

# Intrabronchial orthotopic propagation of human lung adenocarcinoma—characterizations of tumorigenicity, invasion and metastasis

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## Abstract

Using the intrabronchial orthotopic propagation method, we evaluated the biological characteristics of human adenocarcinoma cell lines *in vivo* and examined the expressions of matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) and their related proteins. Nine human lung adenocarcinoma cell lines, including A549, NCI-H23, NCI-H322, NCI-H358, Calu-3, PC-14, LC-2/ad, RERF-LC-KJ and PL16T, were injected into the peripheral bronchi of mice using this method. The mice were sacrificed at 4 and 8 weeks after tumor cell propagation and the lungs and other organs were observed macroscopically and histologically. We classified the adenocarcinoma cell lines, according to their intrapulmonary tumorigenicity, into the following three groups: (A) those that showed a high incidence of intrapulmonary implantation (> 50%) (A549 and NCI-H358). A549 showed mediastinal lymph node metastasis and pleural dissemination; (B) those that showed a low incidence of intrapulmonary implantation (PC-14, NCI-H322, NCI-H23, Calu-3, and LC-2/ad); (C) those that showed no tumorigenicity in the lung (RERF-LC-KJ and PL16T). In order to characterize the biological differences between each cell line, we investigated the expressions of MMP-2 and MMP-9 and their related molecules by northern blot analysis. The expressions of MMP-2 and MMP-9 and their activators (membrane-type 1-MMP and urokinase-type plasminogen activator) were thought to be associated with the growth, invasion and metastasis of the human lung adenocarcinoma cell lines examined. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Orthotopic propagation; Human lung adenocarcinoma; Invasion; Metastasis; Matrix metalloproteinases

## 1. Introduction

Lung cancer is the leading cause of cancer-related death in Japan as well as in other countries. In particular, the incidence of adenocarcinoma, which is one of the major histological subtypes of lung carcinoma, is increasing [1]. The survival rate of patients with lung adenocarcinoma is poor even when they receive treatment in the early stage of the disease, since most adenocarcinomas detected clinically are already advanced and there are no definite biological and histological concepts of non-invasive carcinoma [2]. In order to

treat lung adenocarcinoma based on its clinicopathological characteristics, it is very important to clarify the biological mechanisms of invasion and metastasis.

Since Paget proposed the original ‘Seed and Soil’ hypothesis in 1889, many *in vivo* models of the propagation of human tumors at orthotopic sites in athymic nude mice or SCID mice have been developed using renal cell carcinomas, brain tumors, colorectal carcinomas, liver cell carcinomas, pancreatic carcinomas, and lung carcinomas [3–8]. The orthotopic mouse model of lung cancer was first developed by McLemore et al., who succeeded in growing human lung cancer cell lines in the bronchioloalveolar region by injecting intrabronchial tumor cells [9,10]. Recently, Kozaki et al. established an interesting human lung cancer cell line (NCI-H460-LNM35), which shows consistent lympho-

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genous metastasis via orthotopic propagation, using the orthotopic implantation method [11]. However, the transbronchial propagation of cancer cells in nude or SCID mice is technically difficult and there have been only a few studies of lung carcinomas using the transbronchial orthotopic implantation model.

Many biologically active molecules are thought to be associated with tumor invasion and metastasis. For example, at the beginning of tumor invasion, many tumor cells express matrix metalloproteinases (MMPs) which destroy the basement membrane and extracellular matrix below it. In particular, matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) and their activators and inhibitors are thought to be very important during this initial stage [12–16].

In this study, we focused on lung adenocarcinoma, which shows frequent intrapulmonary metastases clinically. Using the modified intrabronchial propagation method of McLemore et al., we characterized the biological behaviors of nine human lung adenocarcinoma cell lines and compared their biological characteristics. The expressions of MMP-2 and MMP-9 and their activators and inhibitors in these cell lines were then compared.

## 2. Materials and methods

### 2.1. Experimental animals

Male BALB/c nude mice, approximately 4 weeks of age, were obtained from CLEA JAPAN (Hamamatsu, Japan). The mice were free of known pathogens at the time of study, and were housed in sterilized filter-topped cages and fed autoclaved food and water ad libitum.

### 2.2. Human lung tumor cell lines

Nine human lung adenocarcinoma cell lines, A549 [17], PC-14, LC-2/ad [18], RERF-LC-KJ, NCI-H23 [19], NCI-H322 [20], NCI-H358 [21], Calu-3 [22] and PL16T, were used in this study. A549, PC-14, LC-2/ad and RERF-LC-KJ were obtained from the RIKEN Cell Bank (Tsukuba, Japan), NCI-H23, NCI-H322, NCI-H358, and Calu-3 were obtained from the American Type Culture Collection (Rockville, MD) and PL16T was originally established to transfect SV40 large T antigen into very well differentiated adenocarcinoma-like atypical adenomatous hyperplasia [23]. The original histology of these adenocarcinoma cell lines was well differentiated for A549, NCI-H322, NCI-H358 and PL16T, moderately differentiated for LC-2/ad and poorly differentiated for PC-14. That of NCI-H23, Calu-3 and RERF-LC-KJ is unknown. PC-14, RERF-LC-KJ, NCI-H23, NCI-H322 and NCI-H358 were maintained in RPMI 1640 supplemented with 10% fetal

bovine serum, A549 was maintained in D-MEM/F12 supplemented with 10% fetal bovine serum, LC-2/ad was maintained in RPMI 1640/F12 supplemented with 15% fetal bovine serum and PL16T was maintained in MCDB153HAA supplemented with 2% fetal bovine serum, 100 µg/ml EGF, 10 mg/ml insulin, 0.36 mg/ml hydrocortisone, 5 mg/ml transferrin and 20 µg/ml sodium selenite. The cells were incubated at 37 °C in a humidified cabinet in an atmosphere of 5% CO<sub>2</sub> in air and harvested for implantation at 80% confluence using trypsin-EDTA (GIBCO BRL, Grand Island, NY). They were washed three times each with serum-free medium, and resuspended in serum-free medium at a concentration of 10<sup>7</sup> live cells/ml.

### 2.3. Intrabronchial propagation procedure

Animals were anesthetized with 2.5% avertin. A 0.5-cm ventral midline incision was made in the neck superior to the supraclavicular notch. The trachea was exposed and then punctured directly with a 24-gauge intravenous catheter (TERUMO, Tokyo, Japan) which was inserted into the trachea and advanced into the left or right bronchus (Fig. 1). An inoculum of 1.0 × 10<sup>6</sup> tumor cells in a 0.1 ml final volume of serum free medium was injected into the peripheral bronchus. The skin incision was then closed with polypropylene suture

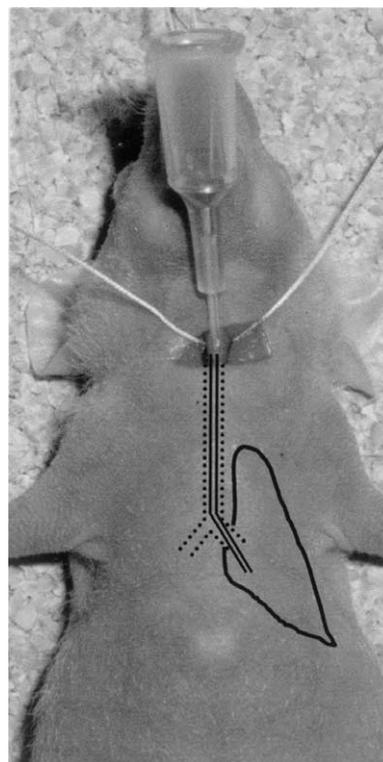


Fig. 1. The intrabronchial propagation technique. The trachea is exposed and then punctured directly with a 24-gauge intravenous catheter which is inserted into the trachea and advanced into the left or right bronchus.

Table 1  
Tumorigenicity of the pulmonary adenocarcinoma cell lines in nude mice

Group	Cell line	T (%)	LN (%)	P (%)	DM (%)
(A) High	A549	6/6 (100)	3/6 (50)	3/6 (50)	0/6 (0)
	NCI-H358	6/9 (67)	0/9 (0)	0/9 (0)	0/9 (0)
(B) Low	PC-14	2/8 (25)	3/8 (38)	0/8 (0)	0/8 (0)
	NCI-H322	2/8 (25)	0/8 (0)	0/8 (0)	0/8 (0)
	NCI-H23	1/6 (17)	0/6 (0)	0/6 (0)	0/6 (0)
	LC-1/ad	2/7 (29)	0/7 (0)	0/7 (0)	0/7 (0)
	Calu-3	1/7 (14)	0/7 (0)	0/7 (0)	0/7 (0)
(C) No	RERF-LC-KJ	0/10 (0)	0/10 (0)	0/10 (0)	0/10 (0)
	PL16-T	0/9 (0)	0/9 (0)	0/9 (0)	0/9 (0)

(A) High: high tumorigenicity; (B) Low: low tumorigenicity; (C) No: no tumorigenicity. T: tumorigenicity (number of mice with implanted nodule/total number of mice in which tumors were propagated), LN: lymph node metastasis, P: pleural dissemination, DM: distant metastasis.

and the animals were returned to their cages after recovering from anesthesia.

#### 2.4. Histopathologic study

Mice were sacrificed on days 28 and 56 after tumor implantation. The heart–lung block, liver, spleen, pancreas, adrenal glands and kidneys were removed, fixed with 10% buffered formalin and embedded in paraffin. The cut sections were stained with hematoxylin and eosin (H and E).

#### 2.5. Northern blot analysis

Total RNA was extracted from each of the cell lines with TRIzol (GIBCO BRL, Rockville, MD) and 20 µg of total RNA was electrophoresed in a 1% agarose gel with 2.2 M formaldehyde. The RNA was then transferred to a nylon membrane and hybridized with <sup>32</sup>P-labeled probes for MMP-2, MMP-9, membrane-type 1 MMP (MT1-MMP), urokinase-type plasminogen activator (uPA), tissue inhibitor of metalloproteinase-2 (TIMP-2), tissue inhibitor of metalloproteinase-1 (TIMP-1) and elongation factor (EF). Human MMP-2, MMP-9, MT1-MMP, uPA, TIMP-2 and TIMP-1 cDNA probes were generated by PCR with the aid of the following sense and anti-sense primer sets, 5'-catcaagggcattcaggag-3' and 5'-ttgctccagttaaagcggc-3' for MMP-2, 5'-gcgctcatgtaccatgtacc-3' and 5'-cgatggcgtcgaagatgttcacg-3' for MMP-9, 5'-cacattaagagactgggc-3' and 5'-cctcctcgtccaccta-3' for MT1-MMP, 5'-agaattcaccaccatcgaga-3' and 5'-ttgctccagttaaagcggc-3' for uPA, 5'-ctggacgttgaggaaaagaag-3' and 5'-tgcttatgggtcctcgtatgc-3' for TIMP-2, 5'-cttccacaggtccacaacc-3' and 5'-cagcctggctcccaggc-3' for TIMP-1. The EF cDNA probe was used as a positive control and the standard was also generated by PCR.

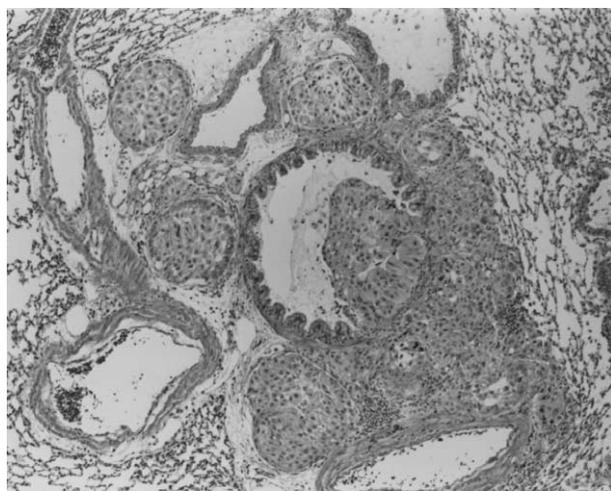
### 3. Results

#### 3.1. Characteristics of *in vivo* tumorigenicity

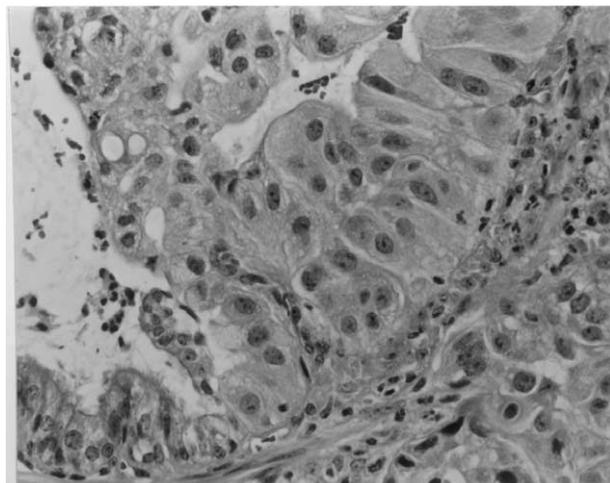
More than 140 intrabronchial implants were performed, and this procedure took approximately 7 min per mouse. The surgery-related mortality was approximately 17%.

After orthotopic propagation, the histology of the developed tumors for all the adenocarcinoma cell lines used was poorly differentiated adenocarcinoma, but the tumor growth of the adenocarcinoma cell lines in the lung was variable. Table 1 shows the results of the tumorigenicity assays for the nine different human lung adenocarcinoma cell lines propagated intrabronchially. We classified the adenocarcinoma cell lines, according to the activity of their intrapulmonary implantation, into the following three groups: (A) those showing a high incidence of intrapulmonary implantation (>50%) (A549 and NCI-H358), (B) those showing a low incidence of intrapulmonary implantation (PC-14, NCI-H322, NCI-H23, Calu-3, and LC-2/ad) and (C) those showing no tumorigenicity in the lung (RERF-LC-KJ and PL16T).

Cell lines classified into group (A) had a high incidence of intrapulmonary tumor development, but the characteristics of the tumors which developed were variable. For example, A549, classified into group (A), developed multiple intrapulmonary implantations (6/6, 100%) and tumor cells also infiltrated the bronchial wall and the lymph vessels, followed by mediastinal lymph node metastases and pleural dissemination (Fig. 2). NCI-H358, also classified into group (A), showed a high incidence of intrapulmonary implantation (6/9, 66.7%), but neither lymph node metastasis nor pleural dissemination (Fig. 3). PC-14, NCI-H322, NCI-H23, Calu-3 and LC-2/ad, classified into group (B), showed a low incidence of intrapulmonary implantation. PC-14 is a characteristic cell line amongst the group (B) tumor cell lines. Although PC-14 had a low incidence of



(a)

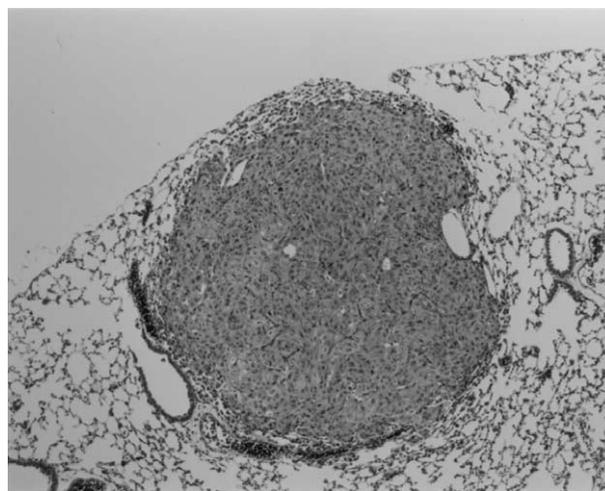


(b)

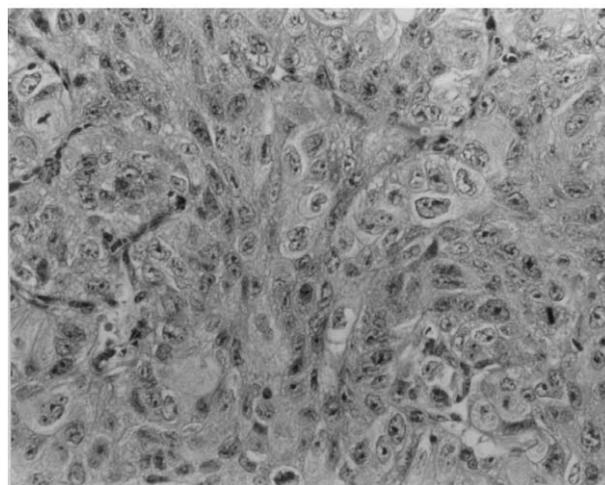
Fig. 2. Implanted A549 tumor nodule invading trachea 28 days following intrabronchial propagation (a) (H.E.,  $\times 40$ ). Histopathologically, the tumor revealed poorly differentiated adenocarcinoma (b) (H.E.,  $\times 200$ ).

intrapulmonary implantation (2/8, 25%), tumor cells in some animals had infiltrated the lymph vessels, and mediastinal lymph node metastases developed without intrapulmonary implantation (3/8, 37.5%) (Fig. 4). With the cell lines other than PC-14 there was no mediastinal lymph node metastasis or pleural dissemination. Another finding was that NCI-H322 produced multiple nodules in the lung, whereas NCI-H23 and Calu-3 produced a solitary nodule. Neither lung nodules nor lymph node metastases were seen in the remaining cell lines, RERF-LC-KJ and PL16T, which were classified into group (C).

All tumor cells proliferated expansively in bronchio-alveolar regions. Histologically, these lesions were poorly differentiated adenocarcinoma but neither invasive nor replacement growth of the alveoli was observed, except for A549 which showed intrabronchial invasion.



(a)



(b)

Fig. 3. Implanted NCI-H358 tumor nodule in the left lobe 28 days following intrabronchial propagation (a) (H.E.,  $\times 40$ ). Histopathologically, the tumor revealed poorly differentiated adenocarcinoma (b) (H.E.,  $\times 200$ ).

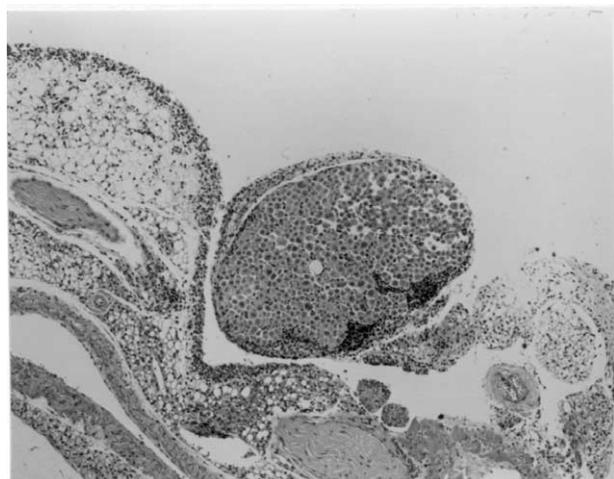
No metastases were observed in the extrathoracic organs with any of the cell lines.

### 3.2. Expression of MMP-2 and MMP-9 and their related molecules

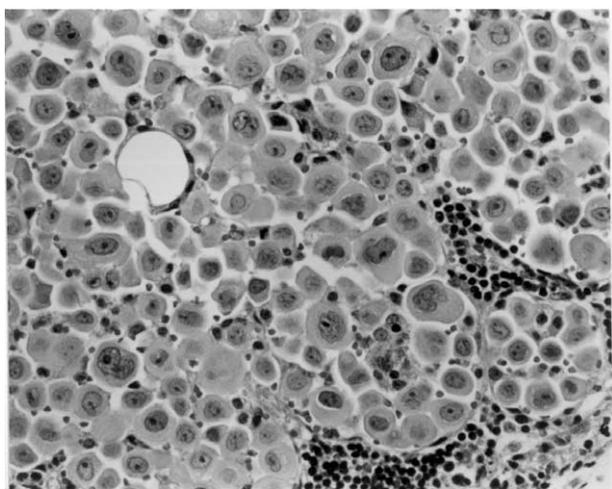
We extracted total RNA from A549, PC-14, NCI-H322, NCI-H23 and RERF-LC-KJ, and examined the expression of MMP-2, MMP-9, MT1-MMP, uPA, TIMP-2 and TIMP-1 mRNA. As shown in Fig. 5, all cell lines showed expression of MT1-MMP and uPA.

## 4. Discussion

This study revealed that the incidence of orthotopic intrapulmonary implantation of pulmonary adenocarcinoma cell lines depended on the biological character-



(a)



(b)

Fig. 4. Implanted PC-14 tumor nodule showing metastasis to the paratracheal lymph node (a) (H.E.,  $\times 40$ ). Histopathologically, the tumor revealed poorly differentiated adenocarcinoma (b) (H.E.,  $\times 200$ ).

istics of each cell line. In group (A), which had a high incidence of tumorigenicity in nude mice, A549 showed not only multiple intrapulmonary implantations but also lymphogenous metastasis to mediastinal lymph nodes. However, NCI-H358 showed a high incidence of intrapulmonary implantation but never metastasized to lymph nodes. Compared to NCI-H358, PC-14 is a very characteristic cell line. Although it is classified as group (B), showing a low incidence of intrapulmonary implantation, the incidence of mediastinal lymph node metastasis was high. These heterogeneous characteristics of each cell line suggest that distinct molecular mechanisms are involved in intrapulmonary implantation (metastasis) and lymphogenic spread or lymph node metastasis.

MMP-2 and MMP-9 have the capacity to degrade type IV collagen and are thought to play important roles

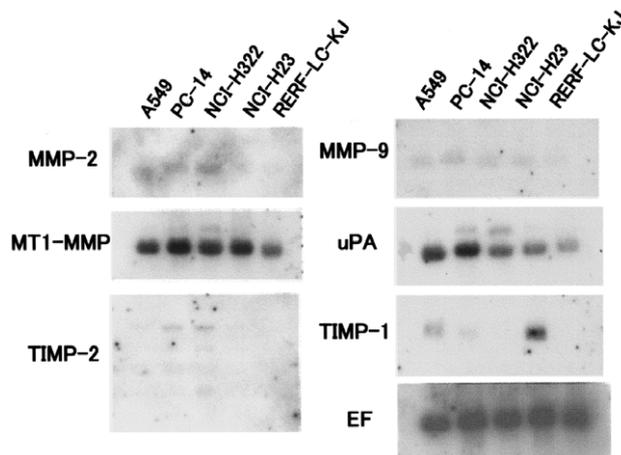


Fig. 5. Northern blot analysis of MMP-2, MMP-9, MT1-MMP, uPA, TIMP-2 and TIMP-1 expression in A549, PC-14, NCI-H322, NCI-H23 and RERF-LC-KJ cell lines. EF: elongation factor.

in tumor invasion and metastasis [12–16]. The correlation between the expression of MMP-2 and MMP-9 and the invasiveness and metastatic potential of human non-small-cell lung cancer (NSCLC) has been examined immunohistochemically [23–28], and some studies have examined gelatinolytic activity or the expressions of MMP-2 and MMP-9 mRNA in surgical samples or human lung cancer cell lines [25,27,29,30]. Several studies have concluded that MMP-2 and/or MMP-9 play important roles in the invasion of NSCLC. In this study, the expressions of MMP-2, MMP-9, MT1-MMP and uPA mRNA were elevated in cell lines which showed development of intrapulmonary implantation or mediastinal lymph node metastases. In particular, MMP-2 mRNA was highly expressed in cell lines producing multiple nodules in the lung and where mediastinal lymph node metastases developed. Our findings suggest that MMP-2 and MMP-9 and their activators (MT1-MMP and uPA) play an important role in the growth, invasion and metastasis of human lung adenocarcinoma cells in vivo.

In summary, using the modified intrabronchial propagation method of McLemore et al., nine human lung adenocarcinoma cell lines were examined and their biological behaviors were classified according to the incidence of tumorigenicity in nude mice. The results suggest that the expression of MMP-related molecules is associated with the growth, invasion and metastasis of human lung adenocarcinoma cells.

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