provides better progression-free survival but not overall survival.[4,5] The purpose of this multi-institutional study was to evaluate prognostic indicators of clinical N2 NSCLC patients treated with concurrent chemoradiotherapy followed by pulmonary resection.

Patients and Methods

We investigated 52 patients with surgical resectable clinical N2 NSCLC who underwent preoperative concurrent chemoradiotherapy before pulmonary resection between 1995 and 2008 at Osaka Prefectural Medical Center for Respiratory and Allergic Disease, Toneyama National Hospital, and Osaka University Hospital. An institutional review board approved this retrospective study and written informed consent for surgical intervention was obtained from each patient. Patients were assessed by a thoracic surgeon, radiation oncologist, and medical oncologist to establish that the cancer was potentially technically resectable. Preoperative pathological N2 disease was shown by mediastinoscopy (n = 35), perbronchial biopsy (n = 13), and thoracoscopy (n = 4) findings.

Patients received 2 cycles of chemotherapy every 4 weeks as follows: PV(M) regimen, cisplatin 80 mg/m^2 on day 1 and vindesine 3 mg/m^2 on days 1 and 8 with or without mitomycin 8 mg/ m^2 on day 1 according to the protocol of Japan Clinical Oncology Group 9209 and 9806[6,7]; nPV regimen, cisplatin 80 mg/m^2 on day 1 and vinorelbine 20 mg/m^2 on days 1 and 8 every 4 weeks according to the protocol described by Naito et al.[8] Radiotherapy directed at the tumor and mediastinal nodes was started on day 2 concurrently in cycle 1 and the total radiation dose was 40 Gy in 20 fractions administered over 4 weeks. For poor responding patients (n = 10), an additional dose of 10 to 20 Gy was administered. Following induction chemoradiotherapy, each case was reevaluated to determine clinical response and indications for resection. A thoracotomy was performed in all patients 6 to 8 weeks after completion of chemoradiotherapy. The surgical procedures included a lobectomy or pneumonectomy with systemic node dissection.

A chi-square test was used to compare the results. Overall survival rates were estimated by the method of Kaplan and Meier, and compared with results of a log-rank test. All patient characteristics were tested multivariately against the primary outcome using a Cox-regression analysis based on the tested variable. All statistical analyses were performed using Statview version 5.0 for Windows (Abacus Concepts, Berkeley, CA). A p-value of <0.05 was considered to be statistically significant.

Table 1 Patient Characteristics (n = 52)

Characteristics	No. of patients
Gender	
Male	43
Female	9
Clinical stage	
IIIA	40
IIIB	12
Clinical T stage	
T1	5
T2	27
T3	8
T4	12
Clinical N stage	
N2	52
Histology	
Adenocarcinoma	34
Squamous cell carcinoma	15
Others	3
Type of mediastinal staging	
Mediastinoscopy	35
Perbronchial biopsy	13
Thoracoscopy	4

Results

The median patient age was 57 years old (range 32–73 years), and their clinicopathologic characteristics are shown in **Table 1**. The median radiation dose was 44 Gy (30–60 Gy), and data for preoperative treatments and surgical procedures are presented in **Table 2**. The overall cardiopulmonary morbidity, early mortality, and 90-day mortality rates were 38% (n = 20), 3.8% (n = 2), and 7.6% (n = 4), respectively. Postoperative cardiopulmonary morbidity rate was not associated with type of chemotherapy (CDDP + VDS vs. CDDP + VDS + MMC vs. CDDP + VNR; morbidity rate 47% vs. 45% vs. 22%; p = 0.38) or radiation dose (40 Gy ≥ vs. 40 Gy<; 46% vs 30%; p = 0.36), while it was greater in patients who underwent a pneumonectomy than in those who underwent a lobectomy (73% vs. 29%, p = 0.01).

Twenty-one patients were alive after a median follow-up period of 4.8 years, and the overall 5-year survival rate was 38% (**Fig. 1A**). Complete pathological response by the tumor was found in 11 patients (21%, see **Table 3A**), though there was no significant difference in survival rate according to tumor pathological response [Complete pathological response (CR-tumor) vs. Non-complete pathological response (NCR-tumor), p = 0.07, **Fig. 1B**).

Table 2 Preoperative treatment and surgical procedures, and results

Procedure/Result	No. of patients
Chemotherapy	
CDDP + VDS	31
CDDP + VDS+MMC	12
CDDP + VNR	9
Radiation dose	
40 Gy ≥	40
40 Gy <	12
Surgical procedure	
Lobectomy	43
Pneumonectomy	9
With combined resection	
Bronchoplasty	8
Rib	4
Pericardium	4
Major vessel	2
Diaphragma	2
Morbidity	
None	32
Pneumonia	7
(ARDS	2)
(Radiation pneumonitis	2)
Atelectasis	4
Empyema	4
(Broncho-fistula	2)
Chylothorax	2
Arrythmia	5
Heart failure	2
Mortality	3
ARDS	2
Hemoptysis	1

CDDP: cisplatin; VDS: vindesine; MMC: mitomycin; VNR: vinorelbine ARDS: adult respiratory distress syndrome

Pathological N0-1 status was found in 29 patients, (56%) and overall survival was better in those with a lower p-N status (Pathological N0-1 status vs. N2 status, p = 0.005, **Fig. 1C**), as the 5-year survival rate was 58% in patients with stage p-N0-1 disease and 0% in those with p-N2 disease. Overall survival did not differ according to the surgical procedure employed (lobectomy vs. pneumonectomy, p = 0.74).

Response to induction therapy by the primary tumor was correlated with postoperative nodal stage (p = 0.01, **Table 3B**). Only one patient with CR-tumor belonged to p-N2 group. The 5-year survival rate was 90% in patients with CR-tumor and p-N0-1 disease, 51% in those with NCR-tumor and p-N0-1 disease, and 0% in those with NCR-tumor and p-N2 disease. In univariate analysis, postoperative nodal stage was a prognostic factor (**Table 3C**). Two variables, including tumor response to

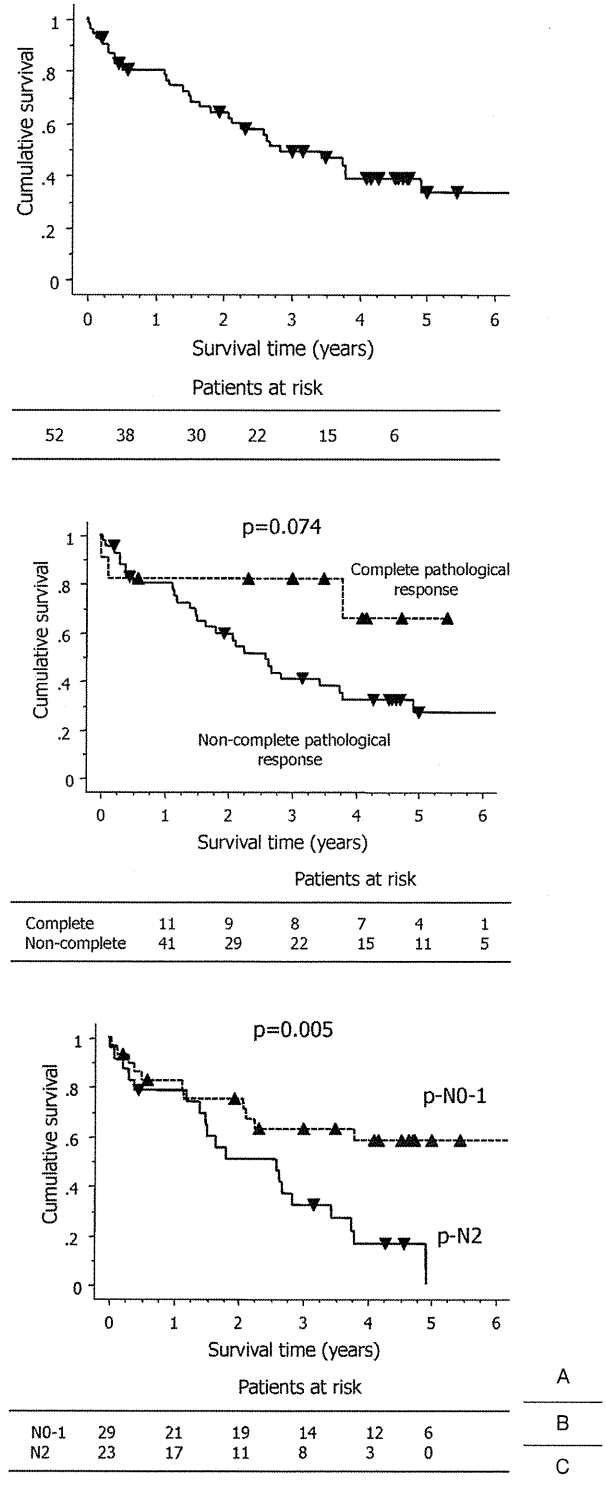


Fig. 1

A: Overall survival for all cases (n = 52).

B: Overall survival according to pathological response of the tumor.

C: Overall survival according to pathological nodal status after chemoradiotherapy.

Table 3

A. Postoperative pathologic results

Characteristics	No. of patients
Pathologic T stage	
T0*	11
T1	16
T2	18
T3	6
T4	1
Pathologic N stage	
N0	25
N1	4
N2	23

*T0, pathological complete response in primary tumor

B. Postoperative pathologic response in tumor and lymph nodes

Pathologic response in tumor	N status	
	p-N0-1	p-N2
Complete	10	1
Non-complete	19	22

*T0, pathological complete response; p = 0.01, chi-square test

C. Univariate analysis of prognostic factors

Variable	Hazard ratio (95% CI)	p value
Tumor response		
Non-complete vs. complete	3.40 (0.81–14.3)	0.09
p-N status		
N2 vs N0-1	2.81 (1.29–6.11)	0.01

D. Multivariate analysis of prognostic factors

Variable	Hazard ratio (95% CI)	p value
Tumor response		
Non-complete vs. complete	1.68 (0.44–6.35)	0.444
p-N status		
N2 vs N0-1	2.40 (1.04–5.57)	0.041

CRT and postoperative nodal stage which were correlated with each other, were analyzed to determine the variables having an effect on survival in NSCLC patients (**Table 3D**). The analysis revealed that postoperative nodal stage was an independent prognostic factor.

Discussion

Cisplatin-vinca alkaloid-based chemotherapy combined with radiation was administered in the present cases, which has been reported to be the most popular and well-tolerated combination for NSCLC in Japan.[6–8] We previously used cisplatin and vindesine in the 1990s, and then changed vindesine to vinorelbine in the 2000s. Chemotherapy regimens with new agents, including paclitaxel, docetaxel, and gemcitabine, have recently been reported to be effective for advanced NSCLC.[9,10] These new agents combined with radiotherapy may also improve overall long-term survival by inducing an increased pathological response by the tumor to induction therapy in patients with local, advanced NSCLC.[11]

Similar to the German Lung Cancer Cooperative Group (GLCCG) trial,[5] we found that preoperative concurrent chemoradiotherapy increased pathological response and mediastinal down-staging, while multivariate analysis showed that pathological N status was an independent predictor of survival. These results suggest that new methods for achieving better control of mediastinal lymph node involvement are needed to improve the overall outcome for patients with locally advanced NSCLC. Along that line, we believe that it is important to accurately select patients in whom preoperative N2 or N3 disease will be controlled by induction chemoradiotherapy for improvement of long-term survival with trimodality therapy. Since N2 disease after chemoradiotherapy has a negative prognostic significance, nodal status should be assessed after induction chemoradiotherapy using computed tomography (CT) and ^{18}F-fluorodeoxy glucose-positron emission tomography (FDG-PET) examinations. When apparent N2 disease is confirmed even after induction chemoradiation, it may be best to avoid a pulmonary resection and treat the patient with definitive chemoradiotherapy. In addition, aggressive staging with EBUS-TBNA as well as a re-mediastinoscopy can be performed, in addition to conventional examinations to exclude such patients from surgical resection.[12–14] On the other hand, response to induction therapy by the primary tumor was shown to be correlated with postoperative down-staging of mediastinal nodes in the present study; thus it is necessary to evaluate using CT or FDG-PET to monitor

treatment response by the primary tumor after induction chemoradiotherapy and predict down-staging of mediastinal nodes. The value of FDG-PET for the primary tumor and its response to therapy should be evaluated in a future study.

The most commonly used dose for induction therapy ranges from 40 to 45 Gy.[2,8] Sonett et al. reported that high dose radiation promotes a high pathologically complete response rate and pulmonary resection may be performed safely after CRT including greater than 59 Gy radiotherapy.[11] In our series, 6 patients received 60 Gy radiotherapy as CRT and each safely underwent pulmonary resection.

The type of surgery is also a determinant of morbidity and mortality. Albain and colleagues recently reported significantly higher mortality rates with a pneumonectomy (26%) as compared to a lobectomy (1%); thus no survival difference was found between the surgical and non-surgical arms in their study,[15] because the increased mortality in patients who required a pneumonectomy after induction chemoradiotherapy adversely affected the overall survival of the surgical group. However, some reports have shown that induction chemoradiotherapy does not increase the risk of morbidity and mortality after a pneumonectomy in properly selected patients.[3,15] In the present study, there was no significant difference for 90-day mortality and overall survival between patients who underwent a pneumonectomy and those who received a lobectomy, whereas cardiopulmonary morbidity and early mortality rates were greater in patients with a pneumonectomy than those with a lobectomy. Notably, a right pneumonectomy after chemoradiotherapy has been reported to be associated with relatively increased risk and should be performed only in selected patients.[16]

Our study has some potential limitations, as it was an observational study performed at 3 different centers and not a randomized trial. Therefore, multicenter prospective randomized trials in Japan are needed for accurate investigation of the effects and risks of trimodality treatment in patients with NSCLC.

Conclusion

Pathological response by mediastinal lymph nodes to preoperative concurrent chemoradiotherapy determined the prognosis in our patients with N2 NSCLC.

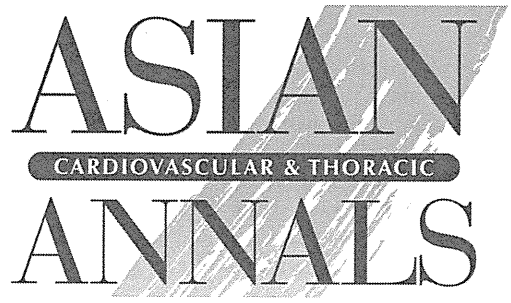
Acknowledgment

We thank Dr Yuko Ohno (professor of Department of Mathematical Health Science, Osaka University Graduate School of Medicine) for her assistance on some statistical work.

References

1) Friedel G, Budach W, Dippon J, et al. Phase II trial of a trimodality regimen for stage III non-small-cell lung cancer using chemotherapy as induction treatment with concurrent hyperfractionated chemoradiation with carboplatin and paclitaxel followed by subsequent resection: a single-center study. J Clin Oncol 2010; **28**: 942-8.
2) Uy KL, Darling G, Xu W, et al. Improved results of induction chemoradiation before surgical intervention for selected patients with stage IIIA-N2 non-small cell lung cancer. J Thorac Cardiovasc Surg 2007; **134**: 188-93.
3) Weder W, Collaud S, Eberhardt WE, et al. Pneumonectomy is a valuable treatment option after neoadjuvant therapy for stage III non-small-cell lung cancer. J Thorac Cardiovasc Surg 2010; **139**: 1424-30.
4) Gilligan D, Nicolson M, Smith I, et al. Preoperative chemotherapy in patients with resectable non-small cell lung cancer: results of the MRC LU22/NVALT 2/EORTC 08012 multicentre randomised trial and update of systematic review. Lancet 2007; **369**: 1929-37.
5) Thomas M, Rübe C, Hoffknecht P, et al. Effect of preoperative chemoradiation in addition to preoperative chemotherapy: a randomised trial in stage III non-small-cell lung cancer. Lancet Oncol 2008; **9**: 636-48.
6) Nagai K, Tsuchiya R, Mori T, et al. A randomized trial comparing induction chemotherapy followed by surgery with surgery alone for patients with stage IIIA N2 non-small cell lung cancer (JCOG 9209). J Thorac Cardiovasc Surg 2003; **125**: 254-60.
7) Kunitoh H, Kato H, Tsuboi M, et al. Phase II trial of preoperative chemoradiotherapy followed by surgical resection in patients with superior sulcus non-small-cell lung cancers: report of Japan Clinical Oncology Group trial 9806. J Clin Oncol 2008; **26**: 644-9.
8) Naito Y, Kubota K, Nihei K, et al. Concurrent chemoradiotherapy with cisplatin and vinorelbine for stage III non-small cell lung cancer. J Thorac Oncol 2008; **3**: 617-22.
9) Pectasides D, Kalofonos HP, Samantas E, et al. An out-patient second-line chemotherapy with gemcitabine and vinorelbine in patients with non-small cell lung cancer previously treated with cisplatin-based chemotherapy. A phase II study of the Hellenic co-operative Oncology Group. Anticancer Res 2001; **21**: 3005-10.
10) Sandler AB, Nemunaitis J, Denham C, et al. Phase III

trial of gemcitabine plus cisplatin versus cisplatin alone in patients with locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol 2000; **18**: 122-30.
11) Sonett JR, Suntharalingam M, Edelman MJ, et al. Pulmonary resection after curative intent radiotherapy (>59 Gy) and concurrent chemotherapy in non-small-cell lung cancer. Ann Thorac Surg 2004; **78**: 1200-5.
12) Surmont V, van Klaveren RJ, Goor C, et al. Lessons to learn from EORTC study 08981: a feasibility study of induction chemoradiotherapy followed by surgical resection for stage IIIB non-small cell lung cancer. Lung Cancer. 2007; **55**: 95-9.
13) Ryu JS, Choi NC, Fischman AJ, et al. FDG-PET in staging and restaging non-small cell lung cancer after neoadjuvant chemoradiotherapy: correlation with histopathology. Lung Cancer 2002; **33**: 179-87.
14) Albain KS, Swann RS, Rusch VW, et al. Radiotherapy plus chemotherapy with or without surgical resection for stage III non-small-cell lung cancer: a phase III randomised controlled trial. Lancet 2009; **374**: 379-86.
15) Refai M, Brunelli A, Rocco G, et al. Does induction treatment increase the risk of morbidity and mortality after pneumonectomy? A multicentre case-matched analysis. Eur J Cardiothorac Surg 2010; **37**: 535-9.
16) Martin J, Ginsberg RJ, Abolhoda A, et al. Morbidity and mortality after neoadjuvant therapy for lung cancer: the risks of right pneumonectomy. Ann Thorac Surg 2001; **72**: 1149-54.



Nutritional status of patients undergoing chemoradiotherapy for lung cancer

Yasushi Shintani, Naoki Ikeda, Tomoshige Matsumoto, Yoshihisa Kadota, Meinoshin Okumura, Yuko Ohno and Mitsunori Ohta

Asian Cardiovasc Thorac Ann 2012;20:172-176
DOI: 10.1177/0218492311435249

Original Article

ASIAN CARDIOVASCULAR & THORACIC ANNALS

Asian Cardiovascular & Thoracic Annals
20(2) 172–176

DOI: 10.1177/0218492311435249
aan.sagepub.com
SAGE

Nutritional status of patients undergoing chemoradiotherapy for lung cancer

Yasushi Shintani[1,2], Naoki Ikeda[2], Tomoshige Matsumoto[3], Yoshihisa Kadota[2], Meinoshin Okumura[1], Yuko Ohno[4] and Mitsunori Ohta[2]

Abstract

Impaired nutrition is an important predictor of perioperative complications in lung cancer patients, and preoperative chemoradiotherapy increases the risk of such complications. The goal of this study was to assess the effect of an immune-enhancing diet on nutritional status in patients undergoing lung resection after chemoradiotherapy. We compared the preoperative nutritional status in 15 patients with lung cancer undergoing lung resection without chemoradiotherapy and 15 who had chemoradiotherapy. Body mass index and lymphocyte counts were lower in patients who had chemoradiotherapy. Although there was no difference in the rate of postoperative morbidity between groups, the chemoradiotherapy patients were more likely to have severe complications postoperatively. After chemoradiotherapy in 12 patients, 6 received oral Impact for 5 days, and 6 had a conventional diet before surgery. Oral intake of Impact for 5 days before surgery modified the decrease in transferrin and lymphocytes after the operation. Preoperative immunonutrition may improve the perioperative nutritional status after induction chemoradiotherapy in patients undergoing lung cancer surgery, and reduce the severity of postoperative complications. These potential benefits need to be confirmed in a randomized controlled trial.

Keywords

carcinoma, non-small-cell lung, chemotherapy, adjuvant, enteral nutrition

Introduction

A successful treatment protocol for patients with locally advanced non-small-cell lung cancer (NSCLC) remains controversial. The results of surgical resection alone for locally advanced NSCLC are poor, thus the option of induction chemoradiotherapy (CRT) has been reported.[1,2] However, preoperative CRT increases the risk of perioperative complications in patients with lung cancer, with failure of antibiotics given for pneumonia being the most common life-threatening complication.[3] We speculated that infections occurring after the operation might be related to poor nutritional status caused by CRT. Although the role of nutrition in the outcome of surgery for lung cancer has been addressed in only a few studies, impaired nutrition is reported to be associated with an increased risk of complications after the operation.[4] The first purpose of this study was to determine the preoperative nutritional variables after CRT in patients with lung cancer.

Perioperative alimentation to reduce postoperative complications and improve surgical outcome has been extensively studied. In addition, the availability of immune-enhancing diets has raised the possibility of decreasing the incidence of complications during the

[1]Department of Thoracic Surgery, Osaka University Graduate School of Medicine, Osaka, Japan
[2]Department of General Thoracic Surgery, Osaka Prefectural Medical Center for Respiratory and Allergic Disease, Osaka University Graduate School of Medicine, Osaka, Japan
[3]Department of Clinical Research and Development, Osaka Prefectural Medical Center for Respiratory and Allergic Disease, Osaka University Graduate School of Medicine, Osaka, Japan
[4]Department of Mathematical Health Science, Osaka University Graduate School of Medicine, Osaka, Japan

Corresponding author:
Yasushi Shintani, MD, PhD, Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, Osaka, Japan
Email: yshintani@thoracic.med.osaka-u.ac.jp

postoperative course. Impact (Ajinomoto Pharma, Tokyo, Japan), which contains arginine, RNA, and ω-3 polyunsaturated fatty acids, has been reported to reinforce immunological function as well as improve nutritional status.[5] Preoperative Impact administration in patients with gastrointestinal cancer was found to decrease the incidence of postoperative complications. Thus, we administered Impact after induction CRT in patients with lung cancer. The second goal of this study was to assess the effect of the immune-enhancing diet provided by Impact in patients undergoing lung resection after CRT.

Patients and Methods

We enrolled 15 patients with surgically resectable clinical N2 or N3 NSCLC, treated at Osaka Prefectural Medical Center for Respiratory and Allergic Disease or Osaka University, who underwent preoperative concurrent chemoradiotherapy before pulmonary resection. They received 2 cycles of cisplatin-based chemotherapy every 4 weeks as well as radiotherapy directed at the tumor and mediastinal nodes, which was started on day 2 of cycle 1. A thoracotomy was performed 6 to 8 weeks after completion of CRT. We compared the perioperative nutritional status of the enrolled patients after CRT with that of 15 randomly selected patients treated without CRT during the same period.

Twelve patients with NSCLC who underwent CRT between March 2008 and May 2010 at Osaka Prefectural Medical Center for Respiratory and Allergic Disease were divided into 2 groups: an immunonutrition group and a conventional diet group. Patients in the immunonutrition group received oral Impact (750–1,000 mL per day) for 5 days before surgery, with the amount determined by nutritionists, based on the diet of each patient during that period. We compared the perioperative nutritional status between these 2 groups. An institutional review board approved this study, and written informed consent for surgical intervention was obtained from each patient.

Measurements of serum protein levels and lymphocyte counts were used in conjunction with other parameters to determine the overall nutritional status of each patient before surgery and on postoperative days 1, 3, and 7. Serum proteins used for nutritional assessment included transferrin, prealbumin, and retinol-binding protein.

The chi-square test, Mann-Whitney *U* test, and Wilcoxon signed-ranks test were utilized to compare the results using Statview version 5.0 for

Table 1. Patient characteristics and preoperative nutritional status according to administration of CRT

Variable	CRT ($n = 15$)	No CRT ($n = 15$)	p Value
Age (years)	62 ± 8	62 ± 6	0.83
Sex (M/F)	10/5	11/4	0.69
Clinical stage			<0.0001
I	9		
II	5	3	
III	1	12	
Operative time (min)	227 ± 48	252 ± 58	0.27
Blood loss (mL)	303 ± 197	591 ± 460	0.30
Pathological stage			0.19
0		4	
I	9	7	
II	5	3	
III	1	1	
Body mass index ($kg \cdot m^{-2}$)	22.9 ± 2.1	20.8 ± 2.1	0.02
Transferrin ($mg \cdot dL^{-1}$)	192 ± 12	158 ± 14	0.38
Pre-albumin ($mg \cdot dL^{-1}$)	27.6 ± 9.5	23.1 ± 5.9	0.14
Retinol-binding protein ($mg \cdot dL^{-1}$)	4.1 ± 1.7	4.2 ± 1.0	0.44
Albumin ($g \cdot dL^{-1}$)	4.1 ± 0.4	3.9 ± 0.3	0.06
White blood cell count	6,267 ± 1971	5,388 ± 1844	0.6
Lymphocytes	1,837 ± 431	981 ± 574	< 0.0001

CRT = chemoradiotherapy.

Windows (Abacus Concepts, Berkeley, CA, USA). A p value <0.05 was considered to be statistically significant.

Results

The preoperative nutritional variables in lung cancer patients with and without induction CRT are listed in Table 1. The clinical stage was more advanced in patients with CRT. Although there was no significant difference in the levels of rapid turnover protein, albumin, and white blood cells, both body mass index and lymphocyte counts were lower in patients with CRT. These data suggest that induction CRT impairs the preoperative nutritional status of patients with lung cancer. Pneumonia, arrhythmia, and prolonged air leakage were the main complications in both groups. Pneumonia occurred in 5 of 15 patients with CRT (both required mechanical ventilation) and in 1 of 15 without CRT. Arrhythmia or prolonged air leakage occurred in 3 patients with CRT and 2 without CRT. Although there was no difference between the 2 groups in the rate of postoperative morbidity, the patients who had CRT were more likely to have a severe complication postoperatively.

All patients in the immunonutrition group were administered Impact without complications, although 2 of them reduced the amount of their conventional diet while taking Impact. There was no significant difference in patient background and nutritional status between these 2 groups (Table 2). Although oral Impact for 5 days before surgery did not cause changes in pre-albumin and retinol-binding protein, immunonutrition alleviated the decrease of transferrin and lymphocytes after the operation, compared to the conventional diet group (Figure 1). There were no deaths in either group. Pneumonia and prolonged air leakage were the main complications in both. Two patients in the immunonutrition group suffered postoperative pneumonia, and 2 in the conventional diet group had both postoperative pneumonia and prolonged air leakage. There was no difference between these groups in the rate of postoperative morbidity.

Discussion

Nutritional assessment is the first step in effective treatment of malnutrition, during which specific data are obtained to create a metabolic and nutritional profile of the patient. The primary goal of nutritional assessment is identification of patients who are at risk of developing malnutrition. Some studies have suggested that certain nutritional indicators such as weight loss and serum albumin concentration may predict complications after an operation, particularly postoperative infections.[6,7] One mechanism by which nutritional status may influence outcome is the effect on respiratory muscle strength in generation of an effective cough.[4] Furthermore, Tewari and colleagues[8] reported that nutritional status is a predictor of long-term survival that is independent of tumor extension and staging.

Table 2. Clinical data of patients given Impact or a conventional diet after induction chemoradiotherapy

Variable	Impact ($n=6$)	No Impact ($n=6$)	p Value
Age (years)	62.3 ± 4.5	62.3 ± 4.5	0.98
Clinical stage			0.29
IIB	0	2	
IIIA	5	3	
IIIB	1	1	
Pathological stage			0.48
0	2	3	
I	2	0	
II	2	1	
III	0	2	
Operative time (min)	257 ± 105	252 ± 66	0.93
Blood loss (mL)	675 ± 403	482 ± 312	0.45
Body mass index ($kg \cdot m^{-2}$)	21.2 ± 3.6	20.5 ± 1.5	0.76
Transferrin ($mg \cdot dL^{-1}$)	222 ± 17.6	205 ± 22.3	0.16
Pre-albumin ($mg \cdot dL^{-1}$)	22 ± 8.6	22 ± 4.8	0.63
Retinol-binding protein ($mg \cdot dL^{-1}$)	4.2 ± 1.8	4.2 ± 1.0	0.97
Lymphocytes	1,018 ± 772	896 ± 421	0.89

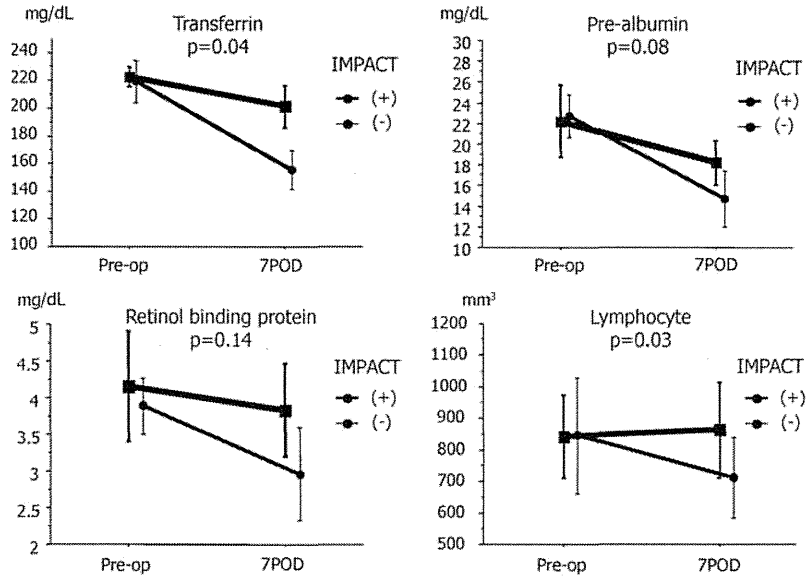


Figure 1. Evaluation of nutritional status in patients given Impact (+) and for those who consumed a conventional diet (−) following induction chemoradiotherapy. Bars indicate mean ± standard error. POD = postoperative day.

We found no difference in preoperative nutritional status between patients with NSCLC undergoing lung resection with and without CRT, based on rapid turnover protein values, while body mass index and lymphocyte counts were different. The patients who received CRT were in more advanced stages and underwent more invasive operations, so we could not precisely compare the nutritional status between the groups. However, these findings suggest that nutritional status might be impaired in patients who undergo CRT. If nutritional depletion has an important relationship with complications after surgery, improving oral intake in the period before the operation might be beneficial.

A 1-liter serving of Impact contains 1,000 kcal, 12.5 g arginine, 2.8 g ω-3 polyunsaturated fatty acids, and 1.23 g RNA, which not only ameliorate nutritional status but also greatly reinforce immunological function.[9] Xu and colleagues[5] reported that a preoperative immunonutrition diet in patients with gastrointestinal cancer improved nutritional status and immunity, and also decreased the incidence of postoperative complications and infections.[5] Lung cancer patients generally eat soon after the operation, thus their nutritional status may not significantly influence the outcome of surgery, and severe nutritional depletion is uncommon in patients with lung cancer.[10] Thus, we focused on patients who received induction CRT, and evaluated the effects of an immune-enhancing diet. Preoperative supplementation with Impact was well tolerated by our lung cancer patients who underwent induction CRT. There were no differences regarding patient background and nutritional status between the 2 groups. Oral intake of Impact for 5 days before the surgical procedure resulted in modification of the decrease in transferrin and lymphocytes after the operation, although there was no difference in the rate of postoperative morbidity between groups. Our findings suggest that preoperative immunonutrition may improve the outcome of patients undergoing lung cancer surgery after induction chemoradiotherapy. Our study has some limitations because it was a preliminary report of a small-scale study, and this potential benefit should be confirmed in a larger randomized controlled trial.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflicts of interest statement

None declared.

Acknowledgment

We thank all members of the nutrition support team at Osaka Prefectural Medical Center for Respiratory and Allergic Disease; especially Kyoko Morimoto, Itsuko Fujii, Kyoko Nishida, Yoko Kondo, and Mika Sakon for their assistance and encouragement.

References

1. Friedel G, Budach W, Dippon J, Spengler W, Eschmann SM, Pfannenberg C, et al. Phase II trial of a trimodality

regimen for stage III non-small-cell lung cancer using chemotherapy as induction treatment with concurrent hyperfractionated chemoradiation with carboplatin and paclitaxel followed by subsequent resection: a single-center study. *J Clin Oncol* 2010; 28: 942–8.
2. Uy KL, Darling G, Xu W, Yi QL, De Perrot M, Pierre AF, et al. Improved results of induction chemoradiation before surgical intervention for selected patients with stage IIIA-N2 non-small cell lung cancer. *J Thorac Cardiovasc Surg* 2007; 134: 188–93.
3. Roberts JR, Eustis C, Devore R, Carbone D, Choy H and Johnson D. Induction chemotherapy increases perioperative complications in patients undergoing resection for non-small cell lung cancer. *Ann Thorac Surg* 2001; 72: 885–8.
4. Jagoe RT, Goodship TH and Gibson GJ. The influence of nutritional status on complications after operations for lung cancer. *Ann Thorac Surg* 2001; 71: 936–43.
5. Xu J, Zhong Y, Jing D and Wu Z. Preoperative enteral immunonutrition improves postoperative outcome in patients with gastrointestinal cancer. *World J Surg* 2006; 30: 1284–9.
6. Busch E, Verazin G, Antkowiak JG, Driscoll D and Takita H. Pulmonary complications in patients undergoing thoracotomy for lung carcinoma. *Chest* 1994; 105: 760–6.
7. Martin-Ucar AE, Nicum R, Oey I, Edwards JG and Waller DA. En-bloc chest wall and lung resection for non-small cell lung cancer. Predictors of 60-day non-cancer related mortality. *Eur J Cardiothorac Surg* 2003; 23: 859–64.
8. Tewari N, Martin-Ucar AE, Black E, Beggs L, Beggs FD, Duffy JP, et al. Nutritional status affects long term survival after lobectomy for lung cancer. *Lung Cancer* 2007; 57: 389–94.
9. Bozzetti F, Braga M, Gianotti L, Gavazzi C and Mariani L. Postoperative enteral versus parenteral nutrition in malnourished patients with gastrointestinal cancer: a randomised multicentre trial. *Lancet* 2001; 358: 1487–92.
10. Hollaus PH, Wilfing G, Wurnig PN and Pridun NS. Risk factors for the development of postoperative complications after bronchial sleeve resection for malignancy: a univariate and multivariate analysis. *Ann Thorac Surg* 2003; 75: 966–72.

Lung Cancer 78 (2012) 212–218

Contents lists available at SciVerse ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

An accurate and rapid detection of lymph node metastasis in non-small cell lung cancer patients based on one-step nucleic acid amplification assay

Masayoshi Inoue[a,*], Kayo Hiyama[d], Kadzuki Nakabayashi[d], Eiichi Morii[b], Masato Minami[a], Noriyoshi Sawabata[a], Yasushi Shintani[a], Tomoyuki Nakagiri[a], Yoshiyuki Susaki[a], Jun Maeda[c], Masahiko Higashiyama[c], Jiro Okami[c], Yuichiro Yoshida[d], Junyi Ding[d], Yasuhiro Otomo[d], Meinoshin Okumura[a]

[a] *Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, Suita, Japan*
[b] *Department of Pathology, Osaka University Graduate School of Medicine, Suita, Japan*
[c] *Department of General Thoracic Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan*
[d] *Central Research Laboratory, Sysmex Corporation, Kobe, Japan*

ARTICLE INFO

Article history:
Received 25 April 2012
Received in revised form 16 July 2012
Accepted 23 August 2012

Keywords:
Lung cancer
Lymph node metastasis
One-step nucleic acid amplification assay
Polymerase chain reaction
Cytokeratin 19
Cytokeratin 7

ABSTRACT

A sublobar resection is currently recognized as an option for early small-sized non-small cell lung cancer (NSCLC), and intraoperative rapid and accurate lymph node assessment is required for a complete resection. To solve this issue, we investigated the clinical utility of one-step nucleic acid amplification (OSNA) assay, an automated rapid molecular diagnostic method and its optimal mRNA marker for detection of lymph node metastasis in lung cancer. We extracted 16 target candidate mRNA markers with high expression in lung cancer from a genetic database, and then quantified their expression levels by quantitative RT-PCR using surgically dissected lymph nodes with or without metastasis. Cytokeratin 19 (CK19), cytokeratin 7 (CK7), stratifin (SFN), and anterior gradient homolog 2 (AGR2) showed significant differences for mRNA expression between metastasis-negative and -positive lymph nodes in quantitative-RT-PCR screening. CK19 and CK7 were finally selected as potential target markers and were quantified using OSNA assay findings of 165 dissected lymph nodes obtained from 49 lung cancer patients. The OSNA assay with CK19 and CK7 were completed within 40 min and their positive predictive value, negative predictive value, and accuracy comparing to pathological diagnosis with hematoxylin–eosin staining and immunohistochemistry were shown to be 95.0%, 99.3%, and 98.8%, and 85.0%, 97.9%, and 96.4%, respectively, using a cut-off value of 250 copies/μL. Among the 165 lymph nodes tested, 1 false negative result was due to massive necrosis of cancer cells and 1 false positive was caused by the allocation bias of cancer cells in the sampling in patient with pleural dissemination. The best performance was observed when CK19 was used as a marker, while the addition of CK7 mRNA as a marker did not increase sensitivity or specificity. In conclusion, an OSNA assay using CK19 could be effective for molecular diagnosis of lymph node metastasis in lung cancer. This is the first report suggesting the potential clinical utility of OSNA assay for intraoperative rapid diagnosis of nodal status in lung cancer.

1. Introduction

Lung cancer is characterized by early metastasis and lymphatic spread with poor survival, and is the leading cause of malignancy related deaths in many parts of the world. The American Cancer Society estimates that 226,160 individuals in the United States will be diagnosed with lung cancer and 160,340 will die in 2012. Surgical intervention is mainly indicated for stage I and II non-small cell lung cancer (NSCLC) without mediastinal lymph node metastasis, while adjuvant chemotherapy is the commonly used evidence-based treatment for p-stage II and IIIA [1]. Thus, accurate diagnosis of lymph node metastasis is vitally important for deciding proper treatment strategies.

Recently, sublobar resection, such as a segmentectomy or wedge resection has been recognized as an option for early small-sized NSCLC, though a lobectomy remains the standard procedure in affected patients [2–5]. In cases treated by sublobar resection, intraoperative lymph node exploration is necessary to avoid incomplete resection, as a proportion of small-sized NSCLC may be locally advanced diseases [6–8]. The intraoperative evaluation of lymph

* Corresponding author at: Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, 2-2-L5 Yamadaoka, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 3152; fax: +81 6 6879 3164.
E-mail address: mi@thoracic.med.osaka-u.ac.jp (M. Inoue).

0169-5002/$ – see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.lungcan.2012.08.018

Table 1
Characteristics of non-small cell lung cancer patients and dissected lymph nodes.

Patients	49
Age	50–82 (median, 70)
Sex	
Male	33
Female	16
Histology	
Adenocarcinoma	36
Squamous cell carcinoma	8
Others	5
p-stage	
IA	12
IB	17
IIA	8
IIB	3
IIIA	4
IIIB	3
IV	2
Dissected lymph nodes	165
Metastasis positive	20
Adenocarcinoma	19
Squamous cell carcinoma	1
Metastasis negative	145

node is conventionally histologically diagnosed by using frozen sections, while only maximum cut-surface is usually applied for the rapid diagnosis. A rapid and accurate nodal evaluation procedure using whole nodal specimen is expected in patients with NSCLC, for whom a sublobar resection is intended.

Molecular diagnosis of nodal metastasis has been attempted for various types of cancer, with detection of nodal involvement by RT-PCR reported to be more accurate as compared with conventional histological diagnosis in lung, esophageal, gastric, and colorectal carcinomas [9–18]. As for lung cancer, quantitative RT-PCR has been applied to lymph node metastasis detection by combining several mRNA markers [13,15]. However, an RT-PCR assay requires 2–3 h to obtain results and is not applicable for intraoperative diagnosis. In the present study, we evaluated the utility of one-step nucleic acid amplification (OSNA) assay and its optimal mRNA marker for lung cancer, which was developed as a rapid mRNA detection system and previously reported to detect lymph node metastasis in breast, gastric and colorectal cancer cases [19–22]. This is the first report suggesting the potential clinical utility of OSNA for lung cancer management.

2. Materials and methods

2.1. Patients and surgical specimens

A total of 165 lymph nodes from 49 NSCLC patients who underwent surgery between 2007 and 2011 at Osaka University Hospital and Osaka Medical Center for Cancer and Vascular Diseases were enrolled in the present study. Following approval by an institutional review board, written informed consent was obtained from each patient. Patient characteristics are shown in Table 1. Resected lymph nodes were divided into 2 halves for routine histopathology and further study, with the latter specimen being stored immediately at −80 °C until the OSNA assay. For validation of marker molecule expression, different 100 primary tumors (75 adenocarcinomas, 22 squamous cell carcinomas, 3 large cell carcinomas) were used.

2.2. Histopathological examination

As shown in Fig. 1, the central portion of half of the lymph node specimen was excised. From this block, 2 frozen sections were cut into 10-μm slices, and subjected to a histopathological examination with hematoxylin–eosin (HE) staining as well as immunohistochemistry. For the immunohistochemistry examination, sections were incubated with AE1/3 (Dako, Glostrup, Denmark) for 30 min at room temperature, then stained using an avidin–biotin complex method with ENVISION + KIT/HRP (DAB) (Dako) according to the manufacturer's instructions. An anti-human CD68 antibody (N1576; Dako) was used to identify epithelioid cells for analysis. To evaluate the frequency of cytokeratin 19 (CK19) and anterior gradient homolog 2 (AGR2) expression in primary tumors, paraffin-embedded primary tumors were sectioned at 5-μm. After deparaffinization and rehydration, sections were trypsinized for 20 min at 37 °C with 0.1% standard calcium chloride solution (pH 8.5), then treated for 20 min with 10% normal rabbit serum. The sections were incubated at 4 °C with a mouse monoclonal anti-human CK19 antibody (RCK108; Dako) or anti-human AGR2 rabbit monoclonal antibody (#2574-1; Epitomics, Burlingame, CA), and stained with ENVISION + KIT/HRP (DAB), in accordance with the manufacturer's instructions.

2.3. Preparation of lymph node lysates and RNA solutions

Lymph node tissues (<600 mg) were homogenized for 90 s in 4 mL of Lynorhag (Sysmex, Kobe, Japan) on ice using a Physicotron Warring blender with an NS-7 shaft (Microtech Nichion, Tokyo, Japan). Homogenates were centrifuged at 10 000 × *g* for 1 min at room temperature and the supernatants were used as lymph node lysates. RNA was purified from the lymph node lysates using an RNeasy mini kit (Qiagen, Hamburg, Germany). Purified RNA was quantified by UV spectrophotometry at 260 and 280 nm. RNA quality was confirmed by identifying 18S and 28S rRNA bands on 1% agarose gels after staining with ethidium bromide.

2.4. Quantitative RT-PCR

Quantitative RT-PCR was carried out using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA). Purified RNA (2 μL) was subjected to quantitative RT-PCR with QuantiTest SYBR Green (Qiagen). The primers were designed using ABI Primer Express Version 2.0 (Applied Biosystems). Forward and reverse primers were prepared for 16 mRNAs and β-actin for control (Supplementary Table 1). The QRT-PCR procedures were performed as previously described [19].

Supplementary Table 1 associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2012.08.018.

2.5. Selection of mRNA markers

Optimal markers for the OSNA assay were selected in 3 phases. In the first screening phase, we used a public microarray data set to select candidate mRNA markers for lung cancer lymph node metastasis. Forty-five lung cancer mRNA expression data points (from GSE7670 and GSE6253) and 10 lymph node (GSE2665) mRNA expression data points selected with Affymetrix U133 ver. 2.0 were obtained from the gene expression database Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/), and the data were normalized by *z*-score transformation. The average transformed values for each mRNA were compared between the lung cancer tissues and lymph nodes, then mRNAs that showed greater than 100-fold differences were selected as candidate markers. In the second phase, we compared the mRNA expression of each gene in metastatic lymph nodes with its expression in non-metastatic lymph nodes to verify the expression profile using clinical samples. Expression of each mRNA marker selected with the above method was evaluated by quantitative RT-PCR using 15 positive and 20 negative lymph nodes from 27 patients. In the third screening phase, the performance of lymph node metastasis detection by

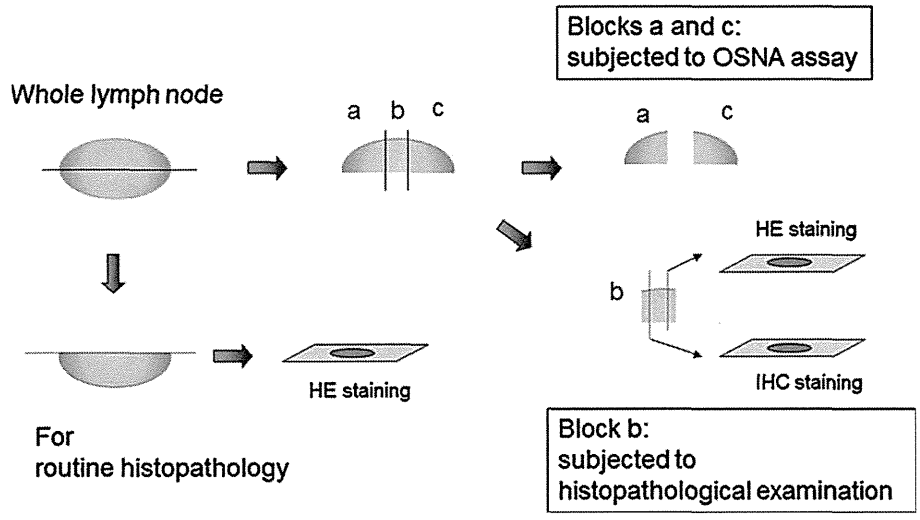


Fig. 1. Schematic representation of lymph node treatment. Fresh samples were divided into at least 4 specimens to determine the accuracy of the assay. Three cut surfaces were examined for pathological diagnosis and 2 distant specimens were subjected to OSNA assays. HE, hematoxylin–eosin staining; IHC, immunohistochemistry.

an OSNA assay with the selected mRNA markers was evaluated by comparison with histopathological examination results.

2.6. OSNA assay

We analyzed all 165 lymph nodes from 49 patients with OSNA assay. The OSNA assay included solubilization of the lymph node specimens, followed by gene amplification using reverse transcription-loop-mediated isothermal amplification (RT-LAMP). The assay was performed using 2 μL of lysate, as previously described [19]. Amplification of CK19 mRNA was monitored by measuring the turbidity of the reaction mixture at 6-s intervals. Threshold time was defined as the time at which turbidity exceeded 0.1. Primers for CK7, designed using Primer Explorer V4 (Fujitsu System Solutions, Tokyo, Japan), were as follow: CK7 FA, 5′-CGTCCCCATGCTTCCCAGCCCTGAAGCCTGGTACCAGACC-3′; CK7 RA, 5′-TTCAGAGATGAACCGGGCCATCCACGCTGGTTCTTGATGTTGT-3′; CK7 F3, 5′-GAGGAGATGGCCAAATGCA-3′; CK7 R3, 5′-GGCGG-CCTCCAACTTG-3′; CK7 LPF, 5′-TGGGCCTGGAGGGTCTCAA-3′; and CK7 LPR 5′-GAGGCTGCAGGCTGAGATCG-3′. To avoid amplification of genomic DNA, the primers were designed to amplify the exon junction regions in each gene.

3. Results

3.1. Screening of candidate mRNA markers from microarray database

Candidate mRNA markers for the OSNA assay were selected by comparison with those expressed in adenocarcinomas, squamous cell carcinomas, and lymph nodes. The expression profiles by histological type are shown in Fig. 2. After marker screening using microarray expression data, 16 mRNA markers, including CK19, CK7, AGR2, SFN, MUC1, FXYD3, CEACAM6, TACSTD1, CK17, CK15, SCGB1A1, SFTPB, SFTPA2, SFTPC, CK6A, and CK6B, with more than 100-fold higher mRNA expression in adenocarcinoma or squamous cell carcinoma in lung cancer tissue, as compared to lymph nodes, were identified. Among the selected mRNA markers, CK19, CK7, SFTPB, and TACSTD1 were previously reported as molecular markers for detection of lymph node metastasis in lung cancer. This indicates that the present method was able to objectively select candidate mRNA markers.

3.2. Evaluation of candidate mRNA markers by quantitative RT-PCR

Screening with quantitative RT-PCR revealed 4 candidates (CK19, CK7, SFN, AGR2) from the 16 mRNA markers that showed significant differences in mRNA expression between metastasis-negative and -positive nodes (Fig. 3). Although FXYD3 and TACSTD1 also showed differences in mRNA expression between positive and negative nodes, the differences in expression levels between the lowest positive and highest negative nodes were small. Expression levels of SFN and CK7 mRNA in metastasis-positive nodes were also relatively low when compared with those of CK19 and AGR2. Thus, we excluded FXYD3, TACSTD1, and SFN from the mRNA markers used in the OSNA assay.

3.3. Expressions of AGR2 and CK19 in primary NSCLC lesion

AGR2, which was selected in quantitative RT-PCR screening, is not generally recognized as a common marker in cancer cells, thus we confirmed its expression using an immunohistochemistry method in 100 primary lesions from NSCLC patients, along with CK19 as a control. We observed 74/75 (98.7%) adenocarcinomas, 21/22 (95.5%) squamous cell carcinomas, and 3/3 (100%) large cell carcinomas with positive staining for AGR2, while CK19 expression was observed in all of the primary lesions. Since definitive positive expression is necessary for target molecules in primary tumors, AGR2 was not considered to be a useful mRNA marker for the OSNA assay.

3.4. Evaluation of CK19 and CK7 mRNA expression by OSNA assay

The expressions of CK19 and CK7 mRNA in metastasis-positive and -negative lymph nodes from lung cancer patients were determined using the OSNA assay, as a combination assay of CK7 with CK19 has been reported to enhance the sensitivity of micrometastasis detection [13,14] (Supplementary Table 2). We separately plotted the mRNA expression levels of histologically metastasis-negative lymph nodes in patients with and without lymph node involvement (Fig. 4), since micrometastasis may be present in patients with locally advanced pN1-2 disease. The cut-off values for each mRNA marker were investigated using ROC curve analysis and we established a cut-off level of 250 copies/μL for each marker,

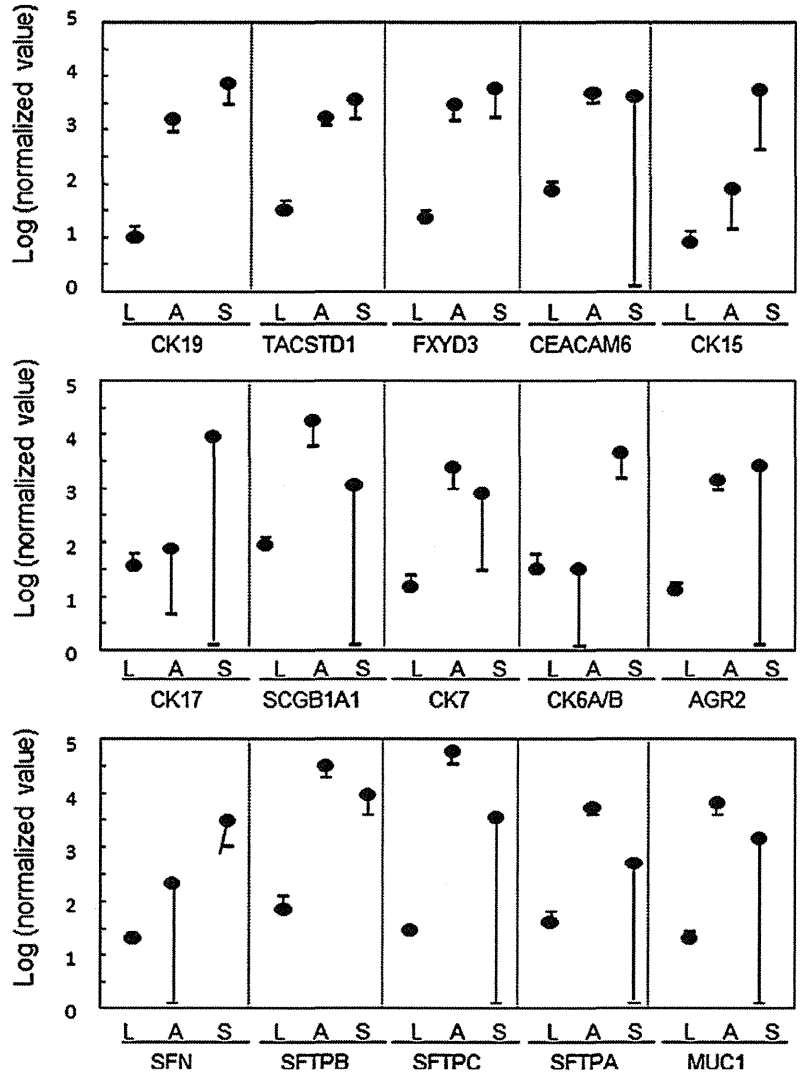


Fig. 2. Expression profiles of candidate mRNA markers extracted from a genetic database. The average normalized value for each mRNA was plotted according to the histological type of non-small cell lung cancer and lymph nodes. mRNA markers with more than 100-fold higher expression in lung cancer, as compared to non-metastatic lymph nodes, are shown. Error bars indicate 1 standard deviation. L: lymph node; A: adenocarcinoma; S: squamous cell carcinoma.

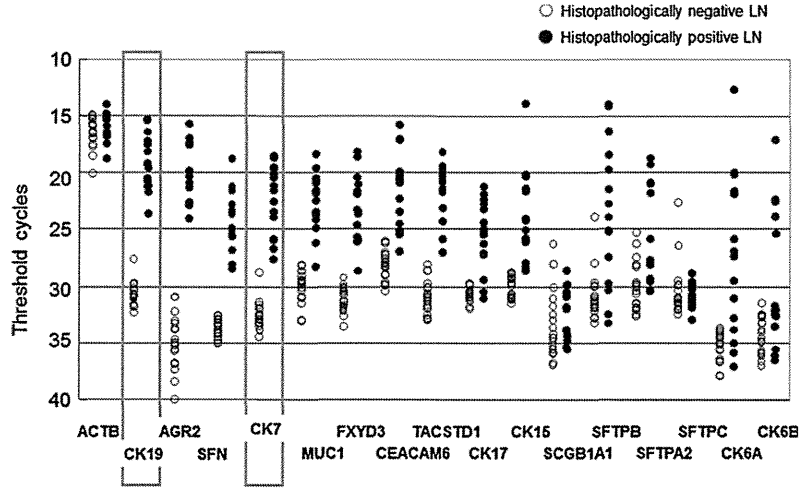


Fig. 3. mRNA expressions of 16 selected molecules, as determined by quantitative RT-PCR using 20 metastasis-negative and 15 metastasis-positive lymph nodes. Metastasis-negative lymph nodes are represented by open circles and metastasis-positive lymph nodes by closed circles. LN, lymph node.

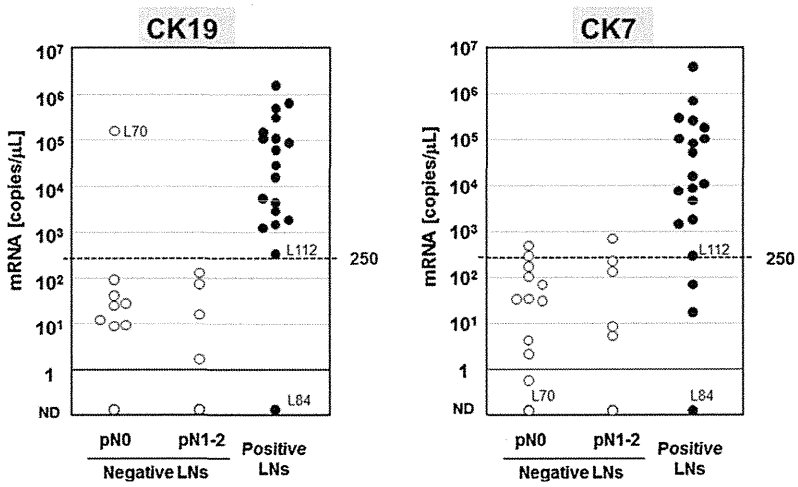


Fig. 4. CK19 and CK7 mRNA expressions shown by OSNA. Metastasis-negative lymph nodes are represented by open circles and metastasis-positive lymph nodes by closed circles. pN0, no pathological nodal metastasis; pN1–2, clear pathological metastasis in hilar (N1) or mediastinal (N2) nodes; LN, lymph node.

which was previously reported to be a reliable cut-off value for CK19 mRNA in breast, gastric, and colorectal cancers [19–22]. For CK19, mRNA amplification was not observed in 144 of 145 pathologically negative lymph nodes, while only 1 node (L70) from a pN0 patient showed significant amplification in the OSNA assay. CK19 mRNA expression was detected in 19 of 20 metastatic lymph nodes, while only 1 pathologically positive node (L84) showed levels below the cut-off. Thus, the positive predictive value, negative predictive value, and accuracy for CK19 were 95.0%, 99.3%, and 98.8%, respectively. On the other hand, for CK7, mRNA amplification was not observed in 142 of 145 pathologically negative lymph nodes, while 3 nodes (2 from pN0, 1 from pN1 cases) showed significant amplification in the OSNA assay. CK7 amplification was also negative in the lymph node that was pathologically negative but CK19 positive in the OSNA assay (L70). CK7 mRNA expression was detected in 17 of 20 pathologically metastatic lymph nodes, while only 3 positive nodes (L84, L95, L96) did not show significant amplification. Thus, the positive predictive value, negative predictive value, and accuracy for CK7 were 85.0%, 97.9% and 96.4%, respectively.

Supplementary Table 2 associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2012.08.018.

3.5. Histopathological examination of discordant samples

Several of the cases showed discordant results between the OSNA assay using CK19 or CK7 and conventional pathological diagnoses, thus we confirmed the histopathology with additional immunohistochemistry for cytokeratin. In an OSNA assay with CK19, 2 discordant samples were found. The pathologically negative lymph node (L70, squamous cell carcinoma) that showed positive CK19 amplification in the OSNA assay had no cancer cells revealed in a further pathological examination. Although the lymph node had epithelioid cells expressing CD68, non-specific AE1/3 and CK19 expressions were faintly observed in those cells (Fig. 5A). The pathologically metastasis positive lymph node (L84, adenocarcinoma) that was negative for CK19 and CK7 amplification in the OSNA assay possessed a necrotic area in the cancer cell cluster that demonstrated AE1/3 expression (Fig. 5B). Also, the lymph node (L112, adenocarcinoma) with borderline CK19 and CK7 amplification shown by the OSNA assay demonstrated micrometastasis in a pathological examination (Fig. 5C). Furthermore, 2 pathologically metastatic lymph nodes with CK19-positive and CK7-negative amplification (L95 and L96) shown in the OSNA assay had positive immunohistochemical staining for CK19. The best performance for detecting lymph node metastasis with the OSNA assay was observed when CK19 was used as a marker for lung cancer, whereas

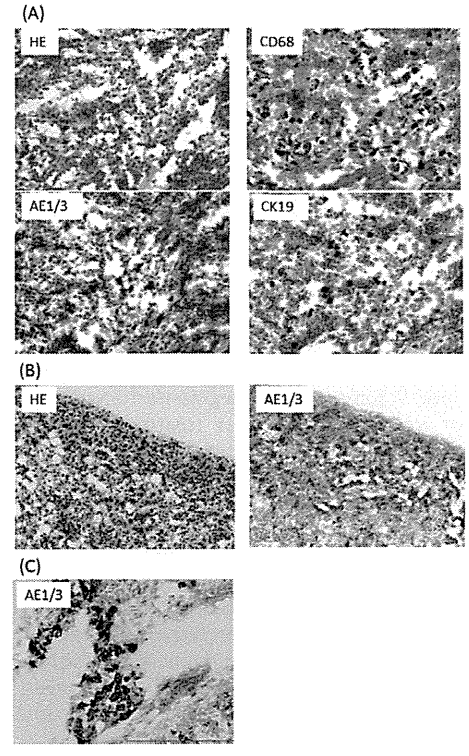


Fig. 5. Histopathological examination of discordant samples. (A) Pathologically negative lymph node (L70) showing positive amplification of CK 19 in OSNA assay. No cancer cells were morphologically detected, and non-specific AE1/3 and CK19 expressions were observed in epithelioid cells, which was confirmed by CD68 expression. (B) Pathologically positive metastatic lymph node (L84) showing negative amplification of CK19 and CK7 in OSNA assay, in which necrosis in the cancer cell cluster and AE1/3 expression were revealed. (C) Lymph node (L112) with borderline CK19 and CK7 amplification in OSNA assay diagnosed as micrometastasis by immunohistochemistry with AE1/3 antibody. HE, hematoxylin–eosin staining.

the addition of CK7 mRNA markers did not increase sensitivity and specificity.

4. Discussion

The present results indicate the possible clinical utility of OSNA assay findings obtained using CK19 mRNA to detect lymph node metastasis in NSCLC. To the best of our knowledge, this is the first study to suggest the availability of OSNA for nodal diagnosis in lung cancer, though several reports have confirmed the usefulness of such an assay for lymph node diagnosis in cases of breast, gastric, and colorectal carcinomas [19–22], with CK19 used as a target molecule for mRNA amplification in each of those studies. In the present study, we initially conducted genome-wide screening of mRNA markers from a genetic database, followed by quantitative RT-PCR to identify other mRNA markers in lung cancer. From those results, CK19 was selected as the most useful mRNA marker. CK19 mRNA was previously used for detection of nodal involvement in lung cancer by quantitative RT-PCR [10–14]. We believe that the diagnostic accuracy and rapidity of an OSNA assay using CK19 is sufficient for clinical settings in lung cancer management.

We assessed CK7, SFN, and AGR2 as candidate mRNA markers in the screening phase using quantitative RT-PCR, and found that their expression levels significantly differed between metastasis-positive and -negative lymph nodes. For detection of metastatic foci in lymph nodes, strongly expressed mRNA markers should be useful to detect small metastatic foci, thus SFN was excluded from the list of candidate mRNA markers. Similar to CK19, AGR2 mRNA was also strongly expressed. Its expression is well recognized in breast, prostate, and colorectal epithelial cells. It has also been reported that AGR2 expression is predominant in adenocarcinoma as compared to squamous cell carcinoma in NSCLC [23]. Because not all primary lesions expressed AGR2, we excluded it from marker for OSNA assay. Although CK7 expression was lower as compared with CK19 and AGR2, CK7 in combination with CK19 was previously reported to enhance the sensitivity of micrometastasis detection and was examined for OSNA assay [13,14]. The reactivity of squamous cell carcinoma to mRNA markers is generally recognized to be worse than that of adenocarcinoma [24,25]. Finally, we found that CK7 mRNA expression did not compensate for CK19 mRNA expression and an OSNA assay with those in combination did not give better results than an assay with CK19 alone.

The use of less invasive pulmonary resection procedures to preserve lung volume, such as segmentectomy for early NSCLC, is a major trend in lung cancer treatment, as the lung is a non-regenerative vital organ. In sublobar resection, intraoperative lymph node sampling for pathological diagnosis is performed using rapid frozen sections in order to avoid an incomplete resection. An OSNA assay requires only 40 min to obtain results and does not need a pathology laboratory. In addition, the whole specimen can be examined rapidly with an OSNA assay, while pathological diagnosis using H–E stained sections is associated with underestimation of metastatic lymph nodes. Intraoperative sentinel lymph node mapping and detection of micrometastasis using RT-PCR for CK19 and CK7 have been investigated [14]. However, the OSNA assay is advantageous for its rapid diagnosis as compared to RT-PCR and may be more useful for intraoperative diagnosis.

The detection sensitivity of OSNA for micrometastasis was not sufficiently evaluated in the present study. Only a case (L112), in which the amplification levels were plotted against the cut-off values for the OSNA assay using CK19 and CK7, can be a considered as a suitable reference sample to consider detection sensitivity, as that lymph node had a micrometastasis greater than 200-μm shown by immunohistochemistry findings with the AE1/3 antibody. Three-cut level and 2-mm interval histopathology results for breast and colorectal cancers, respectively, are reported to be similar to those of an OSNA assay [19–22]. It has also been reported that isolated tumor cells in breast and gastric cancers cannot be detected by an OSNA assay [19,21]. Thus, additional analysis is necessary for detection of micrometastasis using an OSNA assay in lung cancer.

Mediastinal and hilar lymph nodes are frequently associated with anthracosis, particularly in smokers with lung cancer, which is a different nodal condition than observed in patients with breast, gastric, and colorectal carcinomas. Thus, we evaluated whether inflammatory lymph nodes in lung cancer patients are applicable to OSNA, in which mRNA is rapidly extracted using special homogenizing lysis buffer and PCR products are quantified using the turbidity of a magnesium pyrophosphate precipitate. Positive amplification with expected detection sensitivity was observed in the present study. Therefore, we understood that anthracosis in lymph nodes can be ignored in OSNA findings.

Discordant results between the OSNA assay with CK19 and pathological diagnosis were observed for 1 false-positive and 1 false-negative case. L70 was positive in the assay with CK19, while it was metastasis-negative in the pathological diagnosis and no metastatic focus was observed in a further pathological investigation using immunohistochemistry. The faint expression of AE1/3 and CK19 on epithelioid cells detected using immunohistochemistry might contribute to the false-positive result of OSNA assay with CK19. Another possibility is the allocation bias of cancer cells in the sampling, because the lymph node was obtained from a stage IV patient with pleural dissemination. On the other hand, we observed no CK19/CK7 mRNA amplification in necrotic cancer tissue in OSNA findings (L84), while cytokeratin staining was seen with immunohistochemistry. These results indicate the current limitations of molecular assays using mRNA markers. Diagnosis of lymph node involvement following preoperative chemotherapy or radiation treatments should be carefully performed when using an OSNA assay, as necrosis or micrometastasis is frequently observed following preoperative therapy. It is also known that re-evaluation of nodal involvement after induction therapy is very important for deciding operative indications in cases of locally advanced NSCLC [26,27].

In conclusion, OSNA using CK19 mRNA is a promising rapid diagnostic method to detect lymph node metastasis in NSCLC.

Conflict of interest statement

Ms. Hiyama K, Mr. Nakabayashi K, Dr. Yoshida Y, Dr. Ding J, and Dr. Otomo Y are employed by Sysmex Corporation. Department of General Thoracic Surgery, Osaka University Graduate School of Medicine and Department of General Thoracic Surgery, Osaka Medical Center for Cancer and Cardiovascular Diseases received research support of $6000/year during study period. All authors contributing to this work have no other conflict of interest to declare.

Acknowledgment

This study was supported by grant-in-aid for scientific research from Japan Society for the Promotion of Science (no. 21591812).

References

[1] Non-small cell lung cancer. NCCN Guideline for Patients. Version 2012. http://www.nccn.org/index.asp
[2] Schuchert MJ, Kilic A, Pennathur A, Nason KS, Wilson DO, Luketich JD, et al. Oncologic outcomes after surgical resection of subcentimeter non-small cell lung cancer. Ann Thorac Surg 2011;91:1681–8.
[3] Kates M, Swanson S, Wisnivesky JP. Survival following lobectomy and limited resection for the treatment of stage I non-small cell lung cancer ≤1 cm in size: a review of SEER data. Chest 2011;139:491–6.

[4] Kilic A, Schuchert MJ, Pettiford BL, Pennathur A, Landreneau JR, Landreneau JP, et al. Anatomic segmentectomy for stage I non-small cell lung cancer in the elderly. Ann Thorac Surg 2009;87:1662–6.
[5] Okada M, Koike T, Higashiyama M, Yamato Y, Kodama K, Tsubota N. Radical sublobar resection for small-sized non-small cell lung cancer: a multicenter study. J Thorac Cardiovasc Surg 2006;132:769–75.
[6] Inoue M, Minami M, Sawabata N, Utsumi T, Kadota Y, Shigemura N, et al. Clinical outcome of resected solid-type small-sized c-stage IA non-small cell lung cancer. Eur J Cardiothorac Surg 2010;37:1445–9.
[7] Asamura H, Suzuki K, Watanabe S, Matsuno Y, Maeshima A, Tsuchiya R. A clinicopathological study of resected subcentimeter lung cancers: a favorable prognosis for ground glass opacity lesions. Ann Thorac Surg 2003;76:1016–22.
[8] Hashizume T, Yamada K, Okamoto N, Saito H, Oshita F, Kato Y, et al. Prognostic significance of thin-section CT scan findings in small-sized lung adenocarcinoma. Chest 2008;133:441–4.
[9] Nosotti M, Falleni M, Palleschi A, Pellegrini C, Alessi F, Bosari S, et al. Quantitative real-time polymerase chain reaction detection of lymph node lung cancer micrometastasis using carcinoembryonic antigen marker. Chest 2005;128:1539–44.
[10] Wang XT, Sienel W, Eggeling S, Ludwig C, Stoelben E, Mueller J, et al. Detection of disseminated tumor cells in mediastinoscopic lymph node biopsies and lymphadenectomy specimens of patients with NSCLC by quantitative RT-PCR. Eur J Cardiothorac Surg 2005;28:26–32.
[11] Le Pimpec-Barthes F, Danel C, Lacave R, Ricci S, Bry X, Lancelin F, et al. Association of CK19 mRNA detection of occult cancer cells in mediastinal lymph nodes in non-small cell lung carcinoma and high risk of early recurrence. Eur J Cancer 2005;41:306–12.
[12] Wallace MB, Block MI, Gillanders W, Ravenel J, Hoffman BJ, Reed CE, et al. Accurate molecular detection of non-small cell lung cancer metastases in mediastinal lymph nodes sampled by endoscopic ultrasound-guided needle aspiration. Chest 2005;127:430–7.
[13] Saintigny P, Coulon S, Kambouchner M, Ricci S, Martinot E, Danel C, et al. Real-time RT-PCR detection of CK19, CK7 and MUC1 mRNA for diagnosis of lymph node micrometastases in non-small cell lung carcinoma. Int J Cancer 2005;115:777–82.
[14] Melfi FM, Lucchi M, Davini F, Viti A, Fontanini G, Boldrini L, et al. Intraoperative sentinel lymph node mapping in stage I non-small cell lung cancer: detection of micrometastases by polymerase chain reaction. Eur J Cardiothorac Surg 2008;34:181–6.
[15] Xi L, Coello MC, Litle VR, Raja S, Gooding WE, Yousem SA, et al. A combination of molecular markers accurately detects lymph node metastasis in non-small cell lung cancer patients. Clin Cancer Res 2006;12:2484–91.
[16] Xi L, Luketich JD, Raja S, Gooding WE, Litle VR, Coello MC, et al. Molecular staging of lymph nodes from patients with esophageal adenocarcinoma. Clin Cancer Res 2005;11:1099–109.
[17] Kubota K, Nakanishi H, Hiki N, Shimizu N, Tsuji E, Yamagichi H, et al. Quantitative detection of micrometastases in the lymph nodes of gastric cancer patients with real-time RT-PCR: a comparative study with immunohistochemistry. Int J Cancer 2003;105:136–43.
[18] Noura S, Yamamoto H, Ohnishi T, Masuda N, Matsumoto T, Takayama O, et al. Comparative detection of lymph node micrometastases of stage II colorectal cancer by reverse transcriptase polymerase chain reaction and immunohistochemistry. J Clin Oncol 2002;20:4232–41.
[19] Tsujimoto M, Nakabayashi K, Yoshidome K, Kaneko T, Iwase T, Akiyama F, et al. One-step nucleic acid amplification for intraoperative detection of lymph node metastasis in breast cancer patients. Clin Cancer Res 2007;13:4807–16.
[20] Tamaki Y, Akiyama F, Iwase T, Kaneko T, Tsuda H, Sato K, et al. Molecular detection of lymph node metastases in breast cancer patients: results of a multicenter trial using the one-step nucleic acid amplification assay. Clin Cancer Res 2009;15:2879–84.
[21] Yaguchi Y, Sugasawa H, Tsujimoto H, Takata H, Nakabayashi K, Ichikura T, et al. One-step nucleic acid amplification (OSNA) for the application of sentinel node concept in gastric cancer. Ann Surg Oncol 2011;18:2289–96.
[22] Yamamoto H, Sekimoto M, Oya M, Yamamoto N, Konishi F, Sasaki J, et al. OSNA-based novel molecular testing for lymph node metastases in colorectal cancer patients: results from a multicenter clinical performance study in Japan. Ann Surg Oncol 2011;18:1891–8.
[23] Pizzi M, Fassan M, Balistreri M, Galligioni A, Rea F, Rugge M. Anterior gradient 2 overexpression in lung adenocarcinoma. Appl Immunohistochem Mol Morphol 2011;20:31–6.
[24] Camilo R, Capelozzi VL, Siqueira SA, Del Carlo Bernardi F. Expression of p63, keratin 5/6, keratin 7, and surfactant-A in non-small cell lung carcinomas. Hum Pathol 2006;37:542–6.
[25] Chu P, Wu E, Weiss LM. Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. Mod Pathol 2000;13:962–72.
[26] Cerfolio RJ, Bryant AS, Ojha B. Restaging patients with N2 (stage IIIa) non-small cell lung cancer after neoadjuvant chemoradiotherapy: a prospective study. J Thorac Cardiovasc Surg 2006;13:1229–35.
[27] Dooms C, Verbeken E, Stroobants S, Nackaerts K, De Leyn P, Vansteenkiste J. Prognostic stratification of stage IIIA-N2 non-small-cell lung cancer after induction chemotherapy: a model based on the combination of morphometric-pathologic response in mediastinal nodes and primary tumor response on serial 18-fluoro-2-deoxy-glucose positron emission tomography. J Clin Oncol 2008;26:1128–34.

European Journal of Cardio-Thoracic Surgery Advance Access published October 25, 2012

European Journal of Cardio-Thoracic Surgery 0 (2012) 1-5
doi:10.1093/ejcts/ezs553

ORIGINAL ARTICLE

Significance of tumour vessel invasion in determining the morphology of isolated tumour cells in the pulmonary vein in non-small-cell lung cancer

Soichiro Funaki*, Noriyoshi Sawabata, Abulimiti Abulaiti, Tomoyuki Nakagiri, Yasushi Shintani, Masayoshi Inoue, Masato Minami and Meinoshin Okumura

Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, Osaka, Japan

* Corresponding author. Department of General Thoracic Surgery, Osaka University Graduate School of Medicine, (L-5) 2-2 Yamadaoka Suita, Osaka 565-0879, Japan. Tel: +81-6-68793152; fax: +81-6-68793164; e-mail: funaki@thoracic.med.osaka-u.ac.jp, soo1@hotmail.co.jp (S. Funaki).

Received 12 April 2012; received in revised form 22 August 2012; accepted 7 September 2012

Abstract

OBJECTIVES: The existence of clustered isolated tumour cells (ITCs) in the pulmonary vein (PV) of the lungs of patients with lung cancer has been reported to be a prognostic factor. However, the clinical-pathological characteristics related to their presence in the PV remain unclear.

METHODS: We analysed the surgical results and clinical-pathological findings of 130 patients who underwent surgery for non-small-cell lung cancer in regard to blood vessel invasion (BVI), serum carcinoembryonic antigen (CEA) level, maximum standardized uptake value (SUV-max), size of the solid region in computed tomography findings and pathological stage according to an ITC type, i.e. no tumour (N), singular tumour cells (S) and clustered tumour cells (C).

RESULTS: ITCs were detected in 96 (74%) of the patients, with C observed in 43, S in 53 and N in 34. Recurrence was seen in 33 (26%) cases, 21 of which were classified as C, 9 as S and 3 as N. The disease-free survival rate was significantly worse in C cases when compared with the others ($P < 0.01$). The rate of C was high in cases with high serum CEA, advanced p-staging and positive BVI ratio. Furthermore, BVI positive and ITC morphology were strongly related (BVI positive; 79 in C, 40 in S, 9% in N; $P < 0.01$).

CONCLUSIONS: Clustered ITCs were shown to be a prognostic indicator and strongly related to BVI. Our results suggest that determination of BVI has prognostic value, as clustered ITCs with metastatic potential are disseminated from the invaded vein.

Keywords: Blood vessel invasion • Isolated tumour cells • Surgery • Recurrence • Non-small-cell lung cancer

INTRODUCTION

Lung cancer is a leading cause of cancer death in most industrial countries [1]. In addition, an investigation of the causes of cancer deaths indicated that recurrence and distant metastasis occurred in approximately 70% of patients who underwent surgery [2]. Therefore, useful markers are needed for the early detection of distant metastasis and recurrence, with various prognostic biomarkers thus far reported. In blood chemical studies, serum carcinoembryonic antigen (CEA) has been shown to be one of the most useful tumour markers for providing information regarding cancer progression [3], while the presence of blood vessel invasion (BVI) in histopathological findings is also an important prognostic indicator [4, 5]. On the other hand, clinical imaging techniques such as computed tomography (CT) and positron emission tomography (PET) can also provide important information regarding cancer aggressiveness and malignancy. The solid lesion size of tumours in CT findings and maximum standardized uptake value (SUV-max) in PET imaging are helpful for clinical cancer treatment, as those factors are reported to reflect malignancy or metastatic potential [6, 7]. As for other biomarkers, the presence of isolated tumour cells (ITCs) in blood has been recently reported to be useful for determining prognosis, recurrence and metastasis [8-11]. In our previous report, we presented a novel method to enrich ITCs while maintaining their morphological appearance and then found relationships between ITC morphology and clinical backgrounds [12]. In cases with clustered ITCs, the recurrence rate was higher than that in cases with singular or no ITCs. From those results, we speculated that ITCs are shed from the primary tumour, then flow through a drainage vein and circulate throughout the whole body, easily leading to metastasis. In addition, relationships between ITCs and background factors of primary tumours were noted. In the present study, we assessed the relationships among clinical-pathological findings including BVI and morphological characteristics of ITCs in the pulmonary vein (PV) of resected lungs of non-small-cell lung cancer patients.