Free-floating cancer cells in lymph node sinuses of hilar lymph node-positive patients with non-small cell lung cancer

YUSUKE NAKAMURA[1,2], MASAYA MUKAI[3], SHINICHIRO HIRAIWA[4], KYOKO KISHIMA[3], TOMOKO SUGIYAMA[4], TAKUMA TAJIRI[4], SHUNSUKE YAMADA[1,2] and MASAYUKI IWAZAKI[2]

[1]Department of General Thoracic Surgery, Tokai University Hachioji Hospital, Tokyo 192-0032; [2]Division of Thoracic Surgery, Department of Surgery, Tokai University School of Medicine, Isehara-shi, Kanagawa 259-1193; Departments of [3]Digestive Surgery and [4]Pathology, Tokai University Hachioji Hospital, Tokyo 192-0032, Japan

Received December 13, 2017; Accepted April 30, 2018

DOI: 10.3892/mmr.2018.9014

Abstract. Previous studies demonstrated that free-floating cancer cells (FFCCs) in the lymph node sinuses were of prognostic significance for colorectal and gastric cancer. The present study investigated the clinical significance of detecting FFCCs using Fast Red staining for cytokeratin in stage I/II non-small cell lung cancer (NSCLC) patients and hilar lymph node positive NSCLC patients who underwent curative resection. Between 2002 and 2011, a total of 164 patients (including 22 hilar lymph node positive patients) were investigated. Resected lymph nodes were stained for cytokeratin using an anti-cytokeratin antibody. In order to achieve a clear distinction from coal dust, an anti-cytokeratin antibody was labeled with a secondary antibody conjugated with alkaline phosphatase, which was detected by a reaction with Fast Red/naphthol that produced a red color. Patients were considered to be positive for FFCCs (FFCCs+) if one or more than one free-floating cytokeratin-positive cell was detected in the lymph node sinuses, which could not be detected by hematoxylin and eosin staining. Among all 164 patients, a significant difference was observed in 5-year relapse-free survival (5Y-RFS) rates, with 76.9 and 33.3% being achieved by FFCCs- and FFCCs+ patients, respectively (P<0.001). Similarly, the 5-year overall survival (5Y-OS) rate was significantly lower in FFCCs+ patients, with 86.6% being achieved by FFCCs- and 65.8% by FFCCs+ patients, respectively (P=0.014). Among 22 hilar lymph node-positive patients, a significant difference was also observed in 5Y-RFS, with 53.8 and 0.0% being achieved by FFCCs- and FFCCs+ patients, respectively (P=0.006). The 5Y-OS tended to be lower in FFCCs+ patients, with 69.2 and 53.3% being achieved by FFCCs- and FFCCs+ patients, respectively (P=0.463). The findings of the present study suggested the presence of FFCCs in stage I/II NSCLC patients was associated with a poor prognosis. In addition, FFCCs in hilar lymph node-positive patients may potential be a useful marker in foreseeing the recurrence of cancer.

Correspondence to: Dr Yusuke Nakamura, Department of General Thoracic Surgery, Tokai University Hachioji Hospital, 1838 Ishikawa-cho, Hachioji, Tokyo 192-0032, Japan
E-mail: yus_naka@yahoo.co.jp

Key words: free-floating cancer cells, non-small cell lung cancer, lymph node sinuses, hilar lymph node metastasis, prognosis

Introduction

Free-floating cancer cells (FFCCs) are a population of cells that are completely detached from the primary lesions and float freely inside the lymph node sinuses. FFCCs are so small that they are hard to detect using hematoxylin and eosin (H&E) staining, but they can be easily observed by staining for cytokeratin. Previous studies demonstrated that FFCCs in the lymph node sinuses were of prognostic significance for colorectal and gastric cancers (1-3). Mukai *et al* also reported that FFCCs in the lymph node sinuses were prognostic markers for lung cancer (4,5). However, these studies had poor patient selection and inadequate analyses; the authors did not include all patients who underwent resection for primary lung tumors, and analyzed findings from lung, breast, and gastric cancer patients collectively. Thus, the clinical significance of FFCCs in the lymph node sinuses of non-small cell lung cancer (NSCLC) patients currently remains unclear. In addition, some scientists suspect that FFCCs may be the same as lymph node metastases. So, in this study, we investigated whether there were prognostic differences between FFCCs positive and negative groups in hilar lymph node positive patients who underwent resection for primary lung cancer.

In contrast to lymph nodes in the colon or stomach, many of the lymph nodes in the lungs contain coal dust. Since conventional cytokeratin immunostaining uses 3,3'-diaminobenzidine (DAB), a brown stain, as a substrate to detect peroxidase (e.g., iVIEW DAB Detection kit; Roche Diagnostics K.K, Tokyo, Japan), the detection of FFCCs in lymph node sinuses covered with black coal dust is time-consuming. Therefore, we have used an alkaline phosphatase-conjugated secondary antibody and Fast Red/naphthol, which produces a red color in cytokeratin-positive cells, in order to enable the distinction of cytokeratin-positive cells from coal dust (6).

In the present study, we used Fast Red staining to detect FFCCs in the lymph node sinuses of patients with both

stage I/II and hilar lymph node positive NSCLC patients. We investigated various clinicopathological factors to assess the significance of FFCCs in the lymph node sinuses.

Patients and methods

Patients. Stage I/II (the seventh edition of the TNM classification) lung cancer patients (n=168) who underwent lobectomy (pneumonectomy when required) and hilar-mediastinal lymph node dissection between September 2002 and December 2011 at Tokai University Hachioji Hospital were enrolled in the present study. Three patients were excluded because of small cell lung cancers. One patient was excluded because his primary lesion was not stained by cytokeratin immunostaining using AE1/AE3 anti-body. So, 164 patients were investigated finally. Among stage I (n=132) and stage II (n=32) patients, 122 had adenocarcinoma, 30 squamous cell carcinoma, 6 large cell carcinoma, and 6 tumors with other histological types (1 typical cartinoid, 2 adenosquamous carcinoma, 1 pleomorphic carcinoma, 1 mucoepidermoid carcinoma, and 1 unclassified non-small cell carcinoma). Of the 164 patients, 22 had hilar lymph node metastases diagnosed by H&E staining. Of the 164 patients, 36 had recurrent diseases (n=18 and 18 for stages I and II, respectively) and 128 were relapse-free (n=114 and 14 for stages I and II, respectively). Resected lymph nodes were stained for cytokeratin using Fast Red to detect FFCCs in the lymph node sinuses, and clinicopathological features were investigated in FFCCs+ and FFCCs- patients. Relapse-free survival (RFS) and overall survival (OS) were calculated based on pathology reports and electronic medical records stored at Tokai University Hachioji Hospital. In patients with recurrent tumors, RFS and OS were measured from the date of surgery to the date that recurrent tumors were identified using CT, brain-MRI, bone-scan, FDG-PET, or to the date of death; similarly, RFS and OS were measured in non-recurrent patients from the date of surgery to December 31, 2016. All patients were followed up for the duration of the study, including those who were transferred to another hospital during the observation period. At the end of the 5-year period, 130 patients were alive, 25 were deceased, and 9 were lost to the follow-up (94.5% follow-up rate). The present study was approved by the Tokai University Institutional Review Board for Clinical Research (IRB no. 14R-225; Isehara, Japan) and the patients' samples were examined after receiving informed consent from the patients.

The concept and the definition of FFCCs in lymph node sinuses. FFCCs are a population of cells that are completely detached from the primary lesions and float freely inside the lymph node sinuses. Its concept is different from that of lymph node metastasis. In contrast to metastases in the lymph nodes detected by H&E staining, FFCCs are difficult to detect by H&E staining since they are very small in size; cytokeratin immunostaining can be usually used as an alternative to identify FFCCs in the lymph node sinuses. In the present study, FFCCs in the lymph node sinuses were defined as those that i) it is difficult to detect by H&E staining and can be detected by cytokeratin immunostaining; ii) float freely in the lymph node sinuses and do not invasive to and/or are not caught by the apparatus of the lymph nodes such as cortex and paracortex area; and iii) have an intact nucleus and are not damaged.

Immunohistochemistry. In order to achieve the clear distinction of cytokeratin-positive cells from coal dust, lymph node tissues were stained using a mouse monoclonal anti-cytokeratin antibody (Clone: AE1, AE3, PCK26; Roche Diagnostics K.K.) and secondary antibody conjugated with alkaline phosphatase, which was visualized by the reaction with Fast Red/naphthol that produced a red color. Regarding the preparation of tissues for staining, resected lymph nodes were fixed in formalin, cut along the maximum dimension, and then embedded in paraffin. Tissues were cut into 3-μm-thick sections and processed using an automated system (BenchMark®XT; Roche Diagnostics K.K.). Sections were deparaffinized and treated with protease 1 (0.5 U/ml; Roche Diagnostics K.K.) at 37˚C for 4 min, followed by a mouse monoclonal anti-cytokeratin antibody (Clone: AE1, AE3, PCK26; Roche Diagnostics K.K.) at 37˚C for 16 min. Following the reaction with the primary antibody, sections were treated with the secondary antibody conjugated with alkaline phosphatase, and were stained using a detection kit (ultraView Universal Alkaline Phosphatase Red Detection kit; Roche Diagnostics K.K.). Sections were then stained with hematoxylin for the nucleus, dehydrated, and cleared, and coverslips were placed to prepare the samples for analyses.

Tissues were sectioned serially for H&E and cytokeratin staining. FFCCs were detected in the lymph node sinuses based on the definition described above. Patients were categorized as positive for FFCCs when one or more than one freely floating cytokeratin-positive cells were detected in the lymph node sinuses (FFCCs+), and were categorized as negative for FFCCs when none were present (FFCCs-). 5-year RFS (5Y-RFS) and 5-year OS (5Y-OS) rates were calculated in both groups of patients. In addition, FFCCs+ and FFCCs- patients were categorized based on the disease stages (stage I or II) as well as the histological types of their tumors, including adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and others. The frequency of recurrence in FFCCs+ and FFCCs- patients was analyzed to calculate the sensitivity, specificity, false positive rate, false negative rate, positive predictive value, negative predictive value, and accuracy of detecting FFCCs. The first sites of recurrence were analyzed in FFCCs+ and FFCCs- patients.

Statistical analysis. A Kaplan-Meyer survival analysis was used to calculate 5Y-RFS and 5Y-OS rates (7), and the Log-rank test was used to compare the two groups. Hazard ratios (HR) and 95% confidence intervals (CIs) were calculated using Cox's proportional hazard model (8). The chi-squared test was used to compare FFCCs+ and FFCCs- patients for age, sex, histological types of tumors, the presence of recurrent tumors. Fisher's exact test was also used to compare both groups for the primary sites of recurrence because the number is small. In addition, the odds ratio and 95% CI were calculated in FFCCs+ and FFCCs- patients with recurrent tumors. In all cases, $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 22.0 statistical software (IBM Corp., Armonk, NY, USA).

Results

Detection of FFCCs. FFCCs were detected in 11.0% of all patients (n=18), with 4.5% in stage I patients (n=6) and 37.5% in stage II patients (n=12), using Fast Red staining for cytokeratin. FFCCs were observed as single cells and as clusters of several cells (Fig. 1A and B).

Clinical features and Histological types of primary lesions in FFCCs+ and FFCCs- patients. There were no significant differences in age or sex between the groups FFCCs+ and FFCCs-(Table I). Among 18 FFCCs+ patients, there were 12 cases of adenocarcinoma (66.7%), 5 of squamous cell carcinoma (27.8%), 0 of large cell carcinoma (0%), and 1 of tumors with other histological types (5.6%, 1 mucoepidermoid carcinoma). Among 146 FFCCs- patients, there were 110 cases of adenocarcinoma (75.3%), 25 of squamous cell carcinoma (17.1%), 6 of large cell carcinoma (4.1%), and 5 of tumors with other histological types (3.4%, 1 typical cartinoid, 2 adenosquamous carcinoma, 1 pleomorphic carcinoma, and 1 unclassified non-small cell carcinoma). No significant difference was observed between the two groups in terms of the histological types of primary tumors (Table I).

Recurrence in and prognosis of FFCCs+ and FFCCs- in all patients. Among 164 total patients, 18 belonged to the FFCCs+ group and 146 belonged to the FFCCs- group. The 5Y-RFS rate was significantly lower (P<0.001) in the FFCCs+ group (33.3%, n=18) than in the FFCCs- group (76.9%, n=146), with HR of 4.675 and 95% CI of 2.384-9.164 (Fig. 2A). Similarly, the 5Y-OS rate was significantly lower (P=0.014) in the FFCCs+ group (65.8%, n=18) than in the FFCCs- group (86.6%, n=146), with HR of 2.979 and 95% CI of 1.188-7.467 (Fig. 2B).

Recurrence in and prognosis of FFCCs+ and FFCCs- in n1 positive patients. Twenty two patients had hilar lymph node metastases. Among 22 n1 positive patients, 9 had FFCCs and 13 did not have any FFCCs. The 5Y-RFS rate was significantly lower (P=0.006) in the FFCCs+ group (0.0%, n=9) than in the FFCCs- group (53.8%, n=13), with HR of 4.828 and 95% CI of 1.421-16.402 (Fig. 3A). The 5Y-OS rate tended to be lower (P=0.463) in the FFCCs+ group (53.3%, n=9) than in the FFCCs- group (69.2%, n=13), with HR of 1.674 and 95% CI of 0.417-6.721 (Fig. 3B).

Sensitivity, specificity, and accuracy of the detection of FFCCs in recurrent and non-recurrent patients. Among all study patients (n=164), FFCCs were detected in recurrent and non-recurrent patients with 33.3% sensitivity (12/36), 33.3% false positive rate (6/18), 95.3% specificity (122/128), 16.4% false negative rate (24/146), 66.7% positive predictive value (12/18), 83.6% negative predictive value (122/146), and 81.7% accuracy (134/164), with an odds ratio of 10.167 (P<0.001; 95% CI: 3.537-29.225) (Table II).

First sites of recurrence. Among FFCCs+ patients (n=18), 12 had recurrent disease during the 5-year observational period. Tumor recurrence was identified at 14 sites, including multiple simultaneous tumors. There was 1 case of local recurrence (7.1%), 3 of pleural dissemination or carcinomatous pleuritis (21.4%), 5 of lymph node metastasis (35.7%), 0 of liver metastasis (0.0%), 0 of lung metastasis (0.0%), 1 of brain metastasis (7.1%), 4 of bone metastasis (28.6%), 0 of adrenal metastasis (0.0%), and 0 of metastasis in other/unknown sites (0.0%) (Table III). Among FFCCs- patients (n=146), 24 had recurrent disease during the 5-year observational period. Tumor recurrence was identified at 29 sites, including multiple simultaneous tumors. There was 1 case of local recurrence (3.4%), 4 of pleural dissemination or carcinomatous pleuritis (13.8%), 3 of lymph node metastasis (10.3%), 0 of liver metastasis (0.0%), 15 of lung metastasis (51.7%), 3 of brain metastasis (10.3%), 2 of bone metastasis (6.9%), 0 of adrenal metastasis (0.0%), and 1 of metastasis in other/unknown sites (3.4%) (Table III). In this study, lung metastases were detected at a higher frequency in the FFCCs- group than the FFCCs+ group (P<0.001; Table III).

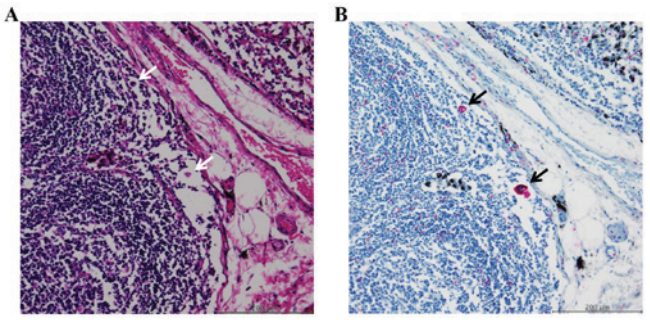

Figure 1. FFCCs in lymph node sinuses detected by Fast Red staining for cytokeratin (arrows). (A) hematoxylin and eosin staining and (B) immunostaining for cytokeratin. FFCCs, free-floating cancer cells. Original magnification, x200.

Discussion

The present study investigated FFCCs and recurrence/prognosis of stage I/II NSCLC, and demonstrated that FFCCs are of prognostic significance in stage I/II NSCLC (Fig. 2A and B). However, some scientists suspect that FFCCs may be the same as lymph node metastases. Thus, this study investigated whether there were prognostic differences between FFCCs positive and negative groups in hilar lymph node positive patients who underwent resection for primary lung cancer, and demonstrated that the 5Y-RFS rate was significantly lower and the 5Y-OS rate tended to be lower in FFCCs+ patients among n1 positive patients (Fig. 3A and B).

FFCCs are a population of cells that are completely detached from the primary lesions and float freely inside the lymph node sinuses. We estimate that FFCCs progress three pathway as follows: i) are caught by the immune systems and die; ii) are settled in lymph nodes and develop into lymph node metastases; and, iii) pass through lymph nodes and the immune systems and circulate into the whole bodies. In this study, the 5Y-RFS rate was significantly lower and the 5Y-OS rate tended to be lower in FFCCs+ patients among n1 positive patients. We think the reason is that some FFCCs progress towards the iii) pathway as stated above.

We think that FFCCs which progress towards the iii) pathway as stated above, may be similar to circulating tumor cells (CTCs). Rack *et al* identified tumor cells that were

Table I. Clinical features and Histological types of primary lesions.

Variable	Total cases (n=164)	FFCC (-) (n=146)	FFCC (+) (n=18)	P-value
Age, median (range), year	66 (30-81)	66 (36-81)	64 (30-79)	P=0.749
Sex, n (%)				
Male	107 (65.2)	95 (65.1)	12 (66.7)	P=0.759
Female	57 (34.8)	51 (34.9)	6 (33.3)	
Histological type, n (%)				
Adenocarcinoma	122 (74.4%)	110 (75.3%)	12 (66.7%)	P=0.302
Squamous cell carcinoma	30 (18.3%)	25 (17.1%)	5 (27.8%)	P=0.342
Large cell carcinoma	6 (3.7%)	6 (4.1%)	0 (0.0%)	P=1.000
Others	6 (3.7%)	5 (3.4%)	1 (5.6%)	P=0.521

FFCC, free-floating cancer cell in lymph node sinuses.

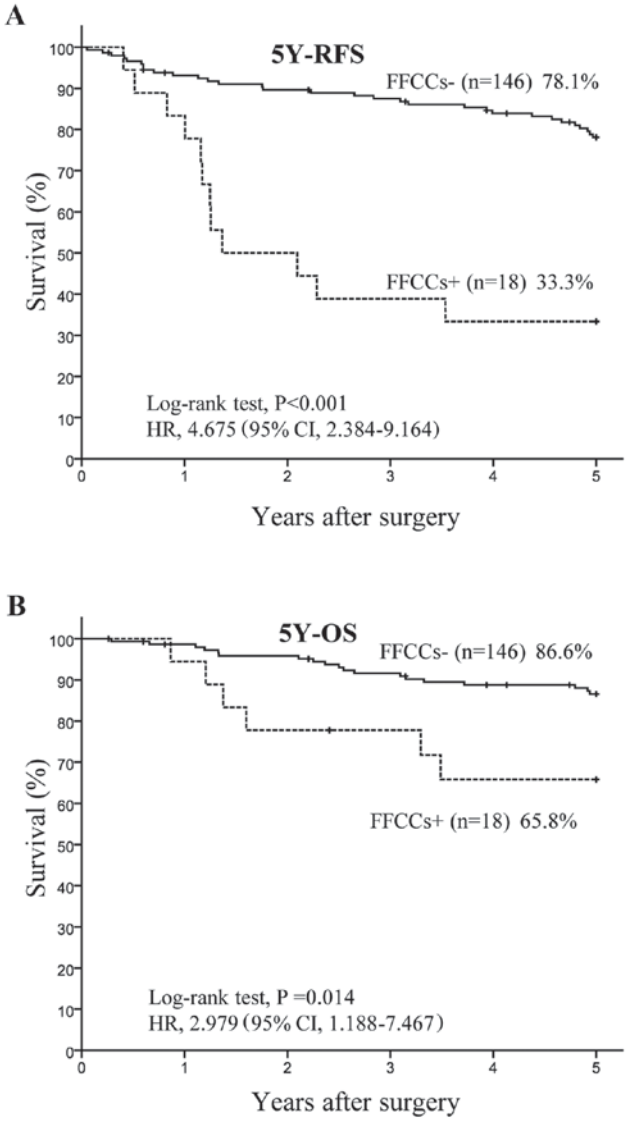

Figure 2. Recurrence in and prognosis of FFCCs+ and FFCCs- in all NSCLC patients. (A) 5Y-RFS rates; (B) 5Y-OS rates; Kaplan-Meyer survival analysis and log-rank test; FFCCs, free-floating cancer cells; NSCLC, non-small cell lung cancer; HR, hazard ratios; CIs, confidence intervals; 5Y-RFS, 5-year relapse-free survival; 5Y-OS, 5-year overall survival.

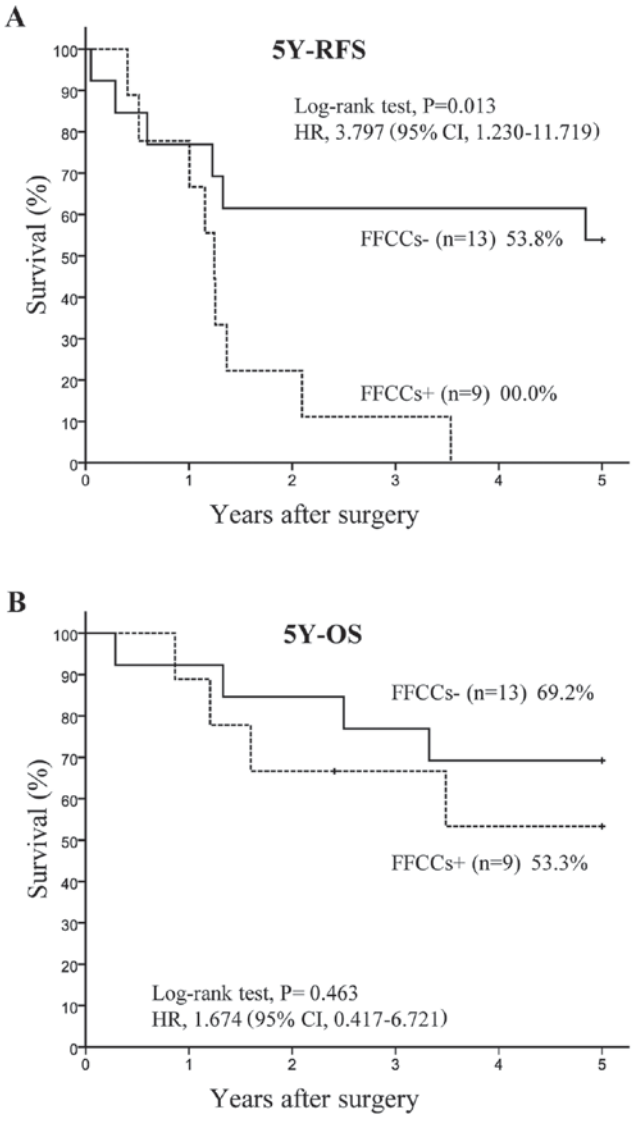

Figure 3. Recurrence in and prognosis of FFCCs+ and FFCCs- in n1 positive NSCLC patients. (A) 5Y-RFS rates; (B) 5Y-OS rates; Kaplan-Meyer survival analysis and log-rank test; FFCCs, free-floating cancer cells; NSCLC, non-small cell lung cancer; HR, hazard ratios; CIs, confidence intervals; 5Y-RFS, 5-year relapse-free survival; 5Y-OS, 5-year overall survival.

Table II. Detection rates of FFCC in the recurrence and the non-recurrence groups.

Total 164 cases (predictive accuracy 81.7%)	Recurrence group (n=36)	Non-recurrence group (n=128)
FFCC(+) 18 cases (PPV 66.7%)	12 cases[a] (Sensitivity 33.3%)	6 cases (FP rate 33.3%)
FFCC(-) 146 cases (NPV 83.6%)	24 cases (FN rate 16.4%)	122 cases (specificity 95.3%)

[a]P<0.001, Odds ratio, 10.167 (95% CI, 3.537-29.225). FFCC, free-floating cancer cell in lymph node sinuses; PPV, positive predictive value; NPV, negative predictive value; FP, false positive; FN, false negative.

Table III. Pattern and site of recurrence/metastasis (N=47).

Variable (number of recurrences)	Total cases (n=43)	FFCC (-) (n=29)	FFCC (+) (n=14)	P-value
Site of recurrence/metastasis, n				
Local	2	1	1	P=1.000
Pleural dissemination/	7	4	3	P=0.666
Carcinomatous pleuritis				
Lymph node	7	3	5	P=0.253
Lung	15	15	0	P<0.001
Brain	5	3	1	P=1.000
Bone	6	2	4	P=0.153
Liver	0	0	0	P=1.000
Others/unknown	1	1	0	P=1.000

FFCC, free-floating cancer cell in lymph node sinuses.

circulating in blood using a cell search system (CellTrack Analyzer), and demonstrated that CTCs predict metastasis and survival in breast cancer patients (9). Furthermore, previous studies demonstrated that CTCs are of prognostic significance in lung cancer patients (10-14). Thus, CTCs and FFCCs may have similar properties despite being present in different locations (blood and lymph node sinuses, respectively). However, that is a mere conjecture and requires more investigations.

The detection of FFCCs using DAB, which stains cytokeratin brown, is complicated by the presence of black coal dust inside the lymph nodes. In the present study, we used Fast Red staining to enable the distinction from coal dust, and so we could find FFCCs more quickly and were not tired relatively.

It seems to be difficult to count the exact number of dissected lymph nodes in the chest, different from the colon and stomach because the lymph nodes in the chest often are not separate but seem to be a lump. However, in this time, we analyzed the number of dissected lymph nodes between the FFCCs+ group and FFCCs- group as exact as possible. Then, there were no significant differences in the number of dissected lymph nodes between the groups FFCCs+ and FFCCs- among both all patients and n1 positive patients (Table IV).

In a previous study on gastric cancer patients, FFCCs were identified in poorly differentiated tissues such as signet ring cell carcinoma and poorly differentiated adenocarcinoma (3). In the present study, we detected FFCCs in all histological types of lung cancers, including adenocarcinoma, squamous cell carcinoma, large cell carcinoma, and others, with no significant differences (Table I). This result indicates that FFCCs originate from any histological type of NSCLC.

Recurrence in lung cancer patients takes various forms, such as metastasis in the lungs, brain, bone, liver, adrenal glands, and lymph nodes as well as pleural dissemination. And, in this study, lung metastases were lower in FFCCs+ patients than FFCCs- patients (Table III). In addition, FFCCs were not detected in stage II (T3N0M0) patients, in which T3 was restricted to metastasis in the same lobe as the location of the primary tumor (data not shown). Thus, lung metastases might be occurred through the respiratory tract which has been found in patients with invasive mucinous adenocarcinoma or papillary adenocarcinoma (15,16), rather than via the lymphatic and vascular systems associated with FFCCs. But, that is a mere conjecture and the evidence as above requires more minute investigations including prospective cohort studies with a large number of patients.

Lung cancer has the highest incidence and mortality rates among all malignant tumors worldwide (17). Stage I/II lung cancer patients sometimes have a poor prognosis, with 5-year survival rates varying between 53 and 92% (18). Recently, the TNM classification became eighth edition, thus we reclassified this study patients according the latest 8th TNM staging system and reanalyzed the 5Y-RFS rates and the 5Y-OS rates on the 8th TNM stage. Then, two patients of FFCCs+ group and four patients of FFCCs- group became stage IIIA (among n1 positive patients, one patients of FFCCs+ group and two patients of FFCCs- group became stage IIIA). 5Y-RFS rates of

Table IV. The number of dissected lymph nodes.

	Total cases	FFCCs (-)	FFCCs (+)	P-value
All patients	(n=164)	(n=146)	(n=18)	
Lymph nodes, median (range)	14 (45)	13 (45)	22 (34)	P=0.156[a]
N1 positive patients	(n=22)	(n=13)	(n=9)	
Lymph nodes, mean (±SD)	17 (±8)	16 (±6)	18 (±10)	P=0.101[b]

FFCCs, free-floating cancer cells in lymph node sinuses; SD, standard deviation; [a]Mann-Whitney U test; [b]T test.

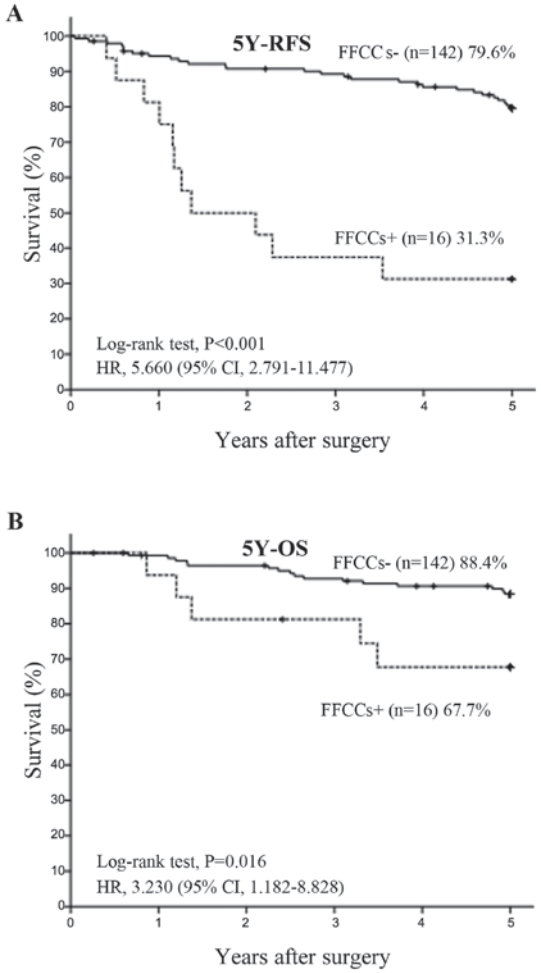

Figure 4. Recurrence in and prognosis of FFCCs+ and FFCCs- in all NSCLC patients (according to the 8th TNM classification version). (A) 5Y-RFS rates; (B) 5Y-OS rates; Kaplan-Meyer survival analysis and log-rank test; FFCCs, free-floating cancer cells; NSCLC, non-small cell lung cancer; HR, hazard ratios; CIs, confidence intervals; 5Y-RFS, 5-year relapse-free survival; 5Y-OS, 5-year overall survival.

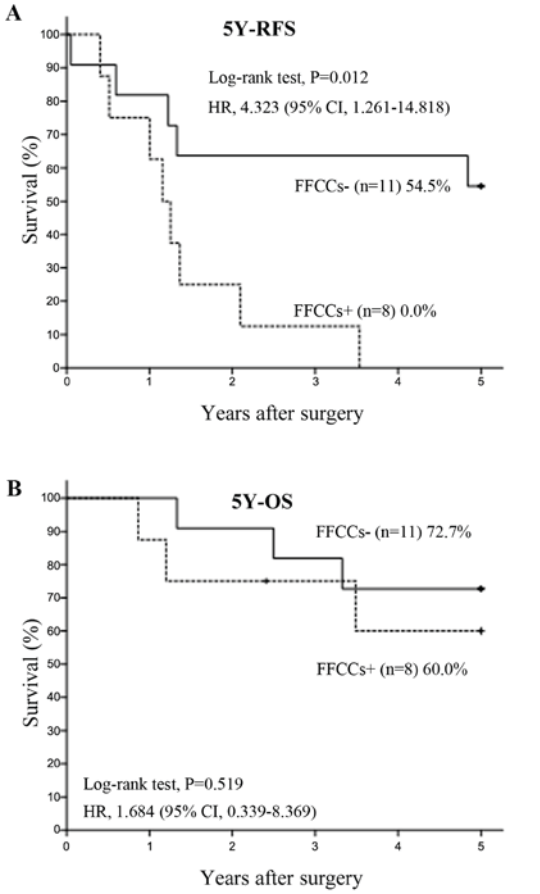

Figure 5. Recurrence in and prognosis of FFCCs+ and FFCCs- in n1 positive NSCLC patients (according to the 8th TNM classification version). (A) 5Y-RFS rates; (B) 5Y-OS rates; Kaplan-Meyer survival analysis and log-rank test; FFCCs, free-floating cancer cells; NSCLC, non-small cell lung cancer; HR, hazard ratios; CIs, confidence intervals; 5Y-RFS, 5-year relapse-free survival; 5Y-OS, 5-year overall survival.

FFCCs+ groups in all patients (Fig. 4A) and n1 positive patients (Fig. 5A) were also significant lower than FFCCs- groups. The 5Y-OS rate of FFCCs+ group in all patients was also significant lower than FFCCs- group (Fig. 4B). The 5Y-OS rate of FFCCs+ group in n1 positive patients also tended to be lower than FFCCs- group (Fig. 5B).

In the present study, FFCCs were detected in 11.0% of stage I/II lung cancer patients (18/164), and their presence was associated with a poor prognosis (Fig. 2A and B). Our results indicate that FFCCs have potential as useful markers for identifying patients at high risk of postoperative recurrence. In addition, the lack of FFCCs may be associated with patients who are at low risk of recurrence based on the high detection specificity and negative predictive value. Therefore, future clinical practice may consider additional postoperative adjuvant chemotherapy and more frequent follow-ups for FFCCs+ patients, and the omission of postoperative adjuvant chemotherapy and less frequent follow-ups for FFCCs- patients.

In conclusion, the presence of FFCCs in stage I/II lung cancer patients was associated with a poor prognosis. In

addition, FFCCs in hilar lymph node positive patients also have potential as a useful marker foreseeing the recurrence.

Acknowledgements

The authors would like to thank laboratory technicians Mr. Machida Tomohisa, Mrs. Nomura Nozomi and Miss Hagiwara Noriko from the Department of Pathology, Tokai University Hachioji Hospital (Hachioji-shi, Japan), and Dr. Suga Atushi (Department of General Thoracic Surgery, Yamato Manicipal Hospital, Yamato-shi, Japan) and Dr. Hamanaka Rurika (Department of General Thoracic Surgery, Ebina General Hospital, Ebina-shi, Japan) who collected the data from patients that were transferred to another hospital during the observation period.

Funding

This study was supported by grants from AstraZeneca K.K. and Eli Lilly Japan K.K.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YN acquired, analyzed and interpreted all of the data, and was a major contributor in writing the manuscript. MM made substantial contributions to conception and design. SH, TS and TT performed the histological examination of FFCCs. KK, SY and MI acquired, analyzed and interpreted all of the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Tokai University Institutional Review Board for Clinical Research (IRB no. 14R-225, Isehara, Japan). This study is a retrospective study using the sample which had already been gotten in the past. It was difficult to obtain consent from some research subjects, because they were deceased. Thus, in accordance with 'Ethical Guidelines for Medical and Health Research Involving Human Subjects' as indicated by the Japanese Ministry of Health, Labour and Welfare and the Japanese Ministry of Education, Culture, Sports, Science and Technology, we made information public including the purpose of utilization of the sample with respect to implementing the research and ensured the opportunities to refuse that the research be implemented for the research subjects.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Mukai M, Sato S, Komatsu N, Nishida T, Shiba K, Ito I, Nakasaki H and Makuuchi H: Correlation between occult neoplastic cells in the lymph node sinuses and recurrence in patients with Dukes' C colorectal cancer. Oncol Rep 10: 1165-1169, 2003.
2. Mukai M, Sato S, Komatsu N, Nishida T, Shiba K, Ito I, Nakasaki H and Makuuchi H: Correlation between occult neoplastic cells in the lymph node sinuses and recurrence in patients with curatively resected Dukes' B colorectal cancer. Oncol Rep 10: 1177-1181, 2003.
3. Sekido Y, Mukai M, Yamazaki M, Tajima T, Yamamoto S, Hasegawa S, Kishima K, Tajiri T and Nakamura N: Occult neoplastic cells in lymph node sinuses and recurrence/metastasis of stage II/III gastric cancer. Oncol Let 7: 53-58, 2014.
4. Mukai M, Sato S, Nakasaki H, Tajiri T, Saito Y, Nishiumi N, Iwasaki M, Tokuda Y, Ogoshi K, Inoue H and Makuuchi H: Occult neoplastic cells in the lymph node sinuses and recurrence of primary breast, lung, esophageal and gastric cancer. Oncol Rep 11: 81-84, 2004.
5. Mukai M, Sato S, Tajima T, Ninomiya H, Wakui K, Komatsu N, Tsuchiya K, Nakasaki H and Makuuchi H: Recurrence and 5-FU sensitivity of stage I/II node-negative breast, lung, or gastric cancer with occult neoplastic cells in lymph node sinuses. Oncol Rep 15: 815-820, 2006.
6. Conner JR, Cibas ES, Hornick JL and Qian X: Wilms Tumor 1/Cytokeratin Dual-Color Immunostaining reveals distinctive staining patterns in metastatic melanoma, metastatic carcinoma, and mesothelial cells in pleural fluids: An effective first-line test for the workup of malignant effusions. Cancer Cytopathol 122: 586-595, 2014.
7. Kaplan EL and Meier P: Nonparametric estimation from incomplete observations. J Am Stat Assoc 53: 457-481, 1958.
8. Cox DR: Regression models and life-tables. J R Stat Soc B 34: 187-220, 1972.
9. Rack B, Schindlbeck C, Jückstock J, Andergassen U, Hepp P, Zwingers T, Friedl TW, Lorenz R, Tesch H, Fasching PA, *et al*: Circulating tumor cells predict survival in early average-to-high risk breast cancer patients. J Natl Cancer Inst 106: pii: dju066, 2014.
10. Igawa S, Gohda K, Fukui T, Ryuge S, Otani S, Masago A, Sato J, Murakami K, Maki S, Katono K, *et al*: Circulating tumor cells as a prognostic factor in patients with small cell lung cancer. Oncol Let 7: 1469-1473, 2014.
11. Naito T, Tanaka F, Ono A, Yoneda K, Takahashi T, Murakami H, Nakamura Y, Tsuya A, Kenmotsu H, Shukuya T, *et al*: Prognostic impact of circulating tumor cells in patients with small cell lung cancer. J Thorac Oncol 7: 512-519, 2012.
12. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, Priest LJ, Greystoke A, Zhou C, Morris K, *et al*: Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. J Clin Oncol 30: 525-532, 2012.
13. Allard WJ, Matera J, Miller MC, Repollet M, Connelly MC, Rao C, Tibbe AG, Uhr JW and Terstappen LW: Tumor cells circulate in the peripheral blood of all major carcinomas but not in healthy subjects or patients with nonmalignant diseases. Clin Cancer Res 10: 6897-6904, 2004.
14. Huang J, Wang K, Xu J, Huang J and Zhang T: Prognostic significance of circulating tumor cells in non-small-cell lung cancer patients: A meta-analysis. PLoS One 8: e78070, 2013.
15. Aokage K, Ishii G, Nagai K, Kawai O, Naito Y, Hasebe T, Nishimura M, Yoshida J and Ochiai A: Intrapulmonary metastasis in resected pathological stage IIIB non-small cell lung cancer: Possible contribution of aerogenous metastasis to the favorable outcome. J Thorac Cardiovasc Surg 134: 386-391, 2007.
16. Gaeta M, Blandino A, Pergolizzi S, Mazziotti S, Caruso R, Barone M and Cascinu S: Patterns of recurrence of bronchioloalveolar cell carcinoma after surgical resection: A radiological, histological, and immunohistochemical study. Lung Cancer 42: 319-326, 2003.
17. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917, 2010.
18. Goldstraw P, Chansky K, Crowley J, Rami-Porta R, Asamura H, Eberhardt WE, Nicholson AG, Groome P, Mitchell A, Bolejack V, *et al*: The IASLC lung cancer staging project: Proposals for revision of the TNM stage groupings in the forthcoming (Eighth) Edition of the TNM classification for lung cancer. J Thorac Oncol 11: 39-51, 2016.