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# RESPIROLOGY

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## ISSUE HIGHLIGHTS

### Invited Review

*Respirology* year-in-review 2007: Clinical science

### Invited Review: Presidents' Series

Pulmonary complications of HIV infection

### Other article highlights include:

Involvement of the p38 MAPK pathway in IL-13-induced mucous cell metaplasia in mouse tracheal epithelial cells

Analysis of gene expression in human bronchial epithelial cells upon influenza virus infection and regulation by p38 mitogen-activated protein kinase and c-Jun-N-terminal kinase

Erythromycin attenuates MUC5AC synthesis and secretion in cultured human tracheal cells infected with RV14

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# RESPIROLOGY

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ORIGINAL ARTICLE

# Torque teno virus DNA titre elevated in idiopathic pulmonary fibrosis with primary lung cancer

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**Torque teno virus DNA titre elevated in idiopathic pulmonary fibrosis with primary lung cancer**
BANDO M, TAKAHASHI M, OHNO S, HOSONO T, HIRONAKA M, OKAMOTO H, SUGIYAMA Y. *Respirology* 2008; **13**: 263–269

**Background and objective:** IPF is an independent risk factor for lung cancer, but the mechanism of this association has not fully been elucidated. The role of Torque teno virus (TTV) in respiratory disease is poorly understood, although it has been shown that infection with TTV is associated with the activity and prognosis of IPF. This study aimed to investigate the prevalence and titre of TTV DNA among patients with IPF and lung cancer.

**Methods:** The presence of TTV DNA was determined by PCR in the sera of patients with both lung cancer and IPF ($n = 22$), patients with IPF only ($n = 35$), and patients with lung cancer only ($n = 142$).

**Results:** TTV DNA was detectable in all patients with both IPF and lung cancer, in 94.3% of the patients with IPF only and 97.2% of the patients with lung cancer only. The TTV DNA titre in the patients with IPF and lung cancer was significantly higher than that in the patients with IPF only or lung cancer only. The percentage of TTV-positive patients with a high TTV titre in the IPF and lung cancer group was significantly higher than that in the IPF only group.

**Conclusions:** These findings are the first report on the association between TTV and the complication of lung cancer in IPF and suggest that TTV infection might be associated with the development of lung cancer in IPF.

**Key words:** chronic inflammation, IPF, latent virus, primary lung cancer, Torque teno virus.

## INTRODUCTION

IPF is a chronic interstitial lung disease with a poor prognosis, the mean survival time after diagnosis being 4–5 years.[1] The main causes of death in IPF patients are respiratory failure, heart failure and lung cancer. IPF is frequently associated with lung cancer which influences the patients' management and prognosis.[2] Aubry *et al.*[3] reviewed the clinical, radiological and pathological findings in patients with primary lung cancer and IPF, and concluded that carcinoma tended to occur in older male smokers and was associated with poor prognosis. IPF is now recognized as an independent risk factor for lung cancer, but the mechanism of this is unclear. There has been increasing evidence for the association of neoplasms with antecedent chronic inflammation. Patients with IPF may have persistent activation of macrophages and lymphocytes,[4,5] which could be a natural reservoir for latent viruses such as cytomegalovirus.[6] Hepatitis C virus (HCV) and Epstein–Barr virus have been implicated in the aetiology of IPF;[7–9] however, these viruses have not been cultured from lung specimens of patients with IPF. In addition, a recent study did not find evidence for an aetiological role of Epstein–Barr virus in the development of lung cancer in IPF patients.[10]

Replicative forms of Torque teno virus (TTV) DNA have been detected in the lung tissue of a viraemic patient with IPF, suggesting an association between TTV infection and IPF.[11] TTV DNA was originally isolated in 1997 from the serum of a patient with post-transfusion hepatitis of unknown aetiology (not A to G), and this virus was named TTV after the initials of

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the first patient in whom it was discovered.[12] TTV is an unenveloped, single-stranded, circular DNA virus with a total genomic length of approximately 3.6–3.9 kb, and it infects human beings worldwide.[13] The ICTV Circoviridae Study Group proposed naming TTV as TTV that is classifiable into a novel genus Anellovirus, unassigned to any family.[14] TTV may replicate in the liver and in bone marrow cells, as circular, double-stranded TTV DNA molecules, which are the replicative intermediate form, and have been detected in these organs.[13] The link between TTV infection and a given pathophysiology remains unproven, although it has been suggested that the viral load is related to the immune status of the host. Recently, Tokita *et al.*[15] demonstrated that a high TTV viral load was independently associated with the complication of hepatocellular carcinoma and may have prognostic significance in patients with HCV-related chronic liver diseases.

The aims of the present study were to investigate the prevalence and titre of TTV DNA in patients with IPF and lung cancer, and to examine the relationship between the extent of TTV viraemia and the complication of primary lung cancer in patients with IPF.

## METHODS

### Patients

Between January 1991 and December 2003, 22 patients with both IPF and primary lung cancer (21 men; age 67.4 ± 8.6 years (mean ± SD), 46–82 years (range)) were admitted to the Division of Pulmonary Medicine, Jichi Medical University Hospital, and these patients were enrolled in this study. To get as many cases as possible, we searched for all patients fitting the criteria for this group from medical records from 1991, and identified 22 patients as study subjects whose serum was preserved at admission and whose consent was obtained for measurements of serum markers. One patient had two lung cancers. Patients with connective tissue disease, asbestos exposure, previous radiation therapy or metastatic disease in the lung were excluded.

Two control groups were employed. The first control group consisted of 35 patients with histologically documented usual interstitial pneumonia in the clinical setting of IPF without lung cancer (IPF only). The clinical characteristics of this group have been previously published.[11] The second control group consisted of 142 patients with primary lung cancer without evidence of IPF (lung cancer only).

The serum samples used for the measurement of TTV DNA titre were all collected before treatment at the initial admission, and the patients had never received steroids, immunosuppressants or chemotherapeutic agents. The survival status of each patient as of September 2005 was established based on hospital clinical records. The survival period was calculated based on the date of diagnosis of lung cancer. Informed written consent was obtained from each patient. The study of protocols were approved by the Committee for Human Subjects at Jichi Medical University Hospital.

### Extraction of nucleic acids from serum

From 50 μL of the serum sample, nucleic acids were extracted using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany), and dissolved in 50 μL of nuclease-free distilled water. Then, nucleic acids were extracted and precipitated with ethanol. DNA species of chromosomal origin, which emerged as a cloudy precipitate immediately after the addition of ethanol, were removed. The remaining nucleic acids were collected by centrifugation and dissolved in 40 μL of nuclease-free distilled water.

### Detection and quantitation of TTV DNA

Two different PCR methods (N22 PCR and untranslated region (UTR) PCR) for the detection of TTV DNA were used.[16–18] N22 PCR can detect primarily TTV of genotypes 1–6 which are classifiable in group 1, whereas UTR PCR can detect essentially all 39 TTV genotypes in groups 1–5 including TTV DNA detectable by N22 PCR.[14,19–21] In N22 PCR, nucleic acids extracted from serum were serially diluted 10-fold in distilled water containing 20 μg of glycogen (Roche Diagnostics GmbH) per millilitre, and the highest dilution in which TTV DNA was detectable by the PCR with semi-nested primers was determined. The primers for the first PCR were NG059 (sense: 5′-ACA GAC AGA GGA GAA GGC AAC ATG-3′) and NG063 (antisense: 5′-CTG GCA TTT TAC CAT TTC CAA AGT T-3′). The conditions for the first PCR were 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 45 s and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. The primers for the second PCR (25 cycles: same conditions as the first PCR) were NG061 (sense: 5′-GGC AAC ATG YTR TGG ATA GAC TGG-3′ (Y = T or C, R = A or G)) and NG063. The first and second PCRs amplified 286-bp and 271-bp DNA fragments, respectively. The amplification products were subject to electrophoresis on a 2.5% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME, USA), stained with ethidium bromide, and observed under UV light. Based on the results, semi-quantification of TTV DNA in $10^n$ copies per 10 μL in serum was performed.

UTR PCR was carried out in the presence of Perkin-Elmer AmpliTaq Gold (Roche Molecular Systems) and nested primers by the method described previously,[18] with slight modifications. Briefly, primers NG472 (sense: 5′-GCG TCC CGW GGG CGG GTG CCG-3′ (W = A or T)) and NG351 (antisense: 5′-GAG CCT TGC CCA TRG CCC GGC CAG-3′ (R = A or G)) were used for the first-round PCR, and primers NG473 (sense: 5′-CGG GTG CCG DAG GTG AGT TTA CAC-3′ (D = G, A or T)) and NG351 (antisense: 5′-CCC ATR GCC CGG CCA GTC CCG AGC-3′) were used for the second-round PCR; the primers were derived from the same well-conserved area in the UTR of the TTV genome as in the original method.[18] The size of the amplification

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**Table 1** Patients whose serum was positive for Torque teno virus (TTV) DNA detectable by N22 PCR and UTR PCR and serum titres, by clinical group

| TTV DNA | IPF only ($n$=35) | Lung cancer only ($n$=142) | IPF and lung cancer ($n$=22) |
|---|---|---|---|
| N22 PCR-positive | 13 (37.1%) | 56 (39.4%) | 9 (40.9%) |
| $\geq 10^3$ copies/mL | 3 (8.6%) | 15 (10.6%) | 4 (18.2%) |
| UTR PCR-positive | 33 (94.3%) | 138 (97.2%) | 22 (100%) |
| $\geq 10^3$ copies/mL | 27 (77.1%)* | 122 (85.9%) | 22 (100%) |
| UTR PCR titre (mean ± SD) ($\times 10^4$ copies/mL) | 2.3 ± 4.2 | 2.5 ± 4.0 | 3.3 ± 3.6† |

*$P < 0.02$ for the comparison between IPF only group and the IPF and lung cancer group.
†Significantly higher than in the IPF only group ($P < 0.05$) and lung cancer only group ($P < 0.05$).
UTR, un-translated region.

product from the first-round PCR was 91 bp and that from the second-round PCR was 71 bp. TTV DNA was quantified by real-time PCR (UTR PCR) which can detect essentially all TTV genotypes in groups 1–5, using 10 μL of the nucleic acid solution as a template, primers NG473-NG352, a doubly labelled probe (NG369-P: 5′-(Fam)-AGT CAA GGG GCA ATT CGG GCT CGG GA-(Tamra)-3′), and the LightCycler-Fast-Start DNA Master Hybridization Probes kit (Roche Diagnostics GmbH). PCR amplification was started with an initial denaturation at 95 °C for 10 min, followed by 50 cycles of denaturation at 95 °C for 10 s and annealing-extension at 62 °C for 30 s. All reactions were carried out in the LightCycler (Roche Diagnostics GmbH). The quantification limit of the system was 3–5 copies per test capillary (20 μL of reaction mixture). Intra- and inter-assay reproducibility was determined by testing on 5 different days 10 independent DNA extractions of two reference TTV-positive sera, and the %CV (coefficient of variance based on the value of the crossing point on the LightCycler) was 0.7 and 1.8, respectively. The overall variation was less than 0.53 log.

### Statistical analyses

The results are presented as mean ± SD. The frequency between groups was compared using the Mann–Whitney $U$-test or chi-squared test. The survival rate between groups was compared by the log rank test.

## RESULTS

### Percentage of patients whose serum was positive for TTV DNA

The percentage of patients in each group whose serum was positive for TTV DNA by N22 PCR or UTR PCR is shown in Table 1. Among the 22 patients with IPF and lung cancer, nine (40.9%) were positive for TTV DNA detectable by N22 PCR, and all patients were positive for TTV DNA detectable by UTR PCR. Among the patients with IPF only or lung cancer only, the TTV DNA-positive rates using N22 PCR were 37.1% and 39.4%, respectively, and the TTV DNA-positive rates using UTR PCR were 94.3% and 97.2%, respectively. The percentage of UTR PCR-positive patients with a high TTV titre ($\geq 10^3$ copies/mL) in the IPF and lung cancer group was significantly higher than that in the IPF only group ($P < 0.02$). The TTV DNA titre by UTR PCR in the patients with IPF and lung cancer ($(3.3 \pm 3.6) \times 10^4$ copies/mL) was significantly higher than those in the patients with IPF only ($(2.3 \pm 4.2) \times 10^4$ copies/mL, $P < 0.05$) or lung cancer only ($(2.5 \pm 4.0) \times 10^4$ copies/mL, $P < 0.05$).

### Demographic and clinical features of patients with IPF complicated with lung cancer

The demographic features and smoking habits of the patients in the three groups were compared (Table 2). The male : female ratio was 21:1 among the patients with IPF and lung cancer and 22:13 among the patients with IPF only, showing a significant difference ($P < 0.01$). The prevalence of smoking in patients with IPF and lung cancer was significantly higher than that in patients with IPF only ($P < 0.01$), and the smoking index (pack-years) was significantly higher among the patients with IPF and lung cancer than among the patients with IPF only ($P < 0.05$) (Table 2). There was no significant difference in age among the three groups.

Among the 23 cancers in the 22 patients with IPF complicated with lung cancer (including double cancer in one patient), 21 cancers (91.3%) were in peripheral lung fields and 65.2% of the cancers were in the non-fibrotic areas. The tumours consisted of adenocarcinomas ($n = 7$), squamous cell carcinomas ($n = 7$), large cell carcinomas ($n = 4$) and small cell carcinomas ($n = 3$). There were no significant differences in the TTV DNA titres by UTR PCR between the four histology groups (Table 3). The clinical stage IV was the most frequent stage, followed by stage IIIA, stage I and stage IIIB. There were also no significant differences in the TTV DNA titres among the clinical stages (Table 3). Smoking index (pack-years) was not correlated with the TTV DNA titre detectable by UTR PCR among the 22 patients (Fig. 1). Among the

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**Table 2** Comparison of the sociodemographic characteristics of patients, by clinical group

| Feature | IPF only ($n$=35) | Lung cancer only ($n$=142) | IPF and lung cancer ($n$=22) |
|---|---|---|---|
| Gender (male/female) | 22/13* | 104/38† | 21/1 |
| Age (years), mean ± SD (range) | 65.1 ± 9.9 (44–81) | 65.0 ± 9.9 (33–85) | 67.4 ± 8.6 (46–82) |
| Smoking history | | | |
| Smokers (%) | 60.0‡§ | 78.2 | 95.5 |
| Pack-years, mean ± SD | 30.3 ± 35.2¶ | 42.0 ± 34.6 | 62.4 ± 40.7 |

*$P < 0.01$ for the comparison between the IPF only group and the IPF and lung cancer group.
†$P < 0.03$ for the comparison between the lung cancer only group and the IPF and lung cancer group.
‡$P < 0.01$ for the comparison between the IPF only group and the IPF and lung cancer group.
§$P < 0.03$ for the comparison between the IPF only group and the lung cancer only group.
¶$P < 0.05$ for the comparison between the IPF only group and the IPF and lung cancer group.

**Table 3** Torque teno virus (TTV) DNA titres of the patients with IPF complicated with lung cancer, by tumour cell type and stage

| | n | TTV UTR PCR titre ($\times 10^4$ copies/mL) |
|---|---|---|
| Histology | | |
| Adenocarcinoma | 7 | 1.7 ± 1.7 |
| Squamous cell carcinoma | 7 | 2.9 ± 3.0 |
| Small cell carcinoma | 3 | 4.6 ± 6.5 |
| Large cell carcinoma | 4 | 5.6 ± 4.7 |
| Other | 1 | 3.7 |
| Stage | | |
| I | 4 | 4.5 ± 2.9 |
| II | 2 | 0.6 |
| IIIA | 5 | 4.2 ± 4.9 |
| IIIB | 3 | 2.0 ± 1.8 |
| IV | 8 | 3.2 ± 4.0 |

The results are presented as mean ± SD.
UTR, un-translated region.

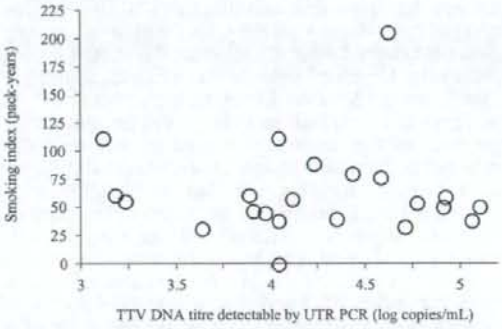


**Figure 1** Correlation between the smoking index and TTV DNA titre among patients with IPF complicated with lung cancer. There was no correlation between the smoking index and the TTV DNA titre detectable by UTR PCR ($n = 22$; $P = 0.96$, $R = -0.012$). TTV, Torque teno virus; UTR, un-translated region.

patients with IPF and lung cancer, mortality was mainly due to progression of the lung cancer and not the IPF. Only one patient with IPF and lung cancer, who had undergone surgery, died of acute exacerbation of IPF. The 5-year survival rate was 38.3% and the median survival period was 16.2 months.

## DISCUSSION

This study has shown that the TTV DNA titre detectable by UTR PCR in patients with IPF and lung cancer was significantly higher than that in patients with IPF only or lung cancer only. In addition, the percentage of UTR PCR-positive patients with a high TTV titre ($\geq 10^3$ copies/mL) in the IPF and lung cancer group was significantly higher than that in the IPF only group. TTV is a newly discovered human virus composed of a circular, single-stranded DNA of approximately 3.6–3.9 kb,[12,13] and it most closely resembles a member (chicken anaemia virus) of the Gyrovirus genus in the Circoviridae family,[22] but it has recently been classified into a novel genus Anellovirus, unassigned to any family.[14,23] TTV is widely distributed in human populations throughout the world and produces chronic viraemia in around 90% of healthy individuals of all ages.[24] Itoh *et al.*[25] demonstrated that TTV DNA was detected by UTR PCR at a high frequency (≥93%) in all age groups. However, it is unknown whether these higher frequencies of PCR positivity are also associated with higher circulatory virus loads in plasma. The natural history and pathogenic potential of TTV are currently under intensive investigation. Biagini *et al.*[26] reported a newborn with primary infection of TTV and clinical symptoms of benign viral rhinitis. Maggi *et al.*[27] demonstrated that the average TTV load was considerably higher in children with bronchopneumonia than in children with milder acute respiratory disease. Recently, Pifferi *et al.* reported that TTV might contribute to the pathogenesis of asthma in children.[28] Infection with TTV is characterized by persistent lifelong viraemia. Infection with TTV has been shown to influence the activity and prognosis of IPF.[11] However, the pathophysiology of TTV in the respiratory tract of infected humans has

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not yet been defined. TTV may replicate in the liver and in bone marrow cells, as circular, double-stranded TTV DNA has been detected in these organs. TTV DNA has also been detected in both frozen normal lung tissue and in cancer tissue obtained by thoracoscopy from a single lung cancer patient with TTV DNA in the serum.

There are two possible explanations for the findings of the present study. One explanation is that an impaired or suppressed immune system is involved in the TTV viral load in patients with IPF complicated with lung cancer. Maggi *et al.*[29] demonstrated that TTV loads were negatively related to the percentages of circulating $CD3^+$ and $CD4^+$ T cells and were positively related to the percentage of circulating B cells, suggesting that TTV might contribute to lymphocyte imbalances and immunosuppressive effects. Furthermore, it has been reported that the TTV viral load is inversely correlated with the CD4 T-cell count among patients infected with HIV type 1,[20,30] and that it may reflect the immune status of these immunocompromised hosts. A possible relationship between an elevated TTV viral load and the level of immunocompetence of the populations studied among TTV-infected patients on maintenance haemodialysis or with diabetes mellitus has also been suggested.[31] Therefore, it is likely that an impaired or suppressed immune system is associated with the elevated TTV viral load in IPF patients with lung cancer. A second explanation is that the high TTV viraemia influences the progression of IPF and promotes the development of lung cancer. Although the precise mechanism for the increased incidence of lung cancer among IPF patients remains unclear, it seems probable that the chronic inflammatory process resulting in remodelling of the lung is an important factor in the development of lung cancer in IPF patients who are also heavy smokers. There has been increasing evidence for the association of neoplasms with antecedent chronic inflammation; for example, gastric cancer in patients with *Helicobactor pylori* infection, malignant lymphoma secondary to chronic pyothorax or colon cancer in patients with ulcerative colitis.[32,33] Recently, Tokita *et al.*[15] demonstrated that a high TTV viral load was independently associated with the development of hepatocellular carcinoma, and may have prognostic significance in patients with HCV-related chronic liver diseases. Although the underlying mechanism by which high TTV viraemia increases the risk for HCC among patients with HCV-related chronic liver disease remains unknown, Moriyama *et al.*[34] reported that the score for irregular regeneration of hepatocytes in TTV-infected cirrhotic patients with chronic hepatitis C was higher than that in non-viraemic patients, suggesting that TTV infection may influence the development of HCV-related HCC. The potential involvement of TTV in the development of lung cancer associated with IPF and its influence on the airway and alveolar epithelial cells can be studied using bio-molecular techniques.

In the present study, the smoking index was significantly higher in patients with IPF and lung cancer than in patients with IPF alone. In addition, the smoking index was not correlated with the TTV DNA titre. These results support the hypothesis that smoking is a predictive factor for cancer in IPF rather than TTV. Turner-Warwick *et al.*[35] first reported the importance of smoking habit as a risk factor for lung cancer among patients with usual interstitial pneumonia using detailed statistical analyses. Several studies have also found an association between cigarette smoking and IPF combined with lung cancer. Nagai *et al.*[36] reported that the relative risk of cigarette smoking and lung cancer development was approximately 3.5 among patients with IPF. Matsushita *et al.*[37] reported that patients with lung cancer and IPF showed a significant predominance of smokers and a significant increase in the smoking index compared with the lung cancer cases without IPF. Smoking, especially heavy smoking, is one of the most important risk factors in the development of lung cancer in patients with IPF, and IPF and smoking may serve as cofactors in the development of lung cancer. On the other hand, smoking itself was associated with an increased risk of IPF in case–control studies.[38–40] Conversely, Hubbard *et al.*[40] provided evidence that the increased prevalence of lung cancer in patients with IPF was independent of the effect of cigarette smoking in a population-based cohort study. To further explore the role of cigarette smoking in the development of lung cancer in patients with IPF, data on the increased lung cancer risk associated with IPF in smokers and non-smokers are needed.

In conclusion, high TTV viraemia was significantly associated with IPF complicated with lung cancer in our study. However, it is not clear whether high TTV viraemia plays a role in the development of IPF and lung cancer; whether it is a cofactor in the progression of lung fibrosis; whether it is a serological marker reflecting the occurrence of lung cancer in IPF patients, or whether it reflects the host's immune system. Prospective studies need to be conducted to elucidate whether a high TTV viral load is associated with the development of lung cancer and whether it has clinical significance in predicting the outcome of patients with IPF complicated with lung cancer. In this context, future studies on the role of high TTV viral loads in patients with respiratory diseases of unknown aetiology are also warranted.

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## Accepted Abbreviations for *Respirology*

| Abbreviation | Full Name | Units |
|---|---|---|
| 6MWD | 6 minute walk distance | m |
| A-a $O_2$ gradient | alveolar-arterial oxygen gradient | |
| AHI | apnoea/hypopnoea index | |
| AIDS | acquired immune deficiency syndrome | |
| ARDS | acute respiratory distress syndrome | |
| BAL | bronchoalveolar lavage | |
| bd | twice daily | |
| BHR | bronchial hyperresponsiveness | |
| BMI | body mass index | $kg/m^2$ |
| BSA | bovine serum albumin | |
| cAMP | cyclic AMP | |
| cDNA | complementary DNA | |
| CPAP | continuous positive airway pressure | |
| CRP | C-reactive protein | mg/L |
| COPD | chronic obstructive pulmonary disease | |
| CT | computed tomography | |
| CXR | chest X-ray | |
| d | day | |
| $DL_{CO}$ | diffusing capacity of carbon monoxide | mL/min/mm Hg |
| DNA | deoxyribonucleic acid | |
| ECG | electrocardiogram | |
| ELISA | enzyme-linked immunosorbent assay | |
| ESR | erythrocyte sedimentation rate | mm/h |
| FACS | fluorescence-activated cell sorter | |
| $FEF_{25-75\%}$ | forced mid-expiratory flow | L/s |
| $FEV_1$ | forced expiratory volume in 1 second | L |
| $FEV_1$% | percent of predicted forced expiratory volume in 1 second | |
| $FEV_1$%FVC | $FEV_1$ as percentage of forced vital capacity | % |
| FRC | functional residual capacity (method of measurement to be specified) | L |
| FVC | forced vital capacity | L |
| FVC% | percent of predicted forced vital capacity | |
| h | hour | |
| Hb | haemoglobin | g/L |
| HIV | human immunodeficiency virus | |
| HPLC | high performance liquid chromatography | |
| Hz | hertz | |
| Ig | immunoglobulin | |
| IL | interleukin | |
| IPF | idiopathic pulmonary fibrosis | |
| IU | international unit | |
| i.v. | intravenous | |
| kg | kilogram | |
| KPa | kilopascals | |
| L | litre | |
| LDH | lactate dehydrogenase | |
| LPS | lipopolysaccharide | |
| m | metre | |

| Abbreviation | Full Name | Units |
|---|---|---|
| $m^2$ | square metre | |
| mAb | monoclonal antibody | |
| MHC | major histocompatibility complex | |
| min | minute | |
| mm | millimetre | |
| mm Hg | millimetre of mercury | |
| MRI | magnetic resonance imaging | |
| mRNA | messenger RNA | |
| MW | molecular weight | |
| *n* | number in study group | |
| °C | degree Celsius | |
| OSA | obstructive sleep apnoea | |
| *p* | probability | |
| $PaO_2$ | partial pressure of arterial oxygen | mm Hg |
| $PaCO_2$ | partial pressure of arterial carbon dioxide | mm Hg |
| PBS | phosphate buffered saline | |
| $PC_{20}$ | provocation concentration of a bronchoconstrictor agonist causing a 20% fall in $FEV_1$ | |
| PCR | polymerase chain reaction | |
| $PD_{20}$ | provocation dose of a bronchoconstrictor agonist causing a 20% fall in $FEV_1$ | |
| PEEP | positive end expiratory pressure | kPa |
| PEF | peak expiratory flow | L/min |
| PET | positron emission tomography | |
| PET FDG | positron emission tomography with fluorodeoxyglucose | |
| RCC | red cell count | $\times 10^9$/L |
| RNA | ribonucleic acid | |
| RV | residual volume (method should be specified) | L |
| s | second | |
| $SaO_2$ | arterial oxygen saturation | % |
| SD | standard deviation | |
| SEM | standard error of the mean | |
| SPECT | single photon emission computed tomography | |
| $T_{\frac{1}{2}}$ | half life | |
| tds | thrice daily | |
| TLC | total lung capacity (method should be specified) | L |
| UV | ultraviolet | |
| $V_A$ | alveolar volume | L |
| VATS | video-assisted thoracoscopic surgery | |
| V/Q | ventilation perfusion | |
| VC | vital capacity | L |
| WCC | white cell count | $\times 10^9$/L |
| µg | microgram | |


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# Identification of Novel Isoforms of the *EML4-ALK* Transforming Gene in Non–Small Cell Lung Cancer

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## Abstract

**The genome of a subset of non–small-cell lung cancers (NSCLC) harbors a small inversion within chromosome 2 that gives rise to a transforming fusion gene, *EML4-ALK*, which encodes an activated protein tyrosine kinase. Although breakpoints within *EML4* have been identified in introns 13 and 20, giving rise to variants 1 and 2, respectively, of *EML4-ALK*, it has remained unclear whether other isoforms of the fusion gene are present in NSCLC cells. We have now screened NSCLC specimens for other in-frame fusion cDNAs that contain both *EML4* and *ALK* sequences. Two slightly different fusion cDNAs in which exon 6 of *EML4* was joined to exon 20 of *ALK* were each identified in two individuals of the cohort. Whereas one cDNA contained only exons 1 to 6 of *EML4* (variant 3a), the other also contained an additional 33-bp sequence derived from intron 6 of *EML4* (variant 3b). The protein encoded by the latter cDNA thus contained an insertion of 11 amino acids between the EML4 and ALK sequences of that encoded by the former. Both variants 3a and 3b of EML4-ALK exhibited marked transforming activity *in vitro* as well as oncogenic activity *in vivo*. A lung cancer cell line expressing endogenous variant 3 of EML4-ALK underwent cell death on exposure to a specific inhibitor of ALK catalytic activity. These data increase the frequency of *EML4-ALK*–positive NSCLC tumors and bolster the clinical relevance of this oncogenic kinase.** [Cancer Res 2008;68(13):4971–6]

## Introduction

Lung cancer is the leading cause of cancer deaths in the United States, with >160,000 individuals dying of this condition in 2006 (1). The efficacy of conventional chemotherapeutic regimens with regard to improving clinical outcome in lung cancer patients is limited. Activating mutations within the epidermal growth factor receptor gene (*EGFR*) have been identified in non–small-cell lung cancer (NSCLC), the major subtype of lung cancer (2, 3), and chemical inhibitors of the kinase activity of EGFR have been found to be effective in the treatment of a subset of NSCLC patients harboring such mutations. However, these somatic mutations of *EGFR* are prevalent only among young women, nonsmokers, and Asian populations (3, 4).

We recently identified a novel transforming fusion gene, *EML4* (echinoderm microtubule-associated protein–like 4)-*ALK* (anaplastic lymphoma kinase), in a clinical specimen of lung adenocarcinoma from a 62-year-old male smoker (5). This fusion gene was formed as the result of a small inversion within the short arm of chromosome 2 that joined intron 13 of *EML4* to intron 19 of *ALK* (transcript ID ENST00000389048 in the Ensembl database[5]). The EML4-ALK protein thus contained the amino-terminal half of EML4 and the intracellular catalytic domain of ALK. Replacement of the extracellular and transmembrane domains of ALK with this region of EML4 results in constitutive dimerization of the kinase domain of ALK and a consequent increase in its catalytic activity (5).

Whereas this *EML4-ALK* fusion gene was detected in 3 of 75 individuals with NSCLC, we further identified another isoform of *EML4-ALK* in two patients of the same cohort (5). In these two individuals, intron 20 of *EML4* was disrupted and joined to intron 19 of *ALK*, with the fusion protein thus consisting of the amino-terminal two thirds of EML4 and the intracellular domain of ALK. This larger version of EML4-ALK was referred to as variant 2, with the original smaller version being termed variant 1. A total of 5 of the 75 (6.7%) patients in the cohort were thus positive for *EML4-ALK*.

Given that detection of *EML4-ALK* cDNA by the PCR would be expected to provide a highly sensitive means for diagnosis of lung cancer, and given that inhibition of the catalytic activity of EML4-ALK may be an effective approach to treatment of this disorder, we have examined whether other isoforms of EML4-ALK are associated with NSCLC. We now describe a third isoform of EML4-ALK (variant 3) that is smaller than variants 1 and 2.

## Materials and Methods

**PCR.** This study was approved by the ethics committees of Jichi Medical University and The Cancer Institute of the Japanese Foundation for Cancer Research. Total cDNA of NSCLC specimens was synthesized with PowerScript reverse transcriptase (Clontech) and an oligo(dT) primer from total RNA purified with the use of an RNeasy Mini RNA purification kit (Qiagen). Reverse transcription-PCR (RT-PCR) to amplify the fusion point of *EML4-ALK* variant 3 mRNA was done with a QuantiTect SYBR Green kit (Qiagen) and the primers 5′-TACCAGTGCTGTCTCAATTGCAGG-3′ and 5′-TCTTGCCAGCAAAGCAGTAGTTGG-3′. A full-length cDNA for *EML4-ALK*

[5] http://www.ensembl.org/index.html

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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variant 3 was amplified from total cDNA of a NSCLC specimen (ID no. 2075) with PrimeSTAR HS DNA polymerase (Takara Bio) and the primers 5′-ACTCTGTCGGTCCGCTGAATGAAG-3′ and 5′-CCACGGTCTTAGGGATCCCAAGG-3′; PCR was done for 35 cycles of 98°C for 10 s and 68°C for 6 min. The fusion point of *EML4-ALK* in the genome was amplified by PCR with genomic DNA of NSCLC specimens, PrimeSTAR HS DNA polymerase, and the primers 5′-GGCATAAAGATGTCATCATCAACCAAGG-3′ and 5′-AGCTTGCTCAGCTTGTACTCAGGG-3′. The nucleotide sequences of the *EML4-ALK* variant 3a and 3b cDNAs have been deposited in DDBJ/EMBL/GenBank under accession nos. AB374361 and AB374362, respectively.

**Fluorescence *in situ* hybridization.** Fluorescence *in situ* hybridization (FISH) analysis of the fusion gene was done with archival pathology specimens and with bacterial artificial chromosomes containing genomic DNA corresponding to *EML4* or *ALK* and their flanking regions as probes. In brief, surgically removed lung cancer tissue was fixed in 20% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness of 3 μm. The sections were placed on glass slides and processed with a Histology FISH Accessory Kit (DakoCytomation) before hybridization with the *EML4* and *ALK* probes and examination with a fluorescence microscope (BX61, Olympus).

**Transforming activity of EML4-ALK variant 3.** Analyses of the function of EML4-ALK variant 3 were done as described previously (5). In brief, the cDNA for EML4-ALK variant 3a or 3b was fused with an oligonucleotide encoding the FLAG epitope tag and then inserted into the retroviral expression plasmid pMXS (6). The resulting plasmids as well as similar pMXS-based expression plasmids for EML4-ALK variant 1, variant 1 (K589M), or variant 2 were individually introduced into mouse 3T3 fibroblasts by the calcium phosphate method for a focus formation assay and assay of tumorigenicity in nu/nu mice. The same set of EML4-ALK proteins was expressed in HEK293 cells and assayed for kinase activity *in vitro* with the YFF peptide (7).

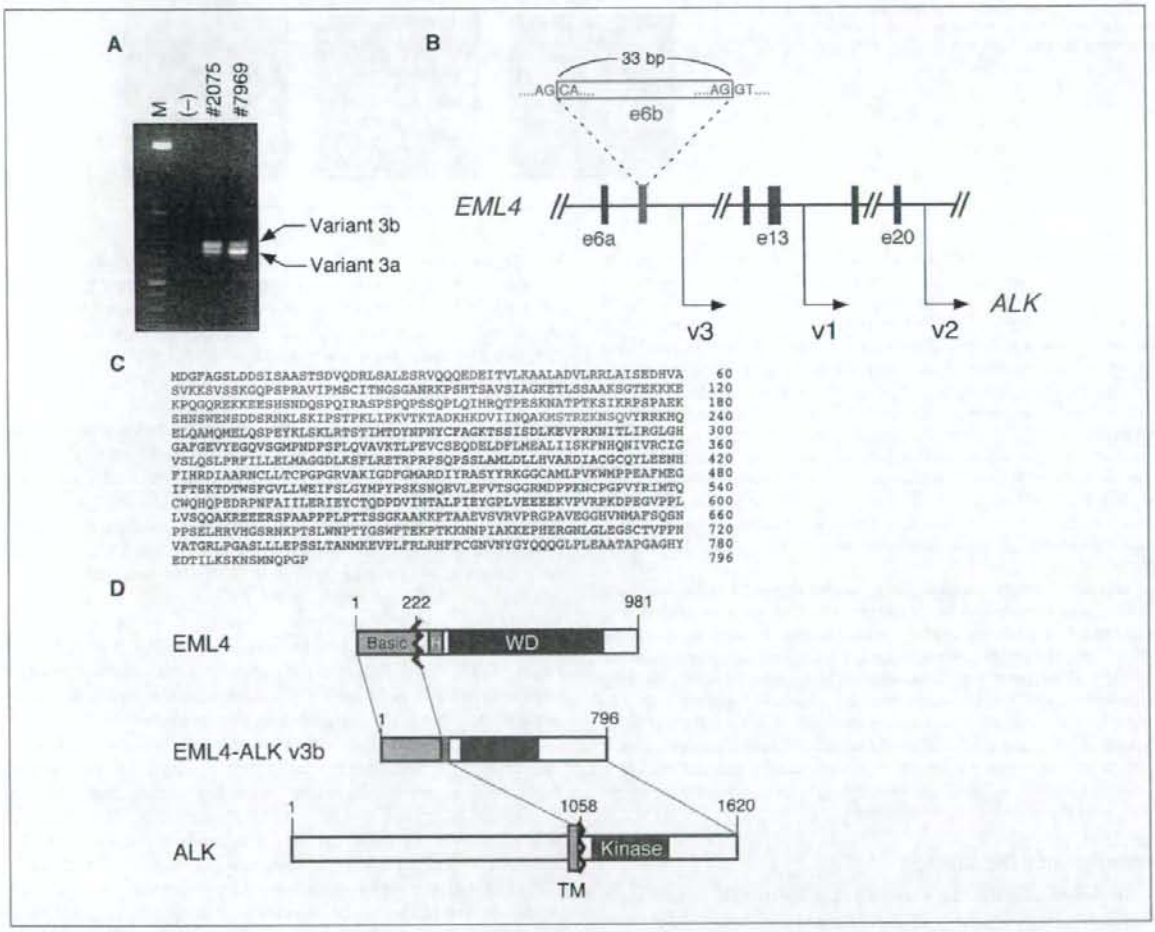


**Figure 1.** Identification of *EML4-ALK* variant 3. *A*, detection of fusion cDNAs linking exon 6 of *EML4* to exon 20 of *ALK* by RT-PCR analysis. Two RT-PCR products of 548 bp (corresponding to variant 3b) and 515 bp (corresponding to variant 3a) were detected by agarose gel electrophoresis with total RNA from two NSCLC specimens (tumor ID nos. 2075 and 7969). *Lane (–)*, no-template control; *lane M*, size markers (50-bp ladder). *B*, genomic organization of *EML4*. Intronic sequences downstream of exons (*e*) 6, 13, and 20 of *EML4* are fused to intron 19 of *ALK* to generate variants (*v*) 3, 1, and 2 of *EML4-ALK*, respectively. Exon-intron boundary sequences as well as the size of exon 6b are indicated. *C*, predicted amino acid sequence of EML4-ALK variant 3b. *Blue*, *green*, and *red*, amino acids corresponding to exons 1 to 6a of *EML4*, exon 6b of *EML4*, and *ALK*, respectively. Amino acid number is indicated on the right. *D*, fusion of an amino-terminal portion of EML4 [which consists of a basic region (*Basic*), HELP domain (*H*), and WD repeats] to the intracellular region of ALK (containing the tyrosine kinase domain) generates EML4-ALK variant 3b. *Green*, the region of the fusion protein encoded by exon 6b of *EML4*. *TM*, transmembrane domain.

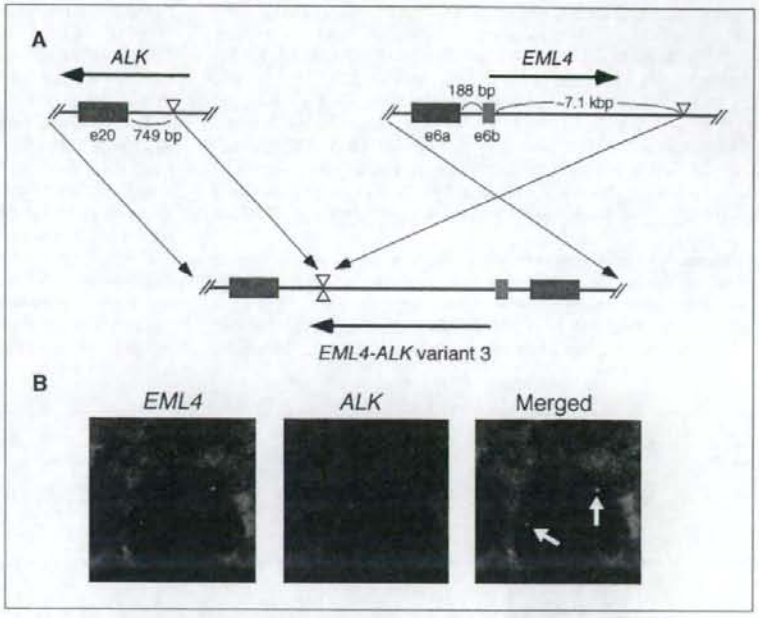


**Figure 2.** Chromosomal rearrangement responsible for generation of *EML4-ALK* variant 3. *A*, schematic representation of the chromosomal rearrangement underlying the generation of *EML4-ALK* variant 3. Exon 6b of *EML4* is located 188 bp downstream of exon 6a. In NSCLC specimen ID no. 7969, *EML4* is disrupted at a position ~7.1 kbp downstream of exon 6b and is ligated to a position 749 bp upstream of exon 20 of *ALK*, giving rise to the *EML4-ALK* (variant 3) fusion gene. *Horizontal arrows*, direction of transcription. *B*, FISH analysis of a representative cancer cell in a histologic section of lung adenocarcinoma (ID no. 7969) with differentially labeled probes for *EML4* (*left*) and *ALK* (*center*). Two fusion signals (*arrows*) and a pair of green (corresponding to *EML4*) and red (corresponding to *ALK*) signals are present in the merged image (*right*).

The cDNA for FLAG-tagged EML4-ALK variant 3b was also inserted into pMX-iresCD8 for the expression of both EML4-ALK and mouse CD8 (8), and the resulting recombinant retroviruses were used to infect mouse BA/F3 cells (9). CD8-positive cells were then purified with the use of a miniMACS magnetic bead–based separation system (Miltenyi Biotec) and cultured in the absence or presence of mouse interleukin-3 (IL-3; Sigma) or 2,4-pyrimidinediamine (Example 3-39, a specific inhibitor of ALK enzymatic activity that was developed by Novartis[6] and synthesized by Astellas Pharma).

Mouse 3T3 fibroblasts and NCI-H2228 lung cancer cells (both from American Type Culture Collection) as well as 3T3 cells expressing v-Ras were plated in 96-well spheroid culture plates (Celltight Spheroid, Sumilon) at a density of $1 \times 10^3$ per well. Cell growth was examined with the WST-1 Cell Proliferation Reagent (Clontech) after culture for 5 d with 2,4-pyrimidinediamine.

**Luciferase reporter assays.** The promoter fragments of *Fos*, *Myc*, and *Bcl-$x_L$* genes were ligated to a luciferase cDNA to generate pFL700 (10), pHXLuc (11), and pBclxl-Luc (12) reporter plasmids, respectively. Luciferase cDNA ligated to the DNA binding sequence for nuclear factor κB (NF-κB) or to the GAS sequence was obtained from Stratagene. HEK293 cells were transfected with these various reporter plasmids together with the expression plasmid for EML4-ALK variant 3b or the empty vector, as described previously (13). The pGL4 plasmid (Promega) for expression of *Renilla* luciferase was also included in each transfection mixture. After culture of the cells for 2 d, luciferase activity in cell lysates was measured with a Luciferase Assay system (Promega).

## Results and Discussion

**Detection of *EML-ALK* variant 3.** The EML4-ALK variant 1 and 2 proteins are produced as a result of genomic rearrangements that lead to the juxtaposition of exons 13 and 20 of *EML4*, respectively, to exon 20 of *ALK*. It is theoretically possible that exon 2, 6, 18, or 21 of *EML4* also could undergo in-frame fusion to exon 20 of *ALK*. We therefore examined whether transcripts of any such novel *EML4-ALK* fusion genes are present in NSCLC cells by RT-PCR analysis with primers that flank each putative fusion point (data not shown). With the primer set for amplification of the *EML4* (exon 6)-*ALK* (exon 20) fusion cDNA, we detected a pair of PCR products in two individuals with lung adenocarcinoma (Fig. 1*A*). Although one of the patients (tumor ID no. 7969) had a smoking index of 540, the other patient (tumor ID no. 2075) had never smoked. Nucleotide sequencing of each PCR product from both patients revealed that the smaller product of 515 bp corresponded to a fusion cDNA linking exon 6 of *EML4* to exon 20 of *ALK*, whereas the larger product of 548 bp contained an additional sequence of 33 bp that was located between these exons of *EML4* and *ALK* and which mapped to intron 6 of *EML4* (Fig. 1*B*). The larger cDNA would thus be expected to encode a fusion protein with an insertion of 11 amino acids between the EML4 and ALK sequences of the protein encoded by the smaller cDNA.

Although we did not detect human mRNAs or expressed sequence tags containing this cryptic exon of *EML4* in the nucleotide sequence databases, it is likely that this exon is physiologic and functional because (*a*) the fusion cDNA containing this exon was identified in two independent patients and in amounts no less than those of the corresponding cDNA without it (Fig. 1*A*); (*b*) the intron-exon boundary sequence for this exon conforms well to the AG-GU rule for mRNA splicing (Fig. 1*B*); and (*c*) *EML4* cDNAs or expressed sequence tags containing this exon were detected in the sequence databases for other species (for instance, GenBank accession no. AK144604 corresponding to a mouse *EML4* cDNA). We thus refer to this cryptic exon as exon 6b and to the original exon 6 as exon 6a (Fig. 1*B*). The novel isoforms of *EML4-ALK* transcripts containing exons 1 to 6a or 1 to 6b of *EML4* were also designated variants 3a and 3b, respectively.

[6] Patent information: Garcia-Echeverria C, Kanazawa T, Kawahara E, Masuya K, Matsuura N, Miyake T, et al., inventors; Novartis AG, Novartis Pharma GmbH, IRM LLC, applicants. 2,4-Pyrimidinediamines useful in the treatment of neoplastic disease, inflammatory and immune system disorders. PCT WO 2005016894. 2005 Feb 24.

To isolate a full-length cDNA for EML4-ALK variant 3, we performed RT-PCR with total cDNA of a positive specimen (ID no. 2075) and with a sense strand primer targeted to the 5′ untranslated region (UTR) of *EML4* mRNA and an antisense strand primer targeted to the 3′ UTR of *ALK* mRNA. One-step PCR analysis yielded cDNA products for both *EML4-ALK* variants 3a and 3b (Fig. 1*C*; Supplementary Fig. S1).

The EML4 protein contains an amino-terminal basic domain followed by a hydrophobic echinoderm microtubule–associated protein–like protein (HELP) domain and WD repeats (14). Given that exons 1 to 6 of *EML4* encode the basic domain, the proteins encoded by the variant 3 cDNAs contain the entire basic domain of EML4 directly linked to the catalytic domain of ALK (Fig. 1*D*). The fact that the basic domain was found to be essential for both the self-dimerization and oncogenic activity of EML4-ALK (5) suggested that the variant 3 isoforms likely also possess transforming activity.

**Chromosome rearrangement responsible for generation of *EML4-ALK* variant 3.** To show the presence of a chromosome rearrangement responsible for the generation of *EML4-ALK* variant

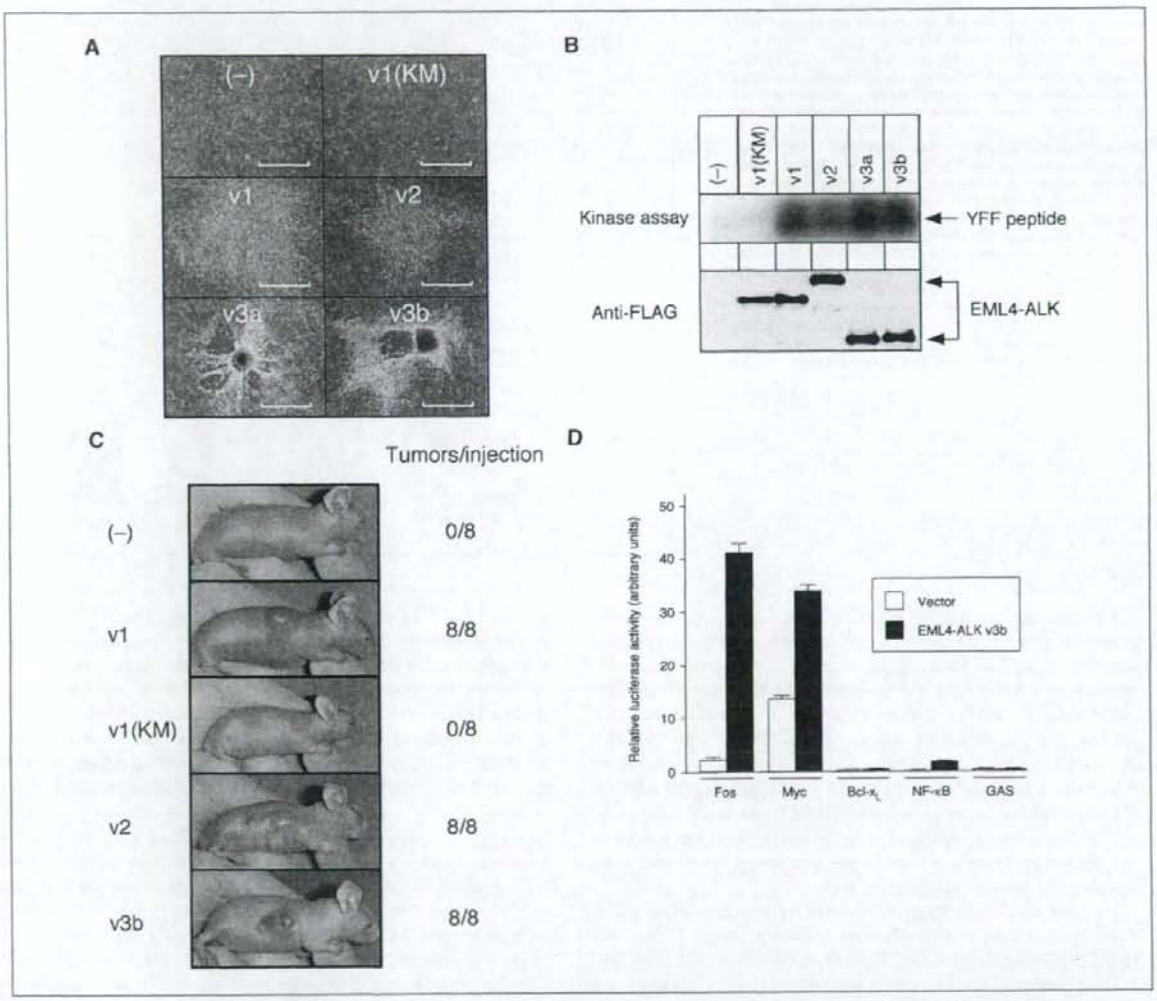


**Figure 3.** Transforming potential of EML4-ALK variants. *A*, focus formation assay. Mouse 3T3 fibroblasts were transfected with the empty expression plasmid [(–)] or with plasmids for wild-type (*v1*) or K589M mutant [*v1(KM)*] forms of variant 1, variant 2 (*v2*), variant 3a (*v3a*), or variant 3b (*v3b*) of FLAG-tagged EML4-ALK. The cells were photographed after culture for 18 d. *Bar*, 1 mm. *B*, *in vitro* kinase assay. HEK293 cells expressing the various FLAG-tagged variants of EML4-ALK were lysed and subjected to immunoprecipitation with antibodies to FLAG, and the resulting precipitates were assayed for kinase activity with the synthetic YFF peptide (*top*) or subjected to immunoblot analysis with antibodies to FLAG (*bottom*). *C*, *in vivo* assay of tumorigenicity. 3T3 cells expressing the indicated EML4-ALK variants were injected s.c. into nu/nu mice, and tumor formation was examined after 20 d. The number of tumors formed per eight injections is indicated on the right. *D*, analysis of EML4-ALK signaling with luciferase-based reporter plasmids. HEK293 cells were transfected with an expression plasmid for EML4-ALK variant 3b (or with the empty vector) together with reporter plasmids containing the promoter fragment of *Fos*, *Myc*, or *Bcl-x$_L$* gene; the DNA binding sequence for NF-κB; or the GAS sequence. Cells were cultured for 2 d, lysed, and assayed for luciferase activity. The activity of firefly luciferase was normalized by that of *Renilla* luciferase. *Columns*, mean of three experiments; *bars*, SD.

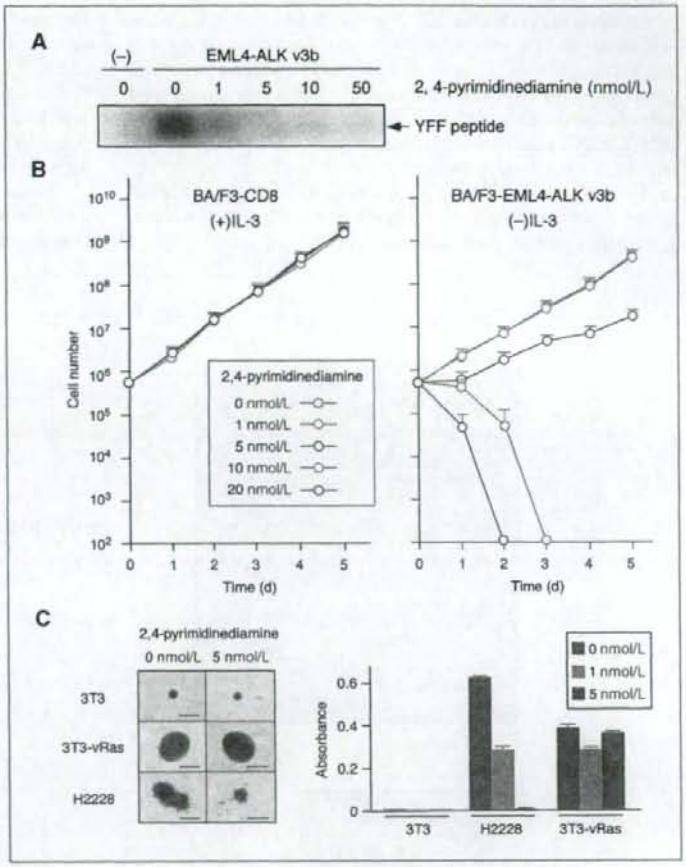


**Figure 4.** Essential role of EML4-ALK kinase activity in malignant transformation. *A*, lysates of HEK293 cells expressing FLAG-tagged EML4-ALK variant 3b (*v3b*) were divided into five equal portions, and each portion was subjected to immunoprecipitation with antibodies to FLAG. The immunoprecipitates were washed with kinase buffer [10 mmol/L HEPES-NaOH (pH 7.4), 50 mmol/L NaCl, 5 mmol/L $MgCl_2$, 5 mmol/L $MnCl_2$, 0.1 mmol/L $Na_3VO_4$] containing 0, 1, 5, 10, or 50 nmol/L of 2,4-pyrimidinediamine and then incubated for 30 min at room temperature for assay of kinase activity with the YFF peptide in the continued absence or presence of 2,4-pyrimidinediamine. The same amount of lysate of cells transfected with the empty vector was also subjected to immunoprecipitation and assayed as a negative control (–). *B*, mouse BA/F3 cells expressing CD8 alone were cultured in the presence of IL-3 (1 ng/mL) and the indicated concentrations of 2,4-pyrimidinediamine (*left*). BA/F3 cells expressing both CD8 and EML4-ALK variant 3b were cultured with the indicated concentrations of 2,4-pyrimidinediamine but without IL-3 (*right*). Cell number was counted at the indicated times. *Points*, mean of three separate experiments; *bars*, SD. *C*, mouse 3T3 fibroblasts expressing (or not) v-Ras or NCI-H2228 cells were cultured in a spheroid culture plate for 2 d, after which 2,4-pyrimidinediamine was added to the culture medium at a concentration of 0, 1, or 5 nmol/L. The cells were photographed after culture for an additional 5 d (*left*). *Bar*, 4 mm. Cell number in each well was also assessed at the same time with the use of the WST-1 assay (*right*). *Columns*, mean of three wells from a representative experiment; *bars*, SD.

3, we attempted to amplify the fusion point between the two genes from the genome of positive NSCLC cells. PCR with primers targeted to regions flanking the putative fusion point yielded a product of ~8 kbp with the genomic DNA of tumor ID no. 7969 (data not shown). Our failure to detect an unambiguous PCR product with genomic DNA of tumor ID no. 2075 may indicate that the breakpoint in intron 6 of *EML4* in this specimen is too distant from exon 6 to be readily amplified by PCR (intron 6 of *EML4* is >16 kbp). Nucleotide sequencing of the PCR product for tumor ID no. 7969 revealed that intron 6 of *EML4* was disrupted at a position ~7.1 kbp downstream of exon 6b and was joined to a point 749 bp upstream of exon 20 of *ALK* (Fig. 2*A*).

We also confirmed the chromosome rearrangement involving *EML4* and *ALK* by FISH analysis of cells from tumor ID no. 7969 (Fig. 2*B*) and tumor ID no. 2075 (data not shown) with differentially labeled probes for the two genes. Both genes map to the short arm of chromosome 2 within a distance of ~12 Mbp. The tumor cells exhibited fusion signals (corresponding to *EML4-ALK*) in addition to a pair of isolated green and red signals (corresponding to the two genes on the normal chromosome 2). The chromosome rearrangement involving the *ALK* locus was further verified with a different set of fluorescent probes (Supplementary Fig. S2).

**Transforming activity of EML4-ALK variant 3.** To compare the transforming potential of variants 1, 2, 3a, and 3b of EML4-ALK, we introduced expression plasmids for each variant into mouse 3T3 fibroblasts for assay of focus formation. No transformed foci were detected for cells transfected with the empty plasmid or with a plasmid for a kinase-inactive mutant (K589M) of EML4-ALK variant 1 (5) in which $Lys^{589}$ in the ATP binding site of the catalytic domain is replaced with Met (Fig. 3*A*). In contrast, variants 3a and 3b of EML4-ALK each exhibited marked transforming activity that was not less than that of variant 1 or 2. To examine directly the tyrosine kinase activity of EML4-ALK variants, we subjected HEK293 cells expressing each of these variants to an *in vitro* kinase assay with a synthetic YFF peptide (7). Again, both variants 3a and 3b exhibited marked kinase activity that was not less than that of variant 1 or 2 (Fig. 3*B*). Similarly, in a tumorigenicity assay with nude mice, 3T3 cells expressing EML4-ALK variant 3b formed large subcutaneous tumors at all injection sites (Fig. 3*C*). Consistent with our previous observations (5), cells expressing variant 1 or 2 of EML4-ALK also formed tumors.

To examine the intracellular signaling pathways activated by EML4-ALK, we linked the luciferase cDNA to the promoter fragment of *Fos*, *Myc*, or *Bcl-$x_L$* gene (10–12); the DNA binding sequence for NF-κB; or the GAS sequence [a target site of the transcription factors signal transducers and activators of transcription (STAT)-1 and STAT3; ref. 15]. The resulting constructs were then introduced into HEK293 cells together with an

expression plasmid for EML4-ALK variant 3b. EML4-ALK variant 3b markedly activated the promoters of the *Fos* and *Myc* genes (Fig. 3*D*), consistent with the transforming potential of EML4-ALK. In contrast, although STAT3 has been shown to be a downstream target of the NPM-ALK fusion protein (16), EML4-ALK did not activate the GAS sequence, suggesting that STAT3 is unlikely to be a major target of EML4-ALK, as was shown in an EML4-ALK–positive lung cancer cell line by a proteomics approach (17). The distinct subcellular localizations of the two ALK fusion proteins [EML4-ALK in the cytoplasm (5) and NPM-ALK in both the nucleus and cytoplasm (18)] may account for this difference. Whereas EML4-ALK did not activate the *Bcl-$x_L$* gene promoter, it induced a small but significant increase in the activity of the NF-κB binding sequence ($P = 1.86 \times 10^{-4}$, Student's $t$ test).

Several compounds have recently been identified as specific inhibitors of the kinase activity of ALK and as potential drugs for the treatment of lymphoma positive for *NPM-ALK* (19). We examined the effects of one such inhibitor, 2,4-pyrimidinediamine, on the transforming potential of EML4-ALK. We first determined the effect of this inhibitor on the kinase activity of EML4-ALK variant 3b immunoprecipitated from transfected cells. 2,4-Pyrimidinediamine inhibited the kinase activity of EML4-ALK in a concentration-dependent manner, with a concentration of 1 nmol/L reducing the kinase activity to <50% of the control value (Fig. 4*A*).

We also introduced EML4-ALK variant 3b and CD8 (or CD8 alone) into the IL-3–dependent hematopoietic cell line BA/F3 (9) and then purified the resulting CD8-positive cell populations. 2,4-Pyrimidinediamine, even at a concentration of 20 nmol/L, did not affect the IL-3–dependent growth of BA/F3 cells expressing only CD8 (Fig. 4*B*), indicating that this agent does not inhibit mitogenic signaling mediated by Janus kinase in BA/F3 cells. Expression of EML4-ALK rendered BA/F3 cells independent of IL-3 for growth, but the cells expressing the fusion protein also rapidly underwent cell death on exposure to 2,4-pyrimidinediamine (Fig. 4*B*).

Finally, we examined the effect of 2,4-pyrimidinediamine on lung cancer cells that express endogenous EML4-ALK variant 3. The human lung cancer cell line NCI-H2228 expresses EML4-ALK variants 3a and 3b (data not shown) and forms spheroids in a three-dimensional spheroid culture system (Fig. 4*C*; ref. 20). Whereas 3T3 fibroblasts are unable to form such spheroids, expression of v-Ras in these cells results in the formation of large spheroids in culture. Whereas 2,4-pyrimidinediamine did not affect the proliferation of 3T3 cells expressing v-Ras in this system, it inhibited the growth of NCI-H2228 cells in a concentration-dependent manner (Fig. 4*C*). These data thus indicate that EML4-ALK is essential for the growth of cancer cells expressing this oncokinase.

In conclusion, we have identified novel isoforms of *EML4-ALK* in two patients with NSCLC. A chromosome inversion within 2p was shown to connect intron 6 of *EML4* to intron 19 of *ALK* and to be responsible for the generation of fusion cDNAs connecting exons 1 to 6a or exons 1 to 6b of *EML4* to exon 20 of *ALK*. Given that fusion cDNAs with or without exon 6b of *EML4* were each present in the two patients, EML4-ALK variant 3a and 3b proteins are likely to be coexpressed in NSCLC cells. Although RT-PCR analysis to detect *EML4-ALK* may provide a highly sensitive means to detect lung cancer, it is important that all variant forms of the fusion gene be assayed with appropriately designed primer sets. Given that all the identified variants possess prominent transforming activity, the newly revealed increased incidence of *EML4-ALK* fusion in NSCLC further increases the importance of the fusion gene as a therapeutic target for this intractable disorder.

## Disclosure of Potential Conflicts of Interest

K. Takeuchi: Consultant, DAKO. The other authors disclosed no potential conflicts of interest.

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# A mouse model for *EML4-ALK*-positive lung cancer

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**EML4-ALK is a fusion-type protein tyrosine kinase that is generated in human non-small-cell lung cancer (NSCLC) as a result of a recurrent chromosome inversion, inv (2)(p21p23). Although mouse 3T3 fibroblasts expressing human EML4-ALK form transformed foci in culture and s.c. tumors in nude mice, it has remained unclear whether this fusion protein plays an essential role in the carcinogenesis of NSCLC. To address this issue, we have now established transgenic mouse lines that express EML4-ALK specifically in lung alveolar epithelial cells. All of the transgenic mice examined developed hundreds of adenocarcinoma nodules in both lungs within a few weeks after birth, confirming the potent oncogenic activity of the fusion kinase. Although such tumors underwent progressive enlargement in control animals, oral administration of a small-molecule inhibitor of the kinase activity of ALK resulted in their rapid disappearance. Similarly, whereas i.v. injection of 3T3 cells expressing EML4-ALK induced lethal respiratory failure in recipient nude mice, administration of the ALK inhibitor effectively cleared the tumor burden and improved the survival of such animals. These data together reinforce the pivotal role of EML4-ALK in the pathogenesis of NSCLC in humans, and they provide experimental support for the treatment of this intractable cancer with ALK inhibitors.**

transgenic mouse | surfactant protein C | molecular targeted therapy

Lung cancer remains the leading cause of cancer deaths, with almost 1.3 million people dying annually from this condition worldwide (www.who.int/cancer/en). Although a variety of chemotherapeutic regimens have been developed to treat this intractable disease, their efficacy is limited and depends on cancer subtype. Non-small-cell lung cancer (NSCLC) accounts for 80–85% of all lung cancer cases and is less sensitive to cytotoxic drugs than is small cell lung cancer. Unless tumor cells are surgically resected at an early clinical stage, individuals with NSCLC can expect a median survival time of less than 1 year (1).

A subset of individuals with NSCLC (mostly nonsmokers, young females, and those of Asian ethnicity) have been shown to harbor mutations in the epidermal growth factor receptor (EGFR) gene (2–4). Such mutations result in constitutive activation of the EGFR tyrosine kinase, the oncogenic potential of which has been demonstrated in a transgenic mouse system (5). Small-molecule drugs that specifically inhibit the catalytic activity of EGFR have been found to exhibit clinical efficacy in the treatment of NSCLC patients, especially in those with an activated EGFR (6, 7).

We recently developed a system for the construction of retroviral cDNA libraries from small quantities of clinical specimens (8–10), and we applied this technology to NSCLC to screen for oncogenes that might be potential drug targets (11). With the use of a focus-formation assay performed with mouse 3T3 fibroblasts, we identified a fusion-type oncogene, *EML4-ALK*, in an NSCLC specimen of a smoker (12). A small inversion within the short arm of chromosome 2 was found to result in the ligation of *EML4* and *ALK*, leading to the production of a fusion protein consisting of the amino-terminal portion of EML4 and the intracellular region of the protein tyrosine kinase ALK. The coiled-coil domain within this portion of EML4 mediates the constitutive dimerization and activation of EML4-ALK, which is responsible for the generation of transformed cell foci in culture and the formation by these cells of s.c. tumors in nude mice. Although the inv (2)(p21p23) rearrangement responsible for the fusion event occurs recurrently in NSCLC patients, it remains to be demonstrated that *EML4-ALK* plays an essential role in the carcinogenesis of NSCLC harboring the fusion gene.

To address this issue, we have now engineered the expression of *EML4-ALK* in lung epithelial cells of transgenic mice. The surfactant protein C gene (*SPC*) is specifically expressed in type 2 alveolar epithelial cells, and a fragment of its promoter has been used widely for establishment of mouse lines that express transgenes specifically in lung epithelial cells (13–15). We therefore generated independent mouse lines in which *EML4-ALK* expression is driven by the *SPC* promoter, and we found that all such mice develop hundreds of adenocarcinoma nodules in both lungs within only a few weeks after birth. Furthermore, inhibition of EML4-ALK activity with a small-molecule drug induced rapid death of the tumor cells.

## Results

**Generation of *EML4-ALK* Transgenic Mice.** To generate mice with lung-specific expression of *EML4-ALK*, we ligated a fragment of the *SPC* promoter (≈3.7 kbp) to a cDNA for EML4-ALK variant 1 with an amino-terminal FLAG epitope tag (12). The cDNA was, in turn, attached to an RNA splicing cassette and a polyadenylation signal (Fig. 1*A*). The transgene construct (≈8.3 kbp) was then injected into pronuclear-stage embryos of C57BL/6J mice, and the resulting progeny were screened for the presence of the transgene by Southern blot analysis. Seven founder mice positive for incorporation of the transgene (copy number per diploid genome ranging from 1 to 30) were obtained (Fig. 1*B* and data not shown). Two transgenic lines (501-3 and 502-4, with 10 and 30 copies of the transgene per genome, respectively) were independently maintained by backcrossing to C57BL/6J mice. To confirm the lung-specific expression of the transgene, we performed RT-PCR analysis to detect *EML4-ALK* mRNA in an $F_1$ mouse of the 502-4 line. The transgene was expressed in lung tissue (containing adenocarcinoma nodules, see below) but not in liver, esophagus, stomach, colon, brain, kidney, or uterus (Fig. 1*C*).

**Detection of Multiple Lung Adenocarcinoma Nodules in the Transgenic Mice.** One founder mouse (503-6, with 3 copies of the transgene per genome) (Fig. 1*B*) died 3 weeks after birth. Postmortem

Author contributions: Y.I., Y.S., and H.M. designed research; M.S., S.T., K.T., Y.L.C., M.E., T.U., H.H., T.H., Y.Y., and Y.I. performed research; and H.M. wrote the paper.

Conflict of interest statement: K.T. is a consultant for Dako.

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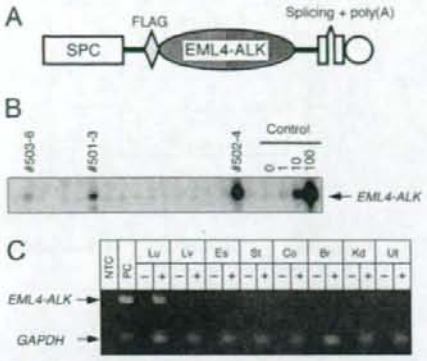


**Fig. 1.** Generation of transgenic mouse lines for *EML4-ALK*. (*A*) A cDNA for FLAG-tagged EML4-ALK was inserted between the *SPC* promoter and both splicing and polyadenylation [poly(A)] signal sequences. (*B*) Genomic DNA was isolated from the tail of founder mice generated from pronuclear-stage C57BL/6J embryos and was subjected to Southern blot analysis with full-length *EML4-ALK* cDNA as a probe. Control samples on the right comprised mouse genomic DNA with 0, 1, 10, or 100 copies of the transgene per diploid genome. The ID numbers of founder mice positive for the transgene are shown at the top. (*C*) Oligo(dT)-primed cDNA was synthesized from total RNA isolated from lung (Lu), liver (Lv), esophagus (Es), stomach (St), colon (Co), brain (Br), kidney (Kd), and uterus (Ut) of an $F_1$ mouse of the 502-4 line, with the reaction being performed in the presence (+) or absence (−) of reverse transcriptase. The cDNA preparations were then subjected to PCR with primer sets for *EML4-ALK* or for *GAPDH*, and the PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide. The positions of the PCR products are indicated on the left. RT-PCR was also performed for a no-template control (NTC) and for a human NSCLC specimen harboring *EML4-ALK* variant 1 as a positive control (PC).

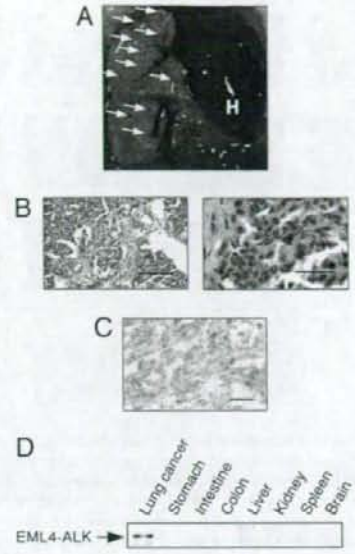


**Fig. 2.** Development of lung adenocarcinoma in *EML4-ALK* transgenic mice. (*A*) Hundreds of adenocarcinoma nodules (arrows) were apparent in the lungs of a founder mouse (503-6) that died 3 weeks after birth. H, heart. (*B*) Microscopic examination of the nodules shown in *A* after staining with H&E. Images at low (*Left*) and high (*Right*) magnification are shown with scale bars of 200 and 40 μm, respectively. Some tumors exhibited obvious scar formation, suggesting that they were invasive carcinomas. (*C*) Immunohistochemical analysis with antibodies to ALK of one of the nodules shown in *A* revealed a pattern of cytoplasmic staining with granular accentuations. (Scale bar, 50 μm.) (*D*) Immunoprecipitates prepared with antibodies to FLAG from the indicated tissues of an $F_1$ mouse of the 502-4 line were subjected to immunoblot analysis with the same antibodies. The position of EML4-ALK is shown at the left.

examination revealed hundreds of nodules in both lungs of this animal (Fig. 2*A*) and that these nodules were filled with adenocarcinoma cells (Fig. 2*B*). Immunohistochemical analysis with antibodies to ALK showed a diffuse cytoplasmic staining with granular accentuations in the neoplastic cells (Fig. 2*C*), consistent with the results of a similar analysis of *EML4-ALK*-positive human tumors (16). The level of immunoreactivity in the lungs of the transgenic mouse, however, was substantially lower than that in *EML4-ALK*-positive human specimens, suggestive of a lower level of expression for the EML4-ALK protein.

Detection of EML4-ALK by immunoblot analysis with antibodies to the FLAG tag confirmed a low-level but lung-specific expression of the kinase (Fig. 2*D*). Pathology and computed tomography (CT) examinations (see below) of the progeny of the maintained transgenic mouse lines (501-3 and 502-4) also revealed the development of multiple adenocarcinoma nodules in their lungs at only a few weeks after birth, demonstrating an essential role for the EML4-ALK kinase in NSCLC carcinogenesis. There was no discernable difference in tumor-forming activity between the 2 transgenic lines. We thus used both of these lines for further analyses.

**Treatment of NSCLC-Positive Transgenic Mice with an ALK-Specific Inhibitor.** To observe the development of NSCLC in the transgenic mice, we performed a series of CT scans of the chest. Multiple large nodules, some with infiltrative profiles of NSCLC, were detected in the lungs of progeny mice [Fig. 3*A*; also see supporting information (SI) Movie S1]. Other progeny with similar CT findings were subjected to pathology examination, confirming that such CT profiles reflected tumor expansion and infiltration in the lungs (data not shown). Examination of other organs of these mice failed to detect metastatic tumor nodules.

Several chemical compounds that specifically inhibit the tyrosine kinase activity of ALK have been identified (17–19). One such 2,4-pyrimidinediamine derivative has a median inhibitory concentration for ALK of <10 nM and a high specificity to ALK (Fig. S1) (20). We therefore examined whether peroral treatment with this compound (10 mg per kg of body weight per day) might inhibit the growth or induce the death of the adenocarcinoma cells in the transgenic mice. CT scans were performed after 0, 11, and 25 days of treatment for all 10 mice in each of the treatment and control (vehicle) groups, and a sequential examination of CT profiles was undertaken for each animal. The tumor mass developed rapidly in both lungs for most of the animals in the control group (Fig. 3*A*; also see Movie S2). Multiple nodules of various sizes newly appeared in the lungs, and the existing nodules became enlarged. In contrast, treatment with the ALK inhibitor greatly reduced the tumor burden in all mice (Fig. 3*B*). A large tumor in the lower lobe of the right lung in mouse 373, for instance, was reduced to ≈30% of its original size (based on the cross-section at the chest level in Fig. 3*B*) after only 11 days of the drug treatment and was almost undetectable by CT after treatment for 25 days (Movie S3). Sequential CT examination of another mouse (381) confirmed the pronounced activity of the ALK inhibitor (Fig. 3*B*; also see Movie S4 and Movie S5).

Mice in both groups were killed for pathology analysis after drug or vehicle administration for 2 months. Although multiple large tumor nodules were readily identified in the lungs of control mice, such nodules were apparent only occasionally in the treated animals (Fig. 3*C*), confirming the marked therapeutic effect of the ALK inhibitor. However, several small nodules were detected in the

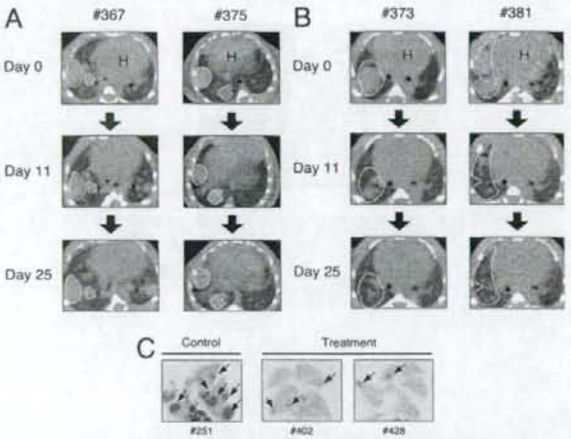


**Fig. 3.** Treatment of *EML4-ALK* transgenic mice with a specific ALK inhibitor. (*A* and *B*) Transgenic mice were subjected to daily peroral administration of vehicle (*A*) or ALK inhibitor (*B*) beginning at 4 weeks of age and were examined by CT scanning of the chest on days 0, 11, and 25. The ID numbers of the mice are shown at the top. H, heart. Tumors (indicated by broken lines) in both lungs underwent progressive enlargement in all control mice but became progressively smaller in all treated animals. (*C*) Macroscopic examination of the lungs from mice of the control and treatment groups at 2 months after the onset of treatment. The tissue was stained with H&E. The ID numbers of the mice are shown at the bottom. Cancer nodules are indicated by arrows. Thy, thymus.

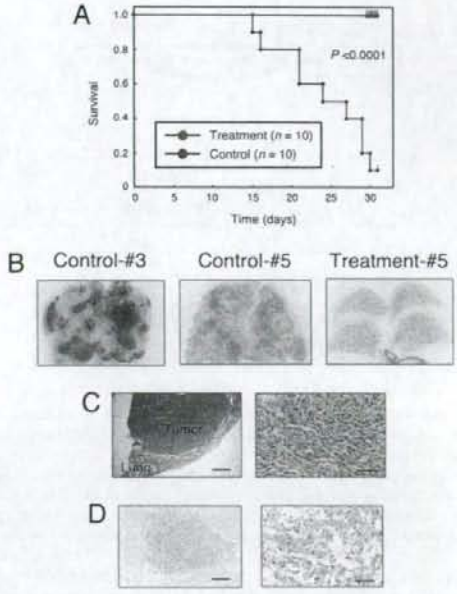


**Fig. 4.** Treatment with the ALK inhibitor of mice injected with EML4-ALK/3T3 cells. (*A*) Nude mice were injected i.v. with $2 \times 10^5$ 3T3 cells expressing EML4-ALK variant 1 and were then immediately subjected to daily peroral administration of vehicle (control, $n = 10$) or ALK inhibitor (treatment, $n = 10$). Survival of the 2 cohorts is shown as a Kaplan–Meier plot and was compared by the log-rank test, with the calculated $P$ value indicated. (*B*) Macroscopic examination of lungs isolated from mice of the control group at death or of the treatment group after treatment for 31 days. The tissue was stained with H&E. Most of the lungs in both control animals were occupied with transformed EML4-ALK/3T3 cells, whereas such cells were rarely observed in the treated animal. (*C*) Microscopic examination of lung tissue from a mouse of the control group after H&E staining. Images of low (*Left*) and high (*Right*) magnification are shown with scale bars of 500 and 50 μm, respectively. (*D*) Immunohistochemical analysis with antibodies to ALK of the nodules of EML4-ALK/3T3 cells that formed in the lungs of a mouse in the control group. Images of low (*Left*) and high (*Right*) magnification are shown with scale bars of 200 and 50 μm, respectively.

treated mice. Microscopic examination of the lungs of control and treated mice confirmed the changes observed by CT scanning and macroscopic analysis (data not shown). Even at this time point, we did not detect metastatic nodules in organs other than the lungs in either control or treated mice, and all animals in these cohorts survived the observation period.

**Treatment of Mice Injected with 3T3 Cells Expressing EML4-ALK.** Given that transgenic mice with lung cancer did not die by 6 months of age (with the exception of the one shown in Fig. 2*A* and another that died at 6 months after birth), we were not able to examine statistically the possible effect of the ALK inhibitor on survival in these animals. We therefore adopted another approach—that of loading mice with a large number of EML4-ALK-positive cells. We previously showed that mouse 3T3 fibroblasts expressing EML4-ALK (EML4-ALK/3T3) undergo transformation and generate s.c. tumors when injected into *nu/nu* mice (12). Such EML4-ALK/3T3 cells ($2 \times 10^5$) were therefore injected i.v. into *nu/nu* mice ($n = 20$), and the ALK inhibitor was administered to half of these animals.

A total of 9 of the 10 untreated mice died within 1 month of injection with the EML4-ALK/3T3 cells (Fig. 4*A*). Postmortem examination of these mice revealed extensive dissemination of EML4-ALK-positive cells into the lungs (>60% of lung tissue was occupied with the transformed EML4-ALK/3T3 cells in all mice) (Fig. 4*B*). Pathology examination of the lungs revealed many nodules of various sizes that were filled with the EML4-ALK/3T3 fibroblasts (Fig. 4*C*). In a separate experiment, we confirmed that injection of parental 3T3 cells did not induce the formation of such nodules in the lungs or affect the survival of mice (data not shown).

To verify that the injected EML4-ALK/3T3 cells continued to express EML4-ALK, we stained tissue sections of the lungs of control mice with antibodies to ALK. All cells within nodules reacted with the antibodies (Fig. 4*D*), giving rise to a diffuse pattern of cytoplasmic staining with granular accentuations. Although the staining profile was similar to that observed for the transgenic mice, the staining intensity in the EML4-ALK/3T3 cell-injected animals was greater than that in the transgenic animals.

Similar to the results obtained with transgenic mice, transformed EML4-ALK/3T3 cells were not detected in any organs other than the lungs of the injected mice, with the exception of 2 animals in the control group (nos. 3 and 7). Given the massive infiltration of EML4-ALK/3T3 cells in the lungs of all mice in the control cohort, these mice likely died from respiratory failure. In the control no. 7 mouse, we detected pronounced infiltration of EML4-ALK/3T3 cells into both the mediastinum (Fig. S2*A*) and the diaphragm (Fig. S2*B*). Given that both of these structures are adjacent to the lungs and that this mouse had an exceptionally high tumor burden in the lungs (>90% of the lungs were occupied with EML4-ALK/3T3 cells; Fig. S2*C*), the presence of EML4-ALK/3T3 cells in the mediastinum and diaphragm was likely the result of direct invasion from the lungs rather than of distant metastasis.

Peroral administration of the ALK inhibitor markedly improved the outcome of mice injected with the transformed EML4-ALK/3T3 cells, with all 10 animals in the treatment group surviving the 1-month observation period ($P < 0.0001$, log-rank test) (Fig. 4*A*). The treated mice also were subjected to pathology analysis after this period, revealing the absence of EML4-

ALK/3T3 nodules from the lungs (Fig. 4*B*) and again demonstrating the high efficacy of the ALK inhibitor.

## Discussion

We have shown here that the EML4-ALK fusion kinase plays an essential role in lung tumorigenesis. Hundreds of adenocarcinoma nodules developed simultaneously within a few weeks after birth in all independent lines of *EML4-ALK* transgenic mice examined. Given that the promoter fragment of *SPC* becomes active only at a late stage of gestation (21), a short period of *EML4-ALK* expression appears to be sufficient for full transformation. Although we did not examine *TP53* and *RB1* for possible abnormalities in the adenocarcinoma nodules of the transgenic mice, with both of these genes being frequently inactivated in human lung cancers (22), it is likely that only one (or at most a few) additional genetic event is required to generate cancer in EML4-ALK-expressing alveolar epithelial cells.

The expression level of EML4-ALK protein in the adenocarcinoma nodules of the transgenic mice was low. Given that the abundance of *EML4-ALK* mRNA in these nodules was found to be greater than that in human *EML4-ALK*-positive NSCLC specimens (data not shown), the expression of EML4-ALK protein appears to be suppressed in the mouse lung epithelial cells, possibly through translational or posttranslational mechanisms. The development of adenocarcinoma even at this low level of protein expression further reinforces the transforming activity of EML4-ALK.

Given the rapid development of NSCLC induced by EML4-ALK, the tumor cells are likely dependent for growth on the tyrosine kinase activity of the fusion protein. Such "oncogene addiction" (23) provides a potential target for the development of treatment strategies. We therefore tested whether inhibition of the enzymatic activity of EML4-ALK might reduce the tumor burden in the transgenic mice. The ALK inhibitor examined proved to be a promising candidate for the treatment of EML4-ALK-positive tumors. Furthermore, given the high sensitivity of the tumors in the transgenic mice to the ALK inhibitor, these animals provide a model system with which to examine the in vivo activity of other compounds or reagents targeted to ALK.

Many of the large tumors in the lungs of the transgenic mice changed to bullae or cysts after treatment with the ALK inhibitor, as revealed both by CT scanning (Fig. S3*A* and Movie S3 and Movie S5) and by pathology examination (Fig. S3*B*). Such a change was not described for treatment of activated EGFR-positive NSCLC in mouse models or humans with EGFR inhibitors (5, 6). A rapid induction of cell death by the ALK inhibitor in the transgenic mice may have triggered a collapse of the tumor burden within each nodule, thereby giving rise to bullae or cysts. Indeed, pathology examination revealed that a large tumor in 1 transgenic mouse (no. 250) became filled with necrotic tissue after treatment (Fig. S3*C*). However, the bullae cysts and necrotic tissue were still surrounded by remaining cancer cells (Fig. S3 *B* and *C*). Similarly, the lining tissue of some bullae cysts in the treated mice appeared to have a high density in CT scans (Fig. S3*A*), suggesting that peripheral cancer cells may survive in the nodules. Furthermore, small foci of cancer cells could be identified in the lungs of transgenic mice in the treatment cohort (Fig. 3*C*). Together, these various observations indicate that the current treatment protocol with the ALK inhibitor did not entirely eliminate tumor cells from the transgenic mice. Indeed, in a separate experiment transgenic mice treated with the 2,4-pyrimidinediamine for 25 days were examined 3 months after cessation of drug administration. Tumors of various sizes regrew in these mice (Fig. S4), indicative of the presence of surviving EML4-ALK-positive cancer cells in the animals after 25 days of drug treatment. Given that we have not tried other protocols or compounds, it remains unknown whether a total cure might be achieved by treatment for a longer period or with a higher dose of the same inhibitor or with a more potent compound. It is also possible that inhibition of additional signaling pathways, such as those mediated by phosphoinositide 3-kinase, mammalian target of rapamycin, or other protein tyrosine kinases (5, 24), may be required for a complete cure.

Despite the rapid growth of multiple tumors in the lungs of the transgenic mice, we failed to detect distant metastasis of such cancer cells in animals killed for analysis or in those that died within the total observation period of 6 months. However, we conclude that the tumors that developed in these mice had malignant characteristics on the basis of the following observations: (*i*) Histological analysis indicated that most tumors were noninvasive papillary adenocarcinomas, with some of them further showing obvious fibrosis and destruction of alveolar structures (Fig. 2*B*), a marker of invasion in human lung adenocarcinoma. (*ii*) Subcutaneous transplantation of tumor nodules that developed in the transgenic mice into the shoulder of *nu/nu* mice resulted in the growth of tumors at 6 of 8 injection sites in the recipient animals (Fig. S5*A*). (*iii*) Tumors that developed in the transgenic mice were shown to keep growing for at least 62 days in in vitro culture (Fig. S5*B*).

It is likely that expression of EML4-ALK (and probably other accompanying genetic changes) alone is not sufficient to render the cancer cells metastatic. It remains to be determined whether additional abnormalities in other oncogenes or tumor suppressor genes, such as *KRAS* or *LKB1* (25, 26), may lead to the generation of metastatic tumors in *EML4-ALK* transgenic mice.

Our present results have reinforced the importance of *EML4-ALK* in the pathogenesis of NSCLC in humans and have provided experimental support for the treatment of such intractable tumors with ALK inhibitors. Given that variants of *EML4-ALK* other than the variants 1 and 2 described in our original study (12) are now being identified (20, 27–29), it will be important to characterize all possible isoforms of *EML4-ALK* in humans to identify precisely the subgroup of patients who are candidates for future treatment with ALK inhibitors. Further to this goal, it will also be important to clarify the genetic changes that accompany the *EML4-ALK* fusion event as well as the downstream targets of EML4-ALK action in human NSCLC.

## Materials and Methods

**Generation of Transgenic Mice.** A cDNA fragment encoding FLAG epitope-tagged EML4-ALK variant 1 (12) was ligated to the *SPC* promoter as well as to splicing and polyadenylation signals (Fig. 1*A*). The expression cassette was injected into pronuclear-stage embryos of C57BL/6J mice (PhoenixBio), and the copy number of the transgene was examined by Southern blot analysis with DNA from the tail of founder animals. All animal procedures were performed with the approval of the scientific committee for animal experiments of Jichi Medical University.

For detection of mRNAs derived from *EML4-ALK* and the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*), total RNA was isolated from the organs of transgenic mice with the use of an RNeasy Mini kit (Qiagen) and was subjected to reverse transcription with SuperScript III reverse transcriptase (Invitrogen) and an oligo(dT) primer. Both reverse transcription and subsequent PCR analysis for each gene were performed as described previously (12).

For analysis of EML4-ALK protein in mice, organ homogenates were prepared with an Nonidet P-40 lysis buffer and subjected to immunoprecipitation with mouse monoclonal antibodies to FLAG (Millipore). The resulting precipitates were then subjected to immunoblot analysis with the same antibodies and a SuperSignal chemiluminescence kit (Pierce Biotechnology).

**Pathology Examination.** For immunohistochemical staining of EML4-ALK in EML4-ALK/3T3 cells, paraffin-embedded sections were depleted of paraffin with xylene, rehydrated with a graded series of ethanol solutions, and stained with mouse monoclonal antibodies to ALK (ALK1; Dako) at a dilution of 1:20 and with an EnVision+DAB system (Dako). The sections were subjected to heat-induced antigen retrieval with Target Retrieval Solution, pH 9.0 (Dako), before exposure to the antibodies. For detection of EML4-ALK in transgenic mice, cryostat sections were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 10 min, treated with Target Retrieval Solution, pH 9.0, and immunostained with the monoclonal antibodies to ALK and the EnVision+DAB system.