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PTK7 expression is associated with lymph node metastasis, ALK and EGFR mutations in lung adenocarcinomas

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Running title: PTK7 expression in lung adenocarcinomas
Abstract
Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer death worldwide. Lung adenocarcinoma is the main tumor type of NSCLC. Recent advances in the molecular characterization and personalized therapies have improved NSCLC patient prognosis. Previous studies showed that protein tyrosine kinase 7 (PTK7) plays an important role in human cancers. However, the role of PTK7 has not been investigated. PTK7 expression was assessed by immunohistochemistry in 95 patients with lung adenocarcinoma. Correlations of PTK7 expression levels with clinicopathological parameters, EGFR mutation and EML4-ALK fusion were examined. Positive PTK7 expression was detected in 47.4% of lung adenocarcinoma. PTK7 expression was associated with gender ($P = 0.024$), lymph node metastasis ($P < 0.001$), ALK mutation ($P = 0.050$), and EGFR mutations ($P = 0.014$). No significant association was found between PTK7 expression and age ($P = 0.831$), differentiation ($P = 0.494$), adenocarcinoma subtype ($P = 0.098$) and Ki67 ($P = 0.473$). Our data suggest that PTK7 plays an oncogenic role in lung adenocarcinoma and may be a molecular marker for lymph node metastasis.

Keywords: PTK7, lung adenocarcinoma, metastasis, EML4-ALK, EGFR mutation
1. Introduction

Lung cancer is the most lethal solid tumor malignancy worldwide. In the United States, an estimated 228,150 estimated new cases of lung cancer and estimated 142,670 deaths are predicted for 2019 (Siegel et al., 2019). Lung cancer is classified into two types for the purposes of therapy: small cell lung cancer and non-small cell lung cancer (NSCLC). Patients with NSCLC at stage I and II are treated surgically and receive chemotherapy and/or radiation therapy, while most patients with stage III and IV NSCLC receive chemotherapy with or without radiation (Miller et al., 2016). Lung adenocarcinoma, the most frequent histological type of NSCLC, is often triggered by an aberration in a driver oncogene in tumor cells, such as epidermal growth factor receptor (EGFR) gene mutation or microtubule-associated protein-like 4 (EML4)-anaplastic lymphoma kinase (ALK) gene fusion. Recently, the identification of these tumor-specific genomic abnormalities has enabled dramatic improvements in the development of targeted therapies for NSCLC. Targeted therapy drugs, such as EGFR inhibitors, ALK inhibitors, and angiogenesis inhibitors, play important roles in the treatment of NSCLC. EGFR inhibitors (EGFR tyrosine kinase inhibitors) have shown a favorable treatment outcome in NSCLC patients harboring EGFR mutations. NSCLC patients harboring EGFR mutations treated with these EGFR inhibitors can show a response rate as high as 80% and approximately 10–14 months of progression-free survival (Wu and Shih, 2018).

Protein tyrosine kinases (PTKs) are a class of receptors associated with the plasma membrane that transduce extracellular signals across the membrane through exhibiting tyrosine-specific protein kinase activity. PTKs are involved in the regulation of cell proliferation, mitogenesis, motility, differentiation, survival, metabolism, adhesion, fasciculation, and morphogenesis. Several studies showed that PTKs also play a key role in oncogenesis. Protein tyrosine kinase 7 (PTK7) was originally identified in normal melanocyte mRNAs and later cloned from colon cancer tissue (Jung et al., 2004). PTK7 consists of immunoglobulin-like extracellular domains, a transmembrane region, and a C-terminal catalytically inactive domain with homology to the tyrosine kinase family. PTK7 is classified as a pseudokinase, and no specific
ligand for PTK7 has been found. PTK7 expression has been investigated in several types of human cancers, including breast cancer (Ataseven et al., 2013, Ataseven et al., 2014), gastric cancer (Lin et al., 2012), prostate cancer (Zhang et al., 2014), and colorectal cancer (Lhoumeau et al., 2015, Tian et al., 2016). These studies indicate that PTK7 plays a vital role in cancers. However, no study has examined PTK7 expression and its clinical significance in NSCLC. In this study, we examined PTK7 expression and analyzed clinicopathological features in lung adenocarcinoma.

2. Materials and methods

2.1 Patients and samples

A total of 95 consecutive patients were included in this study. Patients were diagnosed with lung adenocarcinoma (including two patients with adenosquamous carcinoma) at the Department of Pathology, Taixing Hospital between January 2017 and December 2018. Clinicopathologic characteristics were obtained from medical records. Histological classification was based on the World Health Organization Classification of Tumors, the Pathology and Genetics of Tumors of the Lung, Pleura, Thymus and Heart (2014 version). The study protocol was approved by the Taixing Hospital Clinical Research Ethics Committee.

2.2 Immunohistochemistry

Four-µm sections were cut from formalin-fixed paraffin-embedded (FFPE) archived lung adenocarcinoma tissue blocks. Immunohistochemical staining of PTK7 and Ki67 was performed according to a standard method. The tissue sections were deparaffinized and rehydrated. Antigen retrieval was done by autoclave in 10 mM citrate buffer (pH 6.0) at 120°C for 2 min. After endogenous peroxidase was quenched with aqueous 3% H2O2 for 10 min, the sections were washed with PBS and then incubated at 4°C overnight with primary rabbit polyclonal anti-PTK7 antibody (Abgent, San Diego, CA, USA) at a dilution of 1:100 and anti-Ki67 (Abcam, Cambridge, UK) at a dilution of 1:200 in antibody diluent solution. The sections were then
incubated with secondary antibody (Dako Real Envision Detection System, Dako, UK) for 30 min at room temperature. Color development was performed with 3, 3’-diaminobenzidine. Nuclei were counterstained with hematoxylin. Two pathologists independently assessed the immunostained slides. Any difference in immunohistochemical scores was resolved by a consensus.

IHC scoring and quantification of PTK7. Cytoplasmic staining was considered positive staining. The scoring for percentage of immunoreactive tumor cells was as follows: 0 (0%), 1 (< 20%), 2 (20-50%), and 3 (> 50%). The staining intensity was scored and stratified as follows: 0 (negative), 1 (weak), 2 (moderate), and 3 (strong). A final immunoreactivity score (IRS) was obtained for each of the cases by multiplying the percentage score and the intensity score. Protein expression levels were further analyzed by classifying IRS values as negative (IRS value less than 4) or positive (IRS value equal to or greater than 4).

Immunohistochemical staining of ALK was carried out by Ventana Medical Systems, Inc. and Roche Diagnostics International, Inc. The assay uses the OptiView DAB IHC Detection Kit and OptiView Amplification Kit and is run on the BenchMark ULTRA platforms. Positive cases stained with the VENTANA anti-ALK (D5F3) IHC assay display a strong, granular cytoplasmic signal with any percentage of positive tumor cells.

2.3 Fluorescence in situ hybridization (FISH)

Lung adenocarcinoma samples positive for ALK IHC were subjected to FISH. Interphase FISH was performed for ALK rearrangement with the commercial ALK dual color, break-apart rearrangement probe (GP Medical Technologies, Beijing, China). The 4 μm FFPE tissue sections were deparaffinized using xylene, dehydrated by gradient ethanol, and rehydrated with deionized water. The prepared slides were washed with 2x SSC, treated with 0.1 mol/L HCl, and digested with pepsin (P700; Sigma-Aldrich, St. Louis, MO, USA). The slides and probe mixture were denatured separately. The denatured probe mixture was pipetted onto slides, and hybridization was performed in a wet box at 37°C overnight. After slides were washed, the air-dried slides were restained with 4’, 6-diamindino-2-phenylindole and then
analyzed. Samples were classified as positive for ALK rearrangement when 15% or more of nuclei showed split signals (i.e., red and green signals were separated by ≥ 2 signal diameters) or single red signals (3’ ALK) were observed.

2.4 EGFR mutation analysis
DNA was extracted from FFPE lung adenocarcinoma samples using an FFPE DNA kit (Qiagen, Germany) according to the standard protocol. EGFR mutations were assessed with the amplification refractory mutation system (ARMS) (Amoy Diagnostics, Xiamen, China) according to the manufacturer’s instructions. This kit was used to identify the most common hotspots in EGFR exons 18–21.

2.5 Statistical analysis
The chi-squared test (Fisher’s exact test) was used to analyze the correlations between PTK7 expression and clinicopathological parameters. P-values < 0.05 (two-sided) were considered statistically significant. All analyses were performed by SPSS software (version 16.0; SPSS, Inc., Chicago, IL).

3. Results
3.1 IHC analysis of PTK7 expression in lung adenocarcinoma
We evaluated the expression of PTK7 in 95 lung adenocarcinoma tissue specimens using immunohistochemistry. While negative PTK7 expression was observed in normal alveolar epithelium cells and bronchial cells (Fig. 1A, B), we detected differentially expressed PTK7 in adenocarcinoma cells (Fig. 1C and D). PTK7 staining was localized in the cytoplasm of lung adenocarcinoma cells. In total, 45 out of the 95 (47.4%) lung adenocarcinoma tissue samples showed positive PTK7 expression (Table 1).

3.2 IHC analysis of Ki67 expression in lung adenocarcinoma
We also examined the expression of the Ki67 proliferation index protein using immunohistochemistry (Fig. 2). Ki67 nuclear labeling indices of < 10% and ≥ 10%
were classified as low and high levels (Woo et al., 2009).

### 3.3 IHC and FISH analysis of ALK in lung adenocarcinoma

We also examined the expression of ALK protein in lung adenocarcinoma tissues using immunohistochemistry (Fig. 3A and B). A total of 7 out of 95 (7.4%) lung adenocarcinoma tissue samples were positively stained for ALK. Positive expression of ALK protein in the 7 cases was further confirmed by ALK FISH (Fig. 3C and D). A break-apart signal pattern, one fusion signal and a single red and green signal pattern, was observed in most nuclei in the seven cases.

### 3.4 EGFR mutation in lung adenocarcinoma

We next evaluated EGFR gene mutations in exon 18, 19, 20, and 21 in the lung adenocarcinomas using ARMS. The results identified 47 mutations in the 95 lung adenocarcinomas (49.5%), including 25 cases with exon 19 deletion, 20 mutations of L858R in exon 21, one case with G719A in exon 18, and one case with an insertion in exon 20.

### 3.5 Relationship between PTK7 expression and clinicopathological parameters

The relationship between PTK7 expression and clinicopathological parameters was statistically analyzed. As shown in Table 1, PTK7 expression was associated with sex ($P = 0.024$), lymph node metastasis ($P < 0.001$), ALK mutation ($P = 0.050$), and EGFR mutation ($P = 0.014$). No significant association was found between PTK7 expression and age ($P = 0.831$), differentiation ($P = 0.494$), and Ki67 ($P = 0.473$).

### 4. Discussion

PTKs are oncoproteins that have been implicated in many human cancers. Despite being a pseudokinase, PTK7 expression has been detected in several types of cancer and PTK7 has been shown to function as an oncoprotein in some cancers. Ataseven et al analyzed PTK7 expression in triple-negative breast cancer using immunohistochemistry and identified positive PTK7 expression in approximately 28%
of triple-negative breast cancers (Ataseven et al., 2013). The authors also found that higher levels of PTK7 occurred more frequently in smaller tumors; no other significant associations were described. Lhoumeau et al examined PTK7 protein in a clinically annotated tissue microarray produced from 192 consecutive colorectal cancer patients using immunohistochemistry (Lhoumeau et al., 2015). PTK7 was significantly upregulated in tumoral tissue compared with matched normal mucosae. Overexpression of PTK7 was found in 34% of patients and was associated with a reduced metastasis-free survival in non-metastasis patients. Downregulation of PTK7 by shRNA in HCT116 and HCT15 cells reduced cell migration. Downregulation of PTK7 in a xenograft mouse generated with HCT15 cells also led to reduced tumor growth, while overexpression of PTK7 in PTK7-negative colon cancer cells led to increased metastatic events. PTK7 is also highly expressed in CD44-positive glioblastoma and predicts unfavorable prognosis (Liu et al., 2015). Another study identified DNA Binding 1 (Id1) protein as a potential downstream effector for PTK7. Overexpression of Id1 mostly restored cell proliferation and colony formation in human glioma cell lines silenced for PTK7 by shRNA. These data demonstrate that PTK7 is a versatile coreceptor for cancer-related signaling and support a role for PTK7 as a molecular switch between signaling pathways (Liu et al., 2015).

In this study, we detected PTK7 protein expression in a set of lung adenocarcinoma tissues by immunohistochemistry. Our results revealed positive PTK7 expression in 47.4% lung adenocarcinoma tissue samples. High expression of PTK7 was more often found in female patients and tumors with lymph node metastasis. No relationship was found between PTK7 expression and age, differentiation of tumor, proliferation index Ki67, and lung adenocarcinoma subtype. To the best of our knowledge, only two groups thus far have examined PTK7 in lung cancers (Chen et al., 2014, Kim et al., 2014). However, the results of these studies were contradictory. Chen et al conducted a meta-analysis of lung adenocarcinoma gene expression and identified high expression of PTK7 in lung adenocarcinoma (Chen et al., 2014). The authors further confirmed high expression of PTK7 in lung adenocarcinoma in a tissue microarray using immunohistochemistry. No correlation between PTK7 expression
and clinicopathological parameters was found due to the limited sample size. The authors also demonstrated that shRNA-mediated silencing PTK7 in a panel of lung cancer cell lines decreased cell viability and induced apoptosis. Furthermore, disrupted PTK7 expression in lung cancer cells activated MKK7-JNK signaling (Mapk kinase kinase-7, c-Jun N-terminal kinase/stress-activated protein kinase, SAPK). Loss of PTK7 in some lung adenocarcinoma cell lines led to dysregulation of MKK7-JNK and possibly other signaling pathways, ultimately leading to decreased proliferation and increased apoptosis. These data suggest that PTK7 plays a role as an oncoprotein in lung adenocarcinoma. In contrast, Park et al reported that PTK7 plays a tumor suppressor role in lung squamous cell carcinoma (Kim et al., 2014). The authors found that PTK7 expression was downregulated in lung squamous cell carcinoma tissues and cell lines. Overexpression of PTK7 in lung squamous cell carcinoma cell lines resulted in inhibition of cell proliferation, invasion and migration. We previously reported that PTK7 may be a tumor suppressor in ovarian serous carcinoma (Wang et al., 2014). We found that PTK7 was positively expressed in most normal fallopian tube epithelium (92%) and parts of epithelial ovarian tumors (45%). PTK7 expression was significantly decreased from the benign, the intermediate type, to malignant ovarian epithelial tumor. Survival analysis showed that patients with negative expression of PTK7 had a poorer outcome than patients with positive PTK7 expression. We also previously reported increased PTK7 expression in oral tongue squamous cell carcinoma relative to normal squamous cells (Dong et al., 2017). Patients with high PTK7 expression had a poor overall survival. Together these data suggest that PTK7 functions as an oncoprotein or a tumor suppressor in a tumor cell type-specific manner.

The PTK7 gene is located on chromosomal locus 6p21, which is within the region of frequent copy number gain in an independent lung cancer dataset (Weir et al., 2007). The ALK and EML4 fusion represents a novel target in a subset of NSCLC, especially adenocarcinoma. Approximately 3% to 5% of lung adenocarcinoma patients carry the EML4-ALK fusion (Wong et al., 2009, Wu et al., 2012, Wang et al., 2018). EML4-ALK fusion is characterized with acinar and solid subtypes with mucin secretion (Wang et
Our results showed that high PTK7 expression frequently occurred in tumors with EML4-ALK rearrangement, but not in those with EGFR mutation. Our data showed no relation between PTK7 expression and subtypes of lung adenocarcinoma. Our previous studies and other research results suggest that PTK7 protein is a prognostic marker in some types of cancer (Lin et al., 2012, Chen et al., 2014, Tian et al., 2016, Dong et al., 2017). However, we did not analyze the relationship between PTK7 expression and outcomes of lung adenocarcinoma patients in this study due to a short period time after the operation.

In summary, PTK7 is highly expressed in parts of lung adenocarcinoma. High expression of PTK7 in lung adenocarcinoma is associated with lymph node metastasis and EML4-ALK gene fusion. Our data suggest that PTK7 may function as an oncoprotein in lung adenocarcinoma and may serve as a molecular marker for lymph node metastasis.

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**Figure legends**

Figure 1. PTK7 protein was detected in lung adenocarcinoma tissues using IHC. A: Negative expression of PTK7 in normal alveolar epithelium cells. B: Negative expression of PTK7 in bronchial cells. C: Negative expression of PTK7 in lung adenocarcinoma cells. D: Positive PTK7 expression in lung adenocarcinoma cells. Magnification, 400×.

Figure 2. Positive expression of Ki67 in lung adenocarcinoma. Magnification, 400×.

Figure 3. Detection of EML4-ALK fusion in lung adenocarcinoma tissues.

Representative example of negative EML4-ALK in lung adenocarcinoma cells as shown by immunohistochemistry (A) and confirmed by FISH (C). Representative example of EML4-ALK positive in lung adenocarcinoma cells as detected by immunohistochemistry (B) and confirmed by FISH (D). A and B, magnification 400×; C and D, magnification 1000×.
Table 1 The relationship between PTK7 expression and clinicopathological parameters in lung carcinoma

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Chi-square test