Table I. Clinicopathological characteristics of the patients with adenocarcinoma with an apparent MPP component compared with those with no MPP component or a focal MPP component.

| Clinicopathological characteristic | Apparent MPP component (n=18) (MPP component; >5%) | No or focal MPP component (n=200) (MPP component: 0 or <5%) |
|---|---|---|
| Age | 68.1±2.4 | 64.1±9.5 |
| Gender | | |
| Male | 13 (72.2) | 89 (44.5) |
| Female | 5 (27.8) | 111 (55.5) |
| Size (cm) | 1.8±0.1 | 1.8±0.1 |
| Histological type | MPP-positive | MPP-negative |
| AIS; mixed mucinous/nonmucinous | 0 (0) | 2 (1) |
| AIS; mucinous | 0 (0) | 2 (1) |
| AIS; nonmucinous | 0 (0) | 36 (18) |
| MIA; nonmucinous | 0 (0) | 20 (10) |
| Papillary predominant | 14 (77.7)[a] | 101 (50.5) |
| Acinar predominant | 1 (5.6) | 16 (8) |
| Invasive mucinous adenocarcinoma | 1 (5.6) | 4 (2) |
| Lepidic predominant | 0 (0) | 4 (2) |
| Solid predominant | 0 (0) | 15 (7.5) |
| Micropapillary predominant | 2 (11.1) | 0 (0) |
| Differentiation | | |
| Well | 10 (55.6) | 170 (85) |
| Moderate | 8 (44.4)[a] | 11 (5.5) |
| Poor | 0 (0) | 19 (9.5) |
| Lymphatic invasion | | |
| (+) | 5 (27.8) | 4 (2) |
| (-) | 13 (72.2)[a] | 196 (98) |
| Venous invasion | | |
| (+) | 0 (0) | 4 (2) |
| (-) | 18 (100) | 196 (98) |

[a]P<0.05 vs. the MPP-negative group. Data >3 are presented as the mean ± standard error of the mean. AIS; adenocarcinoma *in situ*; MIA, minimally invasive adenocarcinoma; MPP, micropapillary pattern.

Table II. E-cadherin and vimentin expression in patients with adenocarcinoma in the apparent MPP component group and no MPP component group.

| | | Apparent MPP component group (n=18) | | |
|---|---|---|---|---|
| Antibody | Grade | MPP component | Component without MPP | No MPP group (n=26) |
| E-cadherin | 3+ | 18 | 18 | 21 |
| | 2+ | 0 | 0 | 5 |
| | 1+ | 0 | 0 | 0 |
| | 0 | 0 | 0 | 0 |
| Vimentin | 3+ | 9 | 2 | 6 |
| | 2+ | 7 | 3 | 3 |
| | 1+ | 2 | 1 | 4 |
| | 0 | 0 | 12[a] | 13[a] |

Grade of expression was defined according to the proportion of positive cells as follows: 0, p<5% positive cells; 1+, 5≤p<30% positive cells; 2+, 30≤p<70% positive cells; 3+, p≥70% positive cells. [a]P<0.05 vs. MPP component in apparent MPP component group. MPP, micropapillary pattern.

Table III. Vimentin expression of cancer cells invading in a lymph vessel.

| Case | p of MPP component area (%) | Expression of vimentin | | | | | |
|---|---|---|---|---|---|---|---|
| | | Component without MPP | MPP component | ly-1 | ly-2 | ly-3 | ly-4 |
| 1 | 15≤p<30 | 2+ | 3+ | 3+ | 3+ | | |
| 2 | 5≤p<15 | 1+ | 3+ | 2+ | | | |
| 3 | 5≤p<15 | 0 | 3+ | 2+ | 2+ | 2+ | 2+ |
| 4 | 15≤p<30 | 0 | 3+ | 2+ | | | |
| 5 | 5≤p<15 | 0 | 2+ | 2+ | 2+ | 2+ | 2+ |

p, proportion; ly, lymphatic vessel.

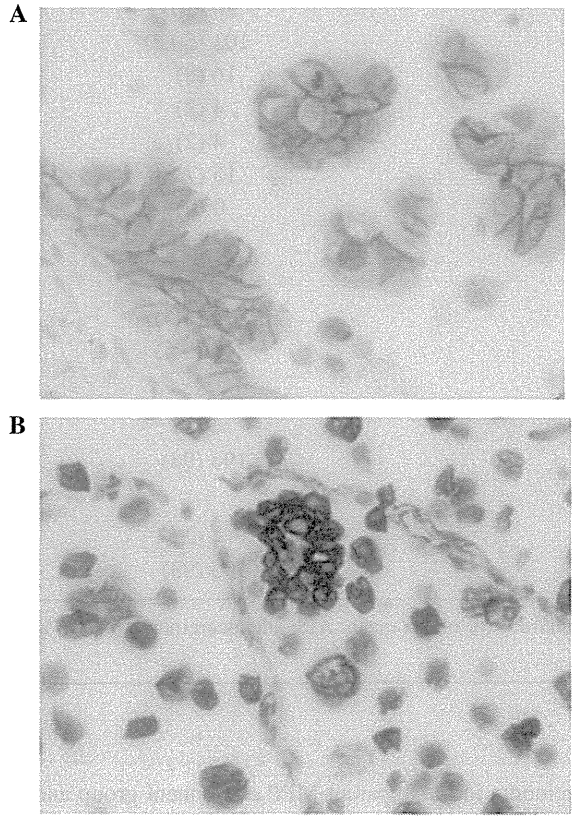


Figure 3. Immunohistochemical (A) E-cadherin and (B) vimentin staining of a micropapillary pattern component in lung adenocarcinoma (magnification, x40).

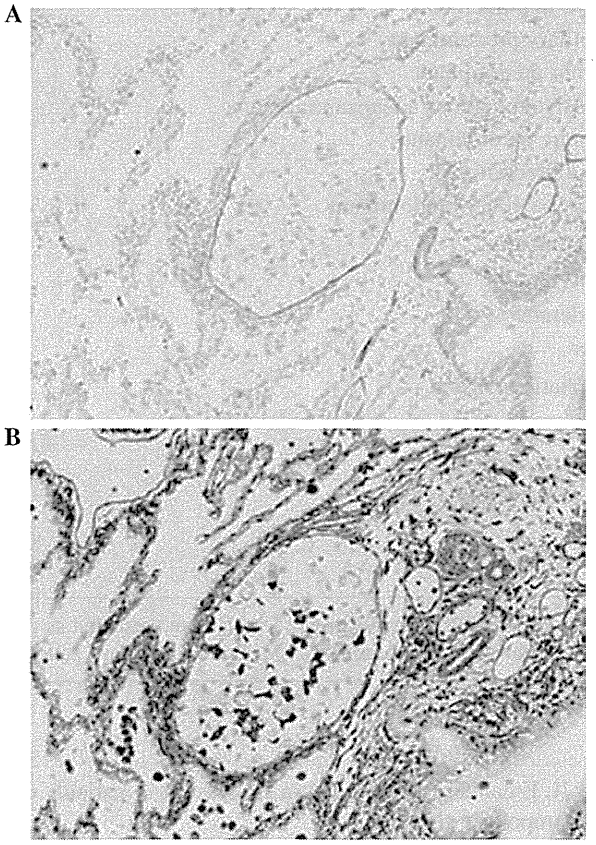


Figure 4. Vimentin expression in the cancer cells in a lymphatic vessel. Serial sections were stained for either (A) D2-40 to identify a lymphatic vessel or (B) vimentin (magnification, x20).

cells, including the expression of vimentin. In the present study, the tumors with an apparent MPP component showed a higher frequency of lymphatic invasion (Table I). Therefore, the present study analyzed the expression of E-cadherin and vimentin in the MPP components and the components without MPP in the patients in the apparent MPP component group, as well as those in the no MPP component group (Table II, Fig. 3). In the patients in the apparent MPP component group, the cancer cells in the MPP components exhibited E-cadherin expression similar to that in components without MPP. However, the cancer cells in the MPP components expressed vimentin more extensively than those in the components without MPP.

In the patients in the apparent MPP component group, cancer cell lymphatic invasion was identified in 5/18 cases. In order to assess which component of the tumor contributed to the cancer cell lymphatic vessel invasion, the level of vimentin expression was analyzed in the MPP and non-MPP components, as well as in the cancer cells in each lymphatic vessel (Table III; Fig. 4). The proportion of the MPP component in the five adenocarcinomas with lymphatic invasion was <25 and these MPP components exhibited vimentin expression

at grade 3+. In three of the adenocarcinomas with no MPP components, vimentin expression was found to be negative, and in two others, vimentin expression was observed to be grade 2+ and 1+. The grade of vimentin expression in each lymphatic vessel was higher than that in the non-MPP component, indicating that the cancer cells detected in the MPP component are also present in the lymphatic vessels.

## Discussion

In the present study, the survival of patients with adenocarcinoma containing an apparent MPP component was found to be worse than that in the patients with adenocarcinoma containing no MPP component or a focal MPP component. Furthermore, adenocarcinoma with an apparent MPP component had a higher frequency of cancer cell lymphatic invasion. These results confirm the findings reported previously (2,3). The poor prognosis of patients with adenocarcinoma with an apparent MPP component may be associated with the higher frequency of lymphatic invasion of the cancer cells in this type of adenocarcinoma.

Kamiya *et al* (4) reported that cancer cells in the MPP component express E-cadherin and exhibit cell-cell adhesion. This is in accordance with the findings of the present study. In addition, to the best of our knowledge, the present study has provided the first evidence that cancer cells in the MPP component express vimentin more extensively than those in the non-MPP component in adenocarcinoma. Vimentin is a marker of mesenchymal cells (8); therefore, this finding suggests that the cancer cells in the MPP component may transform into mesenchymal cells.

The present study also investigated whether cancer cells in lymphatic vessels were derived from the MPP component or the non-MPP component. The results suggested that all of the lymphatic vessels containing cancer cells had cancer cells derived from the MPP component. In each adenocarcinoma exhibiting cancer cell lymphatic invasion, the MPP component occupied <25% of the tumor area. Therefore, it is likely that the cancer cells in the MPP component have a greater invasive potential compared with those in the non-MPP component.

In conclusion, the present study identified that adenocarcinoma with an MPP component had histological predominance of the papillary dominant type and the moderately differentiated type. These findings are consistent with those of previous studies (2,3).

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Short Communication

# Factors Related to Aggressiveness of Some Lung Carcinomas with Peculiar Histological Characteristics

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Keywords
- Adenocarcinoma with micropapillary component
- Large cell neuroendocrine carcinoma
- Pleomorphic carcinoma
- Adhesion molecule, Proliferative activity

**Abstract**

Among non-small cell carcinomas of the lung, some with peculiar histological characteristics are related to significantly worse prognosis including adenocarcinoma with a micropapillary pattern (MPP), large cell neuroendocrine carcinoma (LCNEC), and pleomorphic carcinoma (PC). We have performed studies to clarify factors related to the aggressiveness of these cancers, which revealed the following. 1) Cancer cells in a MPP component have a high ability to invade lymphatic vessels. 2) Abnormal membrane expression of E-cadherin and β-catenin, nuclear β-catenin expression, and the high proliferative potential of LCNECs are associated with their aggressiveness. 3) The aggressiveness of PCs is partly due to decreases in expression of membrane adhesion molecules, such as E-cadherin and β-catenin, not because of the proliferative activity of the cancer cells. Furthermore, the epithelial component of a PC is different from that of an ordinary adenocarcinoma in terms of expression of adhesion molecules.

## ABBREVIATIONS

MPP: adenocarcinoma with Micropapillary Pattern; LCNEC: Large Cell Neuroendocrine Carcinoma; PC: Pleomorphic Carcinoma; p-: Pathological-; PDA: Poorly Differentiated Adenocarcinoma

## INTRODUCTION

Among non-small cell carcinomas of the lung, some with peculiar histological characteristics are associated with significant worse prognosis [1-3], including adenocarcinoma with a Micropapillary Pattern (MPP) (Figure 1A), Large Cell Neuroendocrine Carcinoma (LCNEC) (Figure 1B) and Pleomorphic Carcinoma (PC) (Figure 1C) [1-3]. Analysis of the survival rates of 719 patients with pathological (p)-stage I NSCCs who underwent an operation at our hospital from 2002 to 2010 also confirmed poor prognosis of patients with these cancers (Figure 2). As a result, we attempted to clarify the factors related to their aggressiveness.

We found that cancer cells of an adenocarcinoma with MPP more frequently invade lymphatic vessels as compared to adenocarcinoma without MPP and that the cells population in the lymphatic vessels contains cancer cells derived from the MPP component [4]. Based on those results, we suggested that cancer cells in a MPP component have a high ability to invade lymphatic vessels and that high invasion capacity is associated with the poor prognosis of patients with adenocarcinomas with a MPP component [4].

As for LCNEC and PC, we used an immunohistochemical method to examine the membrane expression of adhesion molecules, such as E-cadherin and β-catenin, as well as the nuclear expression of β-catenin and Ki-67 labeling index as factors related to their aggressiveness because reduced or abnormal expression of adhesion molecules on the cell membrane is associated with the aggressiveness of tumor cells and nuclear β-catenin activates the WNT signaling pathway [5-7]. For these studies, we used the solid components of solid predominant poorly differentiated adenocarcinomas (solid predominant PDAs) as a control. Our findings showed that LCNECs predominantly demonstrated a disrupted pattern of membrane staining for both E-cadherin and β-catenin, while most of the PDAs predominantly showed a linear pattern, i.e., a normal staining pattern [5]. Furthermore, LCNECs were occasionally found to express nuclear β-catenin and their Ki-67 labeling indices were about 4 times greater than those of PDA solid components [5]. We also noted that the disease-free rate of patients with an LCNEC was significant reduced over time as compared to those with a PDA [5]. From these results, we concluded that abnormal membrane expression of E-cadherin and β-catenin, nuclear β-catenin expression, and the high proliferative potential of LCNEC are associated with its aggressiveness.

The study of LCNECs also showed that the membrane expression of E-cadherin and β-catenin was reduced in the solid

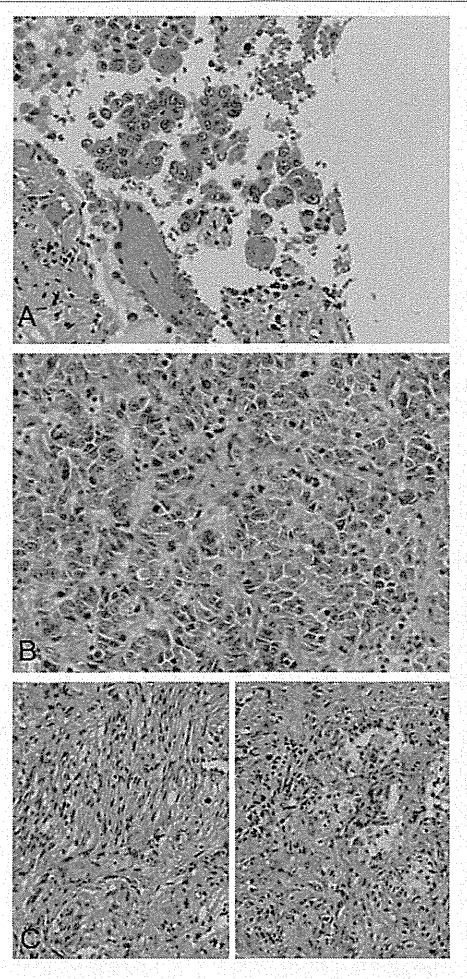


**Figure 1** (A) Histological features of adenocarcinoma with micropapillary pattern.
(B) Large cell neuroendocrine carcinoma.
(C) Pleomorphic carcinoma. (Left) spindle cell pattern. (Right) Poorly differentiated adenocarcinoma component with tubular formation.

and sarcomatous components of PCs as compared to the solid components of PDAs, whereas there was no significant difference regarding Ki-67 labeling index among PC solid components, PC sarcomatous components, and solid components of predominantly solid PDAs [6,7]. These findings indicate that the aggressiveness of a PC is partly due to a decrease in membrane adhesion molecules and not because of the proliferative activity of the cancer cells [6,7]. Furthermore, the epithelial component in a PC may be different from that in an ordinary adenocarcinoma in terms of expression of adhesion molecules [6,7].

Currently, we are examining others such as transforming growth factor (involved in epithelial-stromal transition) and

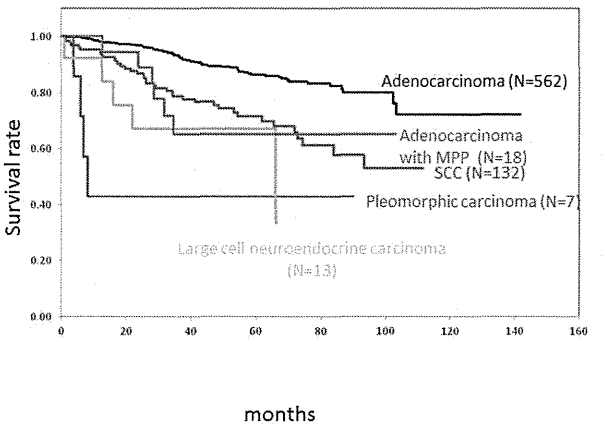


**Figure 2** Survival curves of patients with pathological-stage I lung carcinomas with various histological types.
**Aggravation:** MPP: Micropapillary Pattern

angiogenesis in a search of possible factors related to the aggressiveness of some carcinomas with peculiar histological characteristics that are related to poor prognosis.

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

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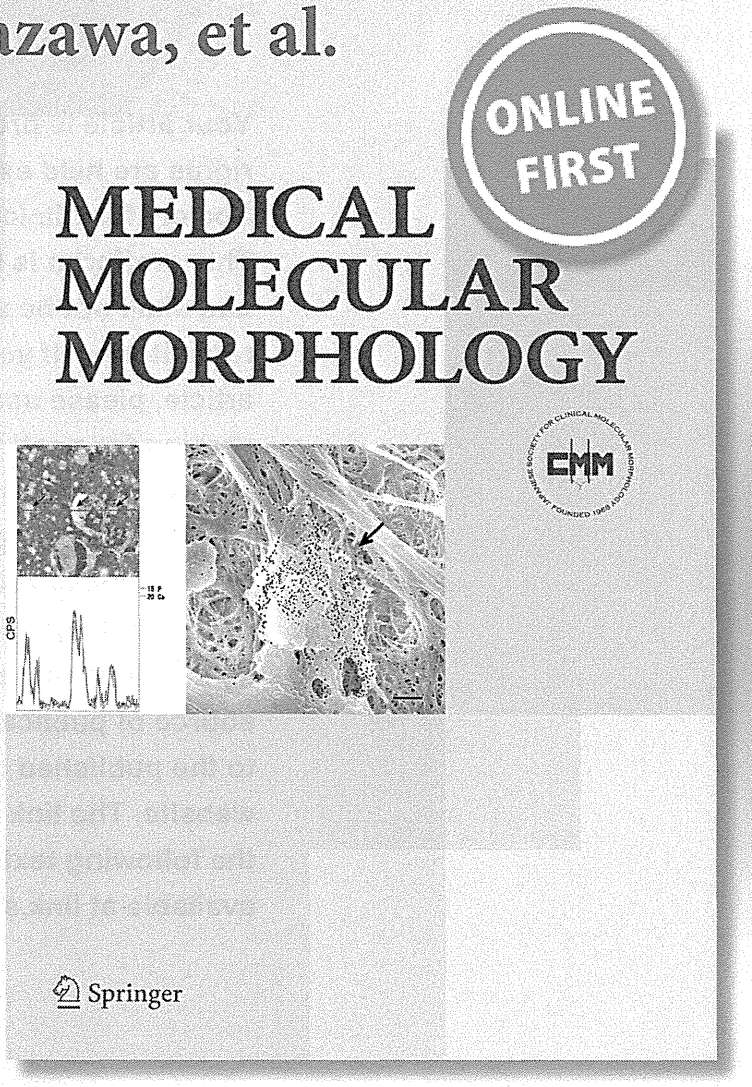


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ORIGINAL PAPER

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

**Hiroshi Hirano · Hajime Maeda · Yukiyasu Takeuchi · Yoshiyuki Susaki · Ryoji Kobayashi · Akio Hayashi · Naoko Ose · Yukie Nakazawa · Toshihiko Yamaguchi · Soichiro Yokota · Masahide Mori**

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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 $\pm$ 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 $816 \pm 59.8$ 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

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**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

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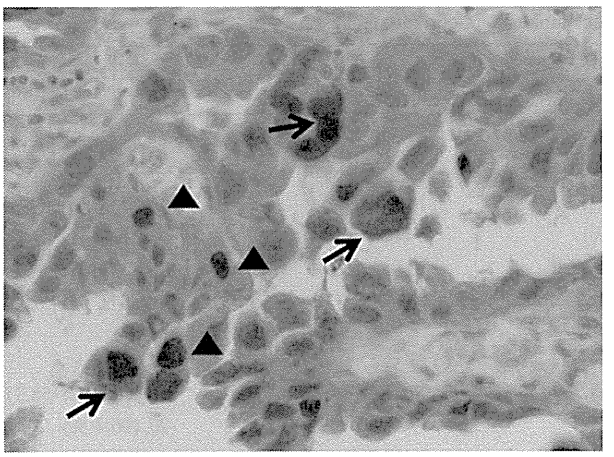

**Fig. 1** Immunohistochemical staining of survivin *arrows* and *arrowheads* indicate cytoplasmic and nuclear staining, respectively

**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.

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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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Research Article

# Immunohisthochemical Analysis of P-Stage I Large Cell Neuroendocrine Carcinoma of the Lung: Analysis of Adhesion Molecules and Proliferative Activity

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- Adhesion molecule
- Proliferative activity
- P-stage I

**Abstract**

A Large Cell Neuroendocrine Carcinoma (LCNEC) of the lung is highly malignant. Reduced or abnormal expression of adhesion molecules, such as E-cadherin and β-catenin, on the cell membrane is associated with the aggressiveness of its tumor cells, while nuclear β-catenin activates the WNT signaling pathway. To examine the mechanism of LCNEC aggressiveness, we used immunohistochemistry to examine the expressions of E-cadherin and β-catenin in the membrane, as well as the nuclear expression of β-catenin and Ki-67 labeling index in 12 pathological (p)-stage I LCNEC specimens. As a control, we used solid-sheet components from 19 p-stages I solid predominant Poorly Differentiated Adenocarcinomas (PDAs), as that tumor is the most aggressive among non-small cell carcinomas of various histological types. The disease-free rate of patients with LCNEC was much lower than that of patients with PDA. In the LCNECs, there was no significant difference in the frequency of membrane-expression of E-cadherin and β-catenin, though all specimens predominantly showed disrupted patterns of membrane staining for both E-cadherin and β-catenin, while 16 of 19 PDAs predominantly showed a linear pattern. Nuclear β-catenin staining was found in 4 of 13 LCNECs, but in none of the PDAs. The Ki-67 labeling index of the LCNEC specimens was about 4-fold greater than that of the PDAs. The present results suggest that abnormal membrane expression of E-cadherin and β-catenin, nuclear β-catenin expression, and high proliferative potential are associated with LCNEC aggressiveness.

## ABBREVIATIONS

LCNEC: Large Cell Neuroendocrine Carcinoma; PDA: Poorly Differentiated Adenocarcinoma; Ki-67 LI: Ki-67 Labeling Index

## INTRODUCTION

Large Cell Neuroendocrine Carcinoma (LCNEC), first described by Travis et al. in 1991, is a highly malignant tumor of the lung that has a very poor prognosis similar to that of small cell carcinoma of the lung [1]. LCNECs show histological features suggesting neuroendocrine differentiation, such as organoid nesting, trabecular growth, and rossette-like and perilobular palisading patterns. Neuroendocrine differentiation is confirmed by immunohistochemical staining of neuroendocrine markers such as synaptophysin, chromogranin A and CD56, as well as ultrastructual observation [2].

E-cadherin is a transmembrane protein that forms cell-cell adhesion complexes with β-catenin. There are two forms of β-catenin, combined and free forms [3]. The combined form

binds with the intracellular domain of E-cadherin and plays an essential role in cell-cell adhesion, while free β-catenin exists in the cytoplasm and can enter the nucleus while activiting the WNT signaling pathway. Reduced expression and dysfunction of E-cadherin, and nuclear β-catenin expression have been reported to be associated with poor prognosis in various cancers, including non-small cell lung cancer [3-6].

A LCNEC is highly malignant, though the related mechanism has not been fully clarified [7]. In order to elucidate the mechanism of high aggressiveness, we investigated the expressions of E-cadherin and β-catenin on the cell membrane and nuclear expression of β-catenin, as well as proliferative activity represented by Ki-67 labeling index using LCNEC specimens obtained form pathological (p)-stage I cases. As a control, p-stage I poorly differentiated adenocarcinomas (PDAs) of the lung where the solid-sheet component occupied more than 80% because adenocarcinomas of the lung consisting predominantly of solid sheets show the worst prognosis among the various reported histological types [8]. Furthermore, LCNECs consist of sheets or nests of tumor cells similar to the solid-sheet component of an adenocarcinoma.

## MATERIALS AND METHODS

### Patients and tissue specimens

Tumors from 12 patients (11 males, 1 female) diagnosed with pathological (p)-stage I large cell endocrine carcinoma (LCNEC) of the lung and from 19 (17 males, 2 females) with p-stage I PDA of the lung were used for this study. Each patient underwent surgical resection of the tumor at Toneyama National General Hospital between 2002 and 2010 and none received neo adjuvant chemotherapy or radiation therapy prior to surgery. In all 19 p-stage I PDAs examined in this study, the PDA area occupied more than 80% of the entire tumor area of each tumor (Table 1). All patients underwent dissection of the bifurcation and ipsilateral mediastinal lymph nodes, and pathological examinations revealed no metastasis in any. Furthermore, computed tomography and

**Table 1:** Clinicopathological data of patients with large cell neuroendocrine carcinoma and poorly differentiated adenocarcinoma.

| Patient No. | Age | Sex | BI | Tumor diameter (mm) | Follow-up period (month) | Recurrence | Percentage of the solid-sheet component |
|---|---|---|---|---|---|---|---|
| Large cell neuroendocrine carcinoma | | | | | | | |
| 1 | 60 | M | 1200 | 19 | 22.6 | Yes | |
| 2 | 72 | M | 1500 | 13 | 1 | Died of the other cause | |
| 3 | 59 | M | 1560 | 25 | 47.2 | Yes | |
| 4 | 69 | M | 1290 | 22 | 13.7 | Yes | |
| 5 | 79 | M | 800 | 27 | 4.9 | Yes | |
| 6 | 75 | M | 1100 | 18 | 5.9 | Yes | |
| 7 | 58 | M | 1200 | 27 | 3.4 | Yes | |
| 8 | 75 | M | 645 | 11 | 23.6 | Yes | |
| Poorly differentiated adenocarcinoma | | | | | | | |
| 1 | 55 | M | 1050 | 50 | 96 | No | 80 |
| 2 | 73 | M | 1000 | 22 | 95.9 | No | 100 |
| 3 | 58 | M | 0 | 30 | 74.9 | No | 80 |
| 4 | 83 | M | 0 | 16 | 72.3 | No | 90 |
| 5 | 65 | M | 1600 | 15 | 71.7 | No | 100 |
| 6 | 54 | M | 1400 | 25 | 89 | No | 100 |
| 7 | 74 | M | 250 | 32 | 75.6 | No | 80 |
| 8 | 73 | M | 1000 | 20 | 75.3 | No | 100 |
| 9 | 63 | M | 1200 | 50 | 73 | No | 80 |
| 10 | 60 | M | 1600 | 20 | 39 | Yes | 90 |
| 11 | 72 | M | 1000 | 8 | 68.8 | No | 100 |
| 12 | 71 | M | 1000 | 12 | 66.4 | No | 80 |
| 13 | 42 | M | 0 | 32 | 60.7 | No | 100 |
| 14 | 81 | M | 975 | 20 | 60.7 | No | 100 |
| 15 | 62 | M | 760 | 35 | 64.5 | No | 80 |
| 16 | 57 | F | 1110 | 28 | 61 | No | 80 |
| 17 | 41 | M | 0 | 20 | 59 | No | 80 |
| 18 | 62 | M | 820 | 12 | 53.9 | No | 80 |
| 19 | 74 | F | 0 | 45 | 53.4 | No | 90 |

BI: Brikemann Index; M: Male; F: Female

magnetic resonance imaging confirmed no metastasis in these patients. All were categorized as p-stage I, according to the TNM classification of the International Union Against Cancer (7th edition) (tumor size <5.0 cm and no lymph node or distal metastasis, or tumor size <3cm and pleural invasion and no lymph node or distal metastasis,) [9]. Clinical information was obtained for each patient by reviewing their medical charts and is presented in Table 1.This study was approved by the Ethics Committee of Toneyama National General Hospital.

## Histology

The largest diameter was used for tumor diameter. The tumors were fixed in 0.01M phosphate-buffered 10% formalin (pH 7.4) and several paraffin-embedded tumor blocks were made from each, then 5-μm in sections, was cut. Some sections were used for hematoxylin-eosin staining and others for immunohistochemistry. LCNEC was diagnosed on the basis of the histological criteria proposed by Travis in 1991 (Figure 1) [1], with immunohistochemistry findings showing neuroendocrine markers such as synaptophysin, chromogranin A and CD56.

## Immunohistochemistry

Immunohistochemical staining was performed using an avidin-streptavidin immunoperoxidase method. A antigen retrieval by incubation of deparaffinized sections in cell condition 1 solution at a mild degree and subsequent immunohistochemical staining were carried out using an automated Benchmark system (Ventana Medical System, Tucson, AZ, USA) according to the manufacturer's instructions. Antibodies used for immunohistochemistry are shown in Table 2.

## Immunohistochemical staining

Positive immunostaining was defined as 5% or more tumor cells with staining. The Ki-67 labeling index (Ki-67 LI) was determined as the percentage of the tumor that was Ki-67-positive by examining 500-1000 tumor cells in the area with the highest labeling index using the Win Roof software program (Mitani Co. Tokyo, Japan). E-cadherin is known to exist on the cell membrane, while β-catenin is located on both the cell membrane and in the nucleus. Immunostaining of E-cadherin and β-catenin on the cell membrane, and in the nucleus was examined in randomly selected fields, then graded according to the percentage (p) of tumor cells stained positively as follows: negative, p <5%; 1+, p≥5-30%; 2+; p≥30-70%; 3+, p>70%. There are two patterns of membrane staining for E-cadherin and β-catenin, membrane-linear and membrane-disrupted. The membrane-linear pattern is seen as a continuous line of staining along the cell membrane and the membrane-disrupted pattern is seen as discontinuous disrupted staining along the cell membrane. For the present study, the predominant pattern was defined as the pattern shown by more than 50% of the membrane-positive tumor cells showed.

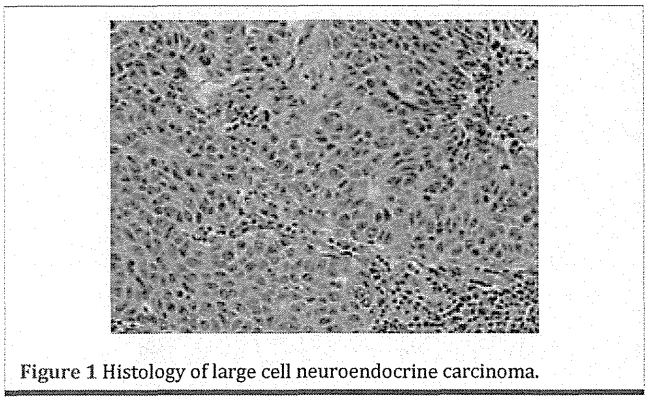

Figure 1 Histology of large cell neuroendocrine carcinoma.

**Table 2:** Antibodies used for the immunohistochemistry.

| Antigen | Clone | Source | Dilution |
|---|---|---|---|
| Chromogranin A | DAK-A3 | Dako Jaoan | 1:200 |
| Synaptophysin | 27G12 | Novocastra | 1:1000 |
| CD56 | 1B6 | Novocastra | 1:100 |
| E-cadherin | 36B5 | Novocastra | 1:50 |
| β-Catein | 17C | Novocastra | 1:80 |
| Ki-67 | Ki-67 | Dako Japan | 1:100 |

**Abbreviations:** Dako Japan, Tokyo, Japan; Novocastra, Newcast upon Tyne, UK.

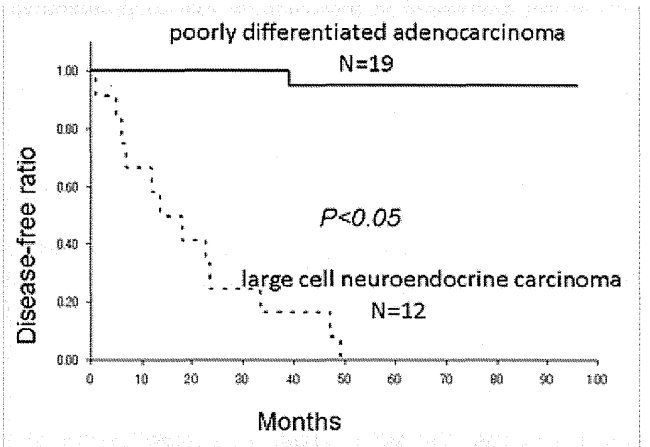


Figure 2 Disease-free rates of patients with large cell neuroendocrine carcinoma and poorly differentiated adenocarcinoma predominantly composed of a solid-sheet component.

## Statistical analysis

Disease-free rates were plotted according to the Kaplan-Meier method and analyzed using a Log-rank test. Data for more than 3 samples are presented as the mean ± S.D. and analyzed using Student's *t*-test. Differences in frequencies between the groups were analyzed using the $\chi^2$ test. All statistical analyses were performed using the Excel Statistics 2012 software package for Windows (SSRI, Tokyo, Japan), with *P* values <0.05 considered to be statistically significant.

# RESULTS

Figure 2 shows the disease-free rates of patients with p-stage I LCNEC and those with p-stage I PDA after surgery. The solid-sheet component occupied more than 80% of the tumor area in all PDAs analzed (Table 1). The disease-free rate of patients with LCNEC rapidly decreased over time and became significantly lower than that of patients with PDA (*P*<0.05).

There was no significant difference in regard to age, sex ratio,

smoking history and Brinkman index for patients with a smoking history between LCNEC and PDA (Table 3). Recurrence occurred in all patients with an LCNEC during the follow-up period, in contrast to in only 1 of 19 patients with a PDA (Table 3).

Immunohistochemical staining for the neuroendocrine markers, such as synaptophysin, chromogranin A and CD56 showed that all LCNECs expressed at least 1 of those markers, supporting the previous diagnosis of LCNEC (data not shown). Results of immunohistochemistry using E-cadherin, β-catenin, and Ki-67 are presented in Table 4 and 5. E-cadherin and β-catenin were stained on the cell membranes of the tumor cells (Figure 3A-D), while β-catenin staining was also observed in the nuclei (Figure 3E). In addition, the membrane-linear pattern was shown as a continuous line of staining along the cell membrane (Figure 3A, 3B), and the membrane-disrupted pattern as discontinuous staining along the cell membrane (Figure 3C, 3D). There was no significant difference in frequency of grade 3+ staining for E-cadherin or β-catenin between the LCNECs and solid-sheet components of the PDAs (Table 5). However, all LCNECs predominantly showed a disrupted pattern of membrane staining for both E-cadherin and β-catenin (Figure 3C and D) while most solid-sheet components of the PDAs predominantly showed a membrane- liner pattern, and the frequencies of predominance of the membrane-disrupted pattern for E-cadherin and β-catenin were significantly greater in LCNECs than in the solid-sheet components of the PDAs (Table 5). Furthermore, the frequency of nuclear staining of β-catenin was significantly greater in the LCNECs than in the solid-sheet components. Finally, the Ki-67 labeling index for the LCNECs was approximately 4-fold greater than that for the solid-sheet components, which was a significant difference (Table 5, Figure 3F).

**Table 3:** Comparison of clinicopathological data of patients with large cell neuroendocrine carcinoma and poorly differentiated adenocarcinoma.

| Item | Large cell endocrine carcinoma (n=12) | Poorly differentiated adenocarcinoma (n=19) | Statistical evaluation |
|---|---|---|---|
| Age | 69.2±8.3 | 64.2±11.6 | |
| Sex | | | |
| Male | 11 | 17 | NS |
| Female | 1 | 2 | NS |
| Smoking History | | | |
| Yes | 11 | 5 | NS |
| No | 1 | 14 | NS |
| Brikeman Index | 917.9±507.5 | 777.1±559.9 | NS |
| Recurrence (+) | | | |
| Yes | 11* | 1 | $P<0.05$ |
| No | 0 | 18 | $P<0.05$ |

**Abbreviations:** *, One patient died of the other cause. NS: Not Significance

**Table 4:** Results of immunohistochemistry.

| No. | E-cadherin | | β-Catenin Membrane | | | Ki-67 labeling index (%) |
|---|---|---|---|---|---|---|
| | Membrane | | Membrane | | Nucleus | |
| | Grade | predominant pattern | Grade | Predominant pattern | Grade | |
| Large cell neuroendocrine carcinomas | | | | | | |
| 1 | (3+) | Disrupted | (3+) | Disrupted | (-) | 92.5 |
| 2 | (3+) | Disrupted | (3+) | Disrupted | (-) | 77.3 |
| 3 | (2+) | Disrupted | (2+) | Disrupted | (-) | 74.7 |
| 4 | (2+) | Disrupted | (3+) | Disrupted | (2+) | 83.1 |
| 5 | (3+) | Disrupted | (3+) | Disrupted | (-) | 62.3 |
| 6 | (3+) | Disrupted | (3+) | Disrupted | (2+) | 99.1 |
| 7 | (3+) | Disrupted | (3+) | Disrupted | (-) | 83.6 |
| 8 | (2+) | Disrupted | (3+) | Disrupted | (-) | 60.3 |
| 9 | (2+) | Disrupted | (3+) | Disrupted | (2+) | 97.9 |
| 10 | (3+) | Disrupted | (3+) | Disrupted | (-) | 95.9 |
| 11 | (2+) | Disrupted | (2+) | Disrupted | (-) | 88.5 |
| 12 | (2+) | Disrupted | (3+) | Disrupted | (2+) | 97.9 |
| Solid-sheet components of poorly differentiated adenocarcinomas | | | | | | |
| 1 | (3+) | Linear | (3+) | Disrupted | (-) | 28.1 |
| 2 | (2+) | Linear | (3+) | Linear | (-) | 22.6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3 | (3+) | Linear | (3+) | Linear | (-) | 14.8 |
| 4 | (3+) | Linear | (3+) | Linear | (-) | 36.2 |
| 5 | (2+) | Disrupted | (1+) | Disrupted | (-) | 55.7 |
| 6 | (2+) | Linear | (3+) | Disrupted | (-) | 39 |
| 7 | (3+) | Linear | (3+) | Linear | (-) | 40.3 |
| 8 | (3+) | Linear | (3+) | Linear | (-) | 22.8 |
| 9 | (1+) | Disrupted | (1+) | Disrupted | (-) | 38.4 |
| 10 | (3+) | Linear | (3+) | Linear | (-) | 11.3 |
| 11 | (2+) | Disrupted | (3+) | Linear | (-) | 4.4 |
| 12 | (3+) | Linear | (3+) | Linear | (-) | 9.6 |
| 13 | (3+) | Linear | (3+) | Linear | (-) | 14.5 |
| 14 | (3+) | Linear | (3+) | Linear | (-) | 10.7 |
| 15 | (3+) | Linear | (3+) | Linear | (-) | 16.1 |
| 16 | (3+) | Linear | (3+) | Linear | (-) | 25.4 |
| 17 | (3+) | Linear | (3+) | Linear | (-) | 20.9 |
| 18 | (3+) | Linear | (3+) | Linear | (-) | 0.6 |
| 19 | (3+) | Linear | (3+) | Linear | (-) | 22.6 |

**Abbreviations:** The staining grade was determined according to the percentage (p) of positive cells: -, p<5%; 1+, 30>p≥5%; 2+; 70%>p≥30%; 3+, p≥70%.

**Table 5:** Comparison of the immunohistochemical results between large cell neuroendocrine carcinomas and solid-sheet components of poorly differentiated adenocarcinomas.

| Items | | Large cell neuroendocrine carcinoma (n=12) | Poorly differentiated adenocarcinoma (n=19) | Statistical evaluation |
|---|---|---|---|---|
| E-cadherin | | | | |
| | Staining grade (3+) | 6/12 (50%) | 14/19 (73.8%) | NS |
| | Staining grade <3+ | 6/12 (50%) | 5/19 (26.3%) | NS |
| | Predominant staining pattern | | | |
| | linear | 0/12 (0%) | 16/19 (84.2%) | *P*<0.05 |
| | disrupted | 12/12 (100%) | 3/19 (15.8%) | *P*<0.05 |
| β-Catenin | | | | |
| | Staining grade (3+) | 10/12 (83.3%) | 17/19 (89.5%) | NS |
| | Staining grade <3+ | 2/12 (16.7%) | 2/19 (10.5%) | NS |
| | Predominant staining pattern | | | |
| | linear | 0/12 (0%) | 15/19 (78.9%) | *P*<0.05 |
| | disrupted | 12/12 (100%) | 4/19 (21.1%) | *P<0.05* |
| | Nuclear expression (+) | 4/13 (30.8%) | 0/20 (0%) | *P<0.05* |
| | | | | |
| Ki-67 labeling index | | 84.4±13.5 | 22.8±14.1 | *P<0.05* |

**Abbreviations:** NS; Not Significant. The staining grade was determined according to the percentage (p) of positive cells: -, p<5%; 1+, 30>p≥5%; 2+; 70%>p≥30%; 3+, p≥70%.

## DISCUSSION

The disease-free rate of patients with an LCNEC was much lower over time, as compared to patients with a PDA, the latter of which is predominantly composed of a solid-sheet component. The prognosis for patients with a PDA composed, predominantly of a solid-sheet component has been reported to be the worst among adenocarcinomas of various histological types [8]. In addition, it has been shown that the prognosis of patients with an LCNEC is as poor as of those with small cell carcinoma of the lung [10], while the prognosis of small cell carcinoma is worse than that of non-small cell carcinoma of the lung [2]. Therefore, the present result is in agreement with these previous studies.

There was no significant difference in regard to the membrane expression of E-cadherin and β-catenin between LCNECs and PDAs. However, all LCNECs predominantly showed the disrupted membrane-staining pattern whereas most solid-sheet components of the PDAs predominantly showed the liner membrane-staining pattern. A disrupted membrane-staining pattern of E-cadherin and β-catenin indicates dysfunction of intercellular adhesion [11]. Therefore, the abnormal staining

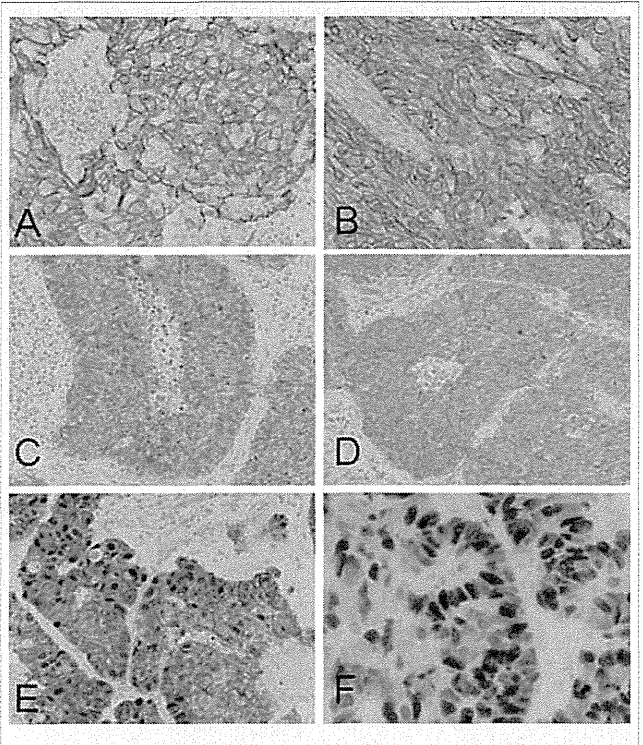


Figure 3 (A) – [Immunohistochemistry findings for E-cadherin, and β-catenin, and Ki-67. Representative linear membrane staining pattern of (A) E-cadherin, and (B) β-catenin in a solid-sheet component of a poorly differentiated adenocarcinoma. Representative disrupted membrane staining pattern of (C) E-cadherin and (D) β-catenin in a large cell neuroendocrine carcinoma. (E) Representative nuclear staining of β-catenin in a large cell neuroendocrine carcinoma. (F) Representative Ki-67 staining in a large cell neuroendocrine carcinoma.

pattern of E-cadherin and β-catenin in LCNEC seems to be correlated to its aggressiveness.

There are two forms of β-catenin, combined and free [12,13]. The combined form binds with the intracellular domain of E-cadherin and plays an essential role in cell-cell adhesion. On the other hand, free β-catenin exists in the cytoplasm and can enter the nucleus, where it activates the WNT signaling pathway and switches on transcription of target genes such as *c-myc* and *cyclin D1*, resulting in proliferation and metastasis of tumor cells [12,14]. Nuclear β-catenin expression was found in 4 of 13 LCNECs, but none of 19 PDAs. Therefore, nuclear β-catenin expression in some LCNECs seems to be associated with their aggressiveness.

Ki-67 LI is considered to be a reliable proliferative marker for estimating malignancy grade [15] Mitosis is frequently found in LCNECs [2]. In agreement with those findings, we found that the Ki-67 labeling index of LCNECs was high, indicating the high proliferative potential of LCNEC tumor cells. In some LCNECs expression of nuclear β-catenin may partly contribute to their high proliferative activity.

## CONCLUSION

In conclusion, the abnormal membrane expression of E-cadherin and β-catenin, as well as nuclear β-catenin expression and high proliferative potential are associated with LCNEC aggressiveness.

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