

Expression of protein tyrosine kinase 6 (PTK6) in nonsmall cell lung cancer and their clinical and prognostic significance

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Aim: The aim of the study was to validate the expression of protein tyrosine kinase 6 (PTK6) in nonsmall cell lung cancer (NSCLC), and to evaluate its clinicopathological and prognostic significance.

Methods: We first conducted a meta-analysis on the mRNA profiling data sets of NSCLC in the Oncomine database. Then, one of the most significantly upregulated tyrosine kinase targets, PTK6, was further validated by immunohistochemistry in 104 primary NSCLC tumors. Furthermore the association between PTK6 expression, the clinical parameters, and overall survival was further analyzed.

Results: Using the Oncomine database, we identified a list of tyrosine kinase genes related to NSCLC, among which PTK6 was the second most overexpressed gene (median rank = 915, $P = 2.9 \times 10^{-5}$). We further confirmed that NSCLC tumors had a higher expression level of PTK6 than normal pulmonary tissues. Moreover, high PTK6 expression correlated positively with shorter overall survival time, but not with other clinicopathological characteristics. In the multivariate Cox regression model, high PTK6 expression was demonstrated to be an independent prognostic factor for NSCLC patients.

Conclusion: Our results validated that PTK6 was found to be overexpressed in a proportion of NSCLC samples, and was associated with a poor prognosis, suggesting that this subgroup of NSCLC patients might benefit from PTK6 inhibitors in the future.

Keywords: nonsmall cell lung cancer, tyrosine kinase, PTK6, target, prognosis

Introduction

Lung cancer ranks among the leading causes of cancer death globally. Mortality related to this malignant disease has risen by 465% during the last 30 years in People's Republic of China.¹ Nonsmall cell lung carcinoma (NSCLC) accounts for approximately 85% of all lung cancer cases. Even among those who have received standard treatments including surgical resection, traditional chemotherapy, radiation therapy, and molecular targeted therapy, a significant proportion of NSCLC patients eventually develop resistance to chemotherapy and recurrence. Therefore, there is an urgent need to discover novel cancer-specific molecular targets and signaling pathways to develop new therapeutic strategies against this devastating malignancy.

Tyrosine kinases, being capable of phosphorylating specific target protein substrates, are involved in the modulation of multiple growth factor signaling during cancer progression.² In recent years, a list of tyrosine kinase inhibitors has been approved, while others are in clinical trials for NSCLC patients.^{3,4} Therefore, identifying new

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tyrosine kinases related to NSCLC would be of important significance in developing novel treatment strategies for this cancer.

In this study, we first performed a meta-analysis on the gene expression profiling data sets of NSCLC specimens from the Oncomine database (Compendia Bioscience, Inc, Ann Arbor, MI, USA), and identified a tyrosine kinase, protein tyrosine kinase 6 (PTK6), was specifically upregulated in NSCLC when compared to normal lung tissues, which might be a novel biomarker and potential therapeutic target for NSCLC. We further validated its expression in NSCLC samples and evaluated its clinicopathological and prognostic significance.

Materials and methods

Data mining

The Oncomine cancer microarray database was used to analyze the mRNA expression profiles of NSCLC tissues relative to their normal controls. Eleven publicly available datasets of gene expression profiles of were chosen in this study. The data mining strategy is based on a published methodology established by Oncomine.⁵ First, significantly upregulated genes in cancer samples compared with the normal tissue counterpart (greater than twofold, $P < 0.05$) were selected. Then, concept filters in the Oncomine database were used to identify known tyrosine kinases differentially expressed in NSCLC.

Samples

Archived formalin-fixed paraffin-embedded NSCLC samples with corresponding adjacent nontumor tissues undergoing surgical resection were obtained from the 89th Hospital, Changhai Hospital, and from the Changzheng Hospital of People's Republic of China between January 2006 and December 2008. General schemes for treatment choice in the sampled NSCLC patients were based on the National Comprehensive Cancer Network NSCLC Clinical Practice Guidelines (Chinese version). Overall survival was calculated from the date of surgery until the date of death or last follow-up. None of the patients had received neoadjuvant chemotherapy, radiation therapy, or immunotherapy. Ethics approval to start this study was obtained from the local Human Research Ethics Committees.

Immunohistochemistry

The immunostaining was performed according to standard SP methods according to the manufacturer's protocol (Zymed®, Life Technologies, Carlsbad, CA, USA).

Antigen retrieval was done by a combination of heat and pressure in sodium citrate buffer. Immunostaining was detected with a rabbit polyclonal antibody against PTK6 (Santa Cruz Biotechnology, Inc, Dallas, TX, USA). Detection was performed using diaminobenzidine as the chromogen. Nonspecific rabbit immunoglobulin G was used as negative control.

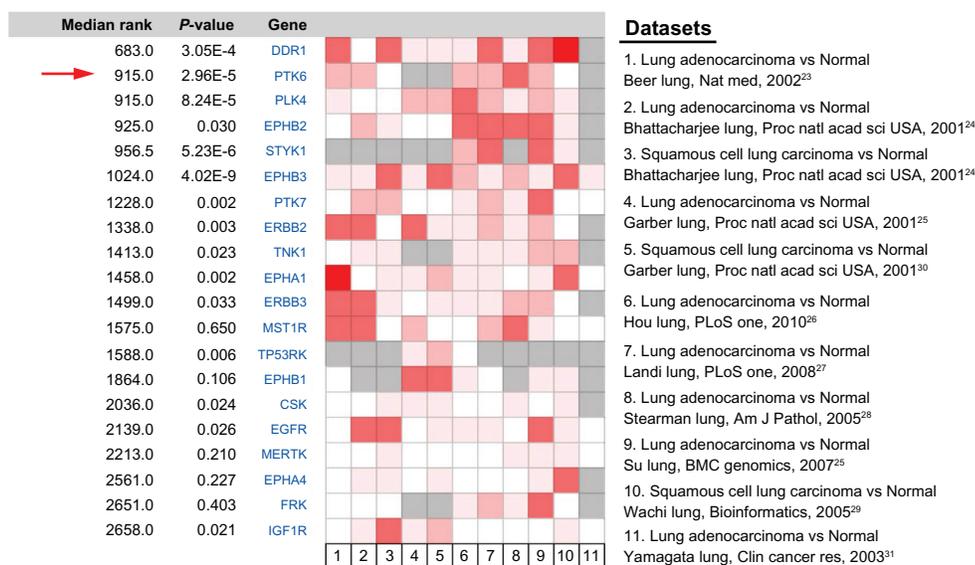
The immunostaining levels of PTK6 protein expression were assessed using a semiquantitative staining index method, as described previously with minor modifications.⁶ The staining intensity results were graded to a four-scale (0 = no staining; 1 = staining obvious only at $\times 400$; 2 = staining obvious at $\times 100$ but not $\times 40$; and 3 = staining obvious at $\times 40$), while the percentages of positive cells were scored into four scales (0 = 0% positive cells; 1 = 1%–33% positive cells; 2 = 34%–66% positive cells; and 3 = 67%–100% positive cells). A staining index ranging from 0 to 9 was generated by multiplying staining intensity and the percentage of positive cells for each sample. The median staining index value was set as a cut-point to delineate PTK6-low and PTK6-high subgroups.

Statistics

The difference in clinicopathological characteristics between PTK6-low and PTK6-high subgroups was compared using the Chi-square test. Overall survival curves were estimated using the Kaplan–Meier method and evaluated using the Log-Rank test. Multivariate analysis on clinicopathological variables and PTK6 expression levels was performed using the Cox proportional hazard regression model. Statistical analyses were performed using the Statistical Package for the Social Sciences 16.0 (SPSS; IBM Corporation, Armonk, NY, USA), and a P -value less than 0.05 was considered statistically significant.

Results

We first queried the Oncomine database to systematically compare the relative gene expression levels between NSCLC and normal pulmonary samples, then upregulating genes belonging to tyrosine kinase targets were further filtered out using a “tyrosine protein kinase, active site – InterPro Protein Domains and Families” concept. The top 20 upregulated tyrosine kinase genes in NSCLC are listed in Figure 1. In the meta-analysis, discoidin domain receptor 1 (DDR1) was the highest ranked gene (median rank = 683) among all of the tyrosine kinase genes with a P -value of 3.05×10^{-4} . Next on the list, with a median rank of 915 and a P -value of 2.96×10^{-5} , was PTK6. Our bioinformatics analysis strongly suggested that



Datasets

1. Lung adenocarcinoma vs Normal Beer lung, Nat med, 2002²³
2. Lung adenocarcinoma vs Normal Bhattacharjee lung, Proc natl acad sci USA, 2001²⁴
3. Squamous cell lung carcinoma vs Normal Bhattacharjee lung, Proc natl acad sci USA, 2001²⁴
4. Lung adenocarcinoma vs Normal Garber lung, Proc natl acad sci USA, 2001²⁵
5. Squamous cell lung carcinoma vs Normal Garber lung, Proc natl acad sci USA, 2001³⁰
6. Lung adenocarcinoma vs Normal Hou lung, PLoS one, 2010²⁶
7. Lung adenocarcinoma vs Normal Landi lung, PLoS one, 2008²⁷
8. Lung adenocarcinoma vs Normal Stearman lung, Am J Pathol, 2005²⁸
9. Lung adenocarcinoma vs Normal Su lung, BMC genomics, 2007²⁵
10. Squamous cell lung carcinoma vs Normal Wachi lung, Bioinformatics, 2005²⁹
11. Lung adenocarcinoma vs Normal Yamagata lung, Clin cancer res, 2003³¹

Figure 1 OncoPrint heat map of upregulated genes in clinical NSCLC samples compared with the normal pulmonary tissues filtered by the “tyrosine protein kinase, active site – InterPro Protein Domains and Families” concept.

Note: PTK6 ranked as the second gene among all of the tyrosine kinase genes with a median rank of 915 and a P-value of 2.96×10^{-5} .

Abbreviations: NSCLC, nonsmall cell lung cancer; PTK6, protein tyrosine kinase 6; EGFR, epidermal growth factor receptor.

PTK6 genes were closely associated with NSCLC, and might be a potential tyrosine kinase target for NSCLC.

We further applied a validation study of PTK6 expression in a panel of clinical samples. As for the staining pattern, the positive staining reaction for PTK6 in normal and cancerous lung tissue cells was observed in the cytoplasm, and in a combination of the nucleus and cytoplasm. Compared with normal lung tissues, NSCLC tissues had a significantly higher staining index of PTK6. For NSCLC tissues, the median staining index of 6 was set as the cut-point to delineate PTK6-low and PTK6-high subgroups. Representative immunostaining images were seen in Figure 2. The associations between PTK6 expression and clinicopathological features of NSCLC are shown in Table 1. No significant association was observed between PTK6 expression with the clinicopathological traits observed in this study. Furthermore, we also found no significant correlation between PTK6 expression with another tyrosine kinase target epidermal growth factor receptor (EGFR) mutation status.

Survival curves were plotted using the Kaplan–Meier method as seen in Figure 3. The results indicated that the patients with a high PTK6 expression had a significantly shorter survival time, compared with those with low expression. High expression of PTK6 protein showed a statistically significant association with survival in univariate analysis (hazard ratio = 2.031, 95% confidence interval = 1.099–3.755, $P = 0.024$ by Log-Rank test). All the NSCLC clinical and pathological factors together with PTK6 expression status were included in a multivariate Cox regression model (Table 2). Our data demonstrated that

high PTK6 expression retained a significant and independent prognostic factor for NSCLC patients (hazard ratio = 2.219, 95% confidence interval = 1.156–4.263, $P = 0.017$ by Log-Rank test).

Discussion

By meta-analysis on the OncoPrint database, we screened and identified a list of upregulated tyrosine kinase genes in NSCLC, including some already confirmed therapeutic

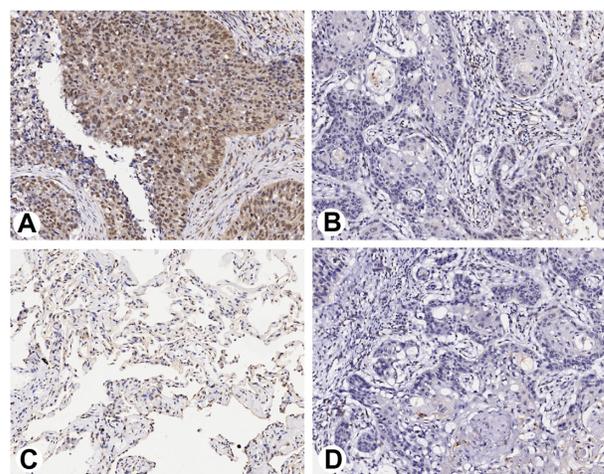


Figure 2 Representative images of PTK6 immunostaining in this study. (A) High level of PTK6 in NSCLC tumors; (B) low level of PTK6 expression in NSCLC tumors; (C) PTK6 expression in adjacent normal lung tissues; (D) negative control for immunostaining (IgG).

Abbreviations: PTK6, protein tyrosine kinase 6; NSCLC, nonsmall cell lung cancer; IgG, immunoglobulin G.

Table 1 Association between PTK6 expression and clinicopathological characteristics of the patients with NSCLC

	N	PTK6		P-value
		Low expression (n = 53)	High expression (n = 51)	
Age (years)				
<60	31	19	12	0.734
≥60	73	34	39	
Gender				
Male	79	41	38	0.170
Female	25	12	13	
Smoking status				
Nonsmoker	32	17	15	0.769
Smoker	72	36	36	
Pathological type				
Squamous cell carcinoma	51	30	21	0.116
Adenocarcinoma	53	23	30	
Tumor size				
T1–T2	89	47	42	0.359
T3	15	6	9	
Lymph node status				
Negative	56	28	28	0.832
Positive	48	25	23	
Tumor grade				
G1–G2	65	33	32	0.96
G3	39	20	19	
TNM stage				
1–2	72	36	36	0.969
3–4	32	17	15	
EGFR mutation				
Absent	85	44	41	0.729
Present	19	9	10	

Abbreviations: PTK6, protein tyrosine kinase 6; NSCLC, nonsmall cell lung cancer; N, number; EGFR, epidermal growth factor receptor.

targets such as EGFR. The first ranking tyrosine kinase gene DDR1 has been demonstrated to contribute to the progression and poor prognosis of NSCLC in previous studies, and could serve as a potential therapeutic target.^{7,8} We further selected one of the top genes, PTK6, for further verification. This tyrosine kinase was chosen for the following reasons: firstly, in the gene expression microarray meta-analysis, PTK6 was the second ranked gene among all of the tyrosine kinase genes, and was shown to be significantly elevated in NSCLC tumors; secondly, the expression of PTK6 in NSCLC has not been widely investigated, and specifically its prognostic value has not been reported; thirdly, commercial antibodies for PTK6 immunohistochemistry are available.

We further evaluated the clinicopathological and prognostic significance of PTK6 expression in NSCLC tumors. Our immunostaining results validated that high expression of PTK6 was detectable in NSCLC tumor samples and was significantly correlated with decreased overall survival.

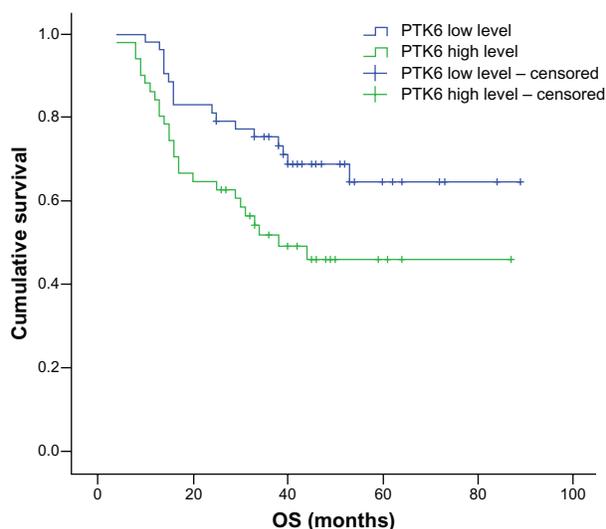


Figure 3 Kaplan–Meier survival curves based on expression of PTK6. **Note:** $P = 0.024$, Log-Rank test.

Abbreviations: PTK6, protein tyrosine kinase 6; Cum, cumulative; OS, overall survival.

Through multivariate analysis, high expression of PTK6 was shown to be an independent prognostic biomarker for poor overall survival. As far as we know, this is the first report of the prognostic significance of PTK6 expression in clinical NSCLC tissue samples. However, different from the previous study,⁹ no significant association with tumor size, lymph node metastasis status, and TNM stage were found in this study. We think the difference between data from the current study and previous report might be a result of the different evaluation criteria of the immunohistochemistry staining adopted. Our findings suggest that high-PTK6 expressed NSCLC tumors could represent a distinct subgroup with more aggressive phenotypes and a poor prognosis, which might benefit from more intensive and targeted treatments including PTK-specific inhibitors.

PTK6 (also known as breast tumor kinase) is a nonreceptor tyrosine kinase, which has been originally cloned from human melanocytes and a metastatic breast tumor sample.^{10,11} High expression of PTK has been found in several cancer types, including breast cancer, melanoma, and colon cancer.^{12,13} Accumulating evidence indicated that PTK6 played an important regulatory role as a proto-oncogene in malignant settings, and was involved directly in the processes of proliferation, migration, and invasion in cancer cells.¹⁴ Harvey et al¹⁵ found that the proliferation of breast carcinoma cells was efficiently suppressed by specifically downregulated PTK6 protein levels using RNA interference. PTK6 has been demonstrated to interact with the ErbB family members EGFR, ErbR2, ErbR3, ErbR4, and enhanced

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Table 2 Univariate and multivariate analysis for prognosis

Variable	Univariate survival analyses		Multivariate survival analyses	
	HR for death (95% CI)	P-value	Adjusted HR for death (95% CI)	P-value
Age	1.411 (0.711–2.801)	0.325	1.238 (0.594–2.580)	0.570
Gender	0.882 (0.434–1.790)	0.728	0.645 (0.208–2.001)	0.448
Smoking status	0.959 (0.506–1.817)	0.897	0.436 (0.163–1.165)	0.098
Pathological type	1.267 (0.696–2.308)	0.439	1.468 (0.691–3.121)	0.318
Tumor size	1.490 (0.690–3.218)	0.310	0.582 (0.224–1.512)	0.266
Lymph node status	3.092 (1.617–5.911)	0.001	3.060 (1.284–7.290)	0.012
Tumor grade	1.135 (0.619–2.081)	0.683	1.207 (0.629–2.313)	0.572
TNM stage	2.324 (1.269–4.255)	0.006	1.151 (0.465–2.848)	0.761
EGFR mutation	3.392 (1.760–6.536)	<0.001	3.172 (1.548–6.501)	0.002
PTK6 expression	2.031 (1.099–3.755)	0.024	2.219 (1.156–4.263)	0.017

Abbreviations: HR, hazard ratios; CI, confidence intervals; EGFR, epidermal growth factor receptor; PTK6, protein tyrosine kinase 6.

EGF-induced proliferation and ERK1/2 activation.^{16–18} It has also been reported that PTK6 could also activate the PI3k/Akt and STAT pathway.¹⁷ Moreover, potential PTK substrates also include a variety of several signaling molecules such as p190RhoGAP, paxillin, Akt, and KAP3A in most human cancer cells.^{19–21} Therefore, considering the overexpression of PTK6 in NSCLC, and its positive association with a poor prognosis, we proposed that specific inhibition of PTK6 kinase activity may provide a potentially novel approach to treat patients with NSCLC in the future. In particular, recently, Zeng et al²² discovered imidazo [1,2-a] pyrazin-8-amines as potent PTK6 kinase inhibitors. The utility of these small molecular PTK6 inhibitors in NSCLC treatment deserves further investigation.

In conclusion, in this study, by meta-analysis on the gene microarray data of NSCLC, we screened and validated a novel tyrosine kinase, PTK6, overexpressed in NSCLC tumors, which was correlated positively to shorter survival. Our findings are, however, preliminary and require other validations on a larger and independent group of NSCLC patients. We also suggest that the exact mechanisms of PTK6 during the malignant progression of NSCLC and its relationship with other confirmed NSCLC tyrosine kinase targets such as ALK deserves further investigation in the future.

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Disclosure

The authors report no conflicts of interest in this work.

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