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ORIGINAL PAPER

# Association of cigarette smoking with the expression of nuclear survivin in pathological Stage IA lung adenocarcinomas

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**Abstract** Survivin is expressed in the cytoplasm and/or nucleus of various types of malignant tumor cells. Cytoplasmic survivin functions as an apoptosis inhibitor, while nuclear survivin is indispensable for complete mitosis completion. To investigate the effect of cigarette smoking on the survivin expression in lung adenocarcinomas at the early developmental stage, we examined the expression of nuclear and cytoplasmic survivin in pathological Stage IA lung adenocarcinomas resected from 38 non-smokers and 44 smokers (current smokers and ex-smokers) using an immunohistochemical method. Labeling indices of nuclear survivin in tumors of smokers were significantly greater than those of non-smokers. The labeling index of nuclear survivin was above 3 % in only 1 (2.6 %) of the 38 tumors of the non-smokers, while the labeling indices in 19 (43.2 %) of 44 tumors of the smokers were above 3 % with a significantly greater frequency. There was no significant difference in the labeling index of nuclear survivin between current smokers and ex-smokers. There was no significant difference in the labeling index of cytoplasmic survivin between tumors of the non-smokers and the smokers. The present results show that cigarette smoking is associated with the higher nuclear surviving expression in lung adenocarcinomas at the early stage, suggesting that cigarette smoking affects the nuclear survivin expression in lung adenocarcinomas at the early developmental stage.

**Keywords** Lung · Adenocarcinoma · Survivin · Nuclear expression

**Abbreviation**
NES Nuclear export signal

## Introduction

Survivin is expressed in the nucleus and/or cytoplasm of cells of a variety of malignant tumors, while its expression is rarely observed in normal differentiated tissues [1–3]. In cytoplasm, survivin functions as an inhibitor of apoptosis while in the nucleus survivin interacts with aurora kinase B and the inner centromere protein (INCENP) to complete mitosis [2, 3].

Recently, the incidence of lung adenocarcinomas has been increasing with cigarette smoking being the most significant risk factor for that as well as other histological types of lung cancer [4, 5]. Dasgupta et al. [6] have reported that the tobacco component nicotine up-regulated survivin in human lung cancer cell lines. Furthermore, Jin et al. [7] have reported that the tobacco components, nicotine and 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone, induced survivin protein synthesis in normal human

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bronchial epithelial cells in vitro and in vivo and showed the involvement of survivin in tobacco-induced malignant transformation of normal human bronchial epithelial cells. These reports suggest that cigarette smoking results in the higher survivin expression in the early developmental stage of lung cancers. Therefore, to examine this possibility, we investigated the association of cigarette smoking with the expression of survivin in lung adenocarcinomas at an early stage. For this, we examined nuclear and cytoplasmic survivin expression in pathological Stage IA resected lung adenocarcinoma specimens obtained from non-smokers and smokers (current smokers and ex-smokers) using an immunohistochemical method.

### Patients and specimens

This study included 82 patients with pathological Stage IA primary lung adenocarcinoma who underwent complete tumor resection at Toneyama National Hospital (Osaka, Japan) between January 2007 and December 2010. Of 82 patients, 38 patients had no experience of smoking and 44 patients were smokers (current smokers or ex-smokers). An ex-smoker was defined as a smoker who quitted smoking before 1 year from the diagnosis of lung cancer, taking time-course of the tumor development into consideration. Smokers except ex-smokers were defined as a current smoker. Numbers of ex-smokers and current smokers were 16 and 28, respectively. None of the patients received neoadjuvant chemotherapy or radiotherapy. All underwent the dissection of the bifurcation and ipsilateral mediastinum lymph nodes, and pathological examinations revealed no metastasis in them. Pathological stage was determined according to the TNM classification of the International Union Against Cancer (7th edition) [8]. The resected tumor specimens were fixed in 0.01 M phosphate-buffered 10 % formalin (pH 7.4) and several paraffin-embedded tissue blocks were made from each. Clinical data including follow-up findings were available for all cases.

The present study was approved by the Toneyama National Hospital Ethics Committee.

### Immunohistochemistry

For immunohistochemical examinations of survivin, one representative tissue block from each obtained tumor was used, with 5-μm-thick sections prepared. Immunohistochemical staining was performed using an avidin-streptavidin immunoperoxidase method with an anti-human survivin rabbit polyclonal antibody (Novus Biologicals, Littleton, Co, USA) at a 500-fold dilution. Antigen retrieval by incubation of deparaffinized sections in the cell condition 1 solution at standard degree and immunohistochemical staining were done using an automated Benchmark system (Ventana Medical System, Tuscon, AZ, USA), according to the manufacturer's instructions.

To estimate a labeling index of nuclear survivin in each tumor, nuclei stained positively or negatively were counted automatically using Win Roof software (Mitani Co, Tokyo, Japan). On the other hand, to estimate a labeling index of cytoplasmic survivin in each tumor, cells with cytoplasm stained positively or negatively were counted by examining each in computer images. When we estimated a labeling index of nuclear survivin in epithelial cells of the bronchioles around a tumor, nuclei stained positively or negatively were counted by examining each in computer images. Labeling indices of nuclear or cytoplasmic survivin were calculated after counting 500–5000 tumor cells.

### Statistical analysis

Statistical analyses were performed using the Excel Statistics 2012 software package for Windows (SSRI, Tokyo, Japan). A $P$ value $<0.05$ was considered to be significant. Frequencies of 2 groups were analyzed using the $\chi^2$ test. Data comprising several values are presented as the mean ± SE and these data for 2 groups were analyzed using the Student's $t$ test. Correlation of 2 variables was estimated by calculation with Pearson's product-moment correlation coefficient followed by a test for no correlation at a significance level of 0.05.

## Result

The clinicopathological characteristics of the non-smoking patients ($n = 38$) and patients having a smoking experience (current smoker and ex-smokers) ($n = 44$) are presented in Table 1. The Brikemann index for the smokers was 816 ± 59.8 and the minimal Brikemann index was 50. There was no significant difference with regard to age, tumor size, follow-up period, and number of recurrences between the non-smokers and smokers. The major histological type of tumor was mixed adenocarcinoma in both groups, and there was no significant difference with regard to histological type between non-smokers and smokers. The ratio of males was significantly greater in the smoker group.

There was no significant difference with regard to age, sex, tumor size, follow-up period, number of recurrence and histological type between ex-smokers and current smokers.

Table 2 shows nuclear and cytoplasmic survivin expression in pathological Stage IA lung adenocarcinoma specimens obtained from non-smokers and smokers (Fig. 1). Labeling indices of nuclear survivin in tumors of smokers were significantly greater than those in tumors of

**Table 1** Clinicopathological characteristics of non-smokers and smokers (ex-smokers and current smokers)

| | Non-smokers (38 cases) | Smokers (44 cases) | Ex-smokers (16 cases) | Current smoker (28 cases) |
|---|---|---|---|---|
| Age | 65 ± 1.8 | 65.3 ± 1.5 | 67.5 ± 10.0 | 64.1 ± 1.9 |
| Sex | | | | |
| Male | 10 (26.3 %)[a] | 36 (81.8 %) | 14 (87.5 %) | 22 (78.6 %) |
| Female | 28 (73.7 %)[a] | 8 (18.2 %) | 2 (12.5 %) | 6 (21.4 %) |
| Size (mm) | 17.3 ± 0.8 | 18.5 ± 0.9 | 16.9 ± 1.3 | 19.3 ± 1.1 |
| Brikemann index | 0 | 816 ± 59.8 | 764.4 ± 97.1 | 846.0 ± 76.4 |
| Outcome | | | | |
| Follow-up period (months) | 39.5 ± 1.9 (range 11.3–57.8) | 38.3 ± 2.0 (range 5.2–58.9) | 32.5 ± 2.4 (range 14.7–50.1) | 42.4 ± 2.3 (range 5.2–58.9) |
| No recurrence | 37 (97.4 %) | 41 (93.2 %) | 14 (87.5 %) | 27 (96.4 %) |
| Recurrence | 1 (2.6 %) | 3 (6.8 %) | 2 (12.5 %) | 1 (3.6 %) |
| Histology of tumors | | | | |
| BAC non-mucinous | 6 | 2 | 1 | 1 |
| Mixed adenocarcinoma | 25 | 31 | 10 | 21 |
| Papillary adenocarcinoma | 6 | 5 | 3 | 2 |
| Acinar adenocarcinoma | 0 | 3 | 2 | 1 |
| Solid adenocarcinoma | 1 | 3 | 0 | 3 |

[a] $P < 0.05$; significant difference from the values for smokers, ex-smokers or current smokers. An ex-smoker was defined as a smoker who quitted smoking before 1 year from the diagnosis of a lung tumor

**Table 2** Nuclear and cytoplasmic survivin labeling indices in tumors of non-smokers and smokers (ex-smokers and current smokers)

| Patients | No. | Nuclear expression | | Cytoplasmic expression |
|---|---|---|---|---|
| | | LI ≥3 % | LI (mean ± SE) | LI (mean ± SE) |
| Non-smokers | 38 | 1 case (2.6 %)[a] | 1.1 ± 0.5 (range 0–17.2)[b,c] | 32.2 ± 5.4 (range 0–92.9) |
| Smokers | 44 | 19 cases (43.2 %) | 5.6 ± 0.5 (range 0–40.4) | 26.7 ± 4.6 (range 0–90.1) |
| Ex-smokers | 16 | 5 cases (31.3 %) | 6.3 ± 2.7 (range 0–35.8) | 27.4 ± 7.0 (range 0–68.1) |
| Current smokers | 28 | 14 cases (50 %) | 5.2 ± 1.5 (range 0–40.4) | 26.3 ± 6.1 (range 0–90.1) |

[a,b] $P < 0.05$, significant difference from the values for smokers, ex-smokers or current smokers

[c] Labeling indices of 37 tumors (mean ± SE; 0.7 ± 0.2) were below 3.0 % and that of 1 was 17.2 %

non-smokers. The labeling index of nuclear survivin in only 1 (2.6 %) of the 38 tumors of the non-smokers was above 3 %. On the other hand, the labeling indices of 19 (43.2 %) of the 44 tumors of the smokers were above 3 % with a significantly greater frequency. In contrast, there was no significant difference with regard to the labeling index of cytoplasmic survivin between the non-smoking and smoking groups. There was no significant difference with regard to labeling indices of nuclear and cytoplasmic survivin between ex-smokers and current smokers.

Since the nuclear survivin labeling indices were below 3 % in 37 of 38 tumors of non-smokers, we analyzed the difference with regard to age, sex, Brikemann index, tumor size, histological type of a tumor and the labeling index for cytoplasmic survivin between the smoking groups showing the nuclear survivin labeling index above and below 3 % (Table 3). However, there was no significant difference between these two groups. In addition, there was no significant correlation at a significant level of 0.05 between Brikemann index and nuclear survivin labeling in tumors with a labeling index greater than 3 % ($r = 0.148$).

In order to examine the effect of smoking on the normal epithelial cells of bronchioles around a tumor, we selected tumors from 9 non-smokers and 11 smokers (a mean ± SE of Brikemann indices: 1229 ± 102) and estimated the expression of nuclear and cytoplasmic survivin in epithelial cells of bronchioles around a tumor. The labeling index (mean ± SE) of nuclear survivin of tumors from 11 smokers was 9.5 ± 1.3 % (range 4.7–19.9 %) and that of tumors from 9 non-smokers was below 0.1 %. Epithelial

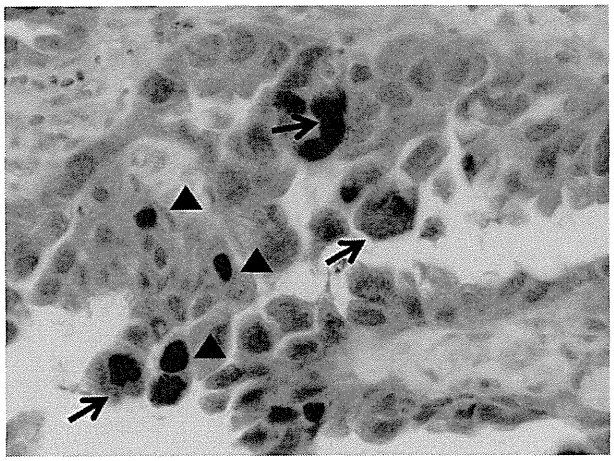

**Fig. 1** Immunohistochemical staining of survivin *arrows* and *arrowheads* indicate cytoplasmic and nuclear staining, respectively

**Table 3** Clinicopathological characteristics of smokers with adenocarcinomas showing the nuclear labeling indices below 3 % and above 3 %

| | Survivin nuclear expression LI ≥3.0 % ($n$ = 19) | Survivin nuclear expression LI <3.0 % ($n$ = 25) |
|---|---|---|
| Age | 67.3 ± 2.0 | 63.8 ± 2.2 |
| Sex | | |
| Male | 15 (78.9 %) | 21 (84 %) |
| Female | 4 (21.1 %) | 4 (16 %) |
| Brikemann index | 947.1 ± 103.6 | 716.9 ± 64.9 |
| Size (mm) | 18.1 ± 1.4 | 18.7 ± 1.1 |
| Survivin expression | | |
| Nuclear labeling index | 12.3 ± 2.4[a] | 0.52 ± 0.13 |
| Cytoplasm labeling index | 36.0 ± 7.9 | 19.7 ± 5.1 |
| Histology of tumor | | |
| BAC non-mucinous | 0 (0 %) | 2 (8 %) |
| Mixed adenocarcinoma | 10 (52.6 %) | 21 (84 %) |
| Papillary adenocarcinoma | 4 (21.1 %) | 1 (4 %) |
| Acinar adenocarcinoma | 2 (10.5 %) | 1 (4 %) |
| Solid adenocarcinoma | 3 (15.8 %) | 0 (0 %) |

*LI* labeling index

[a] $P < 0.05$, significant difference from the values for smokers with adenocarcinomas showing nuclear labeling indices below 3.0 %

cells of bronchioles of smokers showed no histological change compared to those of non-smokers. Bronchiolar epithelial cells of both smokers and non-smokers did not express cytoplasmic survivin, but sometimes expressed nuclear survivin. The labeling indices (mean ± SE) of nuclear survivin in bronchiolar epithelial cells of non-smokers and smokers were 0.067 ± 0.067 (range 0–0.6 %) and 0.582 ± 0.194 (range 0–1.7 %), respectively, being significantly different ($P < 0.05$).

## Discussion

Adenocarcinomas obtained from patients who smoked showed a significantly higher nuclear survivin labeling index as compared to those from non-smokers. Cigarette smoking is known to be a significant risk factor for lung adenocarcinoma development, though the association with lung adenocarcinoma is less than with lung squamous cell carcinoma and small cell carcinoma [5]. Furthermore, tobacco has been shown to up-regulate the survivin expression in normal lung epithelial cells or lung cancer cell lines [7]. Consistently, the labeling index of nuclear survivin in epithelial cells of bronchioles around tumors of smokers was significantly higher than that of non-smokers. Therefore, it is likely that cigarette smoking results in the higher nuclear survivin expression in the early developmental stage of lung adenocarcinomas.

In contrast, we found no effect of cigarette smoking on the cytoplasmic survivin expression. Survivin has five splice variants including the wild type, and their intracellular localization and functions differ [2, 3]. Survivin △Ex3 and survivin 2α have no nuclear export signal (NES) and are retained in the cytoplasm, while wild-type survivin, survivin 2B, and survivin 3B have NES and can move to the nucleus via interaction of NES with the export receptor Crm1 [2, 3]. Unfortunately, the anti-survivin antibodies presently available recognize all survivin variants due to the existence of an identical amino-terminal peptide [1]. Therefore, cigarette smoking may be associated with survivin variants that are able to move to the nucleus and not with those localized only in the cytoplasm.

There was no significant correlation between nuclear survivin labeling index and Brikemann index in the adenocarcinomas with the nuclear survivin labeling index greater than 3 %. This result suggests that the effect of cigarette smoking on nuclear survivin expression is produced by smoking history above a certain Brikemann index level regardless of the levels of that index.

An ex-smoker was defined as a smoker who quitted smoking before 1 year from the diagnosis of lung cancer, taking time-course of the tumor development into consideration. However, there was no significant difference with regard to labeling indices of nuclear and cytoplasmic survivin between ex-smokers and current smokers. This result may imply that cigarette smoking before the development of lung adenocarcinoma can influence the nuclear survivin expression in lung adenocarcinoma at the early stage although the mechanism is unclear.

There was a significantly greater ratio of males in the smoking group than in the non-smoking group, which may be a reflection of the general Japanese smoking population.

There were no significant differences with regard to age, sex, Brikemann index, tumor size, histological type, and cytoplasmic survivin labeling index between the smoking group specimens with the nuclear survivin labeling index above 3 % and those with the index value below 3 %. This suggests that other unknown factor(s) have an influence on the expression of nuclear survivin.

In this study with a relatively small number of patients, prognosis of patients with pathological Stage IA adenocarcinomas was good and the apparent influence of the nuclear survivin on prognosis has not been found. However, the study of Maeda et al. [9] with 1070 patients with clinical Stage IA lung adenocarcinoma who had undergone complete resection of a tumor with systematic lymph node dissection, has shown that a history of heavy smoking was associated with poor prognosis. Furthermore, Shinohara et al. [10] have reported that the nuclear survivin expression is associated with increased recurrence and poor survival in patients with Stage I and II resected nonsmall cell lung carcinoma. We found that cigarette smoking was associated with the higher expression of nuclear survivin in Stage IA lung adenocarcinoma. Therefore, our finding would be helpful in understanding the reason why a history of cigarette smoking produces poor prognosis in patients with Stage IA lung adenocarcinomas.

In conclusion, the present results suggest that cigarette smoking is associated with the nuclear survivin expression in lung adenocarcinomas, suggesting that cigarette smoking results in the higher nuclear survivin expression in lung adenocarcinomas at the early stage.

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ORIGINAL ARTICLE

# Low-dose human atrial natriuretic peptide for the prevention of postoperative cardiopulmonary complications in chronic obstructive pulmonary disease patients undergoing lung cancer surgery†

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## Abstract

**OBJECTIVES**: Lung cancer patients with chronic obstructive pulmonary disease are at an increased risk of respiratory and cardiovascular complications after pulmonary resection. The objective of the present study was to evaluate the clinical effects of low-dose human atrial natriuretic peptide (hANP) on postoperative cardiopulmonary complications in untreated chronic obstructive pulmonary disease patients undergoing lung cancer surgery.

**METHODS**: Of 824 patients who underwent an elective pulmonary resection procedure for lung cancer in two specialized thoracic centres between 2008 and 2011, 202 consecutive patients who had airflow limitation before surgery were included in this retrospective study. The results were compared between patients who did and those who did not receive hANP during the perioperative period. The primary endpoint was the incidence of postoperative cardiopulmonary complications. Postoperative haemodynamics, white blood cell (WBC) counts and C-reactive protein (CRP) levels were also examined. Furthermore, propensity score matching analysis was used to reduce treatment selection bias from patient characteristics.

**RESULTS**: The incidence of postoperative cardiopulmonary complications was significantly lower in the hANP group than in the control group (14 vs 36%, $P < 0.01$). The propensity score matching analysis confirmed the significantly decreased frequency of postoperative cardiopulmonary complications in the hANP group. Patients in the hANP group showed significantly lower WBC counts and serum CRP levels postoperatively.

**CONCLUSIONS**: Treatment with hANP during the perioperative period had a prophylactic effect against postoperative cardiopulmonary complications in chronic obstructive pulmonary disease patients undergoing lung cancer surgery.

**Trial registration number**: JPRN-UMIN000003631.

**Keywords:** Lung cancer surgery • Perioperative care • Postoperative complications

## INTRODUCTION

Lung cancer patients often have chronic obstructive pulmonary disease (COPD), because both conditions are strongly associated with cigarette smoking. A high prevalence of COPD has been reported in patients with a new diagnosis of lung cancer [1, 2]. Since COPD is often underdiagnosed and undertreated [3], a new diagnosis of COPD is often made during the evaluation of patients requiring surgery for lung cancer. Although surgical resection remains the only potentially curative treatment for lung cancer, lung cancer patients with COPD are at high risk of pulmonary resection surgery [4, 5]. Therefore, perioperative management of COPD is an important issue in lung cancer surgery.

Human atrial natriuretic peptide (hANP), a 28 amino-acid peptide hormone synthesized by the cardiac atria, is commercially available in Japan and has been used in the treatment of acute heart failure. hANP has a wide range of cardioprotective effects, including anti-inflammatory and inhibition of sympathetic nervous system activity [6]. Postoperative cardiopulmonary complications are often caused by inflammatory and neurohormonal changes. Since it is now known that hANP has beneficial effects on inflammatory changes and neurohormonal balance [7, 8], it is plausible to expect that hANP would protect against postoperative cardiopulmonary complications.

We previously reported that hANP has a prophylactic effect on postoperative cardiopulmonary complications in elderly

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high-risk patients [9]. However, the effects of hANP for COPD patients have not yet been clear. The objective of this study was to evaluate the effects of hANP on postoperative cardiopulmonary complications in untreated COPD patients undergoing lung cancer surgery.

## MATERIALS AND METHODS

### Study design and population

Of the 824 patients who underwent an elective pulmonary resection procedure for lung cancer at Osaka University Graduate School of Medicine and National Toneyama Hospital from 2008 to 2011, 250 consecutive COPD patients were included in this retrospective surveillance study. Airflow limitation was assessed by spirometry and was defined as a postbronchodilator forced expiratory volume in 1 s/forced vital capacity ($FEV_1/FVC$) < 0.70. COPD severity was classified using the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria, which are based on spirometric findings [10]. Spirometry was performed by a trained technician according to the American Thoracic Society/European Respiratory Society guidelines [11]. Patients diagnosed with respiratory disorders other than COPD, such as asthma, were excluded. The exclusion criteria for the present analysis were history of treatment for COPD, cardiac rhythm other than sinus, previous atrial fibrillation, antiarrhythmic drug use, thyroid dysfunction, renal failure requiring haemodialysis, repeated pulmonary resection and recent pneumonia or myocardial infarction (<1 month). As a result, 48 patients were excluded. Thus, 202 patients were finally enrolled. The results for the patients who did and those who did not receive hANP during the perioperative period were compared. We previously reported that hANP had a prophylactic effect against postoperative cardiopulmonary complications for high-risk patients with elevated preoperative B-type natriuretic peptide (BNP) levels [9]. Therefore, we often used hANP therapy for high-risk COPD patients with elevated BNP levels before surgery. In the hANP group, the subjects received hANP (0.025 μg/kg/min without a bolus (Daiichisankyo Pharmaceutical, Inc., Tokyo, Japan)) for 3 days, which was administered intravenously just before the induction of general anaesthesia. Complete preoperative and follow-up

**Table 1:** Patients' characteristics and preoperative pulmonary function variables[a]

| | hANP group ($n$ = 51) | Control group ($n$ = 151) | $P$-value |
|---|---|---|---|
| Age (years) | 73.5 ± 6.5 | 68.5 ± 9.0 | 0.0004 |
| Males | 45 (88%) | 140 (93%) | 0.32 |
| Hypertension | 39 (77%) | 84 (56%) | 0.008 |
| Dyslipidaemia | 26 (51%) | 40 (27%) | 0.001 |
| Diabetes mellitus | 12 (24%) | 26 (17%) | 0.32 |
| Ischaemic heart disease | 22 (43%) | 27 (18%) | 0.0002 |
| Smoking history | 48 (94%) | 144 (95%) | 0.73 |
| Medication | | | |
| Calcium channel blockers | 30 (58%) | 78 (52%) | 0.42 |
| β-blockers | 4 (8%) | 5 (3%) | 0.18 |
| ACE inhibitors or ARBs | 11 (21%) | 26 (17%) | 0.49 |
| Statins | 16 (31%) | 28 (19%) | 0.06 |
| VATS procedure | 25 (49%) | 78 (52%) | 0.75 |
| Operating time (minutes) | 194 (164–251) | 215 (179–254) | 0.71 |
| Blood loss (ml) | 90 (40–250) | 100 (50–219) | 0.84 |
| Mediastinal lymph node dissection | 38 (75%) | 113 (75%) | 0.96 |
| Use of catecholamines | 5 (10%) | 18 (12%) | 0.68 |
| Surgical procedure | | | |
| Pneumonectomy | 1 (2%) | 3 (2%) | 0.99 |
| Lobectomy | 42 (82%) | 130 (86%) | 0.52 |
| Segmentectomy or wedge resection | 8 (16%) | 18 (12%) | 0.49 |
| Lung cancer staging | | | |
| IA, IB | 40 (78%) | 108 (72%) | 0.34 |
| IIA, IIB | 7 (14%) | 25 (17%) | 0.63 |
| IIIA, IIIB | 4 (8%) | 18 (12%) | 0.81 |
| VC, % predicted | 101 ± 14 | 106 ± 16 | 0.07 |
| $FEV_1$, % predicted | 79.8 ± 12 | 77.0 ± 14 | 0.25 |
| DLco, % predicted | 95.0 ± 23 | 93.8 ± 23 | 0.30 |
| $PaO_2$, Torr | 86.6 ± 8.2 | 86.1 ± 11 | 0.45 |
| $PaCO_2$, Torr | 39.4 ± 5.2 | 39.8 ± 4.1 | 0.76 |
| BNP, pg/ml | 53.6 (30.8–95.4) | 18.8 (10.6–36.1) | <0.0001 |
| COPD-GOLD stages | | | |
| I | 25 (49%) | 77 (51%) | 0.81 |
| II | 23 (45%) | 65 (43%) | 0.80 |
| III | 3 (6%) | 9 (6%) | 0.98 |

[a]Values are shown as numbers (%), means ± SD or medians with an inter-quartile range.
ACE: angiotensin-converting enzyme; ARB: angiotensin receptor blocker; BNP: B-type natriuretic peptide; DLco: diffusion capacity of the lung for carbon monoxide; $FEV_1$: forced expiratory ventilation in 1 s; FVC: forced vital capacity; GOLD: Global Initiative for Obstructive Lung Disease; $PaCO_2$: partial pressure of carbon dioxide in the blood; $PaO_2$: partial pressure of oxygen in arterial blood; VATS: video-assisted thoracoscopic surgery; VC: vital capacity.

data were obtained for all patients. The study protocol was approved by the Institutional Review Boards of both institutions, and all patients provided their written informed consent before participation (trial registration number: JPRN-UMIN000003631). Preoperative evaluations: all the patients underwent pulmonary resections with anterolateral thoracotomy or thoracoscopic surgery, as previously described [12]. White blood cell (WBC) counts and the levels of C-reactive protein (CRP) and BNP were determined before surgery and 1, 3 and 7 days, as well as 1 month, after surgery. All the complications that occurred during the same hospitalization were recorded. Cardiopulmonary complications were defined as previously described [12].

## Statistical analysis

Data are presented as means ± standard deviation (SD) or as medians with an inter-quartile range. All the data were analysed using the SPSS statistical software package (version 11.0; IBM Corporation, Armonk, NY, USA). Categorical variables are shown as the percentage of the sample. Comparisons between the two groups were made by Student's *t*-test for normally distributed variables, by the Mann–Whitney *U*-test for non-normally distributed variables and by the $\chi^2$ test for categorical variables. Probability values <0.05 were considered significant.

In addition, we performed a propensity score matching analysis to reduce treatment selection bias for each group (control group and hANP group). The propensity score for each case was calculated using multilogistic analysis, with age, hypertension, dyslipidaemia, ischaemic heart disease, statin use and BNP levels as variables. The patients were selected by matching propensity scores without clinical information regarding the outcome. Statistical calculations were performed using the R statistical software [13].

# RESULTS

## Subjects

There were a larger number of patients with older age, hypertension, dyslipidaemia and ischaemic heart disease in the hANP

**Table 2:** Patient characteristics with propensity score matching analysis[a]

| | hANP group ($n = 43$) | Control group ($n = 43$) | *P*-value |
|---|---|---|---|
| Age (years) | 72.8 ± 6.7 | 72.9 ± 9.2 | 0.97 |
| Males | 39 (91%) | 37 (86%) | 0.51 |
| Hypertension | 31 (72%) | 29 (67%) | 0.64 |
| Dyslipidaemia | 18 (42%) | 21 (49%) | 0.52 |
| Diabetes mellitus | 10 (23%) | 9 (21%) | 0.80 |
| Ischaemic heart disease | 16 (37%) | 18 (42%) | 0.66 |
| Smoking history | 41 (95%) | 39 (91%) | 0.40 |
| Medication | | | |
| Calcium channel blockers | 21 (49%) | 26 (61%) | 0.28 |
| β-Blockers | 3 (7%) | 1 (2%) | 0.31 |
| ACE inhibitors or ARBs | 8 (19%) | 8 (19%) | - |
| Statins | 10 (23%) | 11 (26%) | 0.80 |
| VATS procedure | 22 (51%) | 29 (67%) | 0.13 |
| Operating time (minutes) | 195 (152–257) | 206 (165–243) | 0.45 |
| Blood loss (ml) | 85 (40–240) | 100 (35–148) | 0.19 |
| Mediastinal lymph node dissection | 32 (74%) | 24 (56%) | 0.72 |
| Use of catecholamines | 4 (10%) | 6 (14%) | 0.43 |
| Surgical procedure | | | |
| Pneumonectomy | 0 (0%) | 0 (0%) | - |
| Lobectomy | 37 (86%) | 33 (77%) | 0.27 |
| Segmentectomy or wedge resection | 5 (12%) | 9 (21%) | 0.25 |
| Lung cancer staging | | | |
| IA, IB | 34 (79%) | 30 (70%) | 0.33 |
| IIA, IIB | 6 (14%) | 7 (16%) | 0.77 |
| IIIA, IIIB | 3 (7%) | 6 (14%) | 0.30 |
| VC, % predicted | 101 ± 13 | 105 ± 14 | 0.18 |
| $FEV_1$, % predicted | 82.8 ± 12 | 82.6 ± 12 | 0.63 |
| DLco, % predicted | 96.0 ± 22 | 94.2 ± 23 | 0.16 |
| $PaO_2$, Torr | 86.5 ± 9.5 | 85.3 ± 9.4 | 0.28 |
| $PaCO_2$, Torr | 38.6 ± 5.1 | 38.5 ± 3.5 | 0.86 |
| BNP, pg/ml | 53.0 (20.7–79.2) | 44.0 (23.7–56.9) | 0.39 |
| COPD-GOLD stages | | | |
| I | 23 (54%) | 25 (58%) | 0.67 |
| II | 18 (42%) | 17 (40%) | 0.83 |
| III | 2 (5%) | 1 (2%) | 0.56 |

[a]Values are shown as numbers (%), means ± SD or medians with an interquartile range.
ACE: angiotensin-converting enzyme; ARB: angiotensin receptor blocker; BNP: B-type natriuretic peptide; DLco: diffusion capacity of the lung for carbon monoxide; $FEV_1$: forced expiratory ventilation in 1 s; FVC: forced vital capacity; GOLD: Global Initiative for Obstructive Lung Disease; $PaCO_2$: carbon dioxide blood partial pressure; $PaO_2$: arterial oxygen blood partial pressure; VATS: video-assisted thoracoscopic surgery; VC: vital capacity.

group, and the hANP group had significantly higher BNP levels before surgery than the control group because of the patient selection, as previously mentioned (Table 1).

## Propensity score matching analysis

Of the 202 patients, 86 were extracted using propensity score matching to confirm the significant effects of hANP during the perioperative period. Their background characteristics were matched, as shown in Table 2.

## Incidence of postoperative cardiopulmonary complications

Postoperative cardiopulmonary complications were identified in 53 (35%) control group patients and 7 (14%) hANP group patients; they are listed in Table 3. The incidence of postoperative cardiopulmonary complications was significantly lower in the hANP group ($P = 0.004$); nevertheless, there were more high-risk patients in the hANP group. The most common complication in both groups was arrhythmias, and the incidence of cardiovascular complications was significantly lower in the hANP group ($P = 0.037$). The incidence of respiratory complications was also significantly lower in the hANP group ($P = 0.044$). In the patients with propensity score matching analysis, the incidence of postoperative cardiopulmonary complications was also significantly lower in the hANP group ($P = 0.0004$, Table 4).

## Operative mortality

The overall operative mortality was 1.0% (two patients). The causes of death were acute respiratory distress syndrome and acute myocardial infarction, both in the control group.

## Haemodynamics

There were no significant differences between the groups in systolic blood pressure, diastolic blood pressure and heart rate during the perioperative period (Fig. 1). There were similar results in the analysis of the propensity score-matched groups.

## Postoperative white blood cell counts and C-reactive protein levels

The hANP group had significantly lower WBC counts and CRP levels than the control group 3 and 7 days, as well as 1 month, after surgery (Fig. 2), even though the period of hANP infusion was only 3 days. There were similar results in the analysis of the propensity score-matched groups.

## DISCUSSION

The present results indicate that low-dose hANP has a prophylactic effect against postoperative cardiopulmonary complications in COPD patients following pulmonary resection for lung cancer. We also performed propensity score matching analysis to reduce treatment selection bias. This analysis clarified the effects of hANP on postoperative cardiopulmonary complications. The present findings indicate that low-dose hANP administration is a valuable treatment option to prevent postoperative cardiopulmonary complications in untreated COPD patients undergoing thoracic surgery.

It has been reported that the sympathetic nervous system, as well as renin-angiotensin activity, is activated in COPD patients [14]. It is well known that enhanced sympathetic nervous system activity is associated with the pathophysiology of cardiovascular events. Several studies have demonstrated an increased risk of cardiovascular complications in COPD patients after lung cancer surgery compared with the general population [4, 5]. It is known that hANP has cardioprotective effects, including inhibition of sympathetic nervous system activity and the renin-angiotensin-aldosterone system [15, 16]. Sezai *et al.* [15] reported that low-dose hANP infusion (0.02 μg/kg/min) did not significantly change haemodynamics, but reduced renin activity

**Table 3:** Postoperative cardiopulmonary complications in COPD patients after lung cancer surgery

| | hANP group ($n = 51$) | Control group ($n = 151$) | *P*-value |
|---|---|---|---|
| All cardiopulmonary complications | 7 (14%) | 53 (35%) | 0.004* |
| Cardiovascular complications | 6 (12%) | 39 (26%) | 0.037* |
| Atrial fibrillation | 5 | 34 | |
| Paroxysmal supraventricular tachycardia | 1 | 2 | |
| Acute myocardial infarction | 0 | 3 | |
| Respiratory complications | 1 (2%) | 17 (11%) | 0.044* |
| Pneumonia | 1 | 13 | |
| Acute respiratory distress syndrome | 0 | 4 | |

*Significant ($P < 0.05$).

**Table 4:** Postoperative cardiopulmonary complications in the patients with propensity score matching analysis

| | hANP group ($n = 43$) | Control group ($n = 43$) | *P*-value |
|---|---|---|---|
| All cardiopulmonary complications | 4 (9%) | 18 (42%) | 0.0004* |
| Cardiovascular complications | 3 (7%) | 11 (26%) | 0.0193* |
| Atrial fibrillation | 3 | 7 | |
| Paroxysmal supraventricular tachycardia | 0 | 1 | |
| Acute myocardial infarction | 0 | 3 | |
| Respiratory complications | 1 (2%) | 8 (19%) | 0.0133* |
| Pneumonia | 1 | 6 | |
| Acute respiratory distress syndrome | 0 | 2 | |

*Significant ($P < 0.05$).

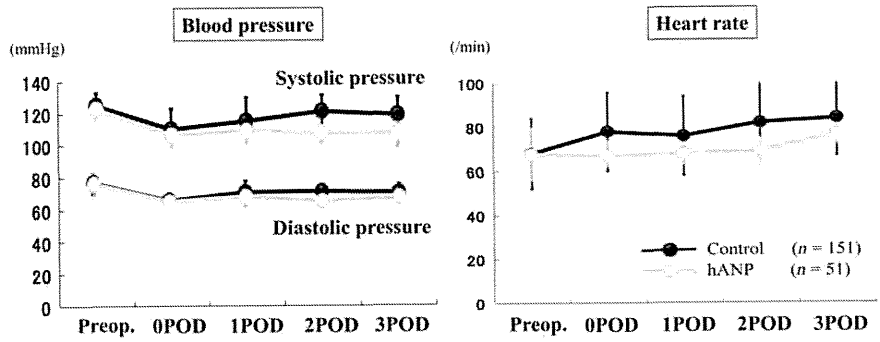


**Figure 1:** Changes in perioperative systolic blood pressure, diastolic blood pressure and heart rate. Each point with bars shows the mean ± SD.

and angiotensin- and aldosterone levels, which prevented postoperative cardiovascular complications after coronary artery bypass grafting [16].

Left ventricular (LV) diastolic dysfunction has often been recognized in COPD patients [17]. LV diastolic dysfunction has also been associated with an increased risk of cardiovascular events [18]. We previously reported that patients with LV diastolic dysfunction before lung cancer surgery have an increased risk of postoperative atrial fibrillation [19]. It is known that hANP has beneficial effects on LV diastolic dysfunction [20]. Nakajima *et al.* [20] reported that atrial natriuretic peptide (ANP) infusion improved LV diastolic function in patients with mild to moderate heart failure. We previously reported that hANP had a prophylactic effect against postoperative atrial fibrillation in patients with elevated preoperative BNP levels [21]. These studies suggest that hANP attenuates sympathetic nervous system activity and impairs LV diastolic function during the perioperative period and exerts cardioprotective effects after surgery, which might lead to a prophylactic effect for COPD patients, preventing cardiovascular complications in the present study.

COPD is not simply a lung disease but a type of systemic inflammatory disease. Cigarette smoking has been shown to induce inflammatory changes in the lung that are characterized by the recruitment and activation of inflammatory cells, including alveolar macrophages and neutrophils [22]. Furthermore, recent reports show evidence that long-term smoking leads to systemic inflammation, possibly through spillover from the pulmonary inflammatory process. COPD patients have higher levels of systemic inflammatory markers, including CRP, fibrinogen, tumour necrosis factor-alpha and interleukin 6 [23]. Furthermore, recent reports suggest that the mechanism linking increased cardiovascular disease and COPD is potentially explained by systemic inflammation, including CRP [24]. ANP has recently been shown to have remarkable anti-inflammatory effects. Several studies have shown that ANP attenuates several pathways of inflammation *in vitro* and *in vivo*, suggesting its role in the regulation of pulmonary function in the setting of acute lung injury and pulmonary inflammation [8, 25]. Xing *et al.* [8] reported that ANP attenuated activation of inflammatory signalling by lipopolysaccharide and tumour necrosis factor-alpha in human pulmonary endothelial cells and protected against bacteria-induced lung injury and pulmonary endothelial barrier dysfunction. Mitaka *et al.* [25] reported that hANP infusion improved pulmonary gas exchange in patients with acute lung injury during mechanical ventilation. Furthermore, we previously reported that hANP had a prophylactic effect not only on cardiovascular but also on pulmonary complications in elderly patients [9]. These

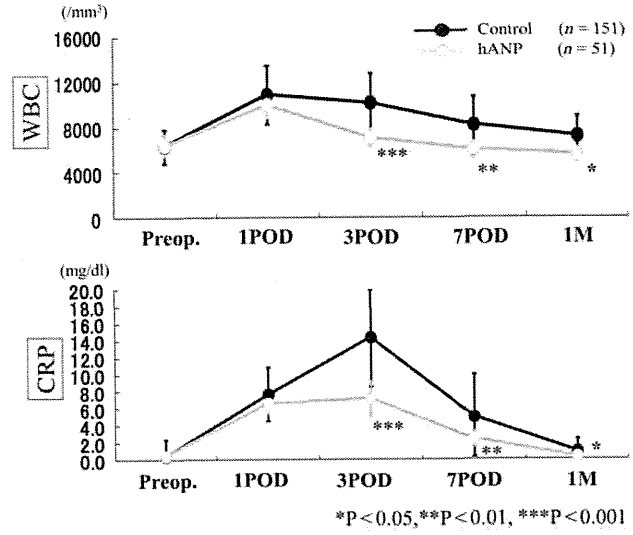


**Figure 2:** Changes in white blood cell counts and C-reactive protein levels in patients undergoing elective pulmonary resection for lung cancer who did and those who did not receive an infusion of hANP. Each point with bars shows the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, POD: postoperative day; M: month; Preop: preoperative.

studies suggest that hANP attenuates systemic inflammatory changes, including pulmonary inflammation, and protects the function of pulmonary endothelial cells after surgery, which might lead to a prophylactic effect for COPD patients, preventing both cardiovascular and respiratory complications in the present study.

This study was a two-institution clinical study, which restricted our ability to generalize the results. In addition, the patients were not assigned to the groups randomly. Furthermore, the number of patients in the study cohort was relatively small. Thus, additional investigations with a larger number of patients from multiple institutions are necessary to allow generalization of the findings obtained here.

The present study is the first to show the prophylactic effects of low-dose hANP on postoperative cardiopulmonary complications in untreated COPD patients undergoing pulmonary resection for lung cancer. Additional studies are warranted to determine whether these effects can be observed in other patients and translated into improved clinical outcomes.

## ACKNOWLEDGEMENT

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**Conflict of interest:** none declared.

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## APPENDIX. CONFERENCE DISCUSSION

***Dr B. Passlick** (Freiburg, Germany)*: What are you planning for the future? Are you going for a randomized trial, or what is the objective?

***Dr Nojiri***: We have already conducted randomized human ANP therapy, and probably next year, we will start the randomized study on lung cancer surgery.

***Dr Passlick***: You mentioned in your abstract that you are able to also decrease respiratory complications. What is the mechanism? How can you explain that?

***Dr Nojiri***: Pulmonary endothelial cells have a high expression of granylate cyclase A receptor, which is the specific receptor of ANP and BNP. ANP has recently been shown to have remarkable anti-inflammatory effects for pulmonary inflammation via the GCA receptor of the pulmonary endothelial cells. Several studies have shown that ANP attenuates activation of inflammatory signalling, such as NFkB and Rho signalling, by LPS or TNF-alpha in *in vitro* and *in vivo* models.

In human studies, Mitaka et al. reported in 1998 that human ANP infusion improved pulmonary gas exchange in patients with acute lung injury during mechanical ventilation.

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# Pneumonectomy after response to gefitinib treatment for lung adenocarcinoma

Yasunobu Funakoshi, Yukiyasu Takeuchi and Hajime Maeda

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# Pneumonectomy after response to gefitinib treatment for lung adenocarcinoma

**Yasunobu Funakoshi, Yukiyasu Takeuchi and Hajime Maeda**

**Abstract**

A 69-year-old Japanese woman who had never smoked had lung adenocarcinoma harboring epidermal growth factor receptor mutation. After 8 months of gefitinib treatment, salvage pneumonectomy was performed. Microscopic examination showed that non-responsive adenocarcinoma remained although necrosis was prominent. Postoperatively, the patient developed empyema that was successfully managed. The postoperative empyema after treatment with gefitinib should be noted, as well as the finding that residual viable tumor cells remained even after the dramatic radiographic response.

**Keywords**

Antineoplastic agents, non-small-cell carcinoma, gefitinib, epidermal growth factor receptor, salvage therapy

## Introduction

Epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKI) have proven efficacy in advanced non-small-cell lung cancer (NSCLC).[1] Here, we report the case of a Japanese woman who underwent salvage pneumonectomy after 8 months of gefitinib treatment. Nonresponsive areas remained even after a dramatic radiographic response.

## Case report

A 69-year-old Japanese woman who had never smoked, presented with a suspicious lesion on chest radiography. A chest computed tomography (CT) scan revealed a mass measuring 5 cm in diameter in the left lower lobe (cT2aN0M0, stage IB; Figure 1(a) and (b)). Fluorodeoxyglucose positron-emission tomography-CT demonstrated increased uptake with a maximum standardized uptake value of 11.7. Her serum level of carcinoembryonic antigen was 78.8 $g \cdot mL^{-1}$. Thrombocytopenia was found; the platelet count was 69,000/$mm^3$. Transbronchial biopsy confirmed adenocarcinoma. She had been initially diagnosed as operable, but pneumonectomy was needed because the tumor invaded the pulmonary artery proximal to the lingula branch and just distal to the second carina. She refused surgery then, and systemic chemotherapy was not recommended because of thrombocytopenia. EGFR-TKI treatment was proposed because an EGFR mutation analysis had revealed an exon 19 deletion. Gefitinib was prescribed at a dose of 250 $mg \cdot day^{-1}$ for first-line therapy. Adverse effects were confined to mild skin toxicity (grade 2). After 8 months of treatment, the patient had a good partial response with a size reduction of 40% on chest CT (Figure 1(c) and (d)), and her serum level of carcinoembryonic antigen decreased to 7.1 $ng \cdot mL^{-1}$. Positron-emission tomography-CT after the treatment showed a striking metabolic response with a reduction of the maximum standardized uptake value to 2.6. We performed salvage pneumonectomy and systemic lymphadenectomy. Complete resection was confirmed (ypT2aN0M0, stage IB). On microscopic examination, necrosis with proliferation

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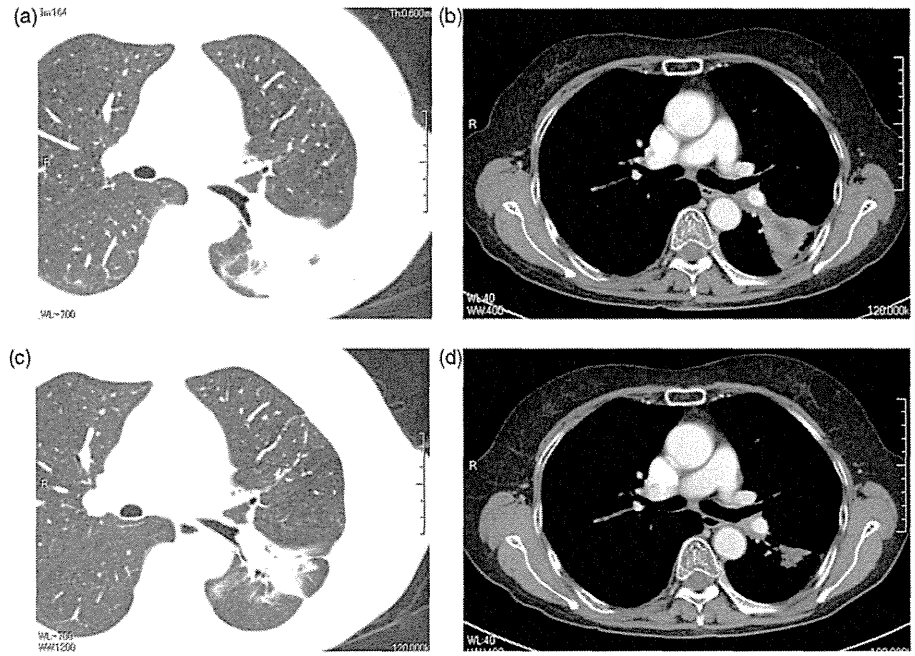


**Figure 1.** Chest computed tomography showing a 50-mm tumor in the left lower lobe before treatment with gefitinib (a) and (b). The shrunken tumor, with a size reduction of 40% after treatment with gefitinib (c) and (d).

of fibroblasts was prominent (Figure 2(a)). However, nonresponsive papillary adenocarcinoma was found (Figure 2(b)), clearly separated from the necrotic areas. Thrombocytopenia resolved after resection of the tumor. Two months after the operation, the patient developed empyema due to methicillin-sensitive *Staphylococcus epidermidis*. It was successfully managed by washing and curettage using video-assisted thoracoscopic surgery. At the time of the reoperation, the bronchial stump had recovered satisfactorily. Adjuvant EGFR-TKI was not applied because of empyema. Twenty-six months after the operation, the patient was alive without recurrence.

## Discussion

EGFR-TKI has dramatic efficacy in more than 70% of advanced NSCLC with EGFR gene mutations.[1] Some patients with inoperable NSCLC demonstrated a downstaging of their cancer to operable status after gefitinib treatment.[2,3] Kappers and colleagues[4] also reported that the response to treatment with erlotinib can be fast, with a complete pathological response achieved after only 3 weeks of treatment. The response to EGFR-TKI, such as gefitinib and erlotinib, is mostly within 3 to 4 weeks. The most common adverse events are skin rash and diarrhea, which are reversible on discontinuation of treatment.[1] Lara-Guerra and colleagues[5] reported that preoperative gefitinib treatment in the short term was well tolerated in terms of minimal toxicity and no additional surgical risk. However, preoperative EGFR-TKI treatment may be associated with impaired wound healing and infection. In this case, it was difficult to extract the drain tube promptly because of postoperative bleeding and then postoperative empyema, perhaps due to antidromic infection from the skin, although skin wound healing was not impaired. The skin rash may be connected to methicillin-sensitive *Staphylococcus epidermidis*. Thus it is necessary to note that wound infection and postoperative empyema may occur as a result of a skin rash, if surgery is planned after treatment with EGFR-TKI.

Takamochi and colleagues[3] reported that despite dramatic radiographic downstaging after gefitinib treatment, most patients had further advanced pathologic stages than their preoperative clinical stages. Hishida and colleagues[2] also stated that initially expressed systemic disease was essentially unchanged even after a dramatic radiologic response to gefitinib. In this case, nonresponsive papillary adenocarcinoma was clearly separated from the fibrous scar, macroscopically as well as microscopically. It should be noted that residual viable tumor cells remained after the dramatic radiographic response to gefitinib treatment.

As well as in the present case, EGFR-TKI treatment could be an option for neoadjuvant therapy in advanced adenocarcinoma. The reoperative EGFR-TKI treatment strategy should be reevaluated in the neoadjuvant setting for early disease as well as locally advanced but operable disease.[2] Although there is a low

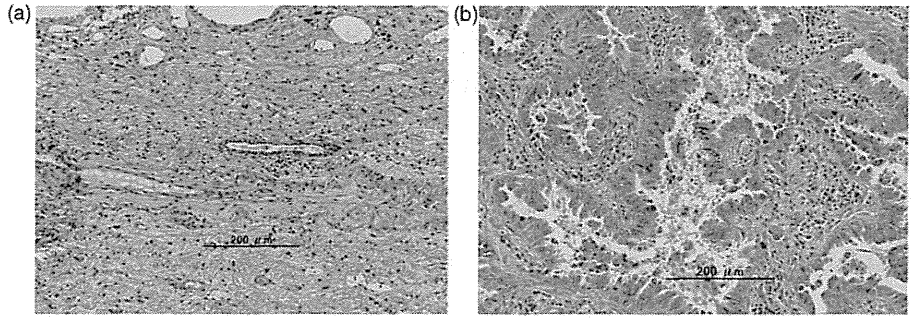


**Figure 2.** Histological findings of the resected tumor (hematoxylin eosin stain). (a) Massive necrosis with proliferation of fibroblasts. (b) Non-responsive papillary adenocarcinoma.

rate of adverse events and high compliance, as well as a satisfactory response rate after 28 days of preoperative treatment in patients with early NSCLC, the safety and feasibility of surgery in patients taking EGFR-TKI for a long period have not yet been confirmed.[5] The optimal duration of EGFR-TKI treatment, the timing of surgery, and the role of adjuvant EGFR-TKI treatment should be investigated in the future.

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## Conflicts of interest statement

None declared.

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# Immunohistochemical studies of pulmonary large cell neuroendocrine carcinoma: A possible association between staining patterns with neuroendocrine markers and tumor response to chemotherapy

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**Objective:** Pulmonary large cell neuroendocrine carcinoma is a rare high-grade malignant tumor. Because large cell neuroendocrine carcinoma is rare, the optimal treatment, including perioperative chemotherapy, has not been defined. We retrospectively analyzed the correlation among the effectiveness of perioperative chemotherapy in treating large cell neuroendocrine carcinoma, pathologic stage, and immunoreactivity to neuroendocrine markers.

**Methods:** A total of 63 patients with pulmonary large cell neuroendocrine carcinoma undergoing surgical resection from 2001 to 2009 were included. The resected tumors were immunohistochemically stained with the 3 neuroendocrine markers synaptophysin, chromogranin A, and neural cell adhesion molecule. We categorized patients who were positive for all 3 markers as the triple-positive group and those who were negative for 1 or 2 markers as the non–triple-positive group.

**Results:** Perioperative chemotherapy resulted in better overall survival than surgery alone ($P = .042$). Multivariate analysis of survival revealed that perioperative chemotherapy was a significant independent prognostic factor (hazard ratio, 0.323; 95% confidence interval, 0.112-0.934; $P = .0371$). Among the patients who received perioperative chemotherapy, the non–triple-positive group had a significantly greater 5-year survival rate than the triple-positive group ($P = .0216$). Moreover, among the non–triple-positive group, a significantly greater 5-year survival rate was observed for the patients who underwent surgery with chemotherapy than for those who underwent surgery without chemotherapy ($P = .0081$). In contrast, no difference was found in 5-year survival between patients with chemotherapy and those without chemotherapy when the tumors were triple positive.

**Conclusions:** Our results suggest that perioperative chemotherapy might benefit the survival of patients with pulmonary large cell neuroendocrine carcinoma, in particular when the tumors are not immunoreactive to all 3 neuroendocrine markers. (J Thorac Cardiovasc Surg 2013;145:839-46)

Pulmonary large cell neuroendocrine carcinoma (LCNEC), proposed as a separate tumor category by Travis and colleagues[1] in 1991, is distinguished from typical carcinoid, atypical carcinoid, and small-cell lung carcinoma (SCLC) by its morphologic and biologic features. In 1999, the World Health Organization classified LCNEC as a variant of large cell carcinoma.[2] Pulmonary LCNEC represents about 2% to 3% of all lung malignancies and is associated with a worse prognosis than other non–SCLC (NSCLC), even in the early stage.[3-6] However, in a recent Japanese study with a large sample size, Asamura and colleagues[7] reported that no prognostic difference was found between pulmonary LCNEC and SCLC.

Several small-scale retrospective studies have demonstrated that perioperative chemotherapy could improve the survival of patients with pulmonary LCNEC. Perioperative chemotherapy is recommended even for patients with resectable stage I LCNEC because of its aggressive course, remarkably dismal prognosis, and high potential for metastasis.[8-11] However, owing to the rarity of this tumor, the incidence, prognosis, and optimal treatment remain to be determined.

In the present study, we retrospectively analyzed the efficacy of perioperative chemotherapy in treating pulmonary LCNEC. Furthermore, we examined the correlation between the sensitivity of LCNEC and perioperative chemotherapy and the immunohistochemical staining patterns of

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**Abbreviations and Acronyms**
CPT-11 = irinotecan
LCNEC = large cell neuroendocrine carcinoma
NCAM = neural cell adhesion molecule
NSCLC = non–small-cell lung carcinoma
SCLC = small-cell lung carcinoma
VP-16 = etoposide

the tumors with 3 immunohistochemical neuroendocrine markers, synaptophysin, chromogranin A, and neural cell adhesion molecule (NCAM). Although our experiences in 2 institutions do not allow us to reach a definite conclusion owing to the small number of subjects, the present preliminary study may be useful in generating a hypothesis to determine the immunohistochemical biomarkers to predict LCNEC's response to perioperative chemotherapy in future prospective multi-institutional trials.

## METHODS

We retrospectively examined the clinical data of 63 patients with pulmonary LCNEC who underwent complete surgical resection from 2001 to 2009. All follow-up data were current as of December 31, 2011. All patients who underwent surgery in 2009 were included in the present study, because more than 2 years have passed since their surgery. The median follow-up period was 32.3 months (range, 2.8-95.3 months). The Hyogo Cancer Center and Kobe University Hospital institutional review boards approved the study, and each participant provided informed consent. LCNEC was diagnosed using the following histopathologic criteria: (1) neuroendocrine morphology such as an organoid, palisading, rosette-like, or trabecular growth pattern; (2) high mitotic count (≥11/10 high-power fields [HPF]); (3) tumor necrosis (often large zone); (4) large cell size with low nuclear/cytoplasmic ratio, vesicular or fine chromatin, and/or frequent nucleoli; and (5) positive immunostaining for 1 or more of the neuroendocrine markers, synaptophysin, chromogranin A, and NCAM.[2]

Immunohistochemical stains were performed on 4-mm-thick, formalin-fixed, paraffin-embedded sections. The deparaffinized sections underwent CC1 buffer pretreatment (pH 8.5, ethylenediaminetetraacetic acid; Roche, Mannheim, Germany) and were immunostained for the markers with the streptavidin-biotin technique with an automated immunostainer (Benchimark; Ventana, Tucson, Ariz) according to the manufacturer's instructions. Antibodies against chromogranin A (polyclonal, 1:500 dilution; Dako, Glostrup, Denmark), synaptophysin (monoclonal, clone 27G12, 1:2 dilution; Nichirei, Tokyo, Japan), CD56 (NCAM; monoclonal, 1:100 dilution; Novocastra, Newcastle, UK), and Ki-67 (monoclonal 1:100 dilution; Dako) were used.

All samples were evaluated by an expert pathologist (C.O.) without knowledge of the patient's outcome. Plural sections, more than 10 sections in most cases, were prepared in each case, and 1 representative specimen involving tumor was selected for immunohistochemistry. The final results were reported as negative (no positive cells) or positive (immunoreactive). Proliferative activity was expressed as the MIB-1 index, which was calculated as the proportion of Ki-67–positive cells by counting 500 to 1000 cancer cells. The mitotic counts were performed using an Olympus BX53 microscope at a magnification of ×400, counting 3 sets of 10 HPF for each tumor. The area with the greatest numbers of mitoses was counted. In the present study, we included pure LCNEC and combined LCNEC, in which at least 1 portion of neuroendocrine differentiation or morphology in NSCLC was LCNEC. The medical records provided information on the patient age, gender, smoking status, pathologic stage, perioperative chemotherapy, and operative procedure. The determination of disease stage was based on the TNM classification using the International Union Against Cancer staging system.[12]

We classified patients into 2 groups to investigate the correlation between the sensitivity of LCNEC to perioperative chemotherapy and the results of immunohistochemical staining of neuroendocrine markers. We categorized the patients who were positive for all 3 neuroendocrine markers (ie, synaptophysin, chromogranin A, and NCAM) as the triple-positive group and those who were negative for 1 or 2 of the markers as the non–triple-positive group. Statistical analyses were performed using JMP, version 8, software (SAS Institute, Cary, NC). Student's *t*-test and the chi-square test were performed to assess the significance of the differences in age, gender, smoking status, surgical procedure, and pathologic stage between the triple-positive and non–triple-positive groups. Survival was calculated using the Kaplan-Meier method, and differences in the distributions were evaluated using the log-rank test. The Cox proportional hazards model was used to evaluate the association between the prognostic factors and survival rate after pulmonary resection, with the hazards ratio and 95% confidence intervals. Significance was set at $P < .05$.

## RESULTS

The clinicopathologic characteristics of the 63 patients with pulmonary LCNEC who underwent surgical resection are listed in Table 1. The patient age ranged from 30 to 84 years (mean age, 67.0 years). Of the 63 patients, 54 (87%) were men, and 58 (92%) were former or current smokers. The surgical procedures included 55 lobectomies, 2 segmentectomies, and 6 wedge resections with complete resection (R0). Of the 55 lobectomies, 8 bronchoplastic procedures were performed and 6 extended resections were required because of tumor invasion into the adjacent tissue, including muscle and rib (n = 3), parietal pleura (n = 1), and vagal nerve (n = 2). Of the 6 patients who underwent extended resection, 5 were treated with chemotherapy. Because these patients had advanced-stage disease and the number of the subjects was small, no correlation was found between the extent of resection and the outcome.

The distribution of pathologic stage was stage IA in 19 patients (30%), stage IB in 16 (25%), stage IIA in 5 (8%), stage IIB in 11 (18%), stage IIIA in 9 (14%), and stage IIIB in 3 patients (5%). The mean MIB-1 index for all patients was 62.7% (range, 5.2%–90.5%), and the mean mitotic count was 71.2/10 HPF (range, 14–153/10 HPF). All 63 patients had tumor necrosis.

Perioperative platinum-based chemotherapy was administered to 23 (37%) of the 63 patients. We have used the criterion of tumor size more than 3 cm in offering chemotherapy for patients with stage I disease since 2004. Thus, 8 of 35 patients with stage I received chemotherapy. Also, 9 of 16 with stage II and 6 of 12 with stage III received chemotherapy. Induction chemotherapy was administered to 3 patients at clinical stage III and adjuvant chemotherapy was administered to 20 patients at clinical stage I/II. Three patients received preoperative mediastinal radiotherapy (40 Gy) combined with induction chemotherapy. No patient

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**TABLE 1. Patient characteristics (n = 63)**

| Factor | Total | Triple positive | Non–triple positive | *P* value |
|---|---|---|---|---|
| Patients (n) | 63 | 31 | 32 | |
| Age (y) | | | | .0473 |
| Mean | 67.0 | 64.4 | 69.5 | |
| Range | 30–84 | 30–78 | 41–84 | |
| Gender | | | | .2578 |
| Male | 54 (87) | 25 (81) | 29 (91) | |
| Female | 9 (13) | 6 (19) | 3 (9) | |
| Smoking status | | | | .1512 |
| Former or current | 58 (92) | 27 (87) | 31 (97) | |
| Never smoked | 5 (8) | 4 (13) | 1 (3) | |
| Surgical procedure | | | | .3416 |
| Lobectomy | 55 (87) | 26 (84) | 29 (90) | |
| Segmentectomy | 2 (3) | 3 (10) | 0 (0) | |
| Wedge resection | 6 (10) | 2 (6) | 3 (10) | |
| Pathologic stage | | | | .6044 |
| IA | 19 (30) | 11 (35) | 8 (25) | |
| IB | 16 (25) | 7 (23) | 9 (28) | |
| IIA | 5 (8) | 3 (10) | 2 (6) | |
| IIB | 11 (18) | 3 (10) | 8 (25) | |
| IIIA | 9 (14) | 5 (16) | 4 (13) | |
| IIIB | 3 (5) | 2 (6) | 1 (3) | |
| MIB-1 index (%) | | | | .5029 |
| Mean | 62.7 | 61.2 | 64.4 | |
| Range | 5.2–90.5 | 5.2–90.0 | 5.8–90.5 | |
| Mitotic counts (/10 HPF) | | | | .3538 |
| Mean | 71.2 | 64.7 | 77.9 | |
| Range | 14–153 | 14–122 | 20–153 | |

Data in parentheses are percentages. *Triple positive,* Positive for synaptophysin, chromogranin A, and neural cell adhesion molecule; *Non–triple positive,* negative for 1 or 2 neuroendocrine markers (synaptophysin, chromogranin A, and neural cell adhesion molecule); *HPF,* High-powered fields.

underwent postoperative radiotherapy. The chemotherapy regimens are listed in Table 2.

The results of immunohistochemical staining for the 3 neuroendocrine markers are summarized in Table 3. Although the percentage of reactive cells ranged very much, the intensity of immunostaining was not so variegated for all 3 neuroendocrine markers. Of the 63 tumors, 40 (63%) were positive for synaptophysin, 36 (57%) for chromogranin A, and 59 (94%) for NCAM. Finally, 31 tumors (49%) were positive for all 3 neuroendocrine markers and 32 (51%) were negative for 1 or 2 markers. The clinicopathologic characteristics and chemotherapy regimens of patients in the triple-positive group and non–triple-positive group are listed in Tables 1and 2, respectively. Although the triple-positive group was significantly younger than the non–triple-positive group, no significant differences were seen in the distribution of other characteristics between the 2 groups. Also, no morphologic differences were found between the 2 groups in the neuroendocrine structures such as rosettes and ribbon-like arrangements, necrosis, mitotic counts, and MIB-1 index.

**TABLE 2. Regimens of perioperative platinum-based chemotherapy (n = 23)**

| Regimen | Triple positive (n = 12) | Non–triple positive (n = 11) |
|---|---|---|
| Induction chemotherapy | | |
| CDDP+VP-16 | 1 | 0 |
| CDDP+VNR | 0 | 1 |
| CBDCA+DOC | 1 | 0 |
| Adjuvant chemotherapy | | |
| CDDP+CPT-11 | 5 | 2 |
| CBDCA+PTX | 2 | 4 |
| CDDP+VNR | 2 | 3 |
| CBDCA+VP-16 | 1 | 1 |

*Triple positive,* Positive for synaptophysin, chromogranin A, and neural cell adhesion molecule; *Non–triple positive,* negative for 1 or 2 neuroendocrine markers (synaptophysin, chromogranin A, and neural cell adhesion molecule); *CDDP,* cisplatin; *VP-16,* etoposide; *VNR,* vinorelbine; *CBDCA,* carboplatin; *DOC,* docetaxel; *CPT-11,* irinotecan; *PTX,* paclitaxel.

The overall 5-year survival rate among the 63 patients was 44.9%. Significantly longer survival was observed for the patients who underwent surgery with chemotherapy than for those who underwent surgery without chemotherapy (74.4% and 32.3%, respectively; $P = .042$; Figure 1, *A*).

Next, we evaluated whether the effects of perioperative chemotherapy were seen in patients with different stages. Although there was a tendency for longer survival for the patients with stage I disease who underwent surgery and chemotherapy compared with those who underwent surgery without chemotherapy, the small number of subjects did not allow us to obtain a statistically significant difference (85.7% and 35.2%, respectively; $P = .1129$; Figure 1, *B*). Similarly, no statistically significant difference in survival between the patients with and without chemotherapy at stage II/III (68.8% and 25.6%, respectively; $P = .1243$; Figure 1, *B*). Multivariate analysis of survival was

**TABLE 3. Immunohistochemical staining of 3 neuroendocrine markers (n = 63)**

| Neuroendocrine marker | Patients (n) |
|---|---|
| Synaptophysin | |
| Positive | 40 (63) |
| Negative | 23 (37) |
| Chromogranin A | |
| Positive | 36 (57) |
| Negative | 27 (43) |
| NCAM | |
| Positive | 59 (94) |
| Negative | 4 (6) |
| Triple positive | 31 (49) |
| Non–triple positive | 32 (51) |

Data in parentheses are percentages. *NCAM,* Neural cell adhesion molecule; *Triple positive,* Positive for synaptophysin, chromogranin A, and neural cell adhesion molecule; *Non–triple positive,* negative for 1 or 2 neuroendocrine markers (synaptophysin, chromogranin A, and neural cell adhesion molecule).

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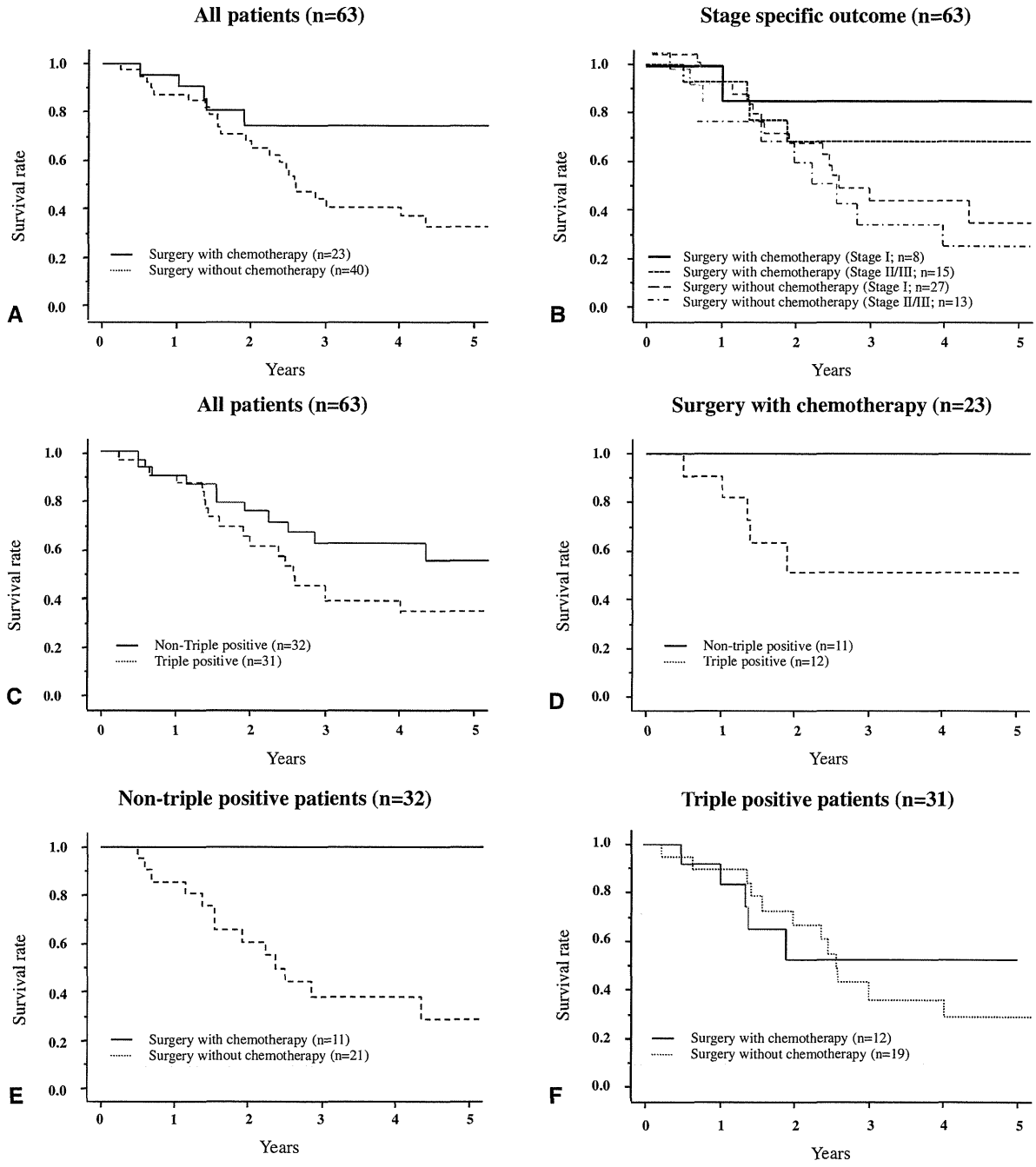


**FIGURE 1.** A, Comparison of survival of patients with large cell neuroendocrine carcinoma who underwent surgery with perioperative chemotherapy and those who underwent surgery alone. B, Comparison of stage-specific survival of patients with large cell neuroendocrine carcinoma who underwent surgery with perioperative chemotherapy and those who underwent surgery alone (stage I vs stage II/III). C, Comparison of survival of the non–triple-positive group and triple-positive group. D, Comparison of survival of the non–triple-positive group and triple-positive group among patients who received perioperative chemotherapy. E, Comparison of survival of non–triple-positive patients who underwent surgery with perioperative chemotherapy and those who underwent surgery without perioperative chemotherapy. F, Comparison of survival of triple-positive patients who underwent surgery with perioperative chemotherapy and those who underwent surgery without perioperative chemotherapy. *Non–triple positive,* Negative for 1 or 2 neuroendocrine markers (synaptophysin, chromogranin A, and neural cell adhesion molecule); *Triple positive,* positive for synaptophysin, chromogranin A, and neural cell adhesion molecule.

**TABLE 4. Multivariate analysis of prognostic factors and survival (Cox proportional hazards model)**

| Variable | HR | 95% CI | *P* value |
|---|---|---|---|
| Age (<75 vs ≥75 y) | 1.030 | 0.466–2.279 | .9409 |
| Gender (male vs female) | 1.091 | 0.400–2.967 | .8659 |
| Pathologic stage (I vs II/III) | 0.645 | 0.286–1.455 | .2904 |
| Surgical procedure (lobectomy vs sublobar resection) | 1.048 | 0.287–3.824 | .9431 |
| Treatment (surgery with chemotherapy vs surgery alone) | 0.323 | 0.112–0.934 | .0371 |

*HR*, Hazard ratio; *CI*, confidence interval.

performed using 5 clinical prognostic factors (age, gender, pathologic stage, surgical procedure, and surgery with or without chemotherapy; Table 4). Patients who underwent surgery with chemotherapy had a significantly better prognosis than those who underwent surgery without chemotherapy (hazards ratio, 0.323; 95% confidence interval, 0.112–0.934; $P = .0371$).

Next, we examined whether the clinical outcome of patients with LCNEC correlated with the immunohistochemical characteristics determined by immunoreactivity for 3 neuroendocrine markers. No significant difference was found in 5-year survival between the triple-positive and non–triple-positive patients (34.0% and 55.3%, respectively; $P = .1312$; Figure 1, *C*). No statistically significant difference was found in survival among the single-positive, double-positive, and triple-positive patients (data not shown). Among the patients who received perioperative chemotherapy, a significantly greater 5-year survival rate was observed in the non–triple-positive group than in the triple-positive group (100% and 51.9%, respectively; $P = .0216$; Figure 1, *D*). Moreover, in the non–triple-positive group, a significantly greater survival rate at 5 years was observed in patients who underwent surgery with adjuvant chemotherapy than in those who underwent surgery without chemotherapy (100% and 34.5%, respectively; $P = .0081$; Figure 1, *E*). In contrast, in the triple-positive group, no difference was found in 5-year survival between the patients who underwent surgery with adjuvant chemotherapy and those who underwent surgery without chemotherapy (51.8% and 28.1%, respectively; $P = .7682$; Figure 1, *F*).

We further analyzed the correlation of chemotherapy benefits and immunoreactivity patterns of neuroendocrine markers in patients with different stages. The patients with stage I and stage II/III did not differ in overall survival in the non–triple-positive group (53.2% and 56.3%, respectively; $P = .8910$; Figure 2, *A*). Survival differences were also not found in the triple-positive group between stage I and stage II/III (36.8% and 28.2%, respectively; $P = .6460$; Figure 2, *B*).

Because a limited number of patients with stage I disease received perioperative chemotherapy, we failed to show a statistically significant survival difference between the patients with and without chemotherapy in the non–triple-positive patients (100% and 40.6%, respectively; $P = .2002$; Figure 2, *C*) and the triple-positive patients (80% and 25.2%, respectively; $P = .2606$; Figure 2, *D*). However, perioperative chemotherapy resulted in a significantly greater 5-year survival rate in the non–triple-positive group patients with stage II/III than in the triple-positive group (100% and 17.9%, respectively; $P = .0074$; Figure 2, *E*). No correlation was found between the use of perioperative chemotherapy and the survival of patients with stage II/III disease in the triple-positive group. In the group of patients with triple-positive tumors, the 5-year survival rate of the patients with chemotherapy and without chemotherapy was 34.3% and 33.3%, respectively ($P = .6108$; Figure 2, *F*).

## DISCUSSION

Neuroendocrine lung tumors comprise a spectrum of epithelial neoplasms ranging from low-grade carcinoid tumor to SCLC. Although most SCLCs show neuroendocrine differentiation on immunohistochemistry or electron microscopy,[13] a significant minority of NSCLCs (approximately 10%–30%) show neuroendocrine differentiation. NSCLCs with neuroendocrine differentiation are considered to result in an especially poor prognosis. Several reports have indicated that NSCLCs with neuroendocrine differentiation were clinically aggressive with greater chemosensitivity; however, other studies have not shown any correlation.[8,14] A 5-year survival rate of 15% to 57% has been reported for all stages of LCNEC.[10,11] Sarkaria and colleagues[11] reported a 5-year survival rate of 37% for patients with stage IB-IIIA LCNEC who did not receive perioperative platinum-based chemotherapy compared with 51% in those patients who received it. Saji and colleagues[10] reported that the 5-year survival rate for patients undergoing perioperative chemotherapy was 87.5% and that of patients without perioperative chemotherapy was 58.5%.[10] Our results were similar.

Thus, we assumed that pulmonary LCNEC might have several features that make it sensitive to chemotherapy and tried to evaluate the association between the 3 neuroendocrine markers that are essential for the diagnosis of LCNEC and the responsiveness to chemotherapy. Positive immunostaining for 1 or more neuroendocrine markers among synaptophysin, chromogranin A, and NCAM is necessary to diagnose pulmonary LCNEC. Synaptophysin is a synaptic vesicle glycoprotein with 4 transmembrane domains; however, its exact function is unknown.[15] Chromogranin A is the major member of the granin family of acidic secretory glycoproteins and plays multiple roles in the secretory process.[16] NCAM, a glycoprotein, is a member of the immunoglobulin superfamily and contributes to the function of cell–cell adhesion.[17] Although all 3 markers are present in neuroendocrine cells, it remains possible