Table 1—*Results of PDT for Central-Type ESLC**

Tumor Size, cm	Lesions, No.	CR	PR	Recurrence After CR†
< 1.0	83	77 (92.8)	6 (7.2)	9 (11.7)
≥ 1.0	31	18 (58.1)	13 (41.9)	3 (16.7)
Total	114	95 (83.3)	19 (16.7)	12 (12.6)

*Data are presented as No. (%) unless otherwise indicated.
†p < 0.001.

maximum longitudinal tumor extent. Of these, 83 lesions (72.8%) were < 1.0 cm and 31 lesions (27.2%) were ≥ 1.0 cm in diameter. The CR and PR rates in the group of patients with lesions < 1.0 cm in maximum diameter were 92.8% (77 of 83 patients) and 7.2% (6 of 83 patients), respectively. Meanwhile, in the group of patients with lesions ≥ 1.0 cm in diameter, the CR and PR rates were 58.1% (18 of 31 patients) and 41.9% (13 of 31 patients), respectively. Neither no change nor partial disease were observed in these groups. There was a significant difference in efficacy between the two groups using the χ^2 test ($p < 0.001$). Recurrences after CR were recognized in 9 of 77 lesions (11.7%) in the group < 1.0 cm and 3 of 18 lesions (16.7%) in the group ≥ 1.0 cm in diameter. The overall 5-year survival rates of the two groups were 57.9% and 59.3%, respectively (Fig. 1). There was no significant difference between the two groups on the basis of the log-rank test ($p = 0.207$).

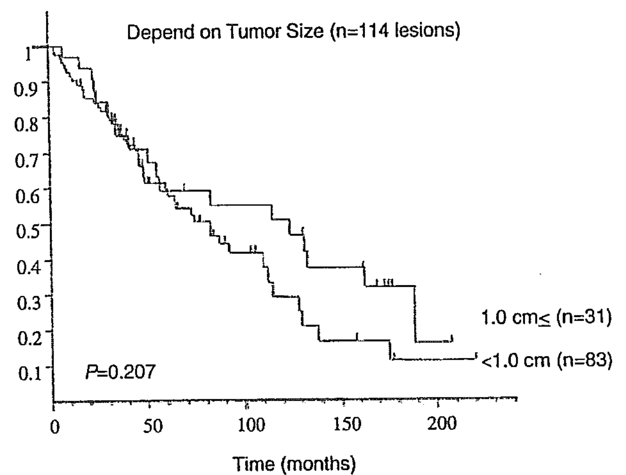


FIGURE 1. The overall 5-year survival rates were 57.9% in the group of patients with tumors < 1.0 cm and 59.3% in the group with tumors ≥ 1.0 cm in diameter, respectively. There was no significant difference between the survival rates of two groups in the Kaplan-Meier curves on the basis of the log-rank test (p = 0.207).

Characteristics of Local Recurrence < 1.0 cm in Diameter After CR

The information on nine patients with nine lesions in the group of patients with lesions < 1.0 cm in diameter who had recurrence after CR had been achieved by initial PDT are presented in Table 2. All patients with recurrence were male, and the age distribution ranged from 64 to 71 years (average age, 67.6 years at the time of initial diagnosis). Evidences of local recurrence were found in nine patients with nine lesions at the site of the primary lesion. The recurrent lesions were located on the trachea in one patient, lobar bronchus in one patient, segmental bronchi in five patients, and subsegmental bronchi in two patients. The average diameter of the nine recurrent lesions was 0.46 cm. All lesions were squamous cell carcinoma, and endoscopic findings showed nodular type in two lesions and superficial type in seven lesions. The disease-free interval of these nine patients ranged from 3 to 18 months (average, 10 months).

Local recurrence at site corresponding to the

Table 2—*Recurrent Cases After PDT for Central-Type ESLC < 1.0 cm in Diameter**

Case No.	Patient Age, yr	Lesion	Size, cm	BF Findings	CR, mo	Recurrence	Additional Treatment	Prognosis
1	66	Segmental bronchus rB^3	0.3	Superficial	18	PM	PDT, OP (RUL)	Alive (24 mo)
2	64	Subsegmental bronchus lB^3 a-b	0.3	Superficial	13	PM	PDT, OP (LPn)	Dead (56 mo), other disease
3	69	Subsegmental rB^{10} a-b	0.4	Superficial	10	PM	PDT Brachy	Alive (41 mo)
4	64	Segmental bronchus lB^{1+2}	0.6	Nodular	5	SS (CIS)	PDT	Alive (24 mo)
5	66	Segmental bronchus rB^1	0.5	Superficial	3	SS (CIS)		Dead (5 mo), other disease
6	69	Segmental bronchus lB^{1+2}	0.4	Superficial	14	SS (CIS)	PDT	Alive (27 mo)
7	71	Trachea	0.3	Nodular	10	SS (CIS)	PDT	Alive (27 mo)
8	70	Lobar bronchus rMid.-low	0.9	Superficial	6	SS (intracartilage)	OP (RML)	Dead (56 mo), other disease
9	69	Segmental lB^{1+2} B^3	0.4	Superficial	11	SS (extracartilage)	Nd-YAG, radiation, OP	Alive (65 mo)

*PM = peripheral margin initially treated; SS = same site initially treated; OP = operation; RML = right middle lobe; RUL = right upper lobe; LPn = left pneumonectomy; BF = bronchofiberscopic.

Table 3—*Cell Feature and Depth of Bronchial Invasion*

Case No.	Before PDT: Cytology (Brush) Type	Before PDT: Pathologic Grade	Recurrence: Cytology (Brush) Type	Recurrence: Pathologic Grade
4	I	1	I	1
5	I	1	I	1
6	I–II	1	I–II	1
7	I	1	II	1
8	I–II	1	II–III	3 (intracartilaginous invasion)
9	I	1	III	3 (extracartilaginous invasion)

peripheral margin of the lesion initially treated by PDT was observed in three patients (cases 1 to 3), while local recurrence at the same site as the initial tumor initially treated was observed in six patients (cases 4 to 9). The local recurrences at the site corresponding to the peripheral lesion were initially located in the subsegmental bronchus in two of three primary lesions. The patients with three local recurrences at the site corresponding to the peripheral margin underwent a second PDT session; however, CR was not obtained in any of these patients. Therefore, additional conventional surgery was performed in two patients and brachytherapy in one patient. The pathologic examinations of two operated patients showed residual tumor at the peripheral site. Right upper lobectomy was performed for case 1, and the resected material revealed superficial tumor invasion peripheral to the right B^3b. Left pneumonectomy was selected for case 2 (ipsilateral double cancer) because an ESLC was located at the bifurcation of left B^3a-b and a malignant lymphoma was in left B^6. This patient died due to malignant lymphoma at 56 months after the initial PDT session. Four patients (cases 4 to 7) with six local recurrences at the same site as the initial tumor local showed superficial tumor invasion (CIS), and a second PDT session was performed in three of four patients. CRs were again obtained in all three patients, who are presently disease free. One double cancer patient who had advanced stomach cancer underwent systemic chemotherapy without a second PDT but died 5 months after the initial PDT session. The pathologic examinations of the two other surgically treated patients (cases 8 and 9) revealed intracartilagious invasion of the bronchial wall. One multiple lung cancer patient (case 8) who received right middle and lower lobectomy after local recurrence died of hemoptysis due to another advanced lung cancer at 56 months after the initial PDT session. At the last follow-up of the nine patients who had local recurrence after CR had been obtained by initial PDT in whom the original primary lesion had been < 1.0 cm

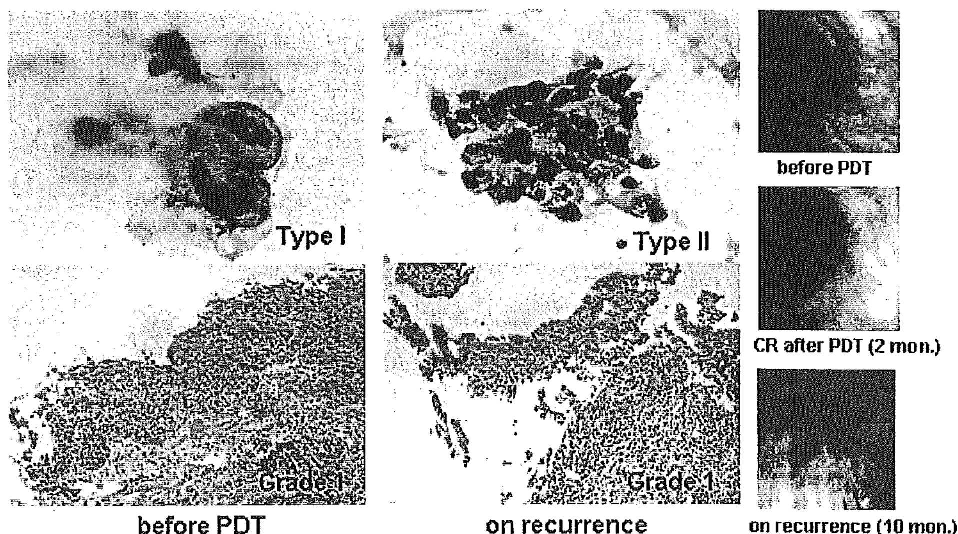


FIGURE 2. The cytopathologic and bronchoscopic findings in case 7. Bronchoscopic findings showed a small nodular tumor at the right side of the tracheal wall before PDT. Redness of the tracheal mucosa was observed on recurrence at 10 months after PDT. Cytologic findings before PDT were classified as type I because cell features showed a round shape and slight increase of nuclear chromatin but low-grade nuclear atypia. The biopsy specimen showed CIS (grade 1). Cytologic findings on recurrence were classified as type II because a sheet formation of polymorphic-shaped cells, increase of nuclear chromatin, and nuclear body were observed. Biopsy specimen on recurrence showed superficial tumor invasion (CIS) beneath the thin epithelial layer (grade 1). A second PDT was performed, and CR was again obtained in this patient.

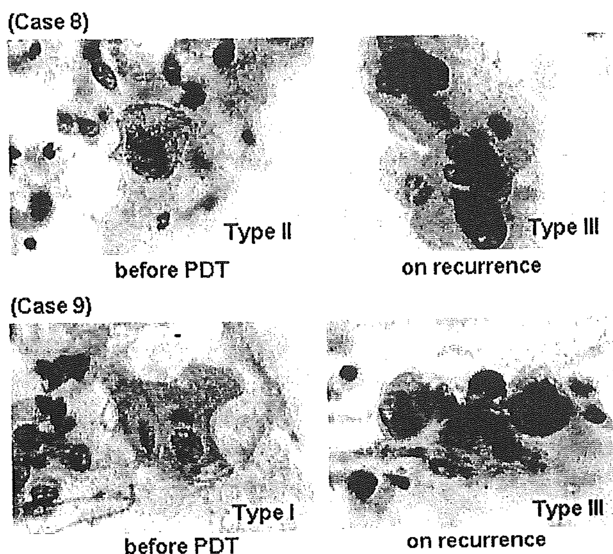


FIGURE 3. The findings of brushing cytology mainly observed in cases 8 and 9 before PDT and on recurrence after CR. Cytologic findings before PDT in case 8 were classified as type II because of a slight increase of nuclear chromatin. Cytologic findings before PDT in case 9 were classified as type I because of low-grade nuclear atypia. The findings of recurrent tumor cells in cases 8 and 9 were classified as type III because of severe increases of nuclear chromatin, high nuclear/cytoplasmic ratio, and high grade of nuclear atypia.

in diameter, three patients had died of other diseases and six patients were alive, and there were no deaths from the primary lesion.

Evaluation of Cytomorphologic Features of Local Recurrences

As mentioned above, local recurrence of the carcinoma at the same site as lesions < 1.0 cm in diameter initially treated successfully by PDT was observed in six out of nine locally recurrent patients (cases 4 to 9). A summary of the cell features and depth of bronchial wall invasion before PDT and after recurrence are shown in Table 3. The brushing cytology specimens before PDT mainly showed type I or II, and biopsy revealed grade 1 in all six cases. The majority of cell features in cases 4 to 7 showed type I or II, and the biopsy specimens showed grade 1 on recurrences. The cytopathologic and bronchoscopic findings of case 7 are shown Figure 2. Populations of type I and II cells were predominant in the recurrent lesions in these cases, which implied that the recurrent tumor was located in a superficial layer of bronchial wall. When the recurrent tumor cells showed type I-II (low-to-moderate atypia) local recurrence at the same site as the initial tumor initially treated, CR could be obtained by a second PDT. In cases 8 and 9, mainly type III cell features were observed in brushing cytology on recurrence (Fig 3). These two cases underwent resection, and the resected specimens revealed intracartilaginous tumor invasion of bronchial wall (grade 3), which implied the residual tumor located in a deep layer of bronchial wall.

DISCUSSION

PDT for cancer using a combination of low-power laser irradiation and tumor specific photosensitizer was first applied clinically by Dougherty et al[12] in 1978 to the skin metastasis of breast cancer. Since then, we performed the first reported endoscopic clinical application of PDT in cooperation with Dougherty and coworkers.[12] In Japan, PDT using porfimer sodium, a tumor-specific photosensitizer and excimer dye laser, was recognized by the government; and from April 1996, hospitals could receive reimbursement for PDT of early stage carcinomas of the lung, esophagus, stomach, and cervix from the national health insurance system.

The best PDT candidates in lung cancer are cases with central-type ESLC because of their endoscopic accessibility; therefore, selection of patient is important to achieve CR. Nagamoto et al[13] demonstrated that no lymph node involvement was found in 59 cancers with a longitudinal extent of < 20 mm; in another study,[14] histology by serial block sectioning showed that there was no nodal involvement in any CIS cases. Nakamura et al[15] retrospectively analyzed resected cases of central-type ESLC to clarify the relation between the endoscopic findings and the histologic extent of tumor. They demonstrated a significant difference is the maximum dimension according to the depth of bronchial invasion between CIS and extramuscular invasion and CIS and invasion into or beyond the cartilaginous layer. Lesions with a maximum diameter < 1.0 cm have a high possibility of being CIS. Their preoperative bronchoscopic diagnosis of centrally located ESLC was correct in 74.0%. In another study, Akaogi et al[16] demonstrated that polypoid or nodular lesions < 1.0 cm and flatly spreading lesions < 1.5 cm in greatest dimension were limited to within the cartilaginous layer without regional lymph node involvement. Also, Furuse et al[5] demonstrated that the length of longitudinal tumor extent was the only independent predictive factor for CR by PDT, and that lesions < 1.0 cm in diameter showed 100% CR. According to these data, therapy for CR requires satisfaction of the following endoscopic conditions: (1) no evidence of lymph node metastasis; (2) the lesion is superficial with a maximum diameter of < 1.0 cm; (3) no invasion into or beyond the cartilaginous layer; (4) the histologic type is squamous cell carcinoma; and

(5) the lesion is located in a position that can be easily irradiated with the laser.

In this study, excellent efficacy with a significant difference of CR rate was seen in patients with lesions < 1.0 cm (92.8%) compared to ≥ 1.0 cm (58.1%) in diameter; however, the overall 5-year survival rate of the two groups showed no significant difference (57.9% vs 59.3%). This may be because it was possible to perform additional alternative modalities such as surgery, second PDT, and brachytherapy to achieve CR after failure of initial PDT or recurrence after PDT. Considering that the 5-year survival rate of pathologic stage Ia (T1N0M0) patients who underwent surgery is approximately 67.0%,[17] our data are favorable because the majority of the PDT group consisted of patients with advanced age and poor cardiopulmonary function. Therefore, we consider that PDT may be used as first-line therapy for central-type ESLC prior to surgery, especially in cases with poor cardiopulmonary function. Also, Edell et al[18] and Cortese et al[19] demonstrated that PDT is an alternative to surgical resection in the management of early superficial squamous cell carcinoma.

In this study, recurrence after CR was recognized in 9 of 77 lesions (11.7%) in the group of patients with lesions < 1.0 cm in diameter. Despite the average diameter of the nine initial lesions being relatively small (0.46 cm), recurrence was recognized in eight of nine lesions (88.9%) within 12 months. Therefore, intensive follow-up studies should be performed until 1 year after PDT even for small primary lesions. The reasons why recurrences after CR were observed in the lesions < 1.0 cm in diameter could be explained by inappropriate estimation of the peripheral margin in cases of local recurrence at the site corresponding to the peripheral margin and insufficient laser irradiation or miss estimation of tumor depth in the cases of local recurrence at the same site as the initial tumor.

From our experiences, to achieve CR with PDT for central-type ESLC, it appears that not only the analysis of cell features but also the comprehension of tumor extent to the peripheral site and tumor invasion to the bronchial wall are of considerable significance. Kurimoto et al[20] demonstrated that endobronchial EBUS was useful to determine the depth of tumor invasion into the bronchial wall, and the accuracy of EBUS from the histopathologic findings was 95.8%. The EBUS image at 20 MHz shows five layers in the cartilaginous portion of bronchial wall. The third to fifth layers are images of cartilage. Therefore, it is feasible to evaluate the depth of invasion using EBUS whether or not the tumor invades into or beyond the cartilaginous layer.. In lesions with an intact third layer on EBUS, CR could be achieved with PDT. Miyazu et al[21] demonstrated that the depth of tumor invasion estimated by EBUS was accurate by histopathologic findings after surgical resection. They found 5 of 14 lesions (35.7%) < 1.0 cm in diameter that showed extracartilaginous invasion on the EBUS image that was later confirmed histopathologically; also, 3 of 5 lesions appeared bronchoscopically superficial but were shown to be extracartilaginous by EBUS. The indications of PDT for centrally located ESLC with a longitudinal extension of < 1.0 cm are unquestionable; meanwhile, we should realize that even < 1.0 cm in diameter can have extracartilaginous invasion. To comprehend the surface extent of superficial tumor invasion in the bronchial lumen, autofluorescence bronchoscopy (AFB) is considered useful.[22–25] The green autofluorescence of the lesion was decreased because of the lack of endogenous fluorophors, thickening of the membrane, and increased microvasculature.[26] We sometimes encountered unexpected surface invasion by AFB.

It is essential to know the extent of the tumor and the depth of bronchogenic carcinoma accurately for the selection of treatment modality. Corresponding to the previous study by Konaka et al,[11] the analysis of cell features is a useful source of information to evaluate the depth of cancer invasion in the bronchial wall. In addition, we believe that it could be beneficial information when choosing the treatment modality, such as recurrence after CR by PDT demonstrated in our study. Additionally, we now perform EBUS and AFB to determine the indications of PDT in all patients who have ESLC for the purpose of achieving 100% CR and reduction of recurrence rate. A comparative study of PDT for the treatment of ESLC before and after the adoption of EBUS and AFB will enable accurate evaluation of the benefits of these new diagnostic tools in the near future.

ACKNOWLEDGMENT: The authors are indebted to Professor J. P. Barron of the International Medical Communications Center of Tokyo Medical University for his review of this article.

References

1 Hayata Y, Kato H, Konaka C, et al. Hematoporphyrin derivative and laser photoradiation in the treatment of lung cancer. Chest 1982; 81:269–277

2 Kato H, Konaka C, Kawate N, et al. Five-year disease-free survival of a lung cancer patient treated only by photodynamic therapy. Chest 1986; 90:768–770

3 Edell ES, Cortese DA. Bronchoscopic phototherapy with hematoporphyrin derivative for treatment of localized bronchogenic carcinoma: a 5-year experience. Mayo Clin Proc 1987; 62:8–14

4 Edell ES, Cortese DA. Bronchoscopic localization and treatment of occult lung cancer. Chest 1989; 96:919–921

5 Furuse K, Fukuoka M, Kato H, et al. A prospective phase II

study on photodynamic therapy with Photofrin II for centrally located early-stage lung cancer. J Clin Oncol 1993; 11:1852–1187
6 Kato H. Photodynamic therapy for lung cancer: a review of 19 years' experience. J Photochem Photobiol 1998; B42:96–99
7 Kato H, Furukawa K, Sato M, et al. Phase II clinical study of photodynamic therapy using mono-L-aspartyl chlorin e6 and diode laser for early superficial squamous cell carcinoma of the lung. Lung Cancer 2003; 42:103–111
8 Niedre M, Patterson MS, Wilson BC. Direct near-infrared luminescence detection of singlet oxygen generated by photodynamic therapy in cells *in vitro* and tissues *in vivo*. Photochem Photobiol 2002; 75:382–391
9 General rules for clinical and pathological records of lung cancer. 4th ed. In: Japan Lung Cancer Society, eds. Tokyo, Japan: Kanehara and Company, 1995; 123–133
10 Niiya H. Toxicity grading criteria of the Japan Clinical Oncology Group. Int J Clin Oncol 1997; 32:61–65
11 Konaka C, Miura H, Ikeda N, et al. The characteristics of early bronchogenic carcinoma evaluated by cytomorphological features. Lung Cancer 2002; 38:267–271
12 Dougherty TJ, Lawrence G, Kaufman JH, et al. Photoradiation in the treatment of recurrent breast carcinoma. J Natl Cancer Inst 1978; 62:231–237
13 Nagamoto N, Saito Y, Ohta S, et al. Relationship of lymph node metastasis to primary tumor size and microscopic appearance of roentgenographically occult lung cancer. Am J Surg Pathol 1989; 13:1009–1013
14 Nagamoto N, Saito Y, Sato M, et al. Clinicopathological analysis of 19 cases of isolated carcinoma in situ of the bronchus. Am J Surg Pathol 1993; 17:1234–1243
15 Nakamura H, Kawasaki N, Hagiwara M, et al. Endoscopic evaluation of centrally located early squamous cell carcinoma of the lung. Cancer 2001; 91:1142–1147
16 Akaogi E, Ogawa I, Mitsui K, et al. Endoscopic criteria of early squamous cell carcinoma of the bronchus. Cancer 1994; 74:3113–3117
17 Mountain CF. Revisions in the international system for staging lung cancer. Chest 1997; 111:1710–1717
18 Edell ES, Cortese DA. Photodynamic therapy in the management of early superficial squamous cell carcinoma as an alternative to surgical resection. Chest 1992; 102:1319–1322
19 Cortese DA, Edell ES, Kinsey JH. Photodynamic therapy for early stage squamous cell carcinoma of the lung. Mayo Clin Proc 1997; 72:595–602
20 Kurimoto N, Murayama M, Yoshioka S, et al. Assessment of usefulness of endobronchial ultrasonography in determination of depth of tracheobronchial tumor invasion. Chest 1999; 115:1500–1506
21 Miyazu Y, Miyazawa T, Kurimoto N, et al. Endobronchial ultrasonography in the assessment of centrally located early-stage lung cancer before photodynamic therapy. Am J Respir Crit Care Med 2002; 165:832–837
22 Lam S, Kennedy T, Unger M, et al. Localization of bronchial intraepithelial neoplastic lesions by fluorescence bronchoscopy. Chest 1998; 113:696–702
23 Ikeda N, Hiyoshi T, Kakihana M, et al. Histopathological evaluation of fluorescence bronchoscopy using resected lungs in cases of lung cancer. Lung Cancer 2003; 41:303–309
24 Sutedja TG, Codrington H, Risse EK, et al. Autofluorescence bronchoscopy improves staging of radiographically occult lung cancer and has an impact on therapeutic strategy. Chest 2001; 120:1327–1332
25 Sutedja TG, Venmans BJ, Smit EF, et al. Fluorescence bronchoscopy for early detection of lung cancer: a clinical perspective. Lung Cancer 2001; 34:157–168
26 Furukawa K, Ikeda N, Miura T, et al. Is autofluorescence bronchoscopy needed to diagnose early bronchogenic carcinoma? J Bronchol 2003; 10:64–69

Quantitative Detection of Lung Cancer Cells by Fluorescence *In Situ* Hybridization*

Comparison With Conventional Cytology

Haruhiko Nakamura, MD, PhD; Idiris Aute, MD, PhD; Norihito Kawasaki, MD; Masahiko Taguchi, MD, PhD; Tatsuo Ohira, MD, PhD; and Harubumi Kato, MD, PhD, FCCP

Study objective: **The aim of this study was to clarify whether fluorescence *in situ* hybridization (FISH) can diagnose lung cancer in various clinical specimens in comparison with conventional cytology.**

Design: **Prospective study.**

Setting: **University hospital in a metropolitan area.**

Patients: **Fifty consecutive patients with abnormal chest radiography or CT scan findings were enrolled. The patients included 32 men and 18 women, with an average age of 64 years. The final definitive diagnosis was made by histologic examination, as follows: 38 primary lung cancers (24 adenocarcinomas, 8 squamous cell carcinomas, 2 large cell carcinomas, and 4 small cell carcinomas); 1 metastatic renal cell carcinoma; and 11 benign lesions.**

Methods: **Four types of clinical specimens were analyzed. Cells obtained by transbronchial brushing and transbronchial fine-needle aspiration using a fiberoptic bronchoscope under fluoroscopy, CT scan-guided percutaneous needle biopsy, and bronchial washings. On every examination, duplicate slides were made for analyses of conventional cytology and FISH.**

Results: **Classifications according to conventional cytology were as follows: class I, 4 patients; class II, 15 patients; class IIIa, 3 patients; class IIIb, 5 patients; and class V, 23 patients. A classification higher than class IIIb was considered to be positive for cancer. For cytology, we found no false-positive cases and 11 false-negative cases. The specificity was 100%, and the sensitivity was 71.8%. By FISH, 34 cases showed aberrant copy numbers in either chromosome 3 or 17. We found no false-positive cases and five false-negative cases. The specificity was 100%, and the sensitivity was 87.1%.**

Conclusion: **The ability of FISH to detect aneusomic lung cancer cells is superior to conventional cytology for the diagnosis of lung cancer.** ***(CHEST 2005; 128:906–911)***

Key words: aneuploidy; aneusomy; cytology; fluorescence *in situ* hybridization; lung cancer

Abbreviations: BW = bronchial washing; FISH = fluorescence *in situ* hybridization; PN = percutaneous needle biopsy; SSC = standard saline citrate; TB = transbronchial brushing

Conventional cytology plays an important role for the diagnosis of lung cancer, especially in the examination of sputum and pleural effusions. In addition, cell specimens obtained by transbronchial brushing (TB)[1] and needle aspiration under fluoroscopy,[2] percutaneous needle biopsy (PN) under CT scanning,[3] and bronchial washings (BWs)[4] provide important information for the differential diagnosis between benign and malignant disease. Cytologic diagnoses are made by experienced cytologists who can properly evaluate the morphologic features of malignant cells. However, this judgment is some-

*From the Department of Chest Surgery (Drs. Nakamura, Kawasaki, and Taguchi), Atami Hospital, International University of Health and Welfare, Atami, Shizuoka, Japan; the Department of Thoracic Surgery (Dr. Aute), The First Affiliated Hospital, Xinjiang Medical University, Urumqi, People's Republic of China; and the Department of Surgery (Drs. Ohira and Kato), Tokyo Medical University, Tokyo Japan.

Supported by grant No. 15591493 from the Ministry of Education, Science, Sports, and Culture in Japan.

Manuscript received September 2, 2004; revision accepted January 13, 2005.

Reproduction of this article is prohibited without written permission from the American College of Chest Physicians (www.chestjournal.org/misc/reprints.shtml).

Correspondence to: Haruhiko Nakamura, MD, PhD, Department of Chest Surgery, Atami Hospital, International University of Health and Welfare, 13-1 Higashikaigan-cho, Atami-city, Shizuoka, Japan 413-0012; e-mail: h.nakamura@iuhw.ac.jp

times difficult when the morphologic changes associated with malignancy are mild. Such cells are usually classified as class III, using the classification of Papanicolaou,[5] which is suggestive of, but not conclusive for, malignancy. This is an ambiguous judgment for clinical decision making. In addition, when one obtains a small number of cells from the lesion, the definitive diagnosis is even more difficult. These limitations of morphology-based conventional cytology have stimulated the search for more objective and quantitative methods for an accurate cytologic diagnosis of cancer.

Aneuploidy is the most common feature of many solid tumors, including lung cancer.[6] Solid tumors are characterized by complicated karyotypes by classic cytogenetics.[7,8] Chromosomal instability[9,10] may cause the uneven distribution of chromosomes during cell division.[11,12] Thus, malignant tumors can be diagnosed by detecting aneuploid, usually hyperdiploid, cells. A rapid and sensitive method for detecting aneusomy of a specific chromosome in an individual cell is fluorescence *in situ* hybridization (FISH). For this purpose, specific centromeric DNA probes enumerated the chromosomes. FISH was originally developed as a method to detect chromosomal aberrations,[13] and is now widely used for gene mapping,[14] the diagnosis of congenital diseases,[15] and detecting specific gene copy number changes in malignant cells.[16–18]

One advantage of FISH in detecting malignant cells is its objective and quantitative evaluation. However, the specificity and sensitivity of FISH in the diagnosis of lung cancer is unclear. We report the results of a prospective study comparing FISH with conventional cytology to detect lung cancer cells.

Materials and Methods

Patients

Fifty consecutive patients who underwent cytologic examination for abnormal chest radiography or CT scan findings at Tokyo Medical University Hospital from July 2003 to January 2004 were enrolled in this prospective study. The patients included 32 men and 18 women, with an average age of 64 years. The final definitive diagnosis was made by histologic examination, as follows: 38 primary lung cancers (24 adenocarcinomas, 8 squamous cell carcinomas, 2 large cell carcinomas, and 4 small cell carcinomas); 1 metastatic renal cell carcinoma; and 11 benign lesions. All patients with lung cancer were staged according to the latest Union Internationale Centre le Cancer criteria.[19] Cases included 10 tumors in stage IA, 5 in stage IB, 1 in stage IIA, 3 in stage IIB, 10 in stage IIIA, 6 in stage IIIB, and 3 in stage IV (Table 1).

Cells gathered from lung lesions were independently analyzed by conventional cytology and FISH. Informed consent for the cytologic examinations and genetic analyses of the specimens were obtained from all patients.

Table 1—*Histology and Stage of Lung Cancer in This Series of Patients**

Case	Age	Gender	Specimen	Histology	Stage
1	59	M	TB	Sq	cIIIA
2	42	F	PN	B	NA
3	43	M	TB	Ad	PIIIA
4	70	M	TB	Ad	CIV
5	77	M	TB	Ad	PIA
6	73	M	PN	Ad	PIA
7	58	M	TN	Ad	PIA
8	71	M	BW	Ad	pIIIA
9	71	M	BW	La	pIB
10	66	F	TN	RCC	NA
11	65	F	TB	Sm	cIIIB
12	68	F	PN	Ad	pIA
13	69	F	BW	Ad	pIV
14	52	F	TB	Ad	pIIA
15	75	F	TN	Sq	pIA
16	37	M	PN	B	NA
17	64	M	PN	Ad	pIIB
18	58	F	TB	B	NA
19	73	F	PN	Ad	pIA
20	62	M	PN	B	NA
21	69	M	TB	B	NA
22	76	M	BW	La	cIIIB
23	76	M	PN	Ad	cIIIB
24	75	M	PN	Ad	pIB
25	65	M	BW	Sm	cIIIA
26	23	M	PN	B	NA
27	74	M	BW	Ad	cIV
28	72	F	PN	B	NA
29	80	M	TB	B	NA
30	56	M	BW	Ad	pIB
31	64	M	TB	B	NA
32	58	M	PN	Ad	cIIIA
33	72	F	PN	Ad	pIA
34	72	M	PN	Ad	cIIIA
35	66	F	TB	Sm	cIIIB
36	61	M	TB	Ad	pIIB
37	72	F	TB	Ad	pIB
38	52	M	PN	B	NA
39	64	M	TB	Ad	pIA
40	79	F	TB	Sq	cIB
41	39	M	TB	B	NA
42	78	M	BW	Sq	cIIIB
43	62	M	TB	Sq	cIIIA
44	57	M	TB	Sq	pIIIA
45	70	M	PN	Ad	pIA
46	55	F	PN	Ad	pIA
47	58	F	TN	Sq	cIIIB
48	66	F	TN	Ad	pIIB
49	62	M	BW	Sm	cIIIA
50	72	F	BW	Sq	cIIIA

*M = male; F = female; TN = transbronchial needle biopsy; Ad = adenocarcinoma; Sq = squamous cell carcinoma; La = large cell carcinoma; Sm = small cell carcinoma; RCC = renal cell carcinoma; B = benign lesion; c = clinical stage; p = pathologic stage; NA = not applicable.

Cell Samples

In this study, the following four types of cell specimens were analyzed: cells obtained by TB (n = 18) and transbronchial fine-needle aspiration (n = 5) using a fiberoptic bronchoscope

under fluoroscopy, CT scan-guided PN using the 19-gauge Tokyo Medical University Needle[3] (n = 17), and BWs (n = 10). On every examination, duplicate specimens were made for simultaneous analyses of conventional cytology and FISH.

For conventional cytology, cells were stained by the Papanicolaou method. [5] Diagnosis was made by cytologists in the Department of Pathology at Tokyo Medical University Hospital. The various classes in conventional cytology are defined as follows: class I, absence of atypical or abnormal cells; class II, atypical cytology but no evidence of malignancy; class III, cytology suggestive of, but not conclusive for, malignancy (IIIa, mild dysplasia; IIIb, advanced dysplasia); class IV, cytology strongly suggestive of malignancy; and class V, cytology conclusive for malignancy.[5]

FISH

For FISH, cells on glass slides were air-dried overnight and stored at −80°C until they were used. Direct fluorochrome-labeled centromeric probes were used for the enumeration of different chromosomes. Spectrum-orange-labeled or Spectrum-green-labeled probes for the respective centromeric regions of chromosomes 3 and 17 were purchased (Vysis Inc; Downers Grove, IL), and dual-color FISH was performed. Slides were denatured by incubation with 70% formamide (two times the standard saline citrate [SSC] solution) at 74°C for 2 min in a water bath. Then, slides were dehydrated through a graded ethanol system (70% for 2 min, 85% for 2 min, and 100% for 2 min). A hybridization solution (10 μL) was applied to each slide, which was coverslipped and sealed with rubber cement. The hybridization solution contained 1 μL each DNA probe in 70% formamide (two times the SSC solution), and 10% dextran sulfate solution (cot I DNA). After incubation for 16 h at 37°C in a humidified chamber, slides were washed (two times SSC solution) for 3 min at 74°C. A di-amidinophenylindole antifade solution (8 μL) was applied to each spot and coverslipped. The slides were observed under a fluorescence microscope that was connected to a cooled charge-coupled device camera and an image analyzer system (CytoVision; Applied Imaging, Ltd; Newcastle, UK).

FISH signal analysis was performed as follows. All cells in a fluorescence microscopy field, except for those with damaged or overlapped nuclei, were evaluated. One hundred cells were counted, and the numbers of each centromeric signal were recorded. If there were < 100 cells on the slide, as many cells as possible were counted. When the percentages of hyperdisomic cells (*ie*, more than three copies for at least one chromosome) were > 10%, we judged the lesion to be malignant.

Comparison of Conventional Cytology and FISH

FISH diagnoses were made without clinical information or the results of conventional cytology. The results of FISH analysis were not shown to the cytologists. Thus, both diagnoses were independently made in a blind fashion.

Statistical Analysis

Differences in the number of countable cells according to the histology of the lung lesions or the cell-gathering methods used were analyzed by the Kruskal-Wallis test. A p value of < 0.05 was considered to be significant.

Results

Cells countable for FISH analyses ranged from 5 to 100 (maximum). Cell counts according to the cell-gathering method did not differ significantly, but the fewest cells were obtained by PN (Fig 1). Although the fewest cells were obtained from small cell carcinomas, no significant difference was seen according to the histologic type of lung cancer (Fig 2).

The results of conventional cytology according to the Papanicolau classification were as follows: class I, 4 cases; class II, 15 cases; class IIIa, 3 cases; class IIIb, 5 cases; and class V, 23 cases (Table 2). Twenty-eight cases showing a higher grade than class IIIb were considered to be positive for lung cancer. By cytology, we found no false-positive cases and 11

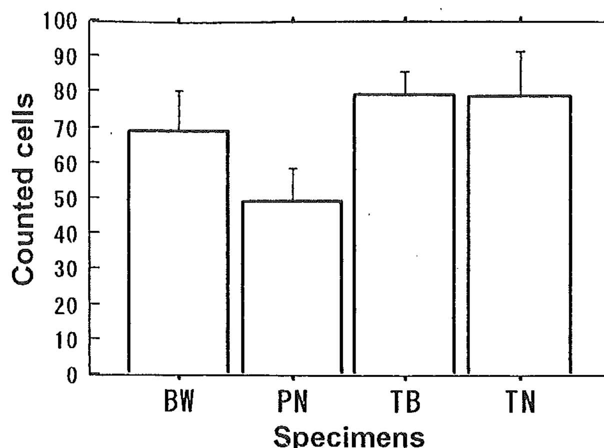


FIGURE 1. Countable cells according to the type of clinical specimen. Although cell counts obtained by PN were the lowest, no statistical significance was obtained by the Kruskal-Wallis test (p = 0.1117). Error bars indicate standard error. TN = transbronchial fine-needle aspiration.

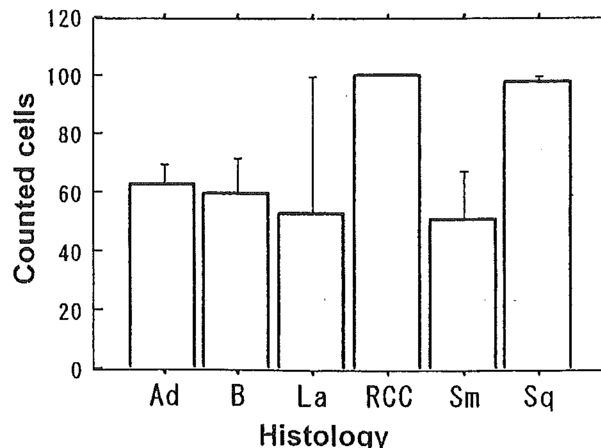


FIGURE 2. Countable cells according to histology. Although cell counts obtained from small cell lung cancer (Sm) were the lowest, no statistical significance was obtained by the Kruskal-Wallis test (p = 0.2369). Error bars indicate SE. Ad = adenocarcinoma; B = benign lesion; La = large cell carcinoma; RCC = renal cell carcinoma; Sq = squamous cell carcinoma.

Table 2—*Results of FISH and Conventional Cytology**

Case	Countable Cells	Three Copies	Four Copies	Five Copies	Six Copies or More	Hyperdisomy, %	FISH	Cytology Stage
		C3/C17	C3/C17	C3/Ch17	C3/C17	C3/C17		
1	100	28/18	10/2	2/2	0/0	40/22	Positive	V
2	8	0/0	0/0	0/0	0/0	0/0	Negative	II
3	100	14/9	4/4	1/1	0/0	18/13	Positive	V
4	100	16/8	0/1	1/0	0/0	17/9	Positive	II†
5	52	2/0	1/0	0/0	0/0	3/0	Negative†	II†
6	100	9/0	1/3	1/0	0/0	10/3	Positive	IIIa†
7	44	7/3	0/0	1/0	0/0	18/7	Positive	II†
8	100	6/6	0/2	2/2	0/0	8/10	Positive	V
9	5	0/0	0/0	0/0	0/0	0/0	Negative†	I†
10	100	17/26	2/5	1/2	0/0	20/33	Positive	II†
11	100	38/37	9/2	3/0	1/0	51/39	Positive	V
12	17	2/3	2/1	1/0	0/0	29/29	Positive	V
13	79	5/11	5/3	4/1	0/0	18/19	Positive	IIIb
14	100	7/3	0/0	0/0	1/0	8/3	Negative†	II†
15	100	9/0	1/3	1/0	0/0	11/3	Positive	II†
16	42	0/1	0/0	0/0	0/0	0/2	Negative	II
17	100	22/16	2/11	3/5	0/1	27/33	Positive	V
18	100	2/0	0/0	0/0	0/0	2/0	Negative	I
19	26	5/8	0/0	0/0	0/0	19/31	Positive	IIIb
20	100	1/1	0/0	0/0	0/0	1/1	Negative	II
21	100	0/0	0/0	0/0	0/0	0/0	Negative	II
22	100	17/14	3/1	2/2	1/1	23/18	Positive	IIIb
23	5	1/1	0/0	0/0	0/0	20/20	Positive	IIIb
24	100	22/23	5/7	1/3	2/1	30/34	Positive	V
25	37	13/10	1/7	1/0	0/0	41/46	Positive	V
26	8	0/0	0/0	0/0	0/0	0/0	Negative	IIIa
27	43	1/1	0/0	0/0	0/0	2/2	Negative†	I†
28	100	0/0	0/0	0/0	0/0	0/0	Negative	I
29	44	0/0	0/1	0/0	0/0	0/2	Negative	II
30	100	8/6	1/1	0/1	0/0	9/8	Negative†	IIIb
31	100	2/0	0/0	0/0	0/0	2/0	Negative	II
32	8	2/3	1/1	1/0	0/0	50/50	Positive	V
33	21	4/5	1/0	0/0	0/0	24/24	Positive	V
34	57	27/31	5/2	2/1	0/1	60/61	Positive	V
35	40	10/9	2/3	4/0	0/2	40/35	Positive	V
36	100	15/15	1/4	1/1	0/0	17/20	Positive	V
37	38	2/4	0/0	0/0	0/0	5/11	Positive	V
38	23	0/1	0/0	0/0	0/0	0/4	Negative	II
39	45	9/9	1/4	2/1	0/0	27/31	Positive	V
40	79	14/12	1/1	1/0	0/0	20/16	Positive	V
41	30	0/1	0/0	0/0	0/0	0/3	Negative	II
42	100	17/6	3/1	0/1	0/0	20/8	Positive	V
43	100	19/18	3/6	4/1	2/0	28/25	Positive	II†
44	100	18/15	1/5	0/1	0/0	19/21	Positive	V
45	100	11/7	0/1	0/0	0/0	11/8	Positive	V
46	14	4/3	0/0	0/0	0/0	29/21	Positive	V
47	100	15/15	0/8	0/1	0/0	15/24	Positive	V
48	48	8/6	0/1	0/0	0/0	17/15	Positive	V
49	25	4/6	1/0	0/0	0/0	20/24	Positive	V
50	100	21/28	5/5	1/1	0/0	27/34	Positive	IIIa†

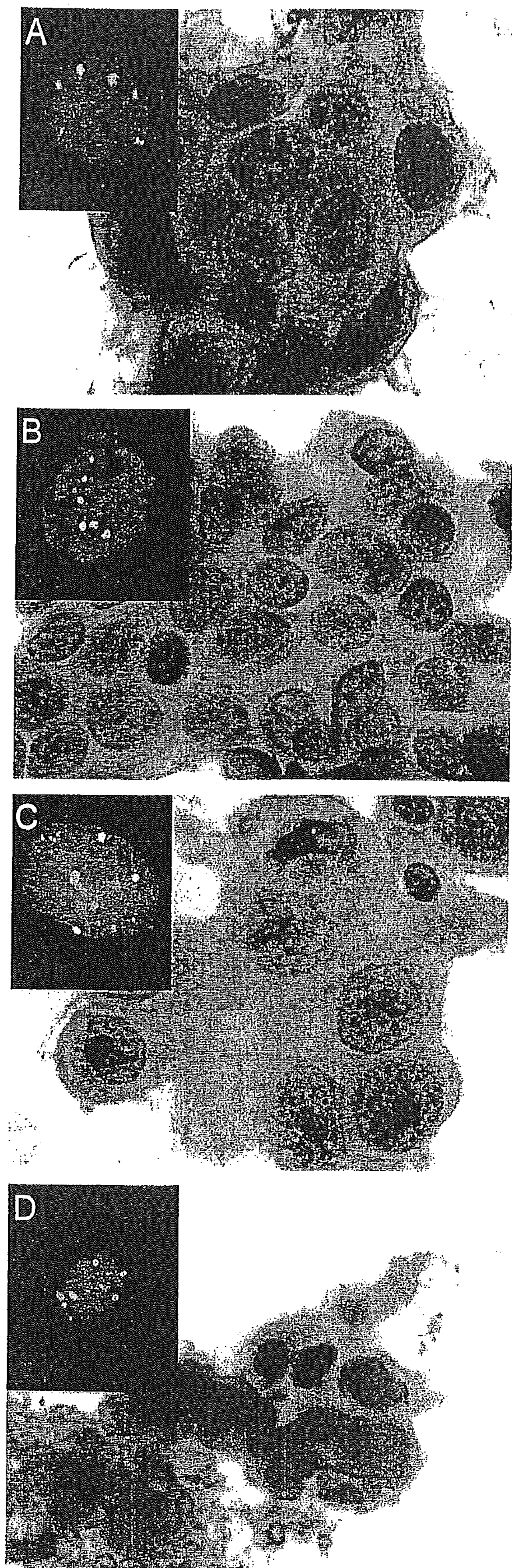
*C3 = chromosome 3; C17 = chromosome 17.

†False-negative result.

false-negative cases. Thus, by conventional cytology, specificity was 100%, and sensitivity was 71.8%.

In FISH analyses, 34 cases showed aberrant copy numbers in either chromosome 3 or 17. Representative findings of conventional cytology and FISH are shown in Figure 3. We found no false-positive cases and five false-negative cases. For FISH analyses, specificity was 100%, and sensitivity was 87.1%.

Seven cases, including one with metastatic renal cell carcinoma, had negative cytology findings and



positive FISH findings. In these cases, the cytologic classifications were as follows: class II, five cases; and class IIIa, two cases. One case was cytology-positive and FISH-negative. In this case, the portion of aneusomic cells were observed to be 8 to 9%, which is just below the predetermined cutoff value.

Discussion

We demonstrated here the usefulness of FISH analyses in diagnosing lung cancer using various clinical specimens. Similar results about the effectiveness of FISH analyses have been reported by several authors. Schenk et al[20] examined 23 patients with lung cancer by FISH with probes specific for chromosomes 3, 8, 11, 12, 17, and 18 for malignant effusions and primary tumors. In that study, chromosomal alterations always consisted of gains in chromosomal signal numbers, and all chromosomes were found to be aneusomic to a similar extent. According to this observation, we used only two probes in the present study, which were specific for chromosomes 3 and 17.

Recently, Sokolova et al[21] analyzed BW specimens from 48 patients with lung cancer by FISH using four probes (*ie*, centromeric region of chromosome 1, 5p15, 8q24 (c-myc), and 7p12 [epidermal growth factor receptor]). In that report, FISH detected 15 of 18 specimens that were falsely negative by cytology. The sensitivity of FISH for the detection of lung cancer was 82% compared with 54% sensitivity by conventional cytology. The same group[22] used a similar FISH probe set to show that significantly higher frequencies of abnormal cells were found in each of the 20 surgical specimens of non-small cell carcinoma (100%) and in the 3 sputum specimens (100%) from lung cancer patients. These probes detected a 4.8 to 7.3% rate of abnormal copy numbers in normal control specimens. In these retrospective studies, FISH detected lung cancer cells in touch preparations of resected tumors and BWs. Thus, we planned a prospective study to compare conventional cytology with FISH using various specimens from lung lesions.

In our study, we determined the cutoff value for the percentage ofhyperdisomic cells to be 10%, be-

Figure 3. Hyperdisomic cells detected by FISH. Red signals are the centromeric region of chromosome 3, and green signals are the centromeric region of chromosome 17.Representative findings of conventional cytology (Papanicolaou stain, original ×400) and FISH in the same cases, as follows: *top left*, *A*: adenocarcinoma (case 39); *top right*, *B*: squamous cell carcinoma (case 1); *bottom left*, *C*: large cell carcinoma (case 24); and *bottom right*, *D*: small cell carcinoma (case 11).

cause we often count ≦6% hyperdisomic cells in normal cell specimens, probably due to counting sister chromatids as two copies. When we set the cutoff value at 10%, a specificity of 100% and a sensitivity of 87.1% were obtained by FISH, whereas the sensitivity of cytology was 71.8%. As a result, we successfully detected seven lung cancer cases that were cytology-negative. Among these cases, two were class IIIa that we could not diagnose as malignant based on morphologic features. FISH may provide decisive information for the detection of malignancies, especially cases with IIIa classification.

Although the sensitivity of FISH is superior to that of conventional cytology, there are some disadvantages to FISH analyses. First, we do not generate information about the histologic type of lung cancer since we cannot observe morphologic features. Second, FISH is expensive. Third, FISH signal counting under fluorescence microscopy is time-consuming. Thus, the present FISH assay system probably can play a complementary role to that of conventional cytology.

We had five cases that we could not correctly diagnose by FISH. There are two possible reasons for our false-negative FISH results. One would be the failure to obtain proper cell material from the lesion, resulting in the absence of cancer cells on the slide. The other would be that the cancer cells were near-diploid, such that we could not detect aneusomy in two target chromosomes. We could probably detect more aneusomic cells using additional suitable probes for other chromosomes or chromosomal regions as reported by Romeo et al,[22] who successfully diagnosed 100% of lung cancer cases by FISH using a set of four probes. In our previous study,[23] chromosomal instability detected by FISH was associated with poor survival in patients with lung cancer. The finding of multiple chromosomal changes by FISH may be used as a prognostic factor and in the selection of patients for different therapeutic programs in the future.

In conclusion, FISH can detect lung cancer cells with aneusomy in various clinical specimens. The sensitivity was superior to that of conventional cytology. FISH should be used in conjunction with conventional cytology.

References

1 Muers M, Boddington M, Cole M, et al. Cytological sampling at fibreoptic bronchoscopy: comparison of catheter aspirates and brush biopsies. Thorax 1982; 37:457–461

2 Schenk D, Bryan C, Bower J, et al. Transbronchial needle aspiration in the diagnosis of bronchogenic carcinoma. Chest 1987; 92:83–85

3 Saji H, Nakamura H, Tsuchida T, et al. The incidence and the risk of pneumothorax and chest tube placement after percutaneous CT-guided lung biopsy: the angle of the needle trajectory is a novel predictor. Chest 2002; 121:1521–1526

4 Ng A, Horak G. Factors significant in the diagnostic accuracy of lung cytology in bronchial washing and sputum samples: I. Bronchial washings. Acta Cytol 1983; 27:391–396

5 Papanicolaou G. Criteria of malignancy. In: Atlas of exfoliative cytology. Cambridge, MA: Harvard University Press, 1954; 13–21

6 Choma D, Daures J-P, Quantin X, et al. Aneuploidy and prognosis of non-small-cell lung cancer: a meta-analysis of published data. Br J Cancer 2001; 85:14–22

7 Nowell P. The clonal evolution of tumor cell populations. Science 1976; 194:23–28

8 Hoglund M, Gisselsson D, Sall T, et al. Coping with complexity. Multivariate analysis of tumor karyotypes. Cancer Genet Cytogenet 2002; 135:103–109

9 Lengauer C, Kinzler K, Vogelstein B. Genetic instabilities in human cancers. Nature 1998; 396:643–649

10 Duesberg P, Rausch C, Rasnick D, et al. Genetic instability of cancer cells is proportional to their degree of aneuploidy. Proc Natl Acad Sci U S A 1998; 95:13692–13697

11 Cahill D, Lengauer C, Yu J, et al. Mutations of mitotic checkpoint genes in human cancers. Nature 1998; 392:300–303

12 Pihan G, Doxsey S. The mitotic machinery as a source of genetic instability in cancer. Semin Cancer Biol 1999; 9:289–302

13 Pinkel D, Straume T, Gray J. Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proc Natl Acad Sci U S A 1986; 83:2934–2938

14 Gingrich J, Shadravan F, Lowry S. A fluorescence *in situ* hybridization map of human chromosome 21 consisting of 30 genetic and physical markers on the chromosome: localization of 137 additional YAC and cosmid clones with respect to this map. Genomics 1993; 17:98–105

15 Kuo W, Tenjin H, Segraves R, et al. Detection of aneuploidy involving chromosomes 13, 18, or 21, by fluorescence *in situ* hybridization (FISH) to interphase and metaphase amniocytes. Am J Hum Genet 1991; 49:112–119

16 Kallioniemi O, Kallioniemi A, Kurisu W, et al. ERBB2 amplification in breast cancer analyzed by fluorescence *in situ* hybridization. Proc Natl Acad Sci U S A 1992; 89:5321–5325

17 Gray J, Collins C, Henderson I, et al. Molecular cytogenetics of human breast cancer. Cold Spring Harb Symp Quant Biol 1994; 59:645–652

18 Hiraguri S, Godfrey T, Nakamura H, et al. Mechanisms of inactivation of E-cadherin in breast cancer cell lines. Cancer Res 1998; 58:1972–1977

19 Mountain C. Revisions in the International System for Staging Lung Cancer. Chest 1997; 111:1710–1717

20 Schenk T, Ackermann J, Brunner C, et al. Detection of chromosomal aneuploidy by interphase fluorescence *in situ* hybridization in bronchoscopically gained cells from lung cancer patients. Chest 1997; 111:1691–1696

21 Sokolova I, Bubendorf L, O'Hare A et al. A fluorescence *in situ* hybridization-based assay for improved detection of lung cancer cells in bronchial washing specimens. Cancer 2002; 96:306–315

22 Romeo M, Sokolova I, Morrison L, et al. Chromosomal abnormalities in non-small cell lung carcinomas and in bronchial epithelia of high-risk smokers detected by multi-target interphase fluorescence *in situ* hybridization. J Mol Diagn 2003; 5:103–112

23 Nakamura H, Saji H, Idiris A, et al. Chromosomal instability detected by fluorescence *in situ* hybridization in surgical specimens of non-small cell lung cancer is associated with poor survival. Clin Cancer Res 2003; 9:2294–2299