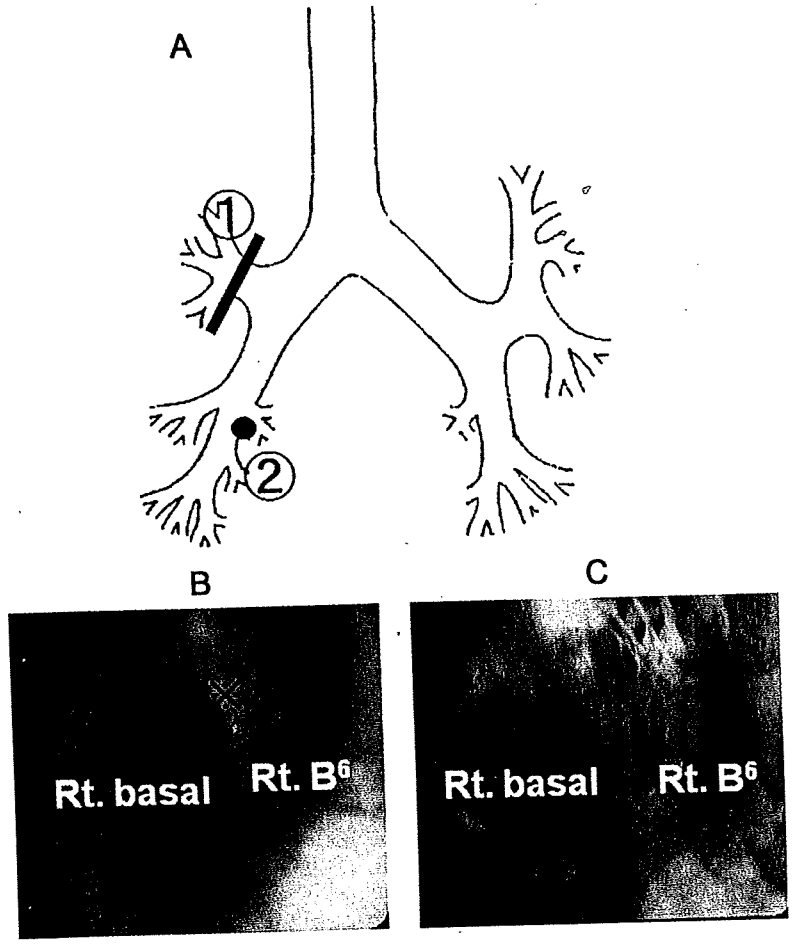

FIGURE 1. *A*, A schema of a 70-year-old man with metachronous multiple primary lung cancer. He underwent a right upper lobectomy for squamous cell carcinoma at S3. Thirty nine months later, centrally located early lung cancer was detected. *B*, Fiberoptic bronchoscopy revealed a thickened lesion. A thickened-type squamous cell carcinoma can be visualized at the bifurcation between right B6 and basal bronchus ※. *C*, Photodynamic diagnosis using SAFE-3000 system and NPe6 was conducted before the PDT. The red fluorescence excited by the diode laser (408 nm) from the SAFE-3000 system revealed the cancerous lesion ※.

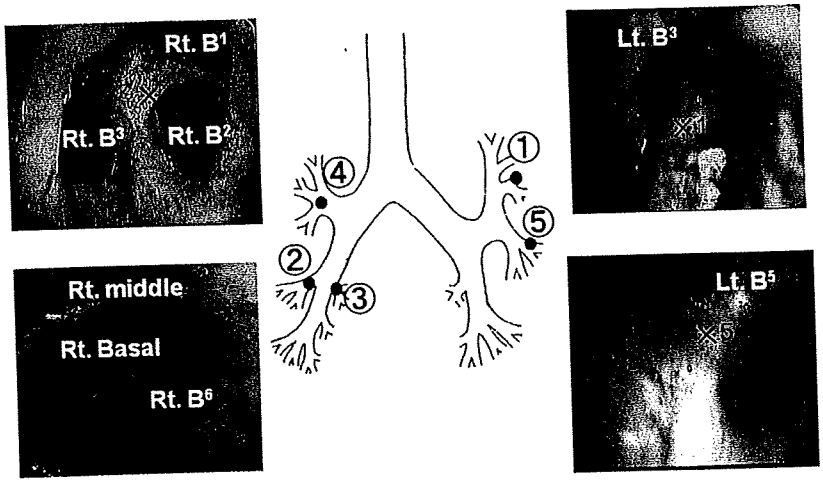

FIGURE 2. A 65-year-old man with metachronous multiple primary lung cancers. Fiberoptic bronchoscopy revealed five thickened-type lesions. First cancer lesions located at the left B3 (※ 1), second lesion at the bifurcation between the right middle lobe bronchus and the basal bronchus (※ 2), third lesion at the bifurcation between the right B6 and the basal bronchus (※ 3), fourth lesion at the right upper bronchus (※ 4) and fifth lesion at left B5 (※ 5). All five metachronous lesions were completely cured using PDT, and a CR was achieved.

Eight months after the PDT, a second tumor at the bifurcation between the right middle lobe bronchus and the basal bronchus was found, and the third tumor was found at the bifurcation between the right B6 and the basal bronchus during a routine bronchoscopic examination. Twenty months after the first PDT, a fourth tumor was detected in the right upper bronchus, and 29 months after the first PDT, a fifth tumor was detected in the left B5. These five lesions were thickened-type, roentgenographically occult lung cancers and were all treated using NPe6-PDT. All five metachronous lesions were completely cured using PDT, and a CR was achieved. No evidence of metastasis or recurrence was noted.

DISCUSSION

The criteria for MPLC were first proposed by Warren and Gates[24] in 1932. The most recent and recognized criteria

for differentiating between MPLC and recurrences are those reported by Martini and Melamed.[18] MPLC is not uncommon, and several authors have recently reported an incidence of 0.2 to 20%.[12–17] Johnson et al.[12,13] reported that the risk of developing a second lung cancer was 2 to 14% per patient per year, and that the risk increased twofold to sevenfold at 10 years after initial diagnosis. Nevertheless, no guidelines have detailed recommendation for the selection and treatment of patients with synchronous or metachronous MPLC. Moreover, CLELCs, which are roentgenographically occult and can be treated using PDT, cannot be detected using high-resolution computed tomography HRCT.[4,5,25–27] Fujimura et al.[11] reported that in 236 patients with roentgenographically occult lung cancers, a second primary lung cancer lesion was detected in 50 patients (22%), and 13 of these 50 patients had a third primary lung cancer lesion, whereas six of these 13 patients had a fifth primary lung cancer lesion. In an analysis of the Mayo Lung Project, only 54 patients with CLELC were identified, and synchronous lesions were found in four patients (7%).[2,28] Our results showed that multiple primary CLELCs were detected in 22 patients (34%, 22 of 64), providing an incidence rate that was higher than in previous reports (Table 1). We hypothesized that the incidence of MPLCs might have increased not only as a result of HRCT but also as a result of sputum cytologic examinations and bronchoscopical examination using fluorescence bronchoscopy, especially among patients with long history of smoking who have undergone surgery for peripheral lung cancers. In this report, eight patients (8 of 64, 11%) with roentgenographically occult secondary primary lung cancers were diagnosed based on sputum cytologic examinations, and their lesions were detected using autofluorescence bronchoscopy after surgical resection for peripheral-type lung cancer.

In 11 patients (11 of 64, 11%), multiple CLELCs were detected using routine bronchoscopical examination. These patients were all heavy smokers (more than 30 pack-years). The incidence of multiple primary CLELCs was similar to that in previous reports.[11,29] As shown Table 4, in case 1, a left lower lobectomy was performed because of peripheral-type lung cancer (adenocarcinoma) and 72 months later, a CLELC was found by abnormal sputum productions. These results suggest that for heavy smokers, there may be higher rate of incidence of squamous cell carcinoma as metachronous cancer compared with nonsmokers.

For heavy smokers with adenocarcinoma, who undergo surgery for primary lung cancer, sputum cytologic examinations should be a required part of their follow-up.[13,30,31] Careful follow-up using HRCT and sputum cytologic examination or fluorescence bronchoscopical examination might also be recommended, as suggested by the case history of patient 1 (Table 4). From our data, postoperative follow-up examinations should consider the incidence of MPLCs at peripheral or central sites in heavy smokers.

For synchronous or metachronous MPLCs, the performance of a pneumonectomy is a predictor of poor long-term survival, and a pneumonectomy should be avoided whenever possible.[15,16] Moghissi and Dixon[32] reported that PDT cured synchronous CLELCs 17 years after a pneumonectomy, and they concluded that PDT can enable long-term survival and serve as a potential cure for MPLCs. We also performed PDT for metachronous lung cancer 17 months after a left pneumonectomy, and the patients was still alive at the time of writing (Table 4). Moreover, as shown Figure 2, PDT cured all five MPLCs in another patient, enabling the patient's cardiopulmonary function to be preserved (Figure 2).

No guidelines detailing recommendations for the treatment of patients with MPLC have been published. Our present results suggest that minimally invasive treatments, such as PDT, should be performed for CLELCs of a synchronous or metachronous nature. Moreover, in heavy smokers who undergo surgery for lung cancer, sputum cytologic examination should be a necessary component of their follow-up care.

In conclusion, PDT is useful for extending the therapeutic options and improving the prognosis of patients with MPLCs. In particular, for patients with long-term history of smoking after surgical resection, careful follow-up with due consideration of the incidence of metachronous primary lung cancers is recommended, and PDT might play an important role for the treatment strategy for MPLC.

ACKNOWLEDGMENTS

Supported in part by a Grant-in-Aid for Scientific Research (C) from Japan Society for the Promotion of Science (JSPS), KAKENHI21591826 (to J. U.).

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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.

(Ann Thorac Surg 2009;88:1583–8)

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-

Accepted for publication April 16, 2009.

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Published by Elsevier Inc

0003-4975/09/$36.00
doi:10.1016/j.athoracsur.2009.04.065

Ann Thorac Surg
2009;88:1583–8

over, these discordant classifications may lead to a non-univocal 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

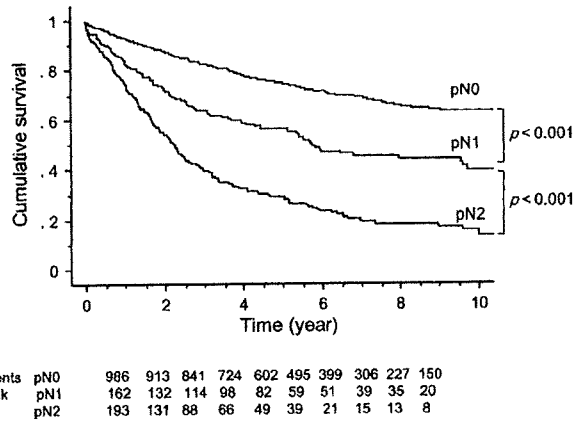

Fig 1. Kaplan-Meier curves show cumulative survival of patients undergoing complete resection for non-small cell lung carcinoma according to pathologic nodal status.

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

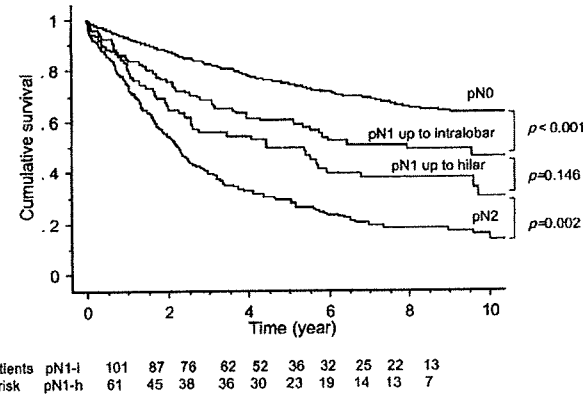

Fig 2. Kaplan-Meier curves show cumulative survival of patients undergoing complete resection for pN1 non-small cell lung carcinoma according to pathologic nodal status. Disease was classified as pN0, intralobar pN1, hilar pN1, and pN2. (Intralobar = No. 12, 13, 14 regional lymph nodes; hilar = No. 11,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

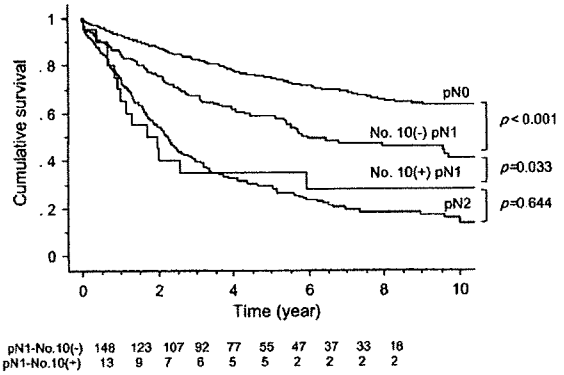

Fig 3. Kaplan-Meier curves show cumulative survival of patients undergoing complete resection for pN1 non-small cell lung carcinoma according to pathologic nodal status. Disease was classified as pN0, pN1 without No. 10 lymph nodes metastasis, pN1 with No. 10 lymph nodes metastasis, and pN.

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.

We are indebted to Professor J. Patrick Barron of the International Medical Communication Center of Tokyo Medical University for his review of this manuscript.

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

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(Received May 31, 2009/Revised July 02, 2009/Accepted July 07, 2009/Online publication August 13, 2009)

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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doi: 10.1111/j.1349-7006.2009.01295.x

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)

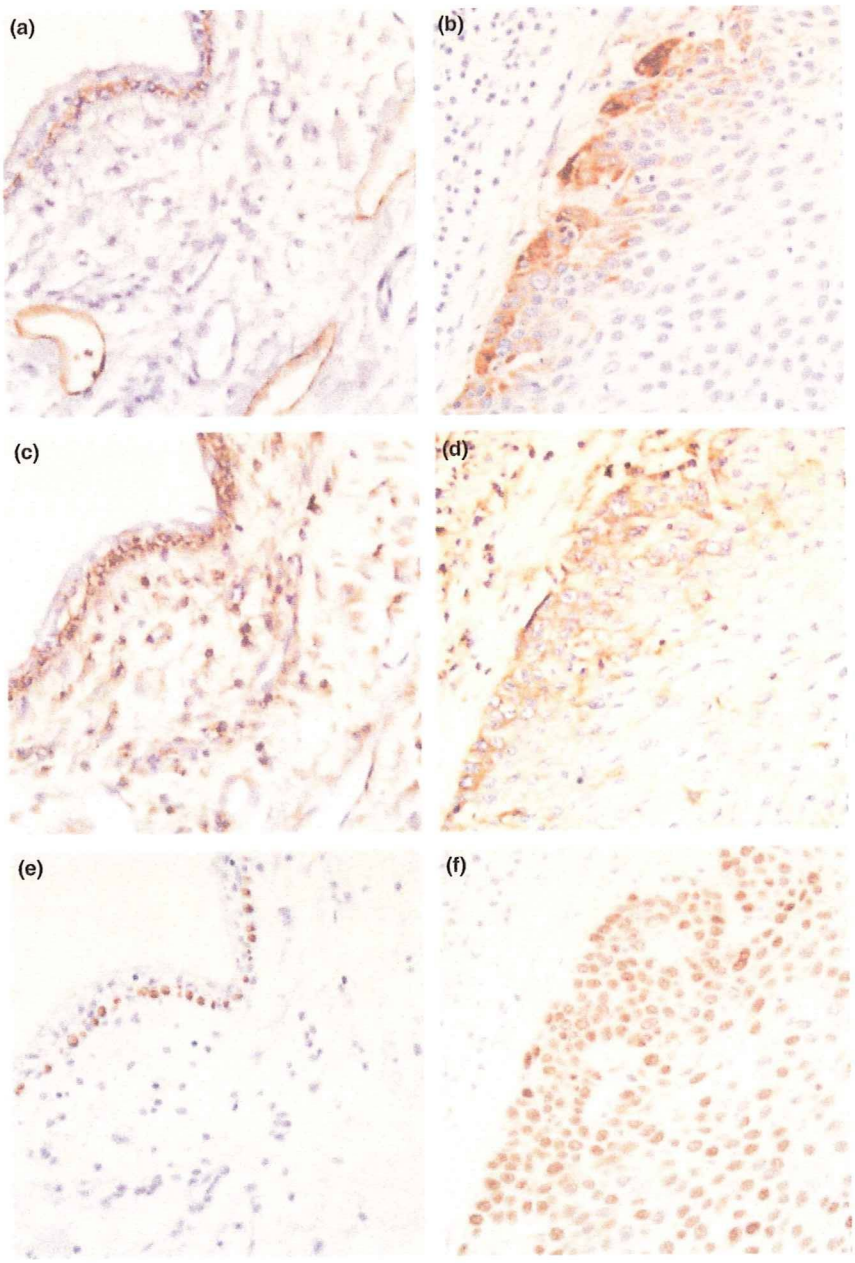

Fig. 1. Immunohistochemical analysis of podoplanin, CD44, and p63 expression in squamous cell carcinomas of the lung and a normal part of the specimen from one patient. (a) Podoplanin expression was detected in the endothelium of lymphatic vessels and in bronchial basal cells. (b) Podoplanin expression 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.

doi: 10.1111/j.1349-7006.2009.01295.x

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 clinicopathological 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.

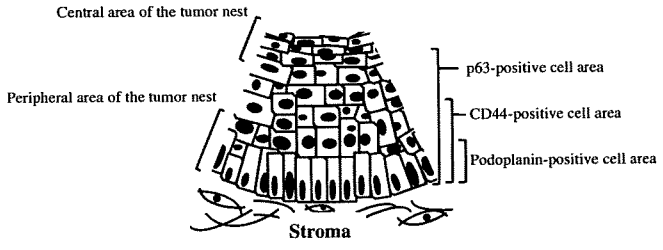

Fig. 2. Schema of the hierarchical distribution pattern of podoplanin, CD44, and p63 within tumor nests. The distribution of podoplanin-positive cells appeared to be more localized to the peripheral area of the tumor nests than the distribution of CD44- and p63-positive cells. The distribution of p63-positive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

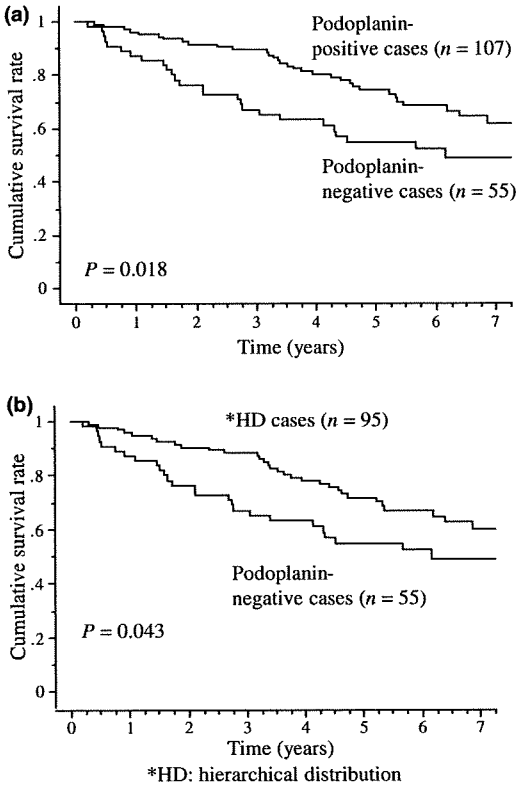

Fig. 3. Kaplan–Meier curves for overall survival. Overall survival curves of patients according to whether their tumor was podoplanin-positive or podoplanin-negative. The 5-year overall survival rates of the former and the latter were 74.4% and 54.8%, respectively. Overall survival curves of patients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

Acknowledgments

The technical support of Hiroko Hashimoto, Manabu Yamazaki, and Shinya Yanagi is gratefully acknowledged. This study was supported in part by a Grant-in-Aid for Cancer Research (19-10) from the Ministry of Health, Labour, and Welfare of Japan, and a Grant-in-Aid for the Third Term Comprehensive 10-year Strategy for Cancer Control from the Ministry of Health, Labour, and Welfare of Japan.

Disclosure Statement

No potential conflicts of interest are disclosed.

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doi: 10.1111/j.1349-7006.2009.01295.x