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Four years experience of the survey on quality control of lung cancer screening system in Japan

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ABSTRACT

Although quality control is essential in mass screening system for early detection of cancer, no global method for quality control has not been established, because the mass screening system in each country is quite different from each other. At present, we have to find appropriate method for each cancer and for each country. In 2000, The Lung Cancer Screening Division (LCSD) of the Miyagi Prefectural Committee for Management of the Cancer Screening System (Miyagi PCMCSS) decided to evaluate annually whether the local governments had appropriate information to evaluate the quality of lung cancer screening systems, announcing that the results would be informed to residents. On the basis of the manual developed by the Ministry of Health, Labor and Welfare, 45 items were selected as indicators for the survey, which could be obtained easily when the screening had been conducted according to the standard method. LCSD of Miyagi PCMCSS sent a questionnaire including the 45 items to the municipalities. According to the reply to the questionnaire, LCSD rated each municipality using a 5-rank classification depend on the number of insufficient items: A: 0; B: 1–4; C: 5–8; D: 9 or more; E: no reply. As the results, 58, 3, 6, 3, and 0 municipalities were categorized in 2002 as A, B, C, D, and E, respectively. In 2003, the number of municipalities changed to 60, 7, 2, 1, and 0. In 2005, the distribution improved more, such as 68, 2, 0, 0, and 0. The detection rate of lung cancer also improved. It is possible for PCMCSS to annually conduct surveys to determine whether the local government has appropriate information to evaluate the quality of lung cancer screening systems. Such surveys improve the distribution of response to better direction.

1. Introduction

In mass screening system for early detection of cancer, quality control is essential. However, no global method for quality control which can be adopted in all countries has not been established, because the mass screening system in each country is quite different from each other. Although there have been some reports concerning quality control of mammography in breast cancer screening [1–3] and cytological diagnosis in cervical cancer screening [4,5], there has been few report about other examinations or other cancers [6,7]. At present, we have to find appropriate method for each cancer and for each country.

In Japan, mass screening for tuberculosis using chest X-ray films had been widely developed since 1950s. In 1982, lung cancer screening, using chest X-ray films for all screenees and sputum cytology for smokers, was introduced under the Health and Medical Services Law for the Aged [8,9]. According to the law, each local municipality conducts lung cancer screening for residents of 40 years old or older. For the quality control of the screening system, The Prefectural Committee for Management of the Cancer Screening System (PCMCSS) was established in each prefecture. Although the committee was expected to conduct appropriate quality control of the cancer screening, the activity of the committee in almost all of the prefectures has been very low. Most of them counted the number of screenees, test-positive rate, and lung cancer detection rate which were submitted from each municipality, but the quality of the data was not tested and no guidance or intervention was conducted to each municipality. The quality control of the screening system totally depended on the individual efforts of those who are

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in charge of screening activities in the municipality or the screening providers which was commissioned lung cancer screening from the municipalities. As a result, quality of the screening system has been quite different according to the municipality. Although PCMCSS does not have enough budget or personnel, some members of the committee thought that additional action should be taken.

In Miyagi Prefecture, Japan, the condition was almost the same as other prefectures until 2000. Some municipalities did not submit the data to PCMCSS, even after several demands to submit. In 2000, The Lung Cancer Screening Division (LCSD) of Miyagi PCMCSS decided to conduct a new survey for the quality control, which began in 2001. We present herein the 4-year results of the survey from 2001 to 2005.

2. Methods

In February 2000, LCSD of Miyagi PCMCSS decided to start the survey annually whether the local governments had appropriate information to evaluate the quality of lung cancer screening systems. On the basis of "The Lung Cancer Screening Manual" developed by the Ministry of Health, Labor and Welfare, Japan, 45 items were selected as indicators for the survey, which were the indispensable for evaluating the quality of the screening. The items could be obtained easily when the screening had been conducted according to the standard screening method. The survey was authorized by Miyagi PCMCSS in March 2001, and the questionnaire was sent to all local governments in June 2001 (Table 1). When the local government did not have the data, they would inquire to the screening provider which was commissioned the lung cancer screening from the local government. In the questionnaire, we notified them that the survey would be conducted annually, and that the results of the survey would be informed to the residents.

After several demand for submission to the municipalities which had not submitted, the questionnaires were finally collected from all municipalities until January 2002. According to the reply to the questionnaire, LCSD rated each municipality using a 5-rank classification: A: all of the items were obtained sufficiently; B: 1–4 items were insufficient; C: 5–8 items were insufficient; D: 9 or more items were insufficient; E: no reply to the survey. The survey was conducted annually since then.

In order to evaluate whether the results in this survey really influenced the quality of lung cancer screening, the municipalities were classified into two groups: Group X (municipalities which had A rank in 2001–2002 survey) and Group Y (municipalities which had B, C, or D rank in 2001–2002 survey). In 2004–2005 survey, all 58 municipalities of Group X also got A rank. On the other hand, 10 of 12 municipalities of Group Y got A rank, and remaining two got B rank. Then, average values of following three indicators were compared between the two groups: (1) the detection rate of lung cancer in all screenees; (2) the ratio of cases who actually underwent further examination to test-positive cases; (3) the ratio of number of clinical stage I lung cancer patients to number of all lung cancer patients.

Mann–Whitney *U* test, Fisher's exact test, and Student's *t*-test were used for statistical analysis. $p < 0.05$ was regarded as statistically significant.

Table 1
The questionnaire of the survey

1 The information about screenees
- 1.1 The table of the number of screenees according to gender and age (5-year stratum)
- 1.2 The number of screenees who was screened in the previous year
- 1.3 The test-positive rate
  - 1.3.1 in chest x-ray
  - 1.3.2 in sputum cytology
  - 1.3.3 in both tests
- 1.4 The case who underwent further examination/The test-positive cases
  - 1.4.1 in chest x-ray
  - 1.4.2 in sputum cytology
  - 1.4.3 in both tests

2 The information about lung cancer patient
- 2.1 The table of the number of lung cancer patients according to gender and age (5-year stratum)
- 2.2 The detection rate of lung cancer
  - 2.2.1 in all screenees
  - 2.2.2 in the screenees who was screened in the previous year
  - 2.2.3 in the screenees who was not screened in the previous year
- 2.3 The standardized detection ratio (the calculation method was in The Lung Cancer Screening Manual)
- 2.4 The detection rate of lung cancer by chest X-ray
  - 2.4.1 in all screenees
  - 2.4.2 in the screenees who was screened in the previous year
  - 2.4.3 in the screenees who was not screened in the previous year
- 2.5 The detection rate of lung cancer by sputum cytology
- 2.6 The detection rate of lung cancer only by sputum cytology (negative result by chest X-ray screening)
- 2.7 The number of clinical stage I lung cancer patients/the number of all lung cancer patients
- 2.8 Positive predictive value
  - 2.8.1 in all screenees
  - 2.8.2 in the screenees who was screened in the previous year
  - 2.8.3 in the screenees who was not screened in the previous year

3 The information of the screening system
- 3.1 Indirect chest X-ray or direct chest X-ray?
- 3.2 Maximum voltage of chest X-ray equipment
- 3.3 Exposure voltage of chest X-ray
- 3.4 Is fluorescent screen used?
- 3.5 Is magnifying paper used?
- 3.6 Is ortho-type film used?
- 3.7 The number of pulmonologist or radiologist/the number of all doctors who performed interpretation
- 3.8 Is double check in interpretation performed as described in The Lung Cancer Screening Manual?
- 3.9 Does compare with previous films of the patients as described in The Lung Cancer Screening Manual?
- 3.10 Does the conference about chest X-ray screening have?
- 3.11 Is the quality of chest X-ray films evaluated?
- 3.12 The number of radiological technicians who work at lung cancer screening
- 3.13 The number of exposures per technician per day
- 3.14 Do the technicians have opportunity to study?
- 3.15 The number of doctors who certified for cytological diagnosis
- 3.16 The number of doctors who mainly diagnose respiratory cytological specimens
- 3.17 The number of cytoscreeners
- 3.18 The number of specimens per cytoscreener per day
- 3.19 Do the cytoscreeners have opportunity to study?
- 3.20 Is double check performed in cytological screening?
- 3.21 Direct smear method or cell collection method?
- 3.22 Are the cytological findings in previous specimens of newly diagnosed patients re-evaluated?
- 3.23 Is the report of the results of further examination collected from the hospitals?

3. Results

In 2002, a total of 70 municipalities were rated, and 58, 3, 6, 3, and 0 municipalities were categorized as A, B, C, D, and E, respectively. All of the rating, with the name of municipality, was proclaimed in the website of Miyagi Prefectural Government [10].

Table 2 shows the rating of the survey in 2001–2002 according to the population of the municipality. The population of the municipalities had no association with the rank they got ($p = 0.143$, Mann–Whitney *U* test).

Each local government commissioned the lung cancer screening to one of several screening providers. Table 3 shows the rating of the survey in 2001–2002 according to the screening providers commissioned. Screening provider P and Q got more A-rank than others ($p < 0.001$, Fisher's exact test).

Table 2
The rating of the survey in 2001–2002 according to the population of the municipality

Population	Rating					Total
	A	B	C	D	E	
1–5,000	6(100%)					6
5,001–10,000	21(91%)		1	1		23
10,001–15,000	11(73%)	1	3			15
15,001–20,000	5(83%)		1			6
20,001–30,000	5(83%)			1		6
30,001–60,000	6(86%)			1		7
60,001–	5(71%)	1	1			7

Table 3
The rating of the survey in 2001–2002 according to the company commissioned

Company	Rating					Total
	A	B	C	D	E	
P	55	2				57
Q	4					4
R			3	1		4
S			2			2
T				2		2
U			1			1

Table 4
The changes of the number of insufficient items and the rank of 15 municipalities in the first 2 years (remaining 55 municipalities had A-rank in both years)

Municipality	Survey in 2001–2002		Survey in 2002–2003		Number of items improved	Number of rank improved
	Number of insufficient items	Rank	Number of insufficient items	Rank		
a	19	D	2	B	17	2
b	11	D	5	C	6	1
c	9	D	0	A	9	3
d	8	C	2	B	6	1
e	8	C	0	A	8	2
f	8	C	1	B	7	1
g	7	C	3	B	4	1
h	7	C	0	A	7	2
i	6	C	9	D	−3	−1
j	2	B	6	C	−4	−1
k	1	B	0	A	1	1
l	1	B	0	A	1	1
m	0	A	1	B	−1	−1
n	0	A	1	B	−1	−1
o	0	A	1	B	−1	−1
Total	87		31		56	10

In 2002–2003, the number of municipalities with each rating changed to 60, 7, 2, 1, and 0, which indicated that 56 more items were newly obtained in the whole prefecture (Table 4). **The results improved year by year. In 2004–2005, the number of municipalities changed to 68, 2, 0, 0, and 0** (Table 5).

Table 6 **shows the results of the changes of the average values concerning quality control in the two groups of municipalities. Although "The detection rate of lung cancer in all screenees" and "The ratio of cases who actually underwent further examination to test-positive cases" of Group Y in 2001–2002 were significantly worse than those of Group X (Student's *t*-test, $p = 0.049$ and 0.036, respectively), those differences disappeared in 2004–2005.**

Table 5
The number of the rank which local municipalities obtained in the 4 years

Year	Rank				
	A	B	C	D	E
2001–2002	58	3	6	3	0
2002–2003	60	7	2	1	0
2003–2004	65	5	0	0	0
2004–2005	68	2	0	0	0

4. Discussion

In February 2000, LCSD in Miyagi PCMCSS decided to start a new survey about quality control of the lung cancer screening. At the beginning, LCSD had to decide what kind of survey they should do, because there was a manual for screening method, but there was not a manual for quality control of screening system in Japan.

When developing the survey, following points were considered. First, the survey should be simple and could be conducted with low cost, because PCMCSS did not have enough budget or personnel. Second, the results of the survey should be concisely informed to the public, showing the comparison to other municipalities, because the residents should know the quality of the screening system in their municipalities to seek better screening system of high quality with reasonable cost. Third, the survey was to evaluate whether the screening system in each municipality achieved at least the minimally required level. Therefore, LCSD of Miyagi PCMCSS decided to conduct the annual survey with questionnaires, whether the local government had enough information to evaluate the quality of their lung cancer screening systems. The questionnaire was prudently made according to "The Lung Cancer Screening Manual", and the survey was authorized by Miyagi PCMCSS in

Table 6
The changes of the average values concerning quality control in the two groups of municipalities

	2001–2002	p value	2004–2005	p value
(1) The detection rate of lung cancer in all screenees (per 1000)				
Group				
X	0.703 (n = 58)	0.049	0.535 (n = 58)	0.979
Y	0.351 (n = 10, 2 unknown)		0.538 (n = 12)	
(2) The ratio of cases who actually underwent further examination to test-positive cases				
Group				
X	0.908 (n = 58)	0.036	0.874 (n = 58)	0.739
Y	0.838 (n = 10, 2 unknown)		0.865 (n = 12)	
(3) The ratio of number of clinical stage I lung cancer patients to number of all lung cancer patients				
Group				
X	0.512 (n = 58)	0.071	0.530 (n = 58)	0.563
Y	0.111 (n = 3, 9 unknown)		0.609 (n = 11, 1 unknown)	

March 2001. In order to avoid misunderstanding, the questionnaire required numerical value rather than Yes–No question, and we could ask to municipalities if the value was strange.

The first survey actually began in June 2001 and finished in January 2002. The result of the survey was satisfactory. Although some municipalities did not submit annual reports of lung cancer screening to LCSD until 2000 despite of several demand to submit, all of the municipalities replied the questionnaire of the survey to LCSD by January 2002. The size of the municipalities did not have an association with the rating, whereas the screening provider commissioned had, which might indicate that the screening providers R, S and T had some problems in quality control.

The results of the survey in successive years were impressive. There were several factors which influenced the results. The most important point was that LCSD declared that the results of the survey would be informed to the residents with comparison to other municipalities. The person in charge in each municipality, especially in municipalities which could not achieve A-rank, inquired many questions to LCSD during the survey. Some people even asked LCSD to postpone the announcement of the results to the residents, which was not accepted. The governor of the local government must feel social pressure when his municipality had lower-rank. After 3 years, there was no municipality which got C, D, or E, regardless of the screening provider commissioned.

By the analysis of the relation between the results of this survey and the three indicators concerning quality of lung cancer screening, two of them were significantly worse in Group Y municipalities than in Group X in 2001–2002. However, in 2004–2005, such differences were not detected. During the years, the rating of Group Y municipalities in the survey had dramatically improved (2001–2002, B: 3; C: 6; D: 3. 2004–2005, A: 10; B:2). Although this survey using the questionnaire did not directly influence these indicators, it might affect the results indirectly.

It is possible for PCMCSS to annually conduct surveys to determine whether the local government has appropriate information to evaluate the quality of lung cancer screening systems. The survey can be developed for lung cancer screening by chest CT. Even in the present system in Japan, such surveys should be done in each prefecture, announcing that the results will be informed to the residents. However, the survey is an only first step in quality control of cancer screening, and more essential, effective, and nationwide surveys should be developed.

Conflict of interest statement

None declared.

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Results of Long-term Follow-up of Photodynamic Therapy for Roentgenographically Occult Bronchogenic Squamous Cell Carcinoma

Chiaki Endo, Akira Miyamoto, Akira Sakurada, Hirokazu Aikawa, Motoyasu Sagawa, Masamai Sato, Yasuki Saito and Takashi Kondo

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CHEST

Original Research

INTERVENTIONAL PULMONOLOGY

Results of Long-term Follow-up of Photodynamic Therapy for Roentgenographically Occult Bronchogenic Squamous Cell Carcinoma

Chiaki Endo, MD, PhD; Akira Miyamoto, MD, PhD; Akira Sakurada, MD, PhD; Hirokazu Aikawa, MD, PhD; Motoyasu Sagawa, MD, PhD; Masamai Sato, MD, PhD; Yasuki Saito, MD, PhD; and Takashi Kondo, MD, PhD, FCCP

Background: **Photodynamic therapy (PDT) is considered a useful and minimally invasive modality for treating centrally located early lung cancer. To date, there has been limited information on the long-term outcome of patients treated with PDT, especially those who are medically operable.**
Methods: **Beginning in 1994, patients with roentgenographically occult bronchogenic squamous cell carcinoma (ROSCC) who met our criteria underwent PDT at Tohoku University Hospital and were followed up through 2006. Our criteria were as follows: (1) ROSCC without distant metastasis; (2) medically operable by means of lobectomy or further resection; (3) longitudinal tumor length of ≤ 10 mm; and (4) superficial bronchoscopic tumor findings.**
Results: **A total of 48 patients with ROSCC underwent PDT. The complete response (CR) rate was 94% (45 of 48 of patients). Nine patients (20%) had local recurrence after CR. A total of 11 deaths was observed, with 6 resulting from multiple primary lung cancer and only 1 from the original ROSCC. The 5-year and 10-year overall survival rates for all 48 patients were 81% and 71%, respectively. The Cox proportional hazard model showed that only metachronous multiple primary lung cancer was an independent poor prognostic factor.**
Conclusions: **PDT is thought to be a first-line modality for patients who have ROSCC with a tumor length of ≤ 10 mm, even if the tumor is medically operable. Most local recurrence can be cured by active therapy such as surgery, radiotherapy, or PDT. Multiple primary lung cancer subsequent to PDT is an important issue from the viewpoint of survival.**

(CHEST 2009; 136:369–375)

Abbreviations: AFB = autofluorescence bronchoscopy; CR = complete response; DFS = disease-free survival; OS = overall survival; PDT = photodynamic therapy; RFS = relapse-free survival; ROSCC = roentgenographically occult bronchogenic squamous cell carcinoma

Photodynamic therapy (PDT) is considered a useful and minimally invasive modality for treating centrally located early lung cancer. Based on the results of a prospective phase II study,[1] PDT is considered a first-line modality for nonsurgical patients with central-type early lung cancer with a longitudinal extent of ≤ 10 mm. The American College of Chest Physicians guidelines[2] stated that there was limited experience using PDT for patients who were surgical candidates. In the current study, patients with centrally located early lung cancers who were medically operable underwent PDT at Tohoku University Hospital, and underwent long-term follow-up.

Materials and Methods

Patients

PDT has been performed at Tohoku University Hospital since 1994. Patients who had roentgenographically occult bronchogenic squamous cell carcinoma (ROSCC) were examined with

bronchoscopy, chest CT scan, upper abdominal CT scan/ultrasonography, brain CT scan/MRI, and bone scintigraphy. Patients were considered candidates for PDT under the following conditions: (1) no metastatic lesions were observed; (2) the longitudinal extent of ROSCC was ≤ 10 mm; (3) the distal edge of ROSCC was visible by bronchoscopy; (4) bronchoscopy findings categorized the tumor as minute or hidden using our classification system (described later); (5) patients were medically operable by means of lobectomy or further lung resection; and (6) informed consent to undergo PDT was obtained. This study was approved by the Institutional Review Board of Tohoku University Hospital.

Bronchoscopic Findings

Our classification of ROSCC bronchoscopic findings has been described previously.[3] In brief, findings were classified into the following three categories: remarkable, minute, and hidden. "Remarkable" meant that a lesion could be identified easily as a tumor > 2 mm in height. "Minute" indicated that a lesion could be identified as a tumor only with difficulty, and was ≤ 2 mm in height. A "hidden" lesion was one that could not be seen using a conventional bronchoscope or that showed no characteristic changes related to carcinoma. Hidden lesions could be detected only by bronchial biopsy, brushing cytology, or autofluorescence bronchoscopy (AFB). Since 1997, AFB (LIFE system; Xilix Technologies; Vancouver, BC, Canada) and endobronchial ultrasonography have also been used for the evaluation of ROSCC. AFB is useful for precisely determining the size of the lesion,[4,5] whereas endobronchial ultrasonography may prove beneficial in evaluating the depth of tumor invasion.[6,7]

PDT

PDT procedures were performed with porfimer sodium (Photofrin; Wyeth Japan KK; Tokyo, Japan) and an excimer dye laser (EDL-1; Hamamatsu Photonics; Hamamatsu, Japan). All patients received 2 mg/kg porfimer sodium IV, 48 h before light irradiation. A quartz fiber was used to carry the laser light through a bronchoscope to the lesions. The total energy of the laser irradiation was 100 J/cm^2, and the duration of irradiation was usually 10 to 20 min. Bronchial toileting to remove necrotic tissues produced by PDT was routinely performed at 7 days after PDT. The lesions were evaluated by brushing cytology and biopsy specimen histology at 1, 2, and 3 months after PDT. Thereafter, patients were assessed with bronchoscopy, sputum cytology, and chest CT scan every 3 months for the first year, and every 6 months from the second through the fifth years. After that, patients were examined annually using sputum cytology and chest CT scan.

Tumor response to PDT was classified into the following two categories: complete response (CR); and non-CR. A CR was defined as the absence of any demonstrable tumor by biopsy or brushing cytology for at least 4 weeks.

Local recurrence was defined as a tumor that recurred at the same lesion as the PDT lesion after CR. Multiple primary lung cancer was defined according to the criteria of Martini and Melamed.[8]

Statistical Analysis

Overall survival (OS), relapse-free survival (RFS), and disease-free survival (DFS) were calculated using the Kaplan-Meier method. OS was defined as the time from PDT to death from any cause. RFS was defined as the time from PDT to local recurrence or death. DFS was defined as the time from PDT to local recurrence or metachronous multiple primary lung cancer or death. For non-CR cases, RFS and DFS times were defined as zero. Subgroup comparisons of survival curves were performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazard model to determine factors significantly associated with survival. A p value of < 0.05 was considered statistically significant.

RESULTS

Baseline Characteristics

A total of 48 ROSCC patients who met our criteria have undergone PDT at Tohoku University Hospital since 1994 and were followed up through 2006. As shown in Table 1, all patients were men, and their average age was 70 years. All patients were current or ex-smokers, and the average smoking index was 50 pack-years. Forty-five of the 48 patients were detected by sputum cytology mass screening,[9,10] and the remaining patients were detected by medical examination for other diseases (arrhythmia, GI disorder, and cerebrovascular disease).

Synchronous multiple primary lung cancer was diagnosed in four patients (Table 2). Three of these patients were treated with surgery; the other patient underwent radiotherapy.

Table 1—*Baseline Patient Characteristics (n = 48)*

Characteristics	Description
Gender	All men
Age,* yr	70 (6.7)
Smoking index,* pack-yr	50 (11.1)
Detection method	Sputum cytology mass screening (45 patients) Medical examination for other diseases (3 patients)
Synchronous multiple primary lung cancer	4 patients

*Values are given as the mean (SD).

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Table 2—*Synchronous Multiple Primary Lung Cancer (n = 4)*

Patient, No.	PDT Lesion	PDT Efficacy	Synchronous Cancer Lesion (Histology)	Treatment for Synchronous Cancer	Prognosis
1	LB^3	CR	RB^1 (Sq)	Surgery (lobectomy)	Alive
2	LUD	CR	RB^3 (Ad)	Surgery (partial resection)	Dead from AMI
3	LB^3	CR	RB^4 (Sq)	Radiotherapy	Dead from synchronous cancer
4	RB^1	CR	RB^5 (Sq)	Surgery (lobectomy)	Alive

Ad = adenocarcinoma; AMI = acute myocardial infarction; LUD = left upper division bronchus; Sq = squamous cell carcinoma.

Tumor Response to PDT

Forty-five of the 48 patients (94%) achieved CR. Non-CR was seen in three patients. Among these, two patients had ROSCCs in segmental bronchi, and the other had disease located in a subsubsegmental bronchus. There was no statistically significant relationship between tumor response and tumor location ($p = 0.64$ [Mann-Whitney *U* test]). Two of the three non-CR cases underwent radiotherapy, and the other one underwent left lung lobectomy. As a result, all of the non-CR patients were free of lung cancer at the last follow-up.

Local Recurrence

Local recurrence after CR was found in 9 of the 45 CR patients (20%) [Table 3]. Among these, ROSCCs were found in segmental bronchi in five cases, in subsegmental bronchi in three cases, and in a subsubsegmental bronchus in the remaining case. There was no statistically significant relationship between local recurrence and tumor location ($p = 0.29$ [Mann-Whitney *U* test]). The relapse-free interval of the nine patients ranged from 3 to 47 months (average, 25 months). Treatments for recurrence were as follows: radiotherapy, five patients; surgery, two patients; additional PDT, one patient; and chemotherapy, one patient. As a result, eight of the nine patients were alive at the last follow-up.

Metachronous Multiple Primary Lung Cancer

During the follow-up period, a total of 10 patients had metachronous second primary lung cancers, and only 1 patient had metachronous third primary lung cancer (Table 4). The time from PDT to metachronous primary lung cancer ranged from 12 to 89 months (average, 45 months). Nine of the 10 patients received some form of active therapy (*ie*, surgical resection, 2 patients; radiotherapy, 2 patients; laser therapy, 2 patients; PDT, 2 patients; and chemotherapy, 1 patient). Five deaths occurred among these 10 patients, all from metachronous lung cancers.

Survival

The median follow-up time for all 48 patients was 63 months. The median follow-up time for living patients was 70 months. Only two patients were lost to follow-up, and their follow-up times were 21 and 27 months.

Among these 48 patients, a total of 11 deaths were observed, with only 1 patient dying from the original ROSCC. Five patients died from metachronous multiple lung cancer, one patient died from synchronous multiple lung cancer, two patients died from myocardial infarction, one patient died from cerebral hemorrhage, and the remaining patient died from an unknown cause. This last case was counted as an original lung cancer death in the survival analysis.

Table 3—*Local Recurrence of PDT*

Patient, No.	PDT Lesion	Months From PDT to Local Recurrence	Treatment for Local Recurrence	Prognosis
1	RB^{1}ai/ii	46	Radiotherapy	Alive
2	LB^{1+2}a+b/c	34	Left lung lobectomy	Alive
3	LB^{3}a/b+c	16	Radiotherapy	Alive
4	LB^9	15	Left lung lobectomy	Alive
5	RB^1	26	Radiotherapy	Alive
6	LB^3	7	Radiotherapy	Alive
7	LB$^{1+2/3}$	29	Chemotherapy	Dead from original lung cancer
8	LB^{3}a/b+c	47	Radiotherapy	Alive
9	LB^6	3	PDT	Alive

See Table 2 for abbreviations not used in the text.

Table 4—*Metachronous Multiple Primary Lung Cancer (n = 10)*

Patient, No.	PDT Lesion	PDT Efficacy	Metachronous Cancer Lesion (Histology)	Treatment for Metachronous Cancer	Time From PDT to Metachronous Cancer, mo	Prognosis
1	RB[1]	Non CR	RB[7] (Sq)	Radiotherapy	38	Dead from metachronous cancer
2	LB[1+2]	CR	LLB (Sq)	PDT	57	Alive
3	LB[3]	CR	RB[5] (Sq)	Laser	50	Alive
4	LB[1+2]	CR	RB[3] (Sq)	Surgery (lobectomy)	71	Dead from metachronous cancer
5	LB[3]	CR	RS[3] (Sq)	Surgery (lobectomy)	29	Alive
6	LB[3]	CR	RLL (NSCLC)	Supportive care	43	Dead from metachronous cancer
7	LB[1+2]	CR	LB[6] (Sq)	Radiotherapy	44	Dead from metachronous cancer
8	RB[10]	CR	RB[7]/RB[8] (Sq)	Laser/Radiotherapy	89	Alive
9	RB[1]	CR	RB[3] (SCLC)	Chemotherapy	12	Dead from metachronous cancer
10	LB[1+2]	CR	LB[6] (Sq)	PDT	14	Alive

LLB = left lingual bronchus; NSCLC = non-small cell lung cancer; RLL = right lower lobe; SCLC = small cell lung cancer. See Table 2 for abbreviations not used in the text.

The 5-year and 10-year OS rates were 81% and 71%, respectively. The relationships among survival, local recurrence, and metachronous multiple primary lung cancer were examined. The 5-year OS rate of patients with local recurrence showed no statistically significant difference from that of patients without local recurrence (log-rank test p = 0.62) [Fig 1]. The 5-year OS rate of patients with metachronous multiple lung cancer was 56%, which was statistically lower (p = 0.031 [log-rank test]) than that of patients without it (88%) [Fig 2]. The 5-year and 10-year RFS rates were 60% and 54%, respectively; the 5-year and 10-year DFS rates were 56% and 43%, respectively.

The Cox proportional hazard model showed that metachronous multiple primary lung cancer was an independent poor prognostic factor (hazard ratio, 1.99; 95% confidence interval, 1.01 to 4.03). Tumor location, local recurrence, and tumor response were not significant prognostic factors (Table 5).

Discussion

The long-term results of PDT for a total of 48 patients with ROSCC are reported in this article. Our cases differed from those in previous studies[1,11–15] in the following respects. First, all patients were medically operable; they selected PDT after fully informed consent. We offered two options (*ie*, surgical resection and PDT) to patients with ROSCC who were medically operable and met our PDT criteria, as mentioned earlier. Most of them selected PDT after fully informed consent, although we did not have the precise number of patients who selected surgery. Second, all lesions were marked by

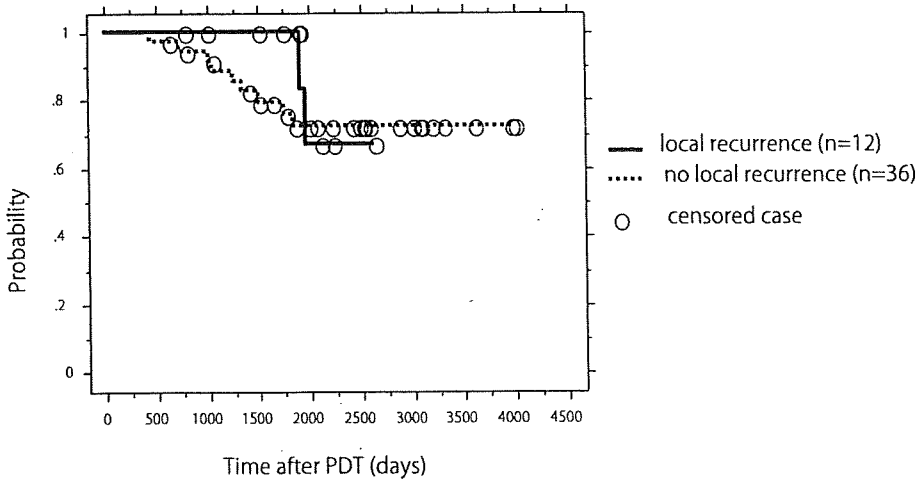

FIGURE 1. OS and local recurrence. The 5-year survival rates of patients with and without local recurrence were 100% and 76%, respectively, indicating no statistically significant difference (log-rank test p = 0.62).

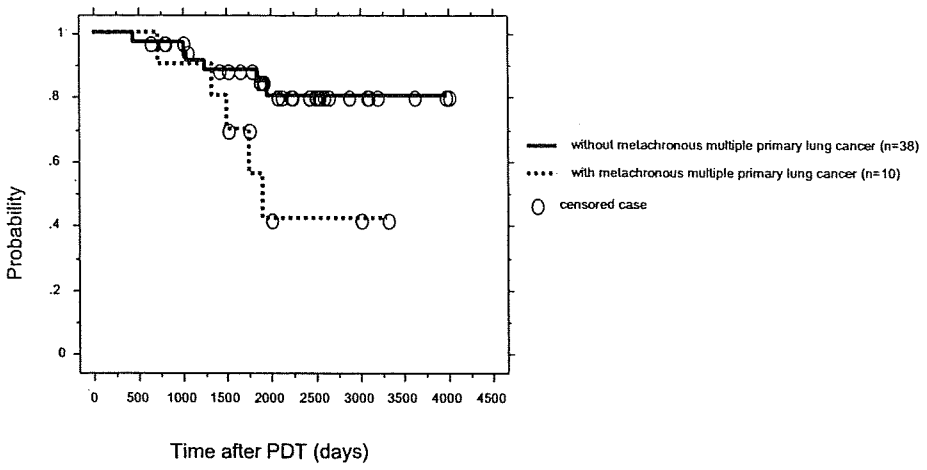

FIGURE 2. OS and metachronous multiple primary lung cancer. The 5-year survival rates of patients with and without metachronous multiple primary lung cancer were 56% and 88%, respectively, indicating a statistically significant difference (log-rank test p = 0.031).

only a superficial change in the bronchial epithelium. In previous articles,[1,13–18] some lesions had nodular findings, which meant that not all cases necessarily possessed superficial changes in the bronchial epithelium. It has been shown[14,19–21] that some tumor qualities make successful treatment by PDT less likely. One of these qualities is tumor invasion into or beyond the cartilaginous layer of the bronchus. We reported previously that the depth of tumor invasion would be within the cartilaginous layer if the bronchoscopic finding was either "minute" or "hidden" according to our classification system.[3] An ROSCC with a bronchoscopic finding of "remarkable" sometimes invades beyond the cartilaginous layer. It was therefore supposed that the tumors found in our patients were less likely to invade beyond the bronchial cartilaginous layer and would therefore be more suitable for PDT. Last, our study followed up patients for a longer period than did those in other reports. Accordingly, the local recurrence rate and the incidence of metachronous multiple primary lung cancer should be more precise. Local recurrence was found only within 4 years of PDT. However, metachronous primary lung cancer was found even after that. To our knowledge, this is the first report where 10-year OS, RFS, and DFS after PDT have been described.

Table 5—*Multivariate Survival Analysis Using the Cox Proportional Hazard Model*

Variables	Hazard Ratio	95% Confidence Interval
Tumor location (segment or more peripheral bronchus)	0.71	0.31–1.94
Local recurrence (+)	0.71	0.16–1.70
Tumor CR	0.69	0.13–3.51
Metachronous multiple primary lung cancer (+)	1.99	1.01–4.03

The Cox proportional hazard model showed that metachronous multiple primary lung cancer was an independent poor prognostic factor (hazard ratio, 1.99; 95% confidence interval, 1.01 to 4.03). Tumor location, local recurrence, and tumor response were not significant prognostic factors. + = positive.

CR was achieved in 94% of patients, with a recurrence rate of 20%. Even if PDT is used only in patients in whom ROSCC is marked by a superficial change of the bronchial epithelium, some patients will have local recurrence. However, most of these patients can be cured by salvage therapy. Both the log-rank test and multivariate survival analysis with the Cox proportional hazard model showed that local recurrence had no influence on survival after PDT. However, the Cox proportional hazard model showed that multiple primary lung cancer was an independent prognostic factor. We have previously reported[22] the frequency and the treatment of multiple primary lung cancer in patients with resected roentgenographically occult lung cancer, where the cumulative rate of postoperative metachronous multiple primary lung cancer was 0.11 5 years after the initial operation, and the OS rates for solitary and multiple resected occult lung cancers 5 years after the initial operation were 0.90 and 0.59, respectively, which is a statistically significant difference ($p < 0.01$). Multicentricity in roentgenographically occult lung cancer is a very important matter in terms of prognosis after the initial treatment. Although the main themes in studying the effectiveness of PDT for lung cancer have so far been tumor response or local

recurrence, we also have to consider metachronous multiple primary lung cancer after PDT and its impact on prognosis.

As stated in the American College of Chest Physicians guidelines,[2] the use of PDT for patients who are surgical candidates has so far remained limited. Because all of our patients were medically operable, the current study could offer new important information. In our previous report[19] on the results of surgical treatment for ROSCC, the 5-year OS rate of the 94 ROSCC patients was 80%, which was comparable to the results of the current study. However, the 10-year OS rate of 207 patients undergoing surgical treatment for ROSCCs was 40%,[23] which was much lower than the 70% 10-year OS rate found in the current study. We do not know the precise reason for a greater 10-year survival after PDT than after surgery. However, the following could be the reason. PDT does not result in the pulmonary function loss, so that adequate therapy, including surgery, can be undertaken for either local recurrence or the subsequent primary lung cancer after PDT. However, pulmonary resection would cause pulmonary function loss, so that an additional resection may be impossible even if surgery is the most curative therapy for local recurrence or the subsequent primary lung cancer after the initial resection. Moreover, quality of life after PDT should be better than that after pulmonary resection.

The current study is one of the largest assessing the effectiveness of PDT for early lung cancer. The design of the study was retrospective and observational, so its information might be limited. However, when PDT is employed with curative intent, its main targets are centrally located, early bronchogenic squamous cell carcinomas, which account for a very small percentage of all lung cancers. Given this fact, even retrospective studies can offer important information. Actually, to date, only one prospective study[1] has been published.

In summary, the current study indicates that PDT is thought to be a first-line modality for patients who have ROSCC with a tumor length of $\leq$ 10 mm, even if they are medically operable. After PDT, a 5-year OS rate of about 80% and a 10-year OS rate of 70% can be expected for these patients after PDT. Most local recurrence can be cured by salvage therapy such as surgery, radiotherapy, or PDT. During long-term follow-up after PDT, lung cancer was the most frequent cause of deaths, and multiple primary lung cancer was the most important issue from the viewpoint of survival. Further study, especially a prospective study, should be conducted to get a robust data of PDT for medically operable patients with ROSCC.

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Results of Long-term Follow-up of Photodynamic Therapy for Roentgenographically Occult Bronchogenic Squamous Cell Carcinoma

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ORIGINAL ARTICLE

Protein phosphatase Dusp26 associates with KIF3 motor and promotes N-cadherin-mediated cell–cell adhesion

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Recent studies have demonstrated essential functions for KIF3, a microtubule-directed protein motor, in subcellular transport of several cancer-related proteins, including the β-catenin–cadherin(s) complex. In this study, we report identification of the protein-phosphatase Dusp26 as a novel regulator of the KIF3 motor. Here we undertake yeast two-hybrid screening and identify Kif3a, a motor subunit of the KIF3 heterotrimeric complex, as a novel Dusp26-binding protein. Co-immunoprecipitation and colocalization experiments revealed that Dusp26 associates not only with Kif3a, but also with Kap3, another subunit of the KIF3 complex. Dephosphorylation experiments *in vitro* and analysis using mutant forms of Dusp26 in intact cells strongly suggested that Dusp26 is recruited to the KIF3 motor mainly by interaction with Kif3a, and thereby dephosphorylates Kap3. Forced expression of Dusp26, but not its catalytically inactive mutant, promoted distribution of β-catenin/N-cadherin, an established KIF3 cargo, to cell–cell junction sites, resulting in increased cell–cell adhesiveness. We also showed that Dusp26 mRNA expression was downregulated in human glioblastoma samples. These results suggest previously unidentified functions of Dusp26 in intracellular transport and cell–cell adhesion. Downregulation of Dusp26 may contribute to malignant phenotypes of glioma.
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Keywords: DUSP; cadherin; KIF3

Introduction

Dual-specificity protein phosphatases (DSPs) form an evolutionarily conserved subgroup of protein-tyrosine phosphatases (PTPs), originally characterized by their ability to catalyse dephosphorylation of protein phospho-Ser/Thr and phospho-Tyr residues (Camps *et al.*, 2000; Alonso *et al.*, 2004; Pulido and Hooft van Huijsduijnen, 2008). Dusp26 (also referred to as LDP-4, MKP-8 and NEAP) is a recently identified DSP protein and its function remains obscure. Expression of Dusp26 mRNA appears restricted to specific tissues such as brain and retina (Wang *et al.*, 2006; Takagaki *et al.*, 2007). The only functional motif identified thus far in Dusp26 is a DSP catalytic domain: Dusp26 does not resemble DSP molecules belonging to the MKP family, which exhibit Rhodanase domains that recognize substrate mitogen-activated protein kinases (MAPKs: Erk, JNK and p38) (Camps *et al.*, 2000). To date, reports regarding Dusp26 function have been limited to its function in the MAPK pathway. Specifically, Dusp26 has been reported to function as a p38-specific phosphatase (Vasudevan *et al.*, 2005; Yu *et al.*, 2007) and an Erk-phosphatase (Hu and Mivechi, 2006). In contrast, Wang *et al.* (2006) suggested that Dusp26 is not an MAPK phosphatase but rather negatively regulates the PI3K–Akt pathway by an unknown mechanism. In addition, Takagaki *et al.* (2007) reported that Dusp26 can potentiate JNK and p38 activation rather than inactivate it in a certain cellular contexts. Thus, the regulatory functions of Dusp26 on MAPK function are controversial. On the basis of these findings, we hypothesized that Dusp26 might interact with unidentified cofactor(s) and/or substrate(s), in addition to proteins in the MAPK cascade.

The KIF3 complex, a microtubule plus-end-directed motor and member of the kinesin superfamily (KIF), functions in transport of membrane organelles and is composed of two motor subunits, Kif3a and Kif3b, and a nonmotor subunit Kap3 (Hirokawa, 2000b). Mice lacking either Kap3 or Kif3a show loss of left–right asymmetry and embryonic lethality due to the proteins' indispensable function in formation of primary cilia and nodal flow during embryogenesis (Takeda *et al.*, 1999; Hirokawa, 2000a; Hirokawa *et al.*, 2006). The KIF3 motor is also implicated in tumorigenesis: cancer-related proteins such as adenomatous polyposis coli (APC),

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β-catenin–cadherin(s) and the Par3 polarity complex have been identified as KIF3-cargos (Jimbo *et al.*, 2002; Nishimura *et al.*, 2004). β-catenin and cadherin associate with the KIF3 motor probably through interaction of β-catenin with Kap3 (Jimbo *et al.*, 2002). Conditional Kap3 knockout mice develop neuroepitherial tumors likely due to defects in post-Golgi transport of the β-catenin/N-cadherin complex to the cell periphery, resulting in abnormal β-catenin accumulation in the cytosol and nucleus (Teng *et al.*, 2005). More recently, an important function for KIF3 in regulating β-catenin-dependent gene expression in response to Wnt signaling by cilia-dependent mechanism has been reported (Corbit *et al.*, 2008).

β-catenin/cadherin(s) subcellular localization is critical for tumorigenesis. Cadherin(s) at the plasma membrane functions in calcium-dependent cell–cell interactions whereas β-catenin links cell adhesion to activities of the actin cytoskeleton. Recent studies reveal that modulation of cell–cell contact affects cell motility and has consequences for cancer metastasis. Furthermore, defects in transport of β-catenin/cadherin(s) to the cell periphery result in aberrant accumulation of β-catenin in the cytosol as well as in the nucleus, where it together with T-cell factor (TCF) functions as a transcriptional mediator of canonical Wnt signaling. Thus proper localization of the β-catenin–cadherin complex at cell–cell junctions is essential for tumor suppression.

Here, using yeast two-hybrid screening, we identified Kif3a, a subunit of the KIF3 microtubule-dependent protein motor, as a Dusp26-interacting protein (Figure 1). We also demonstrate that Dusp26 dephosphorylates the Kap3 subunit of the KIF3 motor and enhances N-cadherin-mediated cell–cell interactions. Finally we analyse tumor samples and show that Dusp26 expression is downregulated in gliomas, suggesting it may have a tumor-suppressive function in brain tumors.

Results

Dusp26 interacts with the KIF3 kinesin motor

To search for factors interacting with Dusp26 we undertook yeast two-hybrid screening of a human embryonic brain cDNA library. One of the clones obtained encoded the Kif3a C terminus (Figure 1a). We did not detect interaction between Kif3a and Dusp25, another DSP molecule showing 24% amino-acid sequence homology with Dusp26 (Figure 1b). Physical interactions between Dusp26 and Kif3a were further confirmed by co-immunoprecipitation in cells trans-

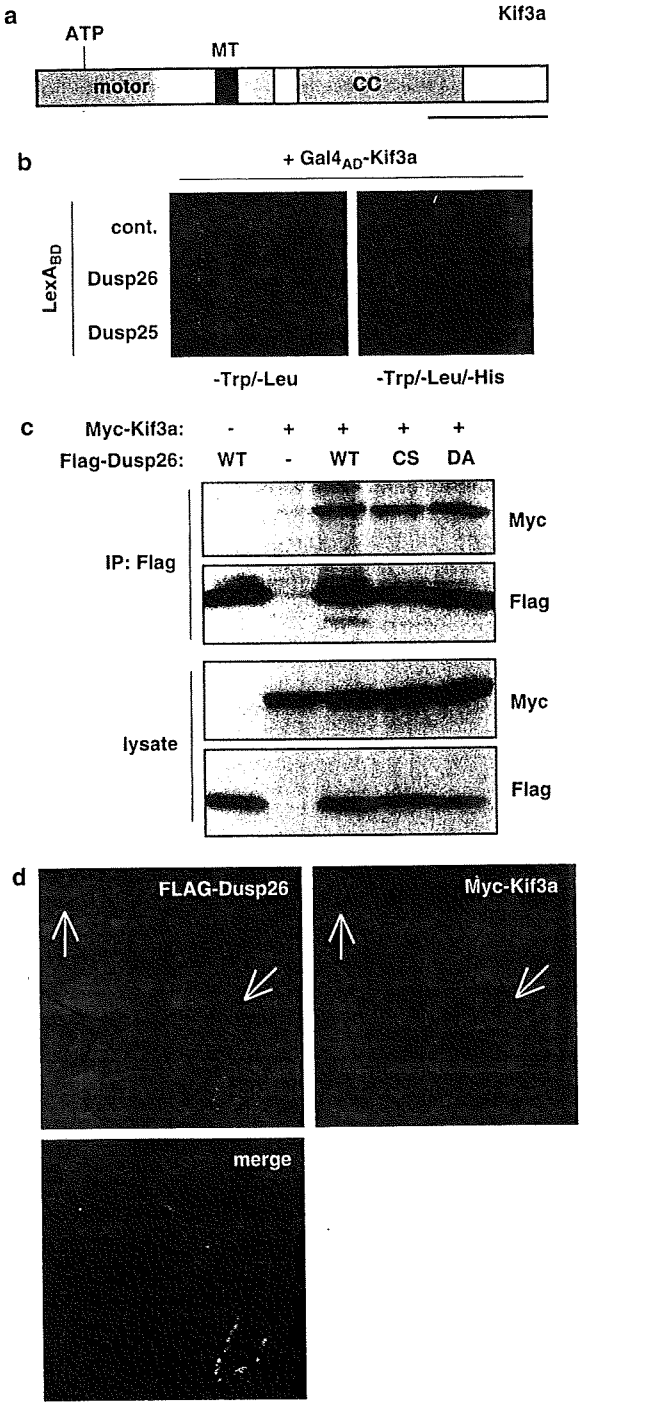


Figure 1 Dusp26 interacts with the Kif3a subunit of KIF3 motor. (**a**) Schematic structure of Kif3a. The motor domain, ATP-binding site (ATP) and microtubule-binding site (MT) therein, and coiled-coiled domain (CC) are shown. The line represents the cDNA fragment isolated in two-hybrid screening using Dusp26 as a bait. (**b**) Two-hybrid analysis showing interaction of Dusp26 with Kif3a fragment isolated in the screening. Dusp25 served as negative control. (**c**) Co-immunoprecipitation of Kif3a with Dusp26. HTO cells were transfected with Flag-tagged versions of wild-type (WT) and inactive mutant forms (CS and DA) of Dusp26, Myc-Kif3a and empty vector in combinations, immunoprecipitated with an anti-Flag antibody, and probed with anti-Myc or anti-Flag antibodies. (**d**) Kif3a and Dusp26 colocalization. HTO cells transfected with Flag-Dusp26 and Myc-Kif3a were immunostained using anti-Flag and anti-Myc antibodies, followed by anti-rabbit IgG-Alexa 488 and anti-mouse IgG-Cy3. Images shown are extended focus views obtained by maximum projection of confocal images along the Z axis. Arrows indicate overlaps of Flag-Dusp26 and Myc-Kif3a outside the Golgi.

fected with Myc-Kif3a with or without Flag-Dusp26 (Figure 1c). Dusp26 protein exhibited conserved Asp and Cys residues, which are required for catalysis in all DSP family members. Substitution of these residues to Ala and Ser (Dusp26-DA and -CS, respectively) did not affect Dusp26 and Kif3a interaction, indicating that association with Kif3a does not require catalytic activity of Dusp26 (Figure 1c). Dusp26 showed cytoplasmic localization and enrichment in Golgi apparatus, as reported previously (Takagaki *et al.*, 2007), and strong colocalization of Dusp26 and Kif3a was observed (Figure 1d).

We next asked whether Dusp26 associates with Kap3, another subunit of the KIF3 motor complex. As shown in Figure 2a, Kap3, as well as Kif3a, was also efficiently co-immunoprecipitated with Dusp26. In a yeast two-hybrid assay, interaction between Dusp26 and Kap3 was relatively weak when compared to that of Dusp26-Kif3a (Figure 2b). Collectively, these results suggest that Dusp26 binds to the KIF3 motor complex, primarily through interaction with the Kif3a subunit.

Dephosphorylation of Kap3 by Dusp26

It has been reported that Kap3 is a phosphoprotein, and that its phosphorylation is regulated during cell cycle (Haraguchi *et al.*, 2006). To elucidate the significance of the Dusp26-KIF3 motor interaction, we asked whether Kap3 was a Dusp26 substrate. To this end, we analysed Kap3 phosphorylation using a recently developed phosphate-binding tag (Phos-tag) that captures phosphomonoester dianions bound to Ser, Thr and Tyr residues (Kinoshita *et al.*, 2006). As shown in Figure 3a, Kap3 expressed in HeLa-TetOff (HTO) cells was identified as two bands. The slower migrating band, which in this assay represents phosphorylated proteins, disappeared following treatment with bacterial λ-phosphatase *in vitro* (Figure 3b), and slightly increased in cells synchronized at G_2/M phase by treatment with

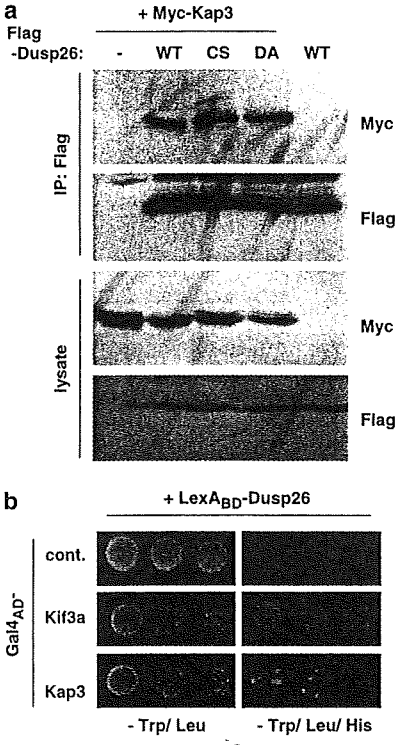


Figure 2 Interaction between Dusp26 and Kap3. (**a**) Co-immunoprecipitation of Kap3 with Dusp26. COS-7 cells were transfected with Flag-Dusp26 or its mutants together with Myc-Kap3 and analysed by co-immunoprecipitation as in Figure 1c. (**b**) Interaction between Dusp26 and Kap3 detected by two-hybrid analysis in yeast. Kif3a served as a positive control.

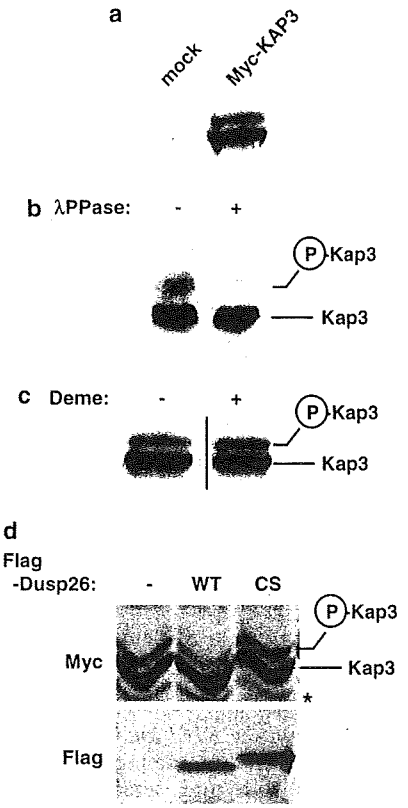


Figure 3 Negative regulation of Kap3 phosphorylation by Dusp26. (**a**) Analysis of Kap3 phosphorylation using Phos-tag/SDS–polyacrylamide gel (PAGE). Lysates from mock- or Myc-Kap3-transfected HTO cells were separated by Phos-tag/SDS–PAGE, under conditions in which phosphorylated proteins migrate more slowly than corresponding nonphosphorylated proteins, and analysed by western blotting using an anti-Myc antibody. (**b**) Identity of slower migrating Kap3 on Phos-tag/SDS–PAGE as phosphorylated Kap3. HTO cells were transfected with Myc-Kap3, lysed, and immunoprecipitated with ani-Myc antibody. Immunoprecipitates were further treated with λ-phosphatase, and subjected to Phos-tag/SDS–PAGE as in A. Myc-Kap3 was detected by western blotting. (**c**) Confirmation of increased Kap3 phosphorylation in cells at G_2/M phase. Kap3 phosphorylation of HTO cells treated with demecolcine (Deme) was analysed as in A. (**d**) Dusp26, but not its inactive mutant (CS), suppresses Kap3 phosphorylation. Myc-Kap3 was introduced into HTO cells together with wild-type (WT) or a mutant form (CS) of Dusp26 and analysed using Phos-tag/SDS–PAGE. An asterisk marks cross-reacted bands.

demecolcine, a microtubule-disrupting reagent (Figure 3c). These findings are consistent with a previous report that Kap3 phosphorylation increases as cells progress toward M-phase (Haraguchi *et al.*, 2006). Strikingly, coexpression of Dusp26 markedly decreased Kap3 phosphorylation, whereas an inactive Dusp26 mutant, Dusp26-CS, had no effect on Kap3 phosphorylation levels (Figure 3d).

To characterize Dusp26-mediated dephosphorylation of Kap3, we constructed several N-terminally truncated mutants of Dusp26 (Figure 4a). Dusp26-ΔN15 and ΔN36 suppressed Kap3 phosphorylation as well as did full-length Dusp26. By contrast, expression of Dusp26-ΔN58 did not decrease Kap3 phosphorylation (Figure 4b), indicating that the Dusp26 N terminus is essential for mediating Kap3 dephosphorylation. Notably, this region is also required for co-immunoprecipitation of Kif3a and Kap3 with Dusp26. As shown in Figure 4c, deletion of the N terminus by 36 or 58 but not 15 amino acids produced gradual decreases in Dusp26/Kif3a and Dusp26/Kap3 co-immunoprecipitations, suggesting that the entire Dusp26 N terminus contributes to the Dusp26/KIF3 interaction. Although the Dusp26 N terminus is required for Kap3 dephosphorylation and Dusp26/KIF3 association together with Figure 3b, we could not exclude the possible structural functions of this region (see below). The interaction between Dusp26 and KIF3 motor at endogenous levels was confirmed by co-immunoprecipitation of Kif3a with Dusp26 from lysate of IMR-32 neuroblastoma cells (Figure 4d). In addition, we also observed co-immunoprecipitation of N-cadherin and β-catenin with Dusp26 (see below). To determine whether Dusp26 dephosphorylates Kap3 directly, we prepared recombinant Dusp26 proteins but found that GST-Dusp26 (full length) expressed in *Escherichia coli* and His-Dusp26 translated *in vitro* using wheat germ extracts were highly insoluble, preventing analysis using these proteins. Thus we performed *in vitro* experiments using recombinant Dusp26-ΔN15 protein. Purified GST-Dusp26-ΔN15 showed vanadate-sensitive catalytic activities against a pNPP substrate in agreement with general features of PTP and DSP enzymes (Figure 4e). Incubation of cell lysate containing Kap3 with GST-Dusp26-ΔN15 *in vitro* under dephosphorylation conditions, that is, in the absence of phosphatase inhibitors, resulted in loss of phosphorylated Kap3 in a vanadate-sensitive manner (Figure 4f). In addition, purified Kap3 was also dephosphorylated by GST-Dusp26-ΔN15 *in vitro*, indicating that Kap3 is a Dusp26 substrate (Figure 4g).

In the course of this study, we found that Dusp26 can be translated from an alternative start site. Dusp26 mRNA including its 5′-untranslated region appeared to be translated mainly from a second or third in-fame ATG codon (Met11 or Met14) (Tanuma *et al.*, unpublished observations). Thus we excluded full-length Dusp26 from further analysis. Note that Dusp26-ΔN15 showed characteristics similar to full-length protein in terms of association with KIF3 and Kap3 dephosphorylation as described previously but slightly different localization from full-length protein (that is, mild enrichment in the Golgi apparatus, as shown in Figure 6).

Dusp26 enhances N-cadherin-mediated cell–cell contacts

To analyse consequences of Dusp26-mediated Kap3 dephosphorylation, stable cell lines expressing Dusp26 (ΔN15) or its inactive mutant (ΔN15CS) were developed by retroviral infection of NIH3T3 cells (Figure 5a). Expression of endogenous Dusp26 in parent NIH3T3 cells was under the detection limit by western blot and immunohistochemical analysis (see below). After drug selection, pools of cells were analysed to avoid clonal variation. As KIF3 was implicated in intracellular transport of N-cadherin, aggregation assays were performed to examine the effect of Dusp26 on cell–cell adhesion. As shown in Figures 5b and c, NIH3T3 cells expressing Dusp26-ΔN15 (NIH3T3-ΔN15) showed more rapid aggregate formation than did cells infected with vector only (NIH3T3-vector) or with Dusp26-ΔN15CS (NIH3T3-ΔN15CS). Increased adhesiveness of NIH3T3-ΔN15 cells was lost when Ca^{2+}-dependent adhesion was blocked by EGTA, suggesting that Dusp26 specifically enhances Ca^{2+}-dependent cell adhesion (Figure 5b). Western blotting of lysates from stable lines indicated little change in total N-cadherin levels in these cells (Figure 5a). Immunostaining revealed that N-cadherin was localized to the cytoplasm, to cell–cell contact sites, to the ruffling membrane and to tips of membrane protrusions in NIH3T3-vector and NIH3T3-ΔN15 cells seeded at subconfluent densities (Figure 6a). In these conditions, colocalization of N-cadherin and Dusp26-ΔN15 was observed at the membrane ruffle but not at cell–cell contact sites. When cells were cultivated at higher density to allow cell–cell contacts, N-cadherin was concentrated at contact sites in NIH3T3-vector cells (Figure 6b). Strikingly, accumulation of N-cadherin at the cell periphery was much more pronounced in NIH3T3-ΔN15 cells and correlated with virtual loss of cytosolic N-cadherin, in contrast to NIH3T3-vector and NIH3T3-ΔN15CS cells. Similar results were obtained when cells were stained for β-catenin (Figure 6c). Overall, these results suggest that Dusp26 promotes N-cadherin-mediated cell–cell adhesion. We also generated cells expressing Dusp26-ΔN58 mutant. Neither cell adhesiveness nor N-cadherin/β-catenin localization was affected by the Dusp26-ΔN58 but its expression was low (Supplementary Figure 1a and data not shown). This was probably due to its misfolding of the mutant protein since recombinant GST-Dusp26-ΔN58 showed little pNPPase activity (Supplementary Figure 1b). Thus it remains not clear whether the Dusp26 N-terminal region is directly involved in association with Kif3a/Kap3 while it is required for mediating both Kap3 dephosphorylation and increased cell adhesiveness.

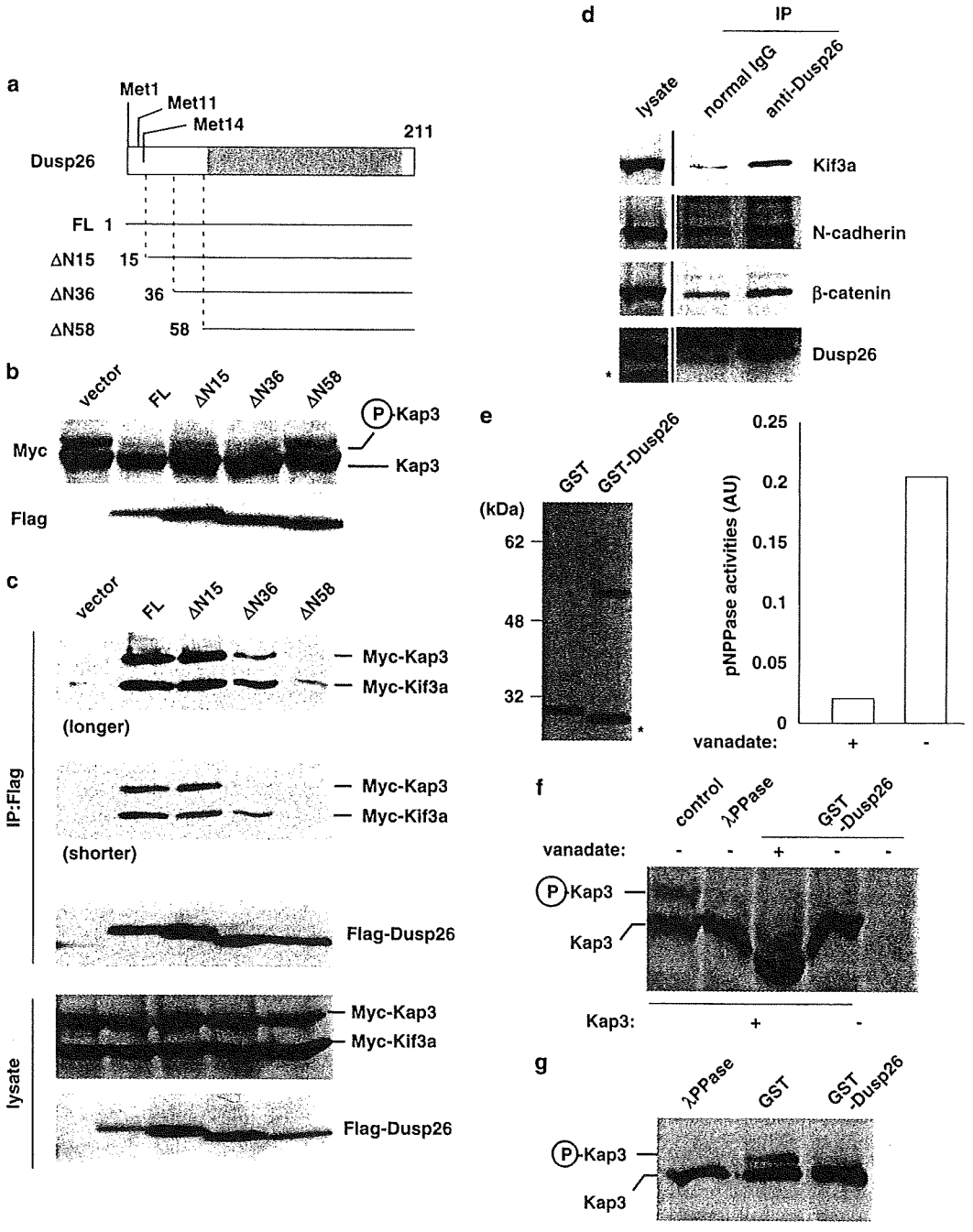
Downregulation of Dusp26 expression in human brain tumors

Previous studies revealed preferential Dusp26 expression in brain and that Dusp26 mRNA is broadly

expressed in neuronal and glial populations throughout the brain, except in hippocampus (Takagaki *et al.*, 2007). We undertook qRT-PCR analysis of human glioblastoma samples and found that Dusp26 mRNA was downregulated in eight of nine patients evaluated (Figure 7a). Together with findings that cell–cell adhesion is enhanced in cells expressing Dusp26, these results suggest tumor-suppressing activities of Dusp26 and raise the possibility that the extent of Dusp26 downregulation correlates with invasive phenotypes of glioblastomas.

Discussion

Cadherin-mediated cell–cell adhesion plays pivotal functions for regulation of cell proliferation, differentiation and polarity, and overall has significant effect on



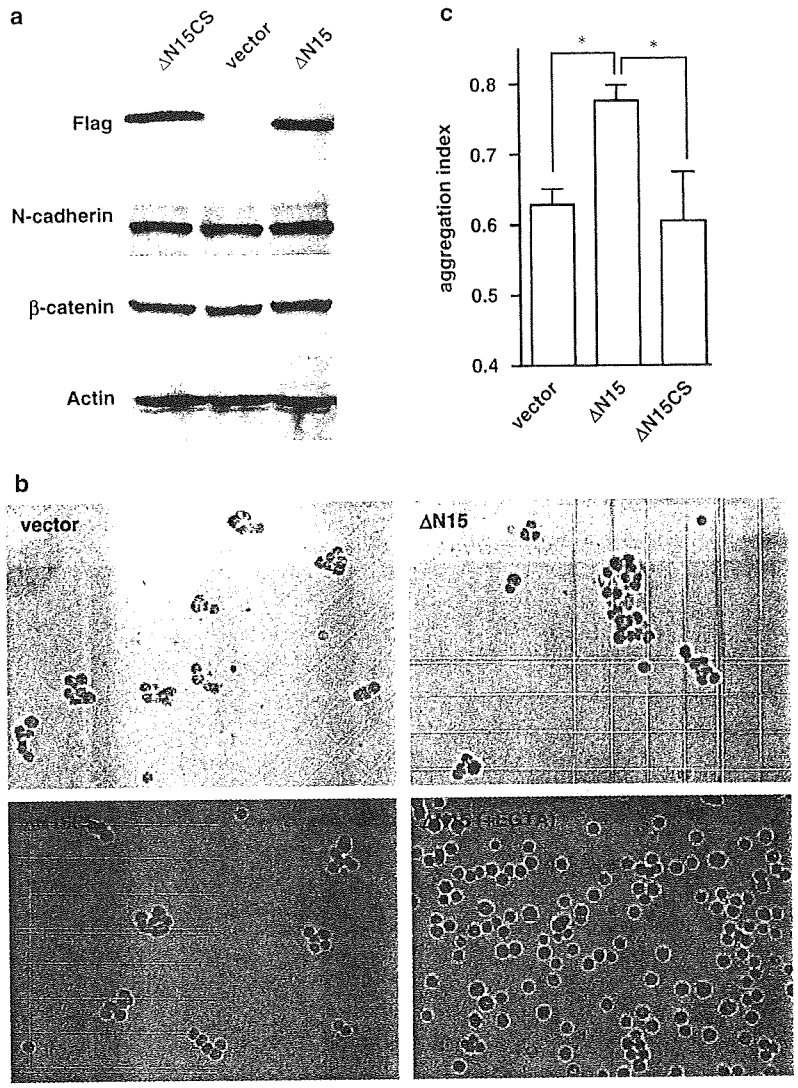


Figure 5 Enhanced cell–cell adhesion of cells expressing Dusp26. (**a**) Western analysis of NIH3T3 cells infected with retroviral vector encoding Dusp26 (ΔN15 form and its inactive mutant; see text for details) or the empty vector. After drug selection, cells were seeded on collagen-coated dishes and immunoblotted with antibodies shown at the left. (**b**, **c**) Enhanced adhesiveness of NIH3T3 cells expressing Dusp26. Aggregation assays were performed as described in 'Materials and methods'. Some representative photographs of assays are shown in (**b**). The extent of cell aggregation was calculated by the aggregation index $(N_0–N_{20})/N_0$, where N_{20} is the total particle number after a 20 min incubation and N_0 is the total particle number at the initiation of incubation, and is shown in (**c**). Data represented are averages of three independent assays with s.d. ($^*P<0.05$).

Figure 4 Dusp26 dephosphorylates Kap3. (**a**) Diagram of wild-type and N-terminally truncated Dusp26 proteins. The dual-specificity protein phosphatase (DSP) catalytic domain is shown by shaded box. (**b**) The Dusp26 N-terminal region contributes to suppression of Kap3 phosphorylation. HTO cells were transfected with Flag-Dusp26 constructs depicted in A together with Myc-Kap3. Kap3 phosphorylation was analysed by Phos-tag/SDS–PAGE as in Figure 3. (**c**) Co-immunoprecipitation of KIF3 subunits with Dusp26 proteins. HTO cells were transfected with full-length or deletion mutants of Flag-Dusp26 with Myc-Kap3 and Myc-Kif3a, immunoprecipitated with an anti-Flag antibody, and immunoblotted. (**d**) Co-immunoprecipitation of KIF3 with endogenous Dusp26. IMR-32 cells were lysed and immunoprecipitated with an anti-Dusp26 antibody or control normal immunoglobulin G (IgG). Western blotting was performed using anti-Kif3a, anti-N-cadherin, anti-β-catenin and anti-Dusp26 antibodies. An asterisk marks cross-reacted and unrelated bands. (**e**) Characterization of purified recombinant Dusp26 protein. The ΔN form of Dusp26 was expressed as a GST-fusion protein in *E. coli* and purified. Left panel shows Coomassie staining of purified Dusp26-ΔN15. Asterisk marks fragment cleaved during the expression/purification process, likely composed of glutathione *S*-transferase (GST) alone. The catalytic activity of the purified enzyme was measured in the presence or absence of vanadate using substrate pNPP and shown in the right panel. (**f**) Dephosphorylation of Kap3 by Dusp26 *in vitro*. *In vitro* dephosphorylation reactions were performed as described in 'Materials and methods', and analysed for Kap3 phosphorylation by Phos-tag/SDS–PAGE followed by western blotting. Severe distortion of the bands, seen in repeated experiments, was likely due to vanadate acting as pseudo-phosphate, and thereby disturbed Phos-tag/protein interactions. (**g**) Dephosphorylation experiments were performed, as in (**f**), using immunopurified Kap3.